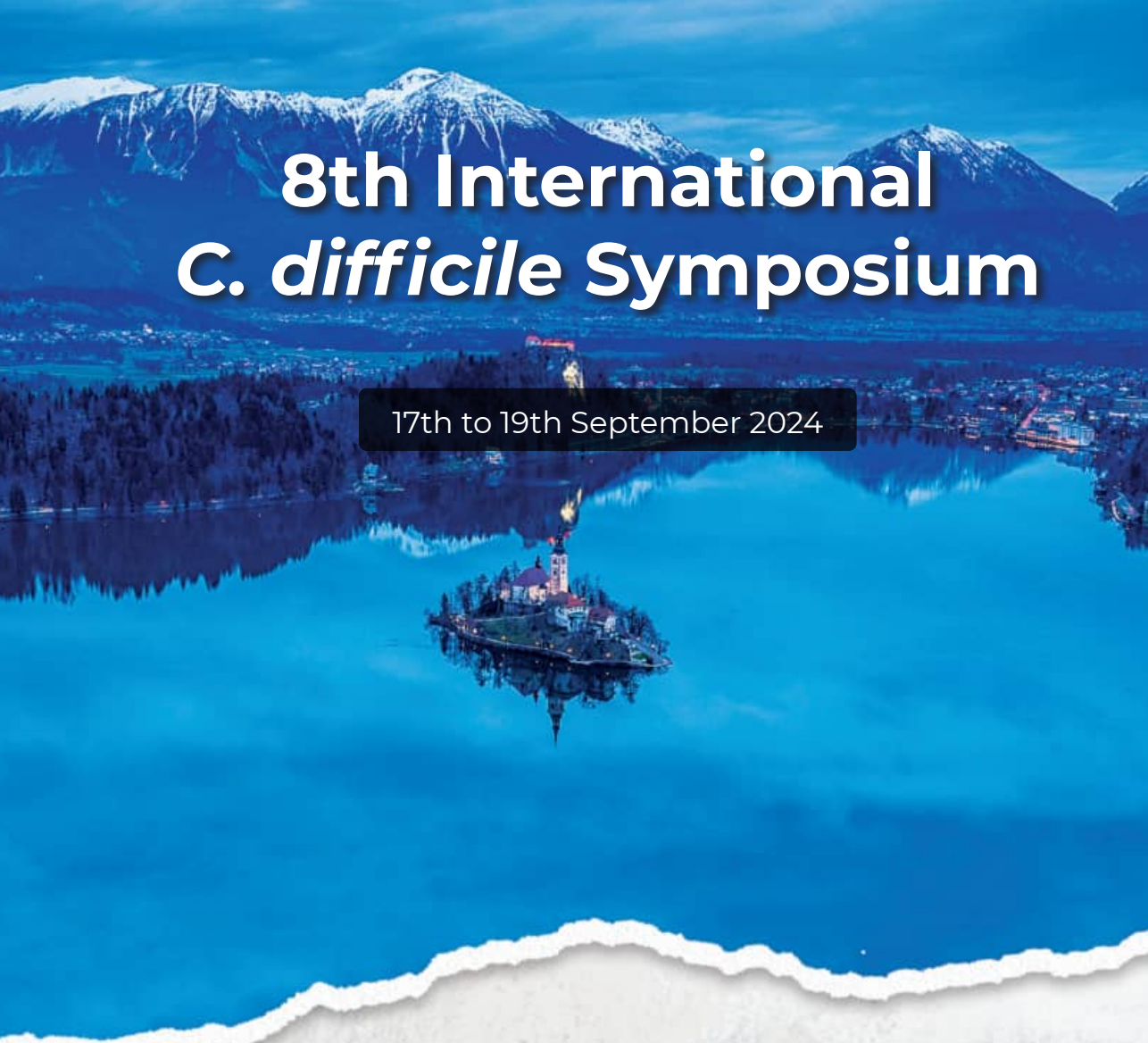

8th International *C. difficile* Symposium

17th to 19th September 2024

NACIONALNI LABORATORIJ ZA ZDRAVJE, OKOLJE IN HRANO

ABSTRACT BOOK

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ABSTRACT BOOK

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8th International *C. difficile* Symposium Abstract book

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WELCOME

Dear friends and colleagues,

It is a great pleasure to welcome you to the 8th International *Clostridium difficile* Symposium (ICDS).

The very first ICDS was organized in 2004. The community was much smaller then, and the first meeting had only 70 participants. This was also the time when the 027/NAP1 strain began to emerge, shifting *C. difficile* infections from an occasional complication associated with antibiotic use to a life-threatening and common condition. As a result, *C. difficile* has quickly gained recognition as an important public health threat.

The ICDS was originally planned as a one-time event, but because of its success, we quickly agreed to hold another meeting in 2007. Since then, the gathering has grown in size and reputation, becoming the primary venue where leading experts meet to discuss advances in research, clinical practices, and development related to *C. difficile*. Since 2010, Bled has served as the traditional location for the meeting.

Over the past two decades, we have seen both continuity and change. Some topics, such as diagnostics, infection control, and treatment, have remained at the forefront of discussion, while others, such as the One Health approach and the role of microbiota, have emerged as new areas of focus. Over the time we have also seen the tremendous development in experimental techniques available to study this fascinating microorganism. Also, the name of the bacterium has changed, from *Clostridium to Clostridioides*.

The *C. difficile* community has grown substantially. Today, the pathogen is well-represented at major microbiological meetings, and dedicated national conferences are becoming common in regions such as South America and Europe. Nevertheless, ICDS remains the premier international gathering. We hope that this year's symposium will inspire new ideas and foster fruitful collaborations.

On this special occasion of the 20th anniversary of the ICDS, we would like to extend our sincere gratitude to all those who have contributed to the symposium's success over the years: our participants, the Organizing Committee members—especially those who have served since the beginning—and our sponsors, whose unwavering support makes this event possible. We hope that you will enjoy the symposium and find it both stimulating and rewarding.

On the behalf of Organizing Committee

Maja Rupnik and Sandra Janezic

GENERAL INFORMATION

CONGRESS VENUE

Rikli Balance Hotel, Cankarjeva cesta 4, 4260 Bled, Slovenia

REGISTRATION DESK OPENING HOURS

Tuesday, September 17: 10:00-16:00 and during coffee breaks
Wednesday, September 18: 8:00 to 9:00 and during coffee breaks
Thursday, September 19: 8:00 to 9:00 and during coffee breaks

MEALS AND SOCIAL EVENTS

Lunch will be provided as part of the registration fee on Wednesday, September 18, and Thursday, September 19. It will be served in the restaurant of the Rikli Balance Hotel.

Coffee breaks will be served in front of the lecture hall and in the poster area.
The **Welcome reception** on Tuesday, September 17, is included in the registration and will be held at the Rikli Balance Hotel.

The **Congress dinner** on Thursday, September 19, is also included in the registration and will take place at the Grand Hotel Toplice.

NAME BADGES

You are required to wear your name badge during all congress scientific sessions as well as during social events.

CERTIFICATE OF ATTENDANCE

Attendance certificates will be available on-site upon request at the Registration desk.

FOR SPEAKERS

Speakers are kindly requested to upload their presentation to the computer in the lecture hall. Technical assistance will be provided. Use of personal computers is not encouraged.

FOR POSTER PRESENTERS

Posters will be displayed throughout the entire meeting. The number of your poster can be found in the Poster overview table. Posters can be set up starting Tuesday, September 17, and should be removed after the Poster session on Thursday, September 19.

INVITED SPEAKERS

Barbut, Frédéric (France)

Fishbein, Skye (USA)

Imwattana, Korakrit (Thailand)

Krutova, Marcela (Czech Republic)

Kuijper, Ed (The Netherlands)

Riley, Tom (Australia)

Rodríguez, César (Costa Rica)

Skinner, Andrew (USA)

Soutourina, Olga (France)

Unnikrishnan, Meera (United Kingdom)

Uzal, Francisco (USA)

ICDS AND ESGCD ATTENDANCE GRANTS

Brajerova, Marie
(Second Faculty of Medicine, Charles University, Czech Republic)

Doyle, Aoife
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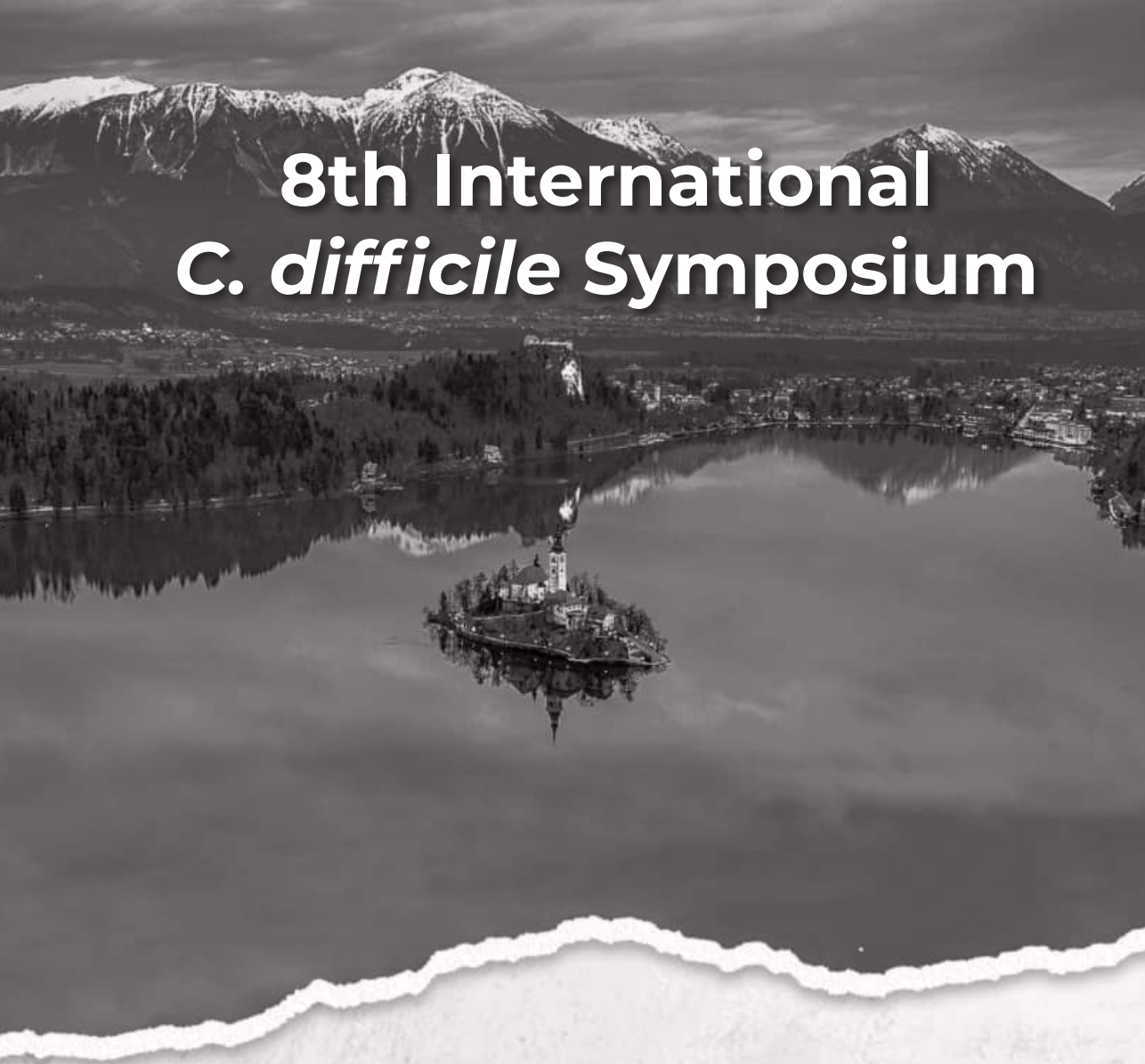
Emadi, Anahita
(Razi Vaccine and Serum Research Institute, Iran)

Eng, Lengsea
(Curtin University, Australia)

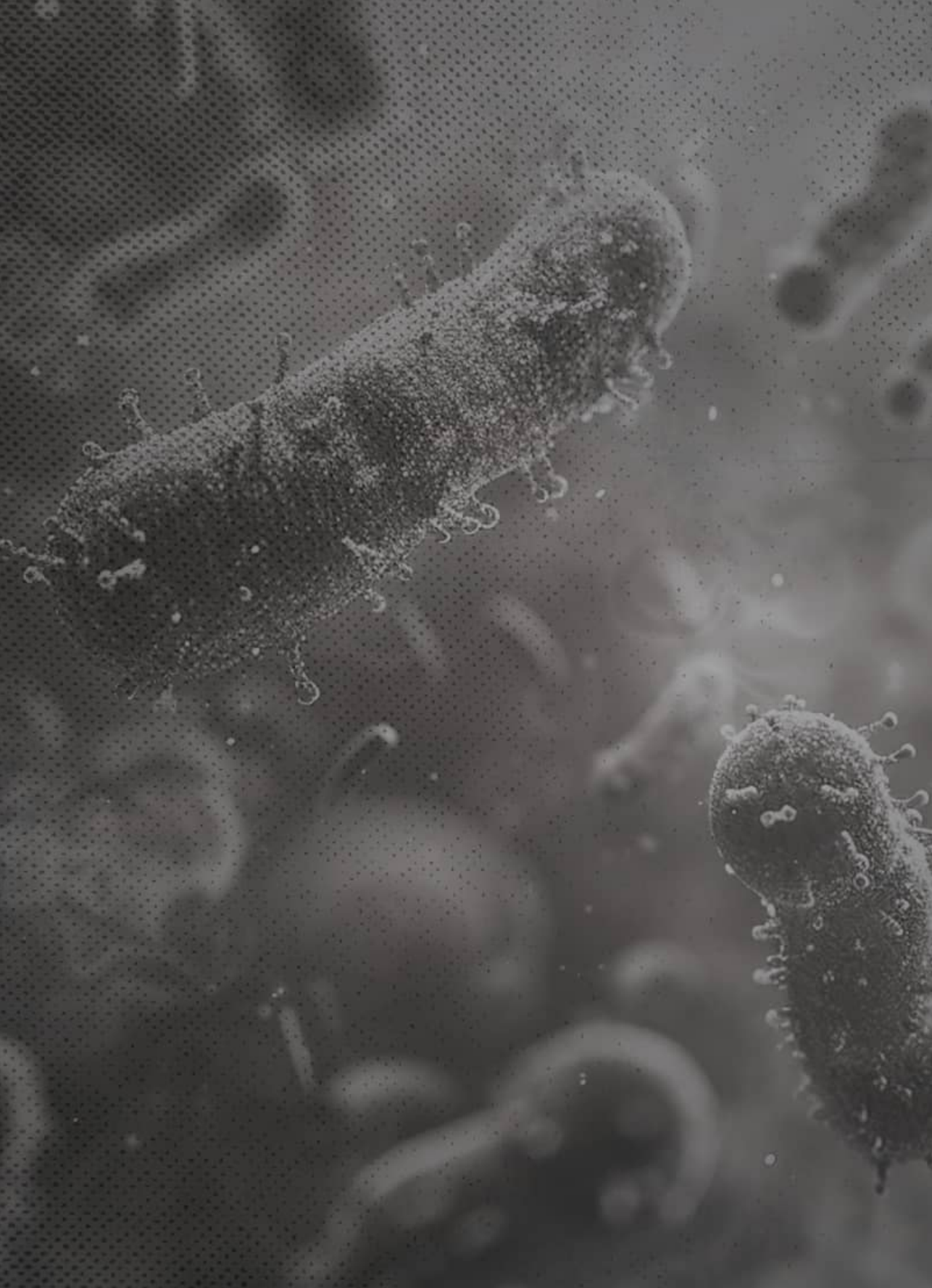
Kanižaj, Lucija
(University Hospital Centre Zagreb, Croatia)

Kobeissy, Philippe Hussein
(Lebanese American University, Lebanon)



8th International *C. difficile* Symposium

ICDS PROGRAMME



TUESDAY, SEPTEMBER 17, 2024

14:15-14:30		**Maja Rupnik**	**WELCOME**
14:30 - 16.20	**SESSION 1**		**DIAGNOSTICS AND SURVEILLANCE | Chair: Tom Riley**
14:30-15:00	INV1	Frédéric Barbut	C. DIFFICILE DIAGNOSTICS - UNANSWERED QUESTIONS
15:00-15:30	INV2	Marcela Krutova	CDI SURVEILLANCE: FROM FRAGMENTS TO GENOMES
15:30-16:00	INV3	Ed Kuijper	BACKGROUND AND CLINICAL RELEVANCE OF ANTIBIOTIC RESISTANCE IN CLOSTRIDIOIDES DIFFICILE
16:00-16:20	OP1	Marie Brajerová	SHOULD WE CONSIDER ACQUIRED RESISTANCE GENES IN CLOSTRIDIOIDES DIFFICILE SURVEILLANCE?
16.20 - 17.00			**COFFEE BREAK**
17.00 - 18.30	**SESSION 2**		**MOLECULAR BIOLOGY AND PHYSIOLOGY | Chair: Paula Salgado**
17:00 - 17:30	INV4	Olga Soutourina	GENETIC TOOLS FOR C. DIFFICILE
17:30 - 17:50	OP2	Joseph A. Sorg	THE IMPACT OF YabG MUTATIONS ON C. DIFFICILE SPORE GERMINATION AND PROCESSING OF SPORE SUBSTRATES
17:50 - 18:10	OP3	Adriana Badilla Lobo	A NOVEL FAMILY OF SMALL PROTEINS REGULATED BY THE SECOND MESSENGERS C-DI-GMP AND C-DI-AMP CONTROLS SPORULATION IN CLOSTRIDIOIDES DIFFICILE
18:10 - 18:30	OP4	Wiep Klaas Smits	STRUCTURE OF THE REPLICATIVE POLYMERASE PolC REVEALS MODE OF ACTION AND MECHANISM OF RESISTANCE OF THE ANTI-CDI AGENT IBEZAPOLSTAT AND RELATED INHIBITORS
19:00			**WELCOME RECEPTION (Hotel Rikli Balance)**

WEDNESDAY, SEPTEMBER 18, 2024

08:30 - 10:10	**SESSION 3**		**CLINICAL TRIALS AND DEVELOPMENTAL RESEARCH | Chair: Aimee Shen**
08:30 - 08:50	OP5	Ken F. Blount	HEALTH-RELATED QUALITY OF LIFE CORRELATED WITH MICROBIOME AND METABOLOME COMPOSITIONS DURING TREATMENT FOR PREVENTION OF RECURRENT CLOSTRIDIOIDES DIFFICILE INFECTION: EXPLORATORY ANALYSIS OF A PHASE 3 STUDY OF FECAL MICROBIOTA, LIVE-JSLM (REBYOTA®)
08:50 - 09:10	OP6	Kevin Garey	IBEZAPOLSTAT PRESERVES KEY CLOSTRIDIUM LEPTUM SPECIES. MICROBIOME RESULTS FROM THE PHASE 2, RANDOMIZED, DOUBLE-BLIND STUDY OF IBEZAPOLSTAT COMPARED WITH VANCOMYCIN FOR THE TREATMENT OF CLOSTRIDIOIDES DIFFICILE INFECTION

09:10 - 09:30	OP7	Rossen Donev	POTENCY OF ANTIBODIES IN OraCAb® TO NEUTRALIZE DIFFERENT C. DIFFICILE TOXINOTYPES - A COMPARATIVE STUDY WITH BEZLOTOXUMAB
09:30 - 09:50	OP8	Ann Marie Stanley	EVALUATION OF THE APPLICABILITY OF AN ULTRASENSITIVE IMMUNE ASSAY FOR TOXIN B DETECTION FOR USE IN C. DIFFICILE CLINICAL TRIALS
09:50 - 10:10	OP9	Erik Dubberke	CLINICAL DEVELOPMENT OF VE303: A RATIONALLY DESIGNED LIVE BIOTHERAPEUTIC PRODUCT TO PREVENT RECURRENT CDI
10.10 - 10.45			**COFFEE BREAK**
10.45 - 12.25	**SESSION 4**		**TREATMENT, PREVENTION, INFECTION CONTROL | Chair: Erik Dubberke**
10:45 - 11:15	INV5	Skye Fishbein	DISSECTING GUT MICROBIOME AND PATHOGEN CONTRIBUTIONS TO CLOSTRIDIOIDES DIFFICILE COLONIZATION OUTCOMES
11:15 - 11:45	INV6	Andrew Skinner	CDI AT THE BED SIDE - CURRENT TREATMENT OPTIONS
11:45 - 12:05	OP10	Mayan Gilboa	IMPACT OF ASYMPTOMATIC CLOSTRIDIOIDES DIFFICILE CARRIAGE SCREENING ON ANTIBIOTIC STEWARDSHIP AMONG HOSPITALIZED PATIENTS
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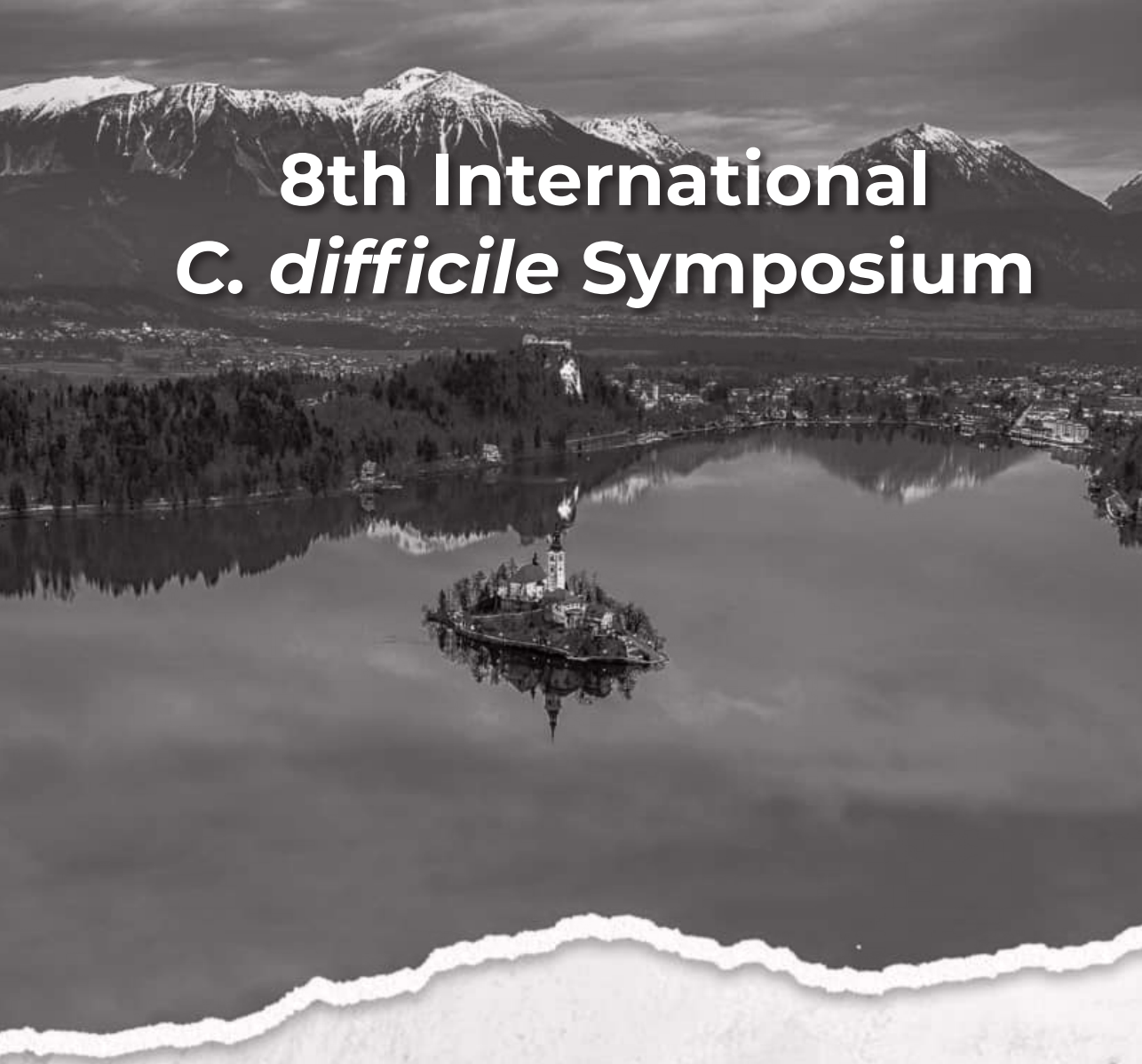
12.30 - 13.00			**POSTER TIME**
13.00 - 14.00			**LUNCH (Hotel Rikli Balance)**
14.00 - 15.00			**POSTER SESSION + COFFEE**
15:00 - 16:30	**SESSION 5**		**C. DIFFICILE VIRULENCE | Chair: Gayatri Vedantam**
15:00 - 15:30	INV7	Meera Unnikrishnan	PROBING THE C. DIFFICILE-HOST INTERFACE USING IN VITRO GUT MODELS
15:30 - 15:50	OP12	Bruno Dupuy	CLOSTRIDIOIDES DIFFICILE BINARY TOXIN CDT INDUCES BIOFILM-LIKE PERSISTING MICROCOLONIES
15:50 - 16:10	OP13	Andrew Hryckowian	BUTYRATE-IMPOSED TRADEOFFS IN CLOSTRIDIOIDES DIFFICILE METABOLISM AND PATHOGENESIS
16:10 - 16:30	OP14	Rajat Madan	NEUTROPHIL HETEROGENEITY IN C. DIFFICILE INFECTION: UNRAVELING THE EFFECTS OF OLFACTOMEDIN-4 IN DISEASE SEVERITY
16.30 - 17.00			**COFFEE BREAK**
17.00 - 18.30	**SESSION 6**		**ROUND TABLE | Moderator: Mark Wilcox**
			FMT -SOONER OR LATER?

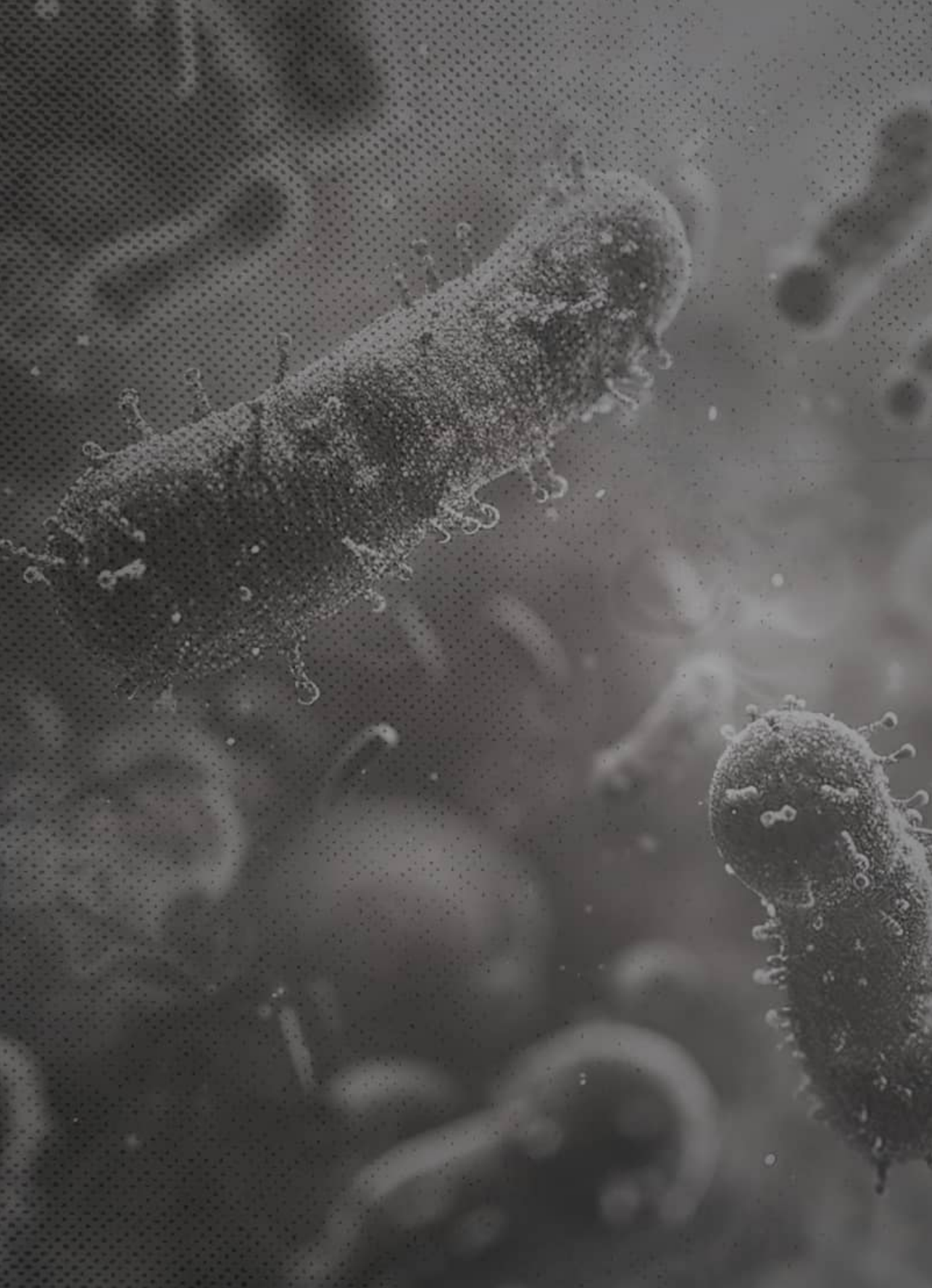
THURSDAY, SEPTEMBER 19, 2024

08:30 - 10:00	**SESSION 7**		**C. DIFFICILE ON THE BENCH | Chair: Johann Peltier**
		Gayatri Vedantam	THE ROLE OF MOUSE MODELS IN ADVANCING CDI DRUG DEVELOPMENT: BENEFITS AND CAVEAT
		TBA	SELECTED PRESENTATIONS ABOUT THE PRACTICAL DIFFICULTIES WHEN WORKING WITH C. DIFFICILE
10.00 - 10.30			**COFFEE BREAK**
10.30 - 12.10	**SESSION 8**		**EPIDEMIOLOGY | Chair: Frédéric Barbut**
10:30 - 11:00	INV8	Thomas Riley	THE RISE AND RISE OF COMMUNITY-ASSOCIATED C. DIFFICILE INFECTION
11:00 - 11:30	INV9	Korakrit Imwattana	C. DIFFICILE AND CDI IN ASIA
11:30 - 11:50	OP15	Diane Pople	NATIONAL C. DIFFICILE SURVEILLANCE USING A NOVEL SENTINEL NETWORK APPROACH
11:50 - 12:10	OP16	Fred Angulo	INCIDENCE OF PUBLIC HEALTH SURVEILLANCE-REPORTED CLOSTRIDIOIDES DIFFICILE INFECTIONS IN THIRTEEN COUNTRIES WORLDWIDE, 2007–2022
12.10 - 13.00			**POSTER TIME**
13.00 - 14.00			**LUNCH (Hotel Rikli Balance)**
14.00 - 15.00			**POSTER SESSION + COFFEE**
15:00 - 16:00	**DISCUSSION SESSION**		**IS C. DIFFICILE A PRIORITY PATHOGEN? Chair: Stuart Johnson**
16:00 - 16:20			**BREAK**
16.20 - 18:00	**SESSION 9**		**ONE HEALTH &VETERINARY FOCUS | Chair: Maja Rupnik**
16:20 - 16:50	INV10	Cesar Rodríguez Sánchez	TRACING C. DIFFICILE THROUGH THE ONE-HEALTH LENS
16:50 - 17:20	INV11	Francisco Uzal	UPDATE ON C. DIFFICILE-ASSOCIATED DISEASE IN ANIMALS
17:20 - 17:40	OP17	Aoife Doyle	AN INVESTIGATION INTO CLOSTRIDIOIDES DIFFICILE RIBOTYPE 078 WITH A ONE HEALTH FOCUS
17:40 - 18:00	OP18	Urska Henigman	SLOVENIAN FARMED MUSSELS HARBOR A DIVERSE POPULATION OF CLOSTRIDIOIDES DIFFICILE
18:00 - 19:30			**NETWORKING**
19:30			**CONGRESS DINNER (Grand Hotel Toplice)**



8th International *C. difficile* Symposium

ABSTRACTS OF INVITED PRESENTATIONS



INV1

CLOSTRIDIOIDES DIFFICILE DIAGNOSTICS - UNANSWERED QUESTIONS

Frédéric Barbut

National Reference Laboratory for Clostridioides difficile

Clostridioides difficile is a Gram-positive, spore-forming anaerobic entero-pathogen responsible for a wide spectrum of clinical features, ranging from mild diarrhea to severe pseudomembranous colitis, septic shock and possible death. *C. difficile* is the first cause of nosocomial infectious diarrhea, but community-associated *C. difficile* infections (CDI) are increasingly reported in patients without the common risk factors (age > 65 years, previous antimicrobial treatment). The main *C. difficile* virulence factors are toxins A (TcdA) and B (TcdB), and, in some cases, the binary toxin (CDT).

Despite increasing awareness of CDI among physicians, there is still a substantial underdiagnosis of CDI both in hospitalized and community patients, mainly due to a lack of clinical suspicion. Patients with unexplained diarrhea should be systematically tested for *C. difficile*, except children less than 3 years old who are frequently colonized. A prompt diagnosis is crucial for both patient management and implementation of infection control measures. Repeated testing in a patient with a first negative result is not useful and test-of-cure at the end of a treatment is not recommended.

Numerous techniques have become commercially available for the CDI diagnosis. The methods fall into 3 groups depending on their targets: some methods detect the free toxins in stools (cell cytotoxicity assay, EIA for toxins, SIMOA based technique), some detect the presence of *C. difficile* irrespective of its capacity to produce toxins (culture, GDH detection), and other detect a strain with the potential of producing toxins in vitro (Nucleic acid amplification technique [NAAT])

Because no standalone method has an adequate positive predictive value at a low CDI prevalence, the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) proposed to implement a 2-step algorithm to overcome this issue. The 2-step algorithm starts with a high-sensitive test like GDH or NAAT as screening method to exclude CDI with a high probability, followed by a high specific test for free toxins for specimens tested positive by the initial NAAT or GDH. Several studies have shown that the bacterial load of *C. difficile* (as determined by the CT in NAAT) is correlated to toxin detection and severity of the disease. According to the Infectious Disease Society of America (IDSA) guidelines, NAAT alone can be considered if appropriate stool selection is guaranteed by laboratories. Over the last decade, several biomarkers such as fecal calprotectin and lactoferrin, which are non specific markers of gastrointestinal inflammation, have been proposed to help interpretation of NAAT-positive /toxin-negative results. Other markers such as proinflammatory cytokines (IL-1ß, IL-8) have been recently studied for assessing disease severity or for helping differentiation between infection and colonization.

INV2

CDI SURVEILLANCE: FROM FRAGMENTS TO GENOMES

Marcela Krutova

Department of Medical Microbiology, Charles University, 2nd Faculty of Medicine and Motol University Hospital, Prague, Czech Republic

Clostridioides (Clostridium) difficile became the main cause of healthcare-associated diarrhoea. The epidemiology of *C. difficile* infections (CDI) has changed dramatically after the emergence and global spread of hypervirulent PCR ribotype (RT) 027 in 2003. The increased number of CDIs with high attributed mortality have led to the implementation of different typing techniques into CDI surveillance to differentiate causative *C. difficile* strains.

In Europe, PCR ribotyping became a commonly used technique. Ribotyping profiles differ based on the length of regions between 16S and 23S rDNA genes and the number of their copies. In addition, dye-labelled primers and capillary electrophoresis separation of PCR amplicons allow automatized ribotype recognition in the WEBRIBO database.

In North America, the leading technique for typing was pulsed-field gel electrophoresis (PFGE) which is based on the digestion of genomic DNA with an infrequent cutting restriction enzyme. The obtained banding patterns are referred to as NAP-field types.

As for many bacterial species, a multilocus sequence typing scheme, when seven housekeeping genes are sequenced and a combination of allele-variants results in sequence type, is also available for *C. difficile*. However, the cost of MLST is higher than in ribotyping and PFGE.

The above techniques allow the characterisation of *C. difficile* isolates and identify suspicious clusters of the same type. However, the subtyping method is needed for the determination of the genetic relatedness of the "same-type" isolates, e.g. a multilocus tandem repeats variant analysis (MLVA) or whole genome sequencing (WGS). MLVA targets variable parts of *C. difficile* genome and WGS provides information on the entire *C. difficile* genome, but the data requires bioinformatic processing.

Several core MLST schemes are now available to facilitate the WGS data analysis but it was shown that the discriminatory power of cgMLST might be insufficient in some ribotypes.

Single nucleotide polymorphism (SNP) analysis is also used to determine the genetic relatedness of *C. difficile* isolates. However, the standardised pipeline is not available and the use of different reference genomes may affect the analysis results.

The lecture will discuss the current trends in *C. difficile* isolates characterisation for surveillance purposes in national and reference laboratories.

INV3

BACKGROUND AND CLINICAL RELEVANCE OF ANTIBIOTIC RESISTANCE IN *CLOSTRIDIOIDES DIFFICILE*

Kuijper Ed[1], *Smits Wiep Klaas*[1]

[1]*Dutch National Expertise Center for Clostridioides difficile infections, Leiden University Center for Infectious Diseases, Leiden and RIVM, Bilthoven*

Antibiotic resistance of *Clostridioides difficile* (*C. difficile*) impacts both the development and spread of *C. difficile* infections (CDI) and the effectiveness of anti-CDI treatment. The importance of association of prior antibiotic use with CDI has been recognized by U.S. Centers for Disease Control and Prevention resulting in *C. difficile* being classified as a "Urgent threat". Among the antibiotic classes that predispose to CDI, cephalosporins, clindamycin, co-amoxicillin-clavulanic acid, and fluoroquinolones are frequently mentioned. It remains unclear whether antibiotic resistance in *C. difficile* is required for infection development, as the gut microbiome's composition and function, particularly the disruption of the ratio between primary and secondary bile acids, also play a crucial role. Antibiotic-resistant *C. difficile* can spread more easily in environments where hospitalized patients are treated with specific antibiotics, such as observed in fluoroquinolone-resistant *C. difficile* PCR ribotype 027 outbreaks. Most antibiotic-resistant *C. difficile* strains result from mutations in existing genes (rifamycins, fluoroquinolones, beta-lactams). In contrast, resistance to tetracyclines, macrolides-lincosamides-streptogramins, linezolid (cfr) and chloramphenicol is primarily transferred via mobile genetic elements with a broad host range

Only a few antibiotics are recommended for the treatment of *Clostridioides difficile* infections (CDI), including vancomycin, fidaxomicin, and to a lesser extent, metronidazole. While decreased susceptibility and resistance to vancomycin have been reported, these findings are challenging for other laboratories to confirm. The mechanism of vancomycin resistance remains largely unknown, with implication of both chromosomal and extrachromosomal determinants. Interestingly, although "van genes" are present in the vast majority of *C. difficile* genomes, they do not always confer glycopeptide resistance to the bacterium. Resistance to fidaxomicin, the most recently approved anti-CDI antibiotic, remains so far uncommon in clinical practice. There is one recent case report of a single T3428G mutation in the rpoB gene that resulted in high level resistance and caused a relapse of CDI, despite reduced bacterial fitness. Metronidazole resistance in *C. difficile* is phenotypically difficult to identify, often being heme-dependent. It is associated with specific PCR ribotypes (e.g., RT 010 and RT027) and is complicated by various genetic factors, including presence of the plasmid pCD-METRO, mutations in heme-responsive genes, and mutations in the promoter of the nimB gene.

INV4

GENETIC TOOLS FOR *C. DIFFICILE*

Olga SOUTOURINA[1]

[1]*Regulatory RNAs in Clostridia group, Microbiology department, Institute for Integrative Biology of the Cell (I2BC), CNRS, Université Paris-Saclay, CEA, 91198, Gif-sur-Yvette, France*

Among human enteropathogens, *Clostridioides difficile* attracted the attention of scientific community due to increased incidence and severity of infections and high rate of recurrences. This pathogen has been renamed several times keeping the « difficile » adjective that highlights original difficulties for this bacterium isolation, growth requiring strictly anaerobic conditions and genetic tool limitations. During the recent years we have seen a real revolution in available tools for manipulation of *C. difficile* genome. The development of next generation sequencing technologies allowed first to generate extremely rich genomics and transcriptomics data that constitute valuable resources for functional gene analysis during current postgenomics era. An efficient genome editing is essential to unravel the secrets of this successful pathogen and the mechanisms by which it adapts to the host. Shortly after the first *C. difficile* genome was sequenced, the adaptation of TargeTron-based technology led to the ClosTron tool that revolutionized the clostridial knock-out genetics. Few years ago, precise manipulation of *C. difficile* genome became possible through allele-coupled exchange method that has been recently improved by adding the inducible expression of a toxin from toxin-antitoxin system as a counter-selection marker or using theophylline-dependent riboswitch to control the editing plasmid replication. Powerful CRISPR-Cas9 and endogenous CRISPR-Cas-based editing strategies have been also successfully adapted for use in *C. difficile*. We can now perform the precise manipulations of the *C. difficile* genome including deletions, insertions and single nucleotide substitutions that appeared not feasible before. « Difficile » genome became now really more accessible for future investigations. Of course, there is still room for improvement, among avenues to be explored we could consider increasing the efficiency of conjugation and homologous recombination or combining several available strategies.

INV5

DISSECTING GUT MICROBIOME AND PATHOGEN CONTRIBUTIONS TO *CLOSTRIDIOIDES DIFFICILE* COLONIZATION OUTCOMES

Skye R. S. Fishbein[1,2*], Anna L. DeVeaux[1*], Sakshi Khanna[1,2*], Aura L. Ferreiro[1,2], James Liao[1,2], Wesley Agee[1], Jie Ning[1,2], Bejan Mahmud[1], Miranda J. Wallace[1,2], Tiffany Hink[3], Kimberly A. Reske[3], Janaki Guruge[1,2], Sidh Leekha1, Erik R. Dubberke[3], Gautam Dantas[1,2,4-6]

[1]*The Edison Family Center for Genome Sciences and Systems Biology, Washington University School of Medicine, St. Louis, Missouri, USA.*
[2]*Department of Pathology and Immunology, Division of Laboratory and Genomic Medicine, Washington University School of Medicine, St. Louis, Missouri, USA.*
[3]*Division of Infectious Diseases; Washington University School of Medicine, St. Louis, Missouri, USA*
[4]*Department of Molecular Microbiology; Washington University School of Medicine, St. Louis, Missouri, USA*
[5]*Department of Biomedical Engineering; Washington University in St Louis, St. Louis, Missouri, USA*
[6]*Department of Pediatrics; Washington University School of Medicine, St. Louis, Missouri, USA*
[*]*these authors contributed equally*

Background and Aims: Gastrointestinal colonization by toxigenic *Clostridioides difficile* is common in healthcare settings and ranges in clinical presentation from asymptomatic carriage to lethal *C. difficile* infection (CDI). Although progression to CDI is influenced by both the gut microbiome and *C. difficile* strain, our understanding of how commensal-pathogen interactions influence *C. difficile* colonization outcomes remains incomplete.

Methods: We used a systems biology approach and gnotobiotic animal models to improve our understanding of why patients colonized with toxigenic *C. difficile* do not necessarily progress to CDI. Specifically, we employed machine learning approaches to identify microbial features that discriminate between carriers and patients with CDI. Following, we used two different gnotobiotic animal models to quantify the commensal and pathogen contribution to disease outcome.

Results: Microbiota-humanization of germ-free mice with fecal samples from toxigenic *C. difficile* carriers revealed a spectrum of virulence among widely circulating clade 1 *C. difficile* lineages and identified commensal Blautia species that correlate with markers of non-pathogenic colonization, including reduced *C. difficile* growth and toxin production. Using gnotobiotic mice engrafted with complex defined communities representative of *C. difficile*-colonized patients, we observed strain-specific CDI severity among clinical clade 1 isolates, including comparable virulence of an ST8 strain to a hypervirulent clade 2 strain. Mice engrafted with a higher diversity community were protected from severe disease and toxin production across all tested strains without suppression of *C. difficile* colonization in vivo.

Conclusions: These results indicate that during instances in which colonization resistance has been breached without overt infection, commensals can attenuate a diversity of otherwise virulent strains without inhibiting their colonization, providing insight into determinants of stable *C. difficile* carriage in the healthcare setting.

INV6

CDI AT THE BED SIDE - CURRENT TREATMENT OPTIONS

Andrew M Skinner[1,2]

[1]*VA Salt Lake City Healthcare System, Salt Lake City, UT, USA;*
[2]*University of Utah School of Medicine, Salt Lake City, UT, USA*

Historically *Clostridioides difficile* infection (CDI) treatment has relied heavily on antimicrobials, primarily metronidazole and vancomycin. However, over the past 10 years, new therapeutic options for CDI have become available including more targeted antimicrobial therapies such as fidaxomicin as well as the addition of adjunctive therapies with the primary goal of providing patients with a durable, long-lasting treatment response.

In 2021, the Infectious Diseases Society of America (IDSA), the Society of Healthcare Epidemiologist of America (SHEA), the American College of Gastroenterology (ACG), and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) all recommended the use of fidaxomicin for primary and recurrent CDI (rCDI). These recommendations were supported by 4 randomized clinical trials which demonstrated a 27% relative reduction in 30-day CDI recurrence when compared to vancomycin. Additionally, these guidelines assessed the monoclonal antibody, bezlotoxumab, which demonstrated a 38% relative reduction in rCDI when given intravenously with standard-of-care (SOC) CDI antibiotics. Thus, bezlotoxumab was recommended as adjunctive therapy for patients at high risk of developing rCDI. Since these recommendations, the US Food and Drug Administration (FDA) approved additional adjunctive therapies for CDI.

The US FDA has now approved the live biotherapeutics, live-jslm and live-brpk as adjunctive therapies following SOC CDI antibiotics. Live-jslm is composed of a standardized consortium of live microbes prepared from human stool given as an enema. Whereas live-brpk is composed of live purified firmicute bacterial spores given as capsules. The proposed mechanism by which these therapies prevent rCDI is by establishment of protective fecal microbiota and restoration of colonization resistance to prevent *C. difficile* germination and subsequent rCDI. In randomized controlled trials, live-brpk reduced CDI recurrence by 68% and live-jslm reduced CDI recurrence by 30% when compared to SOC antibiotic therapy alone.

While initial treatment options remain limited to antibiotics, the addition of new adjunctive therapies greatly reduces the risk of rCDI. Looking forward into the future, additional therapies such as small molecule inhibitors, vaccines, additional biotherapeutics, and newer, more targeted antibiotics are on the horizon.

INV7

PROBING THE *C. DIFFICILE*-HOST INTERFACE USING IN *VITRO* GUT MODELS

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Early interactions between the gut mucosa and the pathogen *Clostridioides difficile* are likely critical in the establishment of *C. difficile* infection, one of the major causes of hospital-acquired diarrhoea worldwide. Although we have significant insight into *C. difficile* pathogenesis through animal infection models, unlike other major intestinal pathogens, interactions between *C. difficile* and the colonic epithelium have not been well defined at a cellular level. This is largely due to the technical challenges associated with co-culturing anaerobes with oxygen requiring epithelia. In recent years however, several in vitro models of the gut have been reported, ranging from simple cell lines to complex 3D organoid models. Additionally, environment control and microfluidics have enhanced our ability to better mimic the gut physiology in vitro. In vitro systems have enabled studies to investigate both *C. difficile* proteins as well as host pathways involved during initial stages of *C. difficile* colonisation and offer excellent potential to study interactions with immune cells and the gut microbiota. I will discuss key models that have been applied to studying *C. difficile* infection and their future potential in deciphering intricate interactions occurring at the gut-microbial interface.

INV8

THE RISE AND RISE OF COMMUNITY-ASSOCIATED *CLOSTRIDIUM DIFFICILE* INFECTION

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Despite remaining a major hospital pathogen, *Clostridium difficile* infections (CDIs) have been increasing in the community world-wide. Non-human reservoirs/sources of *C. difficile* include both production and companion animals, and the environment is being contaminated with strains of *C. difficile* from animal manure. However, the extent of this environmental contamination is poorly understood. Since the 1990s in North America, cephalosporins have been licensed for use in food animals. There has been an amplification of *C. difficile* in these animals since then, with subsequent contamination of soil. Indeed, soil anywhere into which animal manure has been added is now a rich source of *C. difficile*, including soil in home gardens. "Animal" strains of *C. difficile* are now infecting humans. *C. difficile* ribotype (RT) 027 was found in animals in North America in the early 2000s but probably moved from animals to humans a decade earlier around the time that RT027 developed resistance to fluoroquinolones. This strain was likely to have initially caused infections in the community at a time when community-associated (CA) CDI was thought infrequent, and diarrhoea in the community was rarely investigated. The mutation to fluoroquinolone resistance and high use of fluoroquinolones drove RT027 spread in North America, and later Europe once it entered the hospital system. A similar process is now occurring with *C. difficile* RT078, another animal strain causing both CA-CDI and HA-CDI in Europe over the last 15 years. Surveillance data indicate that CA-CDI now comprises a significant fraction of total CDI cases. In the United States, CA-CDI increased 4-fold during the period 1991–2005, and recent studies report higher proportions of CA-CDI, around 40%. A European multi-center study (97 hospitals in 34 European countries) published in 2011 found 14% of 506 cases were classified CA-CDI, however, there are little recent data. In Australia, the most recent report from 2020/21 showed CA-CDI accounted for 80% of all CA-CDI! Individuals with CA-CDI often do not have "classical" risk factors and are generally younger, healthy and female, without contact with hospitalized patients nor prior antimicrobial exposure. In up to 40% of CA-CDI cases, infections were more severe. These findings suggest the need for a One Health approach for effective CDI monitoring, prevention, and control.

INV9

CLOSTRIDIOIDES DIFFICILE AND CDI IN ASIA

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Compared to North America and Europe, little is known about *Clostridioides difficile* and *C. difficile* infection (CDI) in Asia. Most studies originate from the Middle East, East Asia, and Southeast Asia, with fewer from South Asia. The *C. difficile* population in the Middle East resembles Europe and North America, with a high prevalence of toxigenic strains producing toxins A and B (A+B+CDT-) and some binary toxin-producing strains (A+B+CDT+; primarily epidemic ribotype 027 or related types). The epidemiology of *C. difficile* in East and Southeast Asia is unique, with a high prevalence of non-toxigenic *C. difficile* (NTCD) and/or toxigenic strains that produce only toxin B (A-B+CDT-), most of which likely belong to evolutionary clade 4, with only sporadic reports of A+B+CDT+ *C. difficile*. Limited studies suggest that clade 4 *C. difficile* acquired a PaLoc about 500 years ago. Various prevalence studies undertaken more recently confirm a high prevalence and diversity of clade 4 strains in Asia. It remains unclear why epidemic *C. difficile* ribotype 027 failed to spread across Asia. A leading hypothesis was that multidrug-resistant endemic *C. difficile* strains have occupied a niche, preventing *C. difficile* ribotype 027 colonization. One Asian strain, *C. difficile* ribotype 017, has spread successfully worldwide causing outbreaks in many countries. CDI prevalence in Asia appears to be lower, with milder clinical characteristics rarely leading to pseudomembranous colitis or toxic megacolon, and cases usually respond well to oral metronidazole or even no treatment. Possible explanations include the aforementioned unique epidemiology, primarily the high prevalence of NTCD, which could outcompete toxigenic strains for food and habitat. Some host factors may have also played a role, such as the presence of drug-resistant microbiota, capable of protecting the colon even when exposed to antimicrobials. Still, other possible protective factors are yet to be explored. Antimicrobial resistance is common in NTCD in Asia and these strains can also acquire a PaLoc and become toxin producers. In summary, very little is known about *C. difficile* and CDI in Asia, however, these strains of *C. difficile* often lack one or more toxin genes and, predominantly, belong to clade 4. They should not be ignored although more work is required to understand them completely.

INV10

TRACING *CLOSTRIDIOIDES DIFFICILE* THROUGH THE ONE-HEALTH LENS

César Rodríguez (CIET & Faculty of Microbiology, University of Costa Rica)

Clostridioides difficile is a bacterium associated with gastrointestinal infections that relies on spores to persist and spread. This species thrives in the colon of patients undergoing dysbiosis; consequently, *C. difficile* infections (CDI) have been perceived as healthcare-associated for many years. However, we now know that CDI occur and are rising in the community, and we have witnessed how certain human activities have selected strains with epidemic potential. This complex and evolving picture is further complicated by the unexpectedly large diversity of toxigenic and non-toxigenic strains from this species and from other Clostridioides genomospecies isolated from animals, vegetables, and multiple environmental reservoirs, such as soil, sediment, and water bodies, some with minimal human impact. Several of these non-human derived strains are genetically similar to those causing human infections. Hence, CDI seem also to have a zoonotic and foodborne origin and unsuspected transmission routes, exemplifying the One Health-One World paradigm. This talk will review how this knowledge has been constructed and discuss future directions, such as the need for improved cultivation and diagnostic methods, as well as open issues, including the pathogenicity and role in human disease of the TcdB-carrying Clostridioides genomospecies.

INV11

UPDATE ON *CLOSTRIDIOIDES DIFFICILE*-ASSOCIATED DISEASE IN ANIMALS

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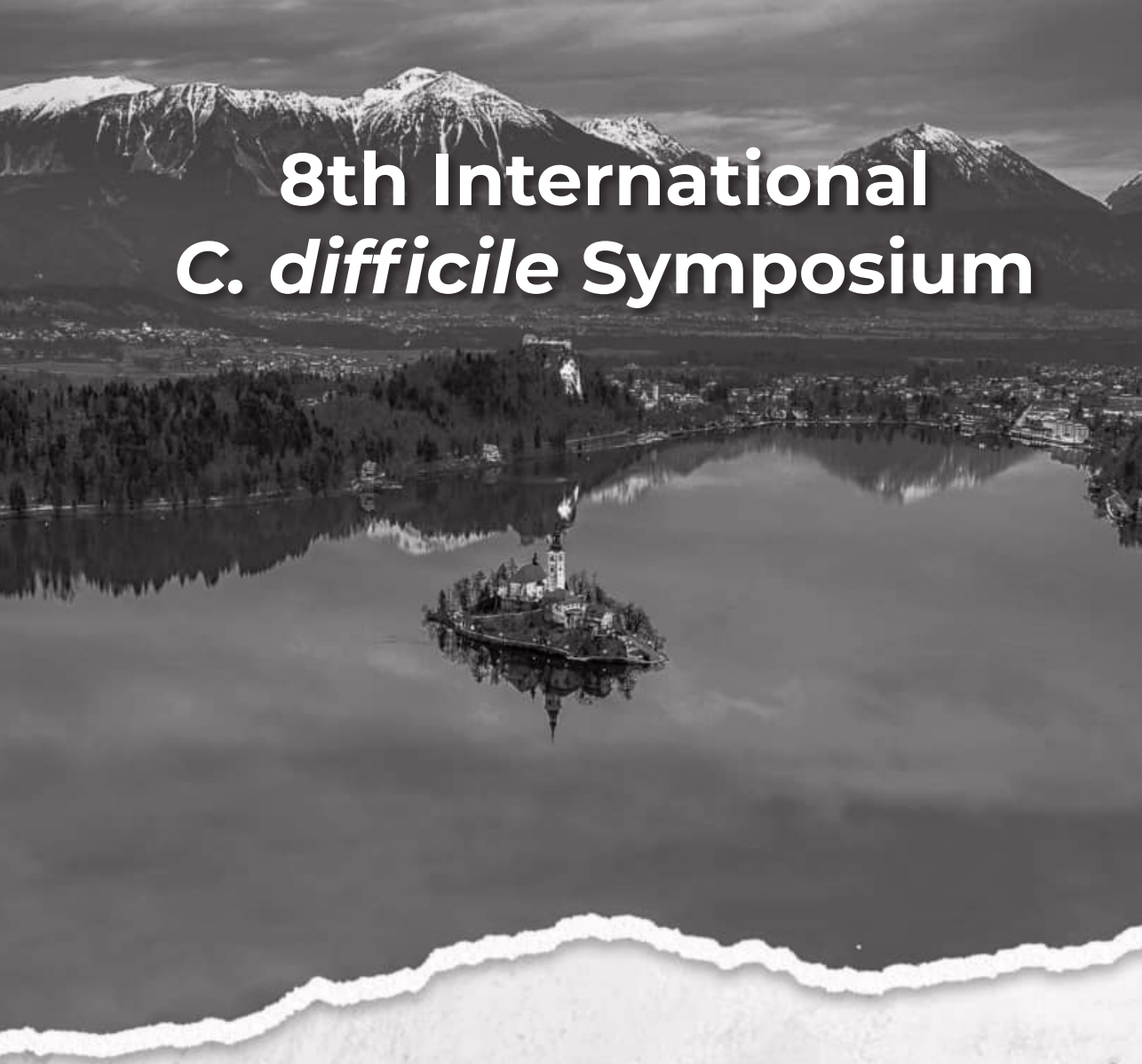
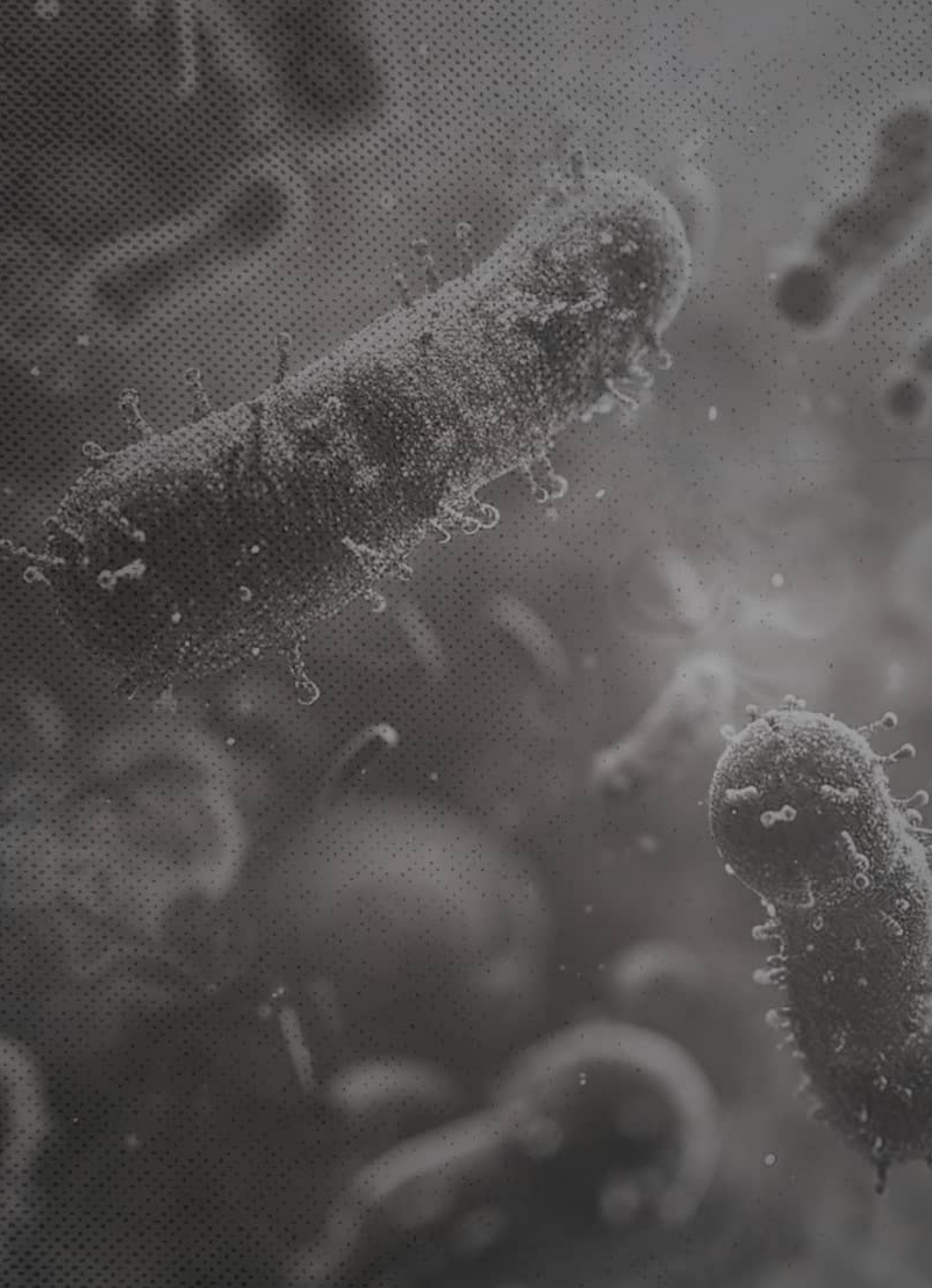
Clostridioides difficile affects, in addition of humans, several animal species, including, but not limited to gerbils, guinea pigs, hamsters, horses, rabbits and pigs. This microorganism has also been isolated from the gastrointestinal tract of a wide variety of animal species in which it is either not associated with disease or its role in disease is not known. In most domestic animals species *C. difficile*-associated disease (CDAD) is typically is antibiotic-associated, although individual cases occur in several species in which no antibiotic association is known. In addition, the great majority of CDAD in pigs are not antibiotic associated. The disease in most animals is not age-associated, with the notable exception of pigs, a species in which CDAD is seen in neonatal animals. CDAD is highly prevalent in horses, a species in which the two main risk factors for the disease are, like in humans, antibiotic therapy and hospitalization. However, recent studies suggest have found that a significant numbers of horses with CDAD have never received antibiotics, which suggests that antibiotic therapy may not be a significant factor in certain geographic regions or environments. Other factors including co-infections with other bacteria or parasites, colon impactions or torsions, transportation, surgical or medical treatment and nasogastric intubation, have been proposed but on the basis of anecdotal evidence rather than case-controlled reports. *C. difficile* infection causes disease more commonly in neonatal piglets up to approximately one week of age, but the disease has also been described in post-parturient sows with mastitis-metritis-agalactia treated with antibiotics. The source of infection for neonatal piglets is the sow and the contaminated environment in the farrowing wards, and transmission can be fecal-oral or even airborne. Experimental evidence indicates that transplacental vertical transmission of *C. difficile* from pregnant sows to piglets is unlikely. Morbidity and mortality are variable. Among laboratory animals, Syrian hamsters are the most susceptible to naturally-acquired disease, although spontaneous or experimental *C. difficile* enteric disease also occurs in gerbils, guinea pigs, mice, rabbits and rats. The role of *C. difficile* in enteric disease of dogs and cats is unclear but evidence indicates that this organism is not a common enteric pathogen. The existence of community-associated and hospital-associated colonization with toxigenic *C. difficile* has been demonstrated in dogs. The prevalence of *C. difficile* in the feces of healthy dogs and cats is variable, ranging approximately between 10 and 40%. However the association between isolation of toxigenic *C. difficile* from feces and diarrhea in dogs is debatable.



8th International *C. difficile* Symposium

ABSTRACTS OF SHORT ORAL PRESENTATIONS



OP1

SHOULD WE CONSIDER ACQUIRED RESISTANCE GENES IN *CLOSTRIDIOIDES DIFFICILE* SURVEILLANCE?

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Background and Aims: *C. difficile* ribotypes 001 and 176 drive an epidemiology of *C. difficile* infection in the Czech Republic. We used whole genome sequencing (WGS) to obtain more information on these ribotypes.

Methods: Thirty-four *C. difficile* isolates (RT001 n=21, RT176 n=13, one of each RT per hospital) were selected for WGS, (Illumina). Genetic relatedness was determined by wgMLST, (Bionumerics v8.1). Antimicrobial resistance (AMR) mechanisms were searched using ResFinder. Isolates with different AMR profiles were sequenced by long-read sequencing (MinION). Hybrid assemblies were annotated in RAST and compared using Easyfig v2.2.5. The presence of inserts in isolates sequenced only by short reads was detected by mapping of raw reads against hybrid assemblies in Geneious v2023.0.4.

Results: In 21 RT001 isolates, wgMLST (3745 loci) showed 0-120 allele differences. A whole genome comparison (hybrid assembly of sensitive and resistant isolate) revealed 13 different inserts, seven contained AMR genes. A 3.6kb insert with tetA(P), tetB(P) genes (1/21 isolates), a 9.3kb insert with the cfrB gene (7/21 isolates, with a partial deletion of the cfrB gene in one isolate), a 10.5kb and a 14kb inserts, both with aac(6')-Ie-aph(2'')-Ia genes (14/21 and 1/21 isolates, respectively), a 23.7kb insert with ermB (17/21 isolates) and a 52.7kb insert (3/21 isolates) with the ermB and cfrB gene.

In 13 RT176 isolates, wgMLST (3298 loci) showed 0-9 allele differences. A whole genome comparison revealed six different inserts, four contained AMR genes. A 17.8kb insert (inserted at two locations in the genome) with the tetM gene (4/13 isolates), a 33.8kb insert with ermB (8/13 isolates), a 57.7kb insert with a putative methyltransferase for macrolide resistance (5/13 isolates) and a 77.3kb insert with the genes cfrE, putative methyltransferase for macrolide resistance (5/13 isolates), and aac(6')-Ie-aph(2'')-Ia (8/13 isolates).

Conclusions: The wgMLST confirmed a clonal relatedness by 0-3 allelic difference of RT001 or 176 isolates from different Czech hospitals. However, different inserts with acquired AMR genes were detected in these isolates. The acquired antimicrobial resistance genes should be considered when clonal relatedness is reported.

OP2

THE IMPACT OF YabG MUTATIONS ON *C. DIFFICILE* SPORE GERMINATION AND PROCESSING OF SPORE SUBSTRATES

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Upon ingestion by the host, *C. difficile* spores germinate into actively growing, toxin-producing vegetative cells. *C. difficile* spore germination is triggered in response to certain bile acids and amino acids [e.g., taurocholic acid (TA) and glycine]. In prior work that identified CspA as the co-germinant receptor, we identified mutation in the promoter or coding region of YabG. YabG is a sporulation-specific protease that processes preproSleC into proSleC and CspBA to CspB and CspA. To understand how the identified mutations in the yabG locus contribute to *C. difficile* spore germination, we introduced these mutations into an isogenic background using our new allelic exchange system. Building upon this, we sought to understand how YabG processes its targets. Spores derived from *C. difficile* ΔyabG, *C. difficile* yabGC$_{207A}$ (catalytically inactive), *C. difficile* yabG$_{A46D}$, *C. difficile* yabG$_{G37E}$, and *C. difficile* yabGP$_{153L}$ strains germinate in response to TA alone and do not require co-germinants. Recombinantly expressed and purified preproSleC incubated with E. coli lysate expressing wild type YabG resulted in the removal of the pre sequence from preproSleC. Of the yabG mutants generated, interestingly only yabG$_{A46D}$ showed any catalytic activity towards purified preproSleC. Moreover, mutation of the YabG processing site in preproSleC (R119A) led to YabG shifting its processing to R115 or R112. Western blot analysis of yabG mutant spores shows less processing of substrates, including, preproSleC and coat proteins. Transmission electron microscopy revealed that the yabG$_{G-8A}$ spores have defects in the coat. Changes in yabG expression under the mutant promoters was analyzed by SNAP-tag, with the yabGG-8A mutation residing in the ribosome-binding site and yabG$_{C-41A}$ mutation residing in the -10 region of the promoter. The C -41 A mutation results in a change from a promoter that is similar to a sigma E-dependent promoter to a promoter that is more similar to that of sigma A. PyabG-SNAP in yabG$_{G-8A}$ has delayed expression, whereas expression of PyabG-SNAP in yabG$_{C-41T}$ is expressed earlier. Overall, our results support and expand upon the hypothesis that YabG is important for germination and spore assembly and, upon mutation of the processing site, YabG can shift where it cleaves substrates.

OP3

A NOVEL FAMILY OF SMALL PROTEINS REGULATED BY THE SECOND MESSENGERS C-DI-GMP AND C-DI-AMP CONTROLS SPORULATION IN *CLOSTRIDIOIDES DIFFICILE*

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Pathophysiology of the human enteropathogen *Clostridioides difficile* is controlled by complex regulatory networks, including RNA-based mechanisms such as riboswitches. Riboswitches are located at the 5' untranslated end of mRNAs and bind ligands, triggering a conformational change that positively or negatively affects the expression of the downstream coding sequence. Sixteen riboswitches responding to the second messenger c-di-GMP are present in *C. difficile*. In this study, we identified five of them upstream of putative genes encoding almost identical small proteins (SP). Detection by immunoblotting of a tagged SP derivative provided evidence for its synthesis and localization, mainly associated with the cellular membrane. RNA-seq analyses showed a decrease of the five SP transcripts in response to c-di-GMP but also to the second messenger c-di-AMP. Furthermore, reporter assays measuring transcriptional regulation of an SP gene in different strain backgrounds suggested that c-di-GMP modulates gene expression through interaction with the riboswitch, whereas c-di-AMP regulates promoter activity. Overexpression of one of the SP genes resulted in hypersporulation consistent with the upregulation of many SigG and SigK-dependent transcripts, as revealed by RNA-seq. In agreement with these data, a strain deleted of the five SP-encoding genes showed a strong reduction in sporulation compared to the wild-type strain with an intermediate phenotype for strains lacking some but not all the SP. A double affinity protein purification assay is in progress to identify possible proteins interacting with the SP and thus elucidate by which mechanism the SP regulate sporulation. Altogether, our data indicate that the genes encoding this new family of small proteins are regulated by both c-di-GMP and c-di-AMP to control *C. difficile* spore formation.

OP4

STRUCTURE OF THE REPLICATIVE POLYMERASE PolC REVEALS MODE OF ACTION AND MECHANISM OF RESISTANCE OF THE ANTI-CDI AGENT IBEZAPOLSTAT AND RELATED INHIBITORS

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Background. DNA replication is an essential process in cells of all living organisms. Bacterial replicative DNA polymerases (that belong to the Pol III family) are distinct from their eukaryotic counterparts and are thus a promising target for the development of new antimicrobial compounds. In contrast to most other bacteria, Bacillota (Firmicutes), including *C. difficile* and Enterococci, encode two Pol III enzymes: DnaE and PolC. The primary replicative polymerase PolC can be targeted by a group of synthetic compounds that includes ibezapolstat. Ibezapolstat demonstrated favorable results in phase 2 clinical trials for the treatment of *C. difficile* infections and is planned to advance to phase 3 international trials.

Methods and Results. We purified and characterized the activity of PolC enzymes from multiple multidrug resistant organisms and showed inhibition by ibezapolstat and a variety of novel PolC inhibitors. Notably, we determined the structure of PolC from *Enterococcus faecium* in complex with a novel PolC inhibitor and in complex with ibezapolstat by cryogenic electron microscopy. These results show an unexpected conformation of the inhibitor in the active site of the polymerase. We have generated spontaneous mutants with up to 16-fold reduced susceptibility to PolC inhibitors; this change correlates with mutations identified in the binding site of the inhibitor compounds. Introduction of these *pol*C mutations in *C. difficile* increases the minimal inhibitory concentration for the PolC inhibitors, in line with the structural homology of PolC within Bacillota.

Conclusion. Together, our data show for the first time that the mode of action and the mechanism of reduced susceptibility to ibezapolstat (and related compounds) are conserved within Bacillota. This paves the way for rational design of new compounds with improved inhibitory activity and pharmacokinetic characteristics, and highlights key regions of *C. difficile pol*C that could be monitored for resistance mutations during clinical trials or clinical use of ibezapolstat.

OP5

HEALTH-RELATED QUALITY OF LIFE CORRELATED WITH MICROBIOME AND METABOLOME COMPOSITIONS DURING TREATMENT FOR PREVENTION OF RECURRENT *CLOSTRIDIOIDES DIFFICILE* INFECTION: EXPLORATORY ANALYSIS OF A PHASE 3 STUDY OF FECAL MICROBIOTA, LIVE-JSLM (REBYOTA®)

Rohan Mishra[1], Adam Harvey[2], Amy Guo[3], Glenn Tillotson[4], Paul Feuerstadt[5,6], Sahil Khanna[7], Thomas Louie[8], Susan N. Elliott[9], Christine Lee[10,11], William D. Shannon[1], Ken F. Blount[2]

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Background and aims: Recurrent *Clostridioides difficile* infection (rCDI) is associated with mortality and morbidity, disruption of the gut microbiome, and worsening health-related quality of life (HRQOL). Microbiota-based approaches have shown to be effective in preventing rCDI, thereby improving patient HRQOL. Fecal microbiota, live-jslm (REBYOTA®; RBL, previously RBX2660), a broad consortium microbiota-based live biotherapeutic approved for preventing rCDI following standard-of-care antibiotics, was efficacious in a phase 3, randomized, placebo-controlled clinical trial (PUNCH™ CD3; NCT03244644). In exploratory analyses, participants adjudicated as clinical responders (those who did not experience rCDI at 8 weeks after RBL or placebo administration) had improved HRQOL and had gut microbiome and bile acid (BA) shifts. The aim of this analysis was to evaluate whether HRQOL changes correlated with gut microbiome and BA changes.

Methods: Responses from Cdiff32 HRQOL questionnaires (a validated CDI-specific survey comprising questions related to mental, physical, and social domains) were obtained from trial participants at multiple time points. A categorical statistical analysis compared Cdiff32 responses between responders administered RBL vs placebo. Fecal samples from both responders and non-responders were sequenced for microbiome composition and analyzed by liquid chromatography mass spectrometry to quantify BAs. Recursive partitioning regression analyses were conducted to assess whether HRQOL outcomes were effective at distinguishing microbiome and BA compositional differences for both responders and non-responders.

Results: RBL responders were more likely than placebo responders to report improved HRQOL, particularly for Cdiff32 mental domain questions. Improvements in HRQOL mental domain scores were associated with changes in microbiome and metabolome composition — a larger improvement in mental HRQOL was associated with: 1) increased relative abundance of Clostridia and Bacteroidia; 2) decreased Gammaproteobacteria and Bacilli; and 3) predominance of secondary rather than primary BAs.

OP5

Conclusions: In this exploratory analysis, RBL administration correlated with multiple effects beyond clinical resolution of diarrheal disease symptoms, including improved patient-reported mental HRQOL and gut microbiome and metabolome changes.

OP6

IBEZAPOLSTAT PRESERVES KEY *CLOSTRIDIUM LEPTUM* SPECIES. MICROBIOME RESULTS FROM THE PHASE 2, RANDOMIZED, DOUBLE-BLIND STUDY OF IBEZAPOLSTAT COMPARED WITH VANCOMYCIN FOR THE TREATMENT OF *CLOSTRIDIOIDES DIFFICILE* INFECTION

Kevin W. Garey, Taryn A. Eubank, M Jahangir Alam, Khurshida Begum, Jacob McPherson, Jinhee Jo, and Michael H. Silverman, for the Ibezapolstat Phase 2 Investigator Group

Background and Aims. Ibezapolstat (IBZ) is Gram-positive selective spectrum antibiotic that inhibits bacterial DNA polymerase IIIC currently in clinical trial development for the treatment of *C. difficile* infection (CDI) in adults. In the open-label, non-comparative, phase 2a study, favorable microbiome changes were observed predicting an anti-CDI recurrence effect. The purpose of this analysis of the phase 2b CDI study was to assess the microbiome effects of IBZ versus vancomycin (VAN).

Methods. Phase 2b (ClinicalTrials.gov, number NCT04247542) was a randomized, double-blind, active-comparator study. Participants with signs and symptoms of CDI and a positive enzyme immunoassay toxin test result were recruited from 12 centers in the USA and randomly assigned (1:1) to receive oral IBZ 450 mg every 12 h or oral VAN 125 mg every 6 h for 10 days. Stool was collected daily for microbiome evaluations (qPCR and metagenomic changes).

Results. Thirty patients were recruited and were assessed in the per protocol analysis (IBZ: n=16; VAN n=14). Fifteen of 16 (93.8%) patients given IBZ (one clinical failure) had a sustained clinical cure versus 12 of 14 (86%) patients given VAN (two recurrences). IBZ patients had stable or increased concentrations of Bacteroides, *C. leptum* and *C. coccoides* without an increase in Enterobacterales. Decreased concentrations of these microbiome species were observed in VAN-treated patients, especially noted in the two patients with CDI recurrence.

Conclusions. In the phase 2b study, IBZ had a favorable microbiome effect increasing concentrations of *C. leptum* and other beneficial commensals while on therapy. Patients with CDI recurrence on VAN had significant disruption to the microbiome. These results warrant further development in phase 3 trials.

OP7

POTENCY OF ANTIBODIES IN ORACAB® TO NEUTRALIZE DIFFERENT *C. DIFFICILE* TOXINOTYPES - A COMPARATIVE STUDY WITH BEZLOTOXUMAB

Rossen Donev[1], Joanna Giles[1], Joanna McGlashan[2], April Roberts[2], Michael Maynard-Smith[2]

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Background and aims: *C. difficile* infection (CDI) is a toxin-mediated infection in the colon. Thus, it would be beneficial to design an antibody therapy that neutralize these toxins at the site of their production. We previously described a highly potent oral therapeutic, OraCAb®, that contains ovine polyclonal antibodies against TcdA and TcdB, within a formulation to protect the antibodies from digestion/inactivation in the GI tract. In the current study we aimed to compare the potency of the antibodies in OraCAb® to neutralize different *C. difficile* toxinotypes to the monoclonal antibodies in Bezlotoxumab (Zinplava®), which is currently used for treatment of CDI. Stability of antibodies and protease inhibitors in OraCAb® was also determined.

Methods: TcdB from 5 different toxinotypes (0, III, V, IX and X) were purified as previously described (Roberts et al., Infect Immun. 2012;80:875-882). Neutralization potency of the antibodies and their stability in the formulation were determined using cells-based toxin-neutralization assay. Activity of trypsin inhibitors in the formulation was measured by L-BAPNA peptide assay.

Results: Anti-TcdB in OraCAb® completely neutralized all 5 toxinotypes tested with EC50 values in µg/mL as follow: 20.01 (toxinotype V), 22.48 (toxinotype III), 32.27 (toxinotype IX), 238.10 (toxinotype X) and 21.96 (toxinotype 0). Bezlotoxumab did not neutralize toxinotypes IX and X and achieved maximum neutralization of 50% at 20.40 µg/mL for toxinotype V. Neutralization of toxinotypes III and 0 at 50% by Bezlotoxumab was estimated at concentrations over 200 and 500 µg/mL, respectively. BLASTP was used to compare the sequence from toxinotype 0 used to raise the anti-TcdB in OraCAb® with toxinotype VIII. The alignment showed 99% homology between the two sequences that warrants the effective neutralization of toxinotype VIII by OraCAb®. The antibodies and trypsin inhibitors in OraCAb® were stable for the entire tested period of 3 years at 2 to 8°C. At ambient temperature (21±4°C), the antibodies showed detectable deterioration 6 months after the manufacture of the formulation, while the trypsin inhibitors showed detectable loss of potency after 18 months.

Conclusions: Using polyclonal antibodies against *C. difficile* toxins is a robust strategy to neutralize majority, if not all, of the TcdB toxinotypes isolated from humans worldwide.

OP8

EVALUATION OF THE APPLICABILITY OF AN ULTRASENSITIVE IMMUNE ASSAY FOR TOXIN B DETECTION FOR USE IN *C. DIFFICILE* CLINICAL TRIALS

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Background and aims. Laboratory tests to confirm *C. difficile* infection have different limitations with differing implications for results in clinical trials settings. Nucleic acid amplification testing approaches are very sensitive but lack specificity and are unable to distinguish colonization from active disease. Toxin enzyme-linked immune assays are very specific but lack sensitivity, which means cases can potentially be missed. Cell cytotoxicity neutralization assay (CCNA) is a gold standard with high sensitivity and specificity but is not standardized and has limited availability. Ultrasensitive immune assays with sensitivity levels comparable with CCNA have already been developed and may have utility in clinical studies to detect low levels of toxin without requiring cell culture. We aimed to develop a sensitive, quantitative assay for the detection of toxin B from human stool based on a Meso Scale Discovery S-PLEX platform.

Methods. In total, 100 antibody pairs were screened for recognition of 5 purified recombinant toxin B toxinotypes representative of the 5 major clades of *C. difficile* (RT027, RT078, RT014, RT017, RT023) along with differential recognition of stool samples with high, low, and negative activity, as defined by CCNA. The top antibody pairs were further evaluated in secondary and tertiary screens that included assessment of a full calibrator curve (RT027), additional sample screening, therapeutic antibody interference, cross-reactivity with toxin A, dilutional linearity and spike recovery.

Results. Using the final selected antibody pair, the assay had spike recovery and dilutional linearity of 75%-125% for stools processed at 10% (w/v) and a 2-fold minimum required dilution. The preliminary lower limit of detection was 0.23 pg/mL; the estimated lower limit of quantification was 2 pg/mL (both in-well).

Conclusion. We have developed a quantitative *C. difficile* toxin B assay with a good spike recovery and dilutional linearity, along with favorable sensitivity. Assay performance is being further characterized using a set of clinical and contrived samples to compare with results from commercially available assays and an in-house CCNA assay. Future work also includes further qualification and validation of the assay, and evaluation in a non-interventional study to assess the value of highly sensitive toxin B detection in future clinical studies.

OP9

CLINICAL DEVELOPMENT OF VE303: A RATIONALLY DESIGNED LIVE BIOTHERAPEUTIC PRODUCT TO PREVENT RECURRENT CDI

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Background. Fecal microbiota products (FMP) are efficacious to prevent recurrent *Clostridioides difficile* infection (rCDI), including fecal microbiota transplantation (FMT), RBX2660, and SER-109. However, owing to their derivation there are regulatory and logistical challenges to their implementation: the composition is variable, there is risk for pathogen transfer, and the number of people willing and eligible to be feces donors is limited. Cultivated live biotherapeutic products (LBP) have the potential to be equally efficacious but overcome these hurdles.

Methods: The development of VE303, a cultivated consortia of 8 clostridial species will be described.

Results: Initial experiments in mice of several cultivated consortia identified VE303 to be able to treat CDI. This consortia was similarly efficacious as FMT in the mouse model. Phase 1 human studies in healthy volunteers found colonization was related to dose and duration of VE303 administration, and oral vancomycin-induce perturbation of the gut microbiome. The phase 2 blinded, randomized controlled trial compared high-dose VE303 (total dose 1.1 x 1011 CFU), low-dose VE303 (total dose 2.2 x 1010 CFU), and placebo. Inclusion criteria included people with rCDI (at least 1 prior episode of CDI in addition to the qualifying CDI episode in the prior 6 months) or primary CDI at high risk for rCDI (≥65 years of age and any of creatine clearance <60 ml/min/1.73m2, proton pump inhibitor use, history of CDI > 6 month prior). Study drug was administered for 14 days after completion of CDI treatment for the qualifying CDI episode. Recurrent CDI developed within eight weeks of completing qualifying CDI treatment antibiotics in 4/29 (13.8%), 10/27 (37.0%), and 10/22 (45.5%) of people who received high-dose VE303, low-dose VE303, and placebo, respectively (p=.006 high-dose vs. placebo, p=.30 low-dose vs. placebo). There was a dose-response relationship between with VE303 strain colonization, and VE303 strain colonization was associated with clinical efficacy. VE303 was well tolerated compared to placebo. A multi-national phase 3 trial of VE303 (RESTORATiVE303) recently opened for enrollment.

Summary: FMPs are efficacious at preventing recurrent CDI. VE303, a cultivated LBP, was well tolerated and had a similar efficacy to FMPs in its phase 2 study. The phase 3 trial of VE303 is open for enrollment.

OP10

IMPACT OF ASYMPTOMATIC *CLOSTRIDIOIDES DIFFICILE* CARRIAGE SCREENING ON ANTIBIOTIC STEWARDSHIP AMONG HOSPITALIZED PATIENTS

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Background and aim: *Clostridioides difficile* (CD), a leading cause of healthcare-associated infections, is strongly associated with antibiotic use. Despite the varying risk levels of different antibiotics, any use can increase infection risk. The utility of screening asymptomatic patients for CD carriage status is currently debated. This study assesses how notifying clinicians of carriage status influences antibiotic stewardship in prescribing practices.

Methods: Since June 2017, patients admitted to internal medicine wards were screened for CD carriage using CD toxin-B PCR via rectal swabs. In Phase I (June-August 2017), clinicians were blinded to the carriage status. In Phase II (June- August 2018), clinicians were informed of the carriage status. Additionally, meetings on antibiotic stewardship, led by an infectious disease specialist, emphasized the treatment of carriers and reducing high-risk antibiotic treatment. We analyzed changes in antibiotic use, treatment duration, and antibiotic types in carriers before and after carriage status was revealed compared to non-carriers using a difference-in-differences analysis.

Results: Phase I identified 52 carriers, and Phase II found 91. These findings were compared with non-carriers hospitalized in the same periods (979 in Phase I and 1396 in Phase II). After adjusting for age, gender, Charlson Comorbidity Index, Norton score, and immunosuppression status, Phase II showed a significant reduction in the duration of antibiotic use among carriers compared to non-carriers (OR 0.81 CI 0.67-0.97), specifically in quinolone use (OR 0.38 CI 0.25-0.55) yet there was no change in the odds of receiving any antibiotic during the hospitalization (OR 0.77 CI 0.45-1.64) or use of Cephalosporins (OR 1.03 CI 0.74-1.46).

Conclusions: Screening for CD carriage at admission and informing clinicians significantly decreases antibiotic usage in carriers, particularly in treatment duration and quinolone use, compared to non-carriers.

OP11

EXPERIMENTAL COLONISATION WITH NON-TOXIGENIC *C. DIFFICILE* IN HEALTHY VOLUNTEERS

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Background. We developed a human model for *C. difficile* colonisation in which we experimentally expose healthy adult volunteers to a non-toxigenic strain of *C. difficile* (NTCD). We aim to better understand susceptibility to colonisation with *C. difficile* by varying the dose and pre-treatment with antibiotics before administration of spores in healthy adult individuals.

Methods. Production of NTCD spores for experimental human administration was carried out according to the principles of Good Manufacturing Practices (GMP) at the bacteriology laboratory of the Leiden University Medical Center (LUMC). The product contains 104 and 107 spores of a well-characterised NTCD strain (L-NTCD03) and is administered orally in capsules. The trial is currently ongoing and was designed as a randomised double-blind controlled clinical trial (start date: Nov 13, 2023), with an adaptive dose, now including a total of 69 subjects. In phase 1, 24 subjects received a capsule with low spore dose (104), high spore dose (107) or placebo for 5 days. In phase 2, 22 subjects were additionally pretreated with 1 day vancomycin (4dd 250mg) 7 days before above mentioned NTCD or placebo exposure, and in phase 3, 23 subjects were pretreated with 5 days of vancomycin 7 days before NTCD or placebo exposure.

Results. So far, the administration of NTCD spores was safe and well tolerated. A total of 33 related adverse events (AEs) were recorded during the first two phases of the trial. The majority of the AEs was gastro-intestinal (31/33) and mild of severity (29/33), and all of the related AEs were self-limiting. There were no severe or serious related AEs recorded. Colonisation was not observed in phase 1. Preliminary results from phase 2 show colonisation in 6/19 (32%) subjects distributed over both dosing groups. Results of phase 3 as well as the deblinding of the study is expected in July 2024 and will be presented.

Conclusions. Interim safety analysis showed that exposure of healthy volunteers to 5 days of NTCD spores was safe and well tolerated. Our preliminary analyses show that vancomycin pre-treatment is required to obtain colonisation of healthy volunteers with L-NTCD03.

OP12

CLOSTRIDIOIDES DIFFICILE BINARY TOXIN CDT INDUCES BIOFILM-LIKE PERSISTING MICROCOLONIES

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Summary. Clinical symptoms of *Clostridioides difficile* infection (CDI) ranges from diarrhea to pseudomembranous colitis, and in some cases recurrent colitis. A major challenge in managing CDI is the high rate of relapse. Several studies correlate production of CDT binary toxin by clinical strains of *Clostridioides difficile* (CD), with higher relapse rates. The mechanism of action of CDT in host cells is known, however its role during CDI is still unclear. To understand the physiological role of CDT during CDI, we established 2 hypoxic relevant intestinal models, Transwell and Intestine-on-chip that were challenged with the epidemic strain UK1 CDT+ and its isogenic CDT- mutant. We showed that CDT binary toxin induces mucin-associated microcolonies that increase CD colonization and display biofilm-like properties by enhancing CD resistance to vancomycin but not to fidaxomicin, a biofilm disrupting antibiotic. Interestingly, biofilm-like CDT-induced microcolonies were formed in the cecum and colon of mice. Our study shows that CDT toxin induces biofilm-like microcolonies increasing CD colonization and persistence.

OP13

BUTYRATE-IMPOSED TRADEOFFS IN *CLOSTRIDIOIDES DIFFICILE* METABOLISM AND PATHOGENESIS

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Background and aims: The gut microbiome engenders colonization resistance against the diarrheal pathogen *Clostridioides difficile* (*Cd*), but the molecular basis of colonization resistance is incompletely understood. Our work aims to understand how *Cd* responds to butyrate, a prominent end-product of gut microbiome community metabolism that is associated with colonization resistance to *Cd*.

Methods: Our work leverages a broad palette of tools including murine models of CDI, human stool samples, bacterial cell culture, bacterial genetics, and a variety of molecular and systems biology techniques.

Results: We showed that diets that increase gastrointestinal butyrate concentrations led to reduced *Cd* burdens and lower mortality in mice. These findings are recapitulated in humans, where butyrate is associated with colonization resistance against *Cd*. To better understand these effects, we exposed diverse *Cd* strains to butyrate. We showed that exogenously-applied butyrate negatively impacts *Cd* growth and is internalized into *Cd* cells, where it interferes with *Cd's* own butyrate production pathways. We further demonstrated that butyrate-dependent growth inhibition in *Cd* coincides with increased sporulation and toxin release from *Cd* cells. Finally, by growing *Cd* in a variety of defined growth media, we provide evidence that distinct molecular and genetic responses underlie the butyrate-dependent growth inhibition, sporulation, and toxin release phenotypes.

Conclusions: Our data support a conceptual model where *Cd* senses butyrate, a prominent metabolic end-product of the microbiome, as a signal of a competitive gut environment. *Cd* growth is impaired by butyrate and *Cd* responds to butyrate by releasing its toxins and producing spores – relevant for maintaining a dysbiotic gut microbiome and for transmission between hosts. Future work to disentangle the molecular and genetic mechanisms underlying these growth and virulence phenotypes will likely lead to new strategies to restrict *Cd* growth in the gut and minimize its pathogenesis during CDI.

OP14

NEUTROPHIL HETEROGENEITY IN *C. DIFFICILE* INFECTION: UNRAVELING THE EFFECTS OF OLFACTOMEDIN-4 IN DISEASE SEVERITY

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Background and Aims: Neutrophils are dominant cells in the innate immune response to CDI, and intensity of neutrophil-mediated inflammation is a key driver of CDI outcomes. Excessive tissue and blood neutrophils are associated with worse histopathology and adverse outcomes. Although neutrophils are heterogenous in nature, there are no studies that examine neutrophil populations in CDI. The main aim of our study was to define the various neutrophil subtypes in bone marrow (BM), blood and colon of *C. difficile*-infected host, and determine their impact on CDI pathogenesis.

Methods: We utilized intestinal epithelial cell (IEC)-neutrophil co-cultures, pre-clinical murine models of CDI and samples from CDI patients and controls. We performed unbiased single-cell transcriptomics and spectral flow cytometry to illuminate various CDI-induced neutrophil populations and define biological pathways that could exacerbate CDI-associated IEC damage.

Results: Our data show that neutrophils activated with LPS exacerbate *C. difficile*-induced IEC injury. Pseudotime trajectory analysis, pathway enrichment analysis, and module scoring of gene signatures of transcriptomics data reveal various neutrophil subtypes in BM, blood and both prior to and after infection. CDI expands various neutrophil clusters that exhibit gene signatures associated with tissue damage, and our data suggest that TNF signaling could be a crucial driver for development of these inflammatory neutrophil populations. Additionally, we identified a pathogenic neutrophil population marked by Olfactomedin-4 (*Olfm4*) expression. During acute CDI, OLFM4+ cells aggregate to areas of damaged epithelium, and their numbers correlate with IEC damage score. *In vitro*, OLFM4+ neutrophils exacerbate toxin-mediated IEC damage, and in mice, CDI increases number of OLFM4+ neutrophils and circulating OLFM4 protein. Similarly, patients with CDI have higher amounts of circulating OLFM4 in blood compared to non-CDI controls. Finally, OLFM4-/- mice had faster resolution of diarrhea and better survival, compared to WT mice, despite similar pathogen burden.

Conclusion: We have created the first transcriptomics atlas of CDI-induced neutrophils and identified novel neutrophil populations with pathogenic potential. Our studies also suggest that OLFM4+ neutrophils are a possible target for developing new host-directed CDI therapies.

OP15

NATIONAL *C. DIFFICILE* SURVEILLANCE USING A NOVEL SENTINEL NETWORK APPROACH

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Background: *Clostridioides difficile* surveillance in England has been based on ribotyping since 2007 (https://www.gov.uk/guidance/clostridium-difficile-ribotyping-network-cdrn-guide-to-services). National surveillance will migrate to a whole genome sequence based system in 2024. To maximise efficiency, we have explored whether using a network of sentinel laboratories can provide a representative picture of *C. difficile* strain types across England. We describe how *C. difficile* surveillance in England is planned to change, notably based on a review of a network analysis of NHS hospitals which, combined with mathematical modelling of inter-hospital spread, can determine an optimised selection of sentinel sites.

Methods: We analysed primary data on hospital admissions from periods before and after the C-19 pandemic, to understand the underlying network of patient movements between acute NHS hospitals in England. Weighted, directed networks were built for all patients and linked surveillance data were used to assess movements of *C. difficile* infection (CDI) patients. We developed a mathematical model simulating the inter-hospital pathogen spread across the network. Using these simulated outbreaks and empirical hospital networks, we identified an optimised set of well-connected sentinel hospitals enabling early detection of novel strains, adapting the algorithm to incorporate real-world sequencing constraints by capping/excluding specified hospitals as sentinel sites.

Results: Mathematical modelling identified 20 out of ~150 hospitals in England that together are expected to result in the fastest detection, and so likely reflect key routes of (healthcare related) dissemination of *C. difficile* strains. Analytical outputs included empirical hospital networks, measures of network characteristics, simulated time-to-importation for each setting for each possible index case of an inter-hospital outbreak, and a prioritised list of sentinel hospitals which minimises the first detection time nationally across outbreak scenarios.

The new sentinel surveillance system will collect the first 10 specimens from each reporting site each month and undertake both PCR-ribotyping and WGS based analysis. Hospitals/laboratories will still be able to refer non-sentinel samples for typing, using currently accepted criteria (clusters of CDI or increases in CDI frequency, severity, mortality or relapse rate).

Conclusions: This novel approach to national surveillance enables the selection of sentinel sites to efficiently provide rapid identification of novel *C. difficile* strains within real-world logistical constraints. The networks provide insight on relationships between hospitals across England which is also potentially applicable to interventions to control inter-hospital spread of other healthcare-acquired infection pathogens.

OP16

INCIDENCE OF PUBLIC HEALTH SURVEILLANCE-REPORTED *CLOSTRIDIOIDES DIFFICILE* INFECTIONS IN THIRTEEN COUNTRIES WORLDWIDE, 2007–2022

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Background and Aim: *Clostridioides difficile* infection (CDI) is an important cause of morbidity and mortality worldwide. Public health surveillance systems are an important data source for estimating CDI disease burden. CDI surveillance can be population-based or hospital-based, yielding estimates of population-based CDI incidence (cases/100,000 population per year) and hospital based CDI incidence (cases/10,000 patient-days) or CDI admission rates (cases/1,000 hospital admissions), respectively. The aim was to identify publicly available national CDI surveillance data worldwide and compare incidence estimates to understand the global burden and inform preventive strategies.

Methods: We searched websites of national governmental agencies responsible for CDI surveillance for data summaries and interactive dashboards. We also searched PubMed for articles reporting CDI surveillance data. We summarized surveillance methods and temporal trends in CDI incidence from 2007–2022.

Results: We identified 13 countries with CDI data; 10 countries conducted population-based surveillance and 10 countries conducted hospital-based surveillance. There was substantial heterogeneity in surveillance systems among countries. The United States (US) had the most comprehensive CDI surveillance systems, and the highest reported CDI incidence. From 2011-2021, population-based CDI incidence declined in England, Finland, Ireland, Northern Ireland, Scotland, Sweden, the US, and Wales, but not in Canada and Norway. In the US, the proportion of cases that were healthcare-associated declined while community-associated increased. Hospital-based CDI incidence declined in Scotland, Sweden, and the US, but not in Australia, Canada, Israel, and the Netherlands, and CDI admission rates declined in Canada, and the US, but not in Belgium, and Germany. Hospital-onset hospital-based CDI incidence declined in Canada, Israel, England, and the US, but not in Australia, Denmark, or the Netherlands.

Conclusions: These data indicate declines in CDI incidence in several, but not all, countries. Inconsistent CDI case definitions and surveillance approaches between countries limit the interpretability of cross-country comparisons. Despite the declines in CDI incidence in some countries, the CDI burden remains high, and the need persists for CDI prevention strategies in community and healthcare settings.

OP17

AN INVESTIGATION INTO *CLOSTRIDIOIDES DIFFICILE* RIBOTYPE 078 WITH A ONE HEALTH FOCUS

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Background and Aims: *Clostridioides difficile* places an increasing burden on healthcare services because of emerging hypervirulent strains such as RT078/ST11 that may be resistant to commonly prescribed antimicrobials e.g. quinolones. Pigs can be colonised by RT078/ST11, and may act as a reservoir for human *C. difficile* infection (CDI) in susceptible individuals. These food-producing animals are the highest consumers of veterinary antimicrobials (including quinolones and tetracyclines) in many countries [1]. We investigated the relationship between *C. difficile* from pigs, environmental and food sources, and CDI cases in hospitals.

Methods: Whole genome sequencing (WGS) was performed on confirmed RT078 isolates collected between 2018 and 2021 in Ireland from pigs (n=60) on 5 farms, the environment (n=42), food (n=3), and human cases of CDI (n=81) from 2 clinical centres that were >200km apart. Ridom Seqsphere+ software was used to determine clusters, and antimicrobial resistance (AMR) determinants.

Results: All isolates (n=186) were identified as *C. difficile* ST11 following sequencing. Bioinformatic analysis revealed 8 clonal groups (CGs), containing isolates of clinical and non-clinical origin differing by ≤2 SNPs in the core genome. Of these 8 CGs, 1 CG contained pig and environmental isolates, 3 CGs contained human and environmental isolates, 2 CGs contained pig and human isolates, 1 CG contained human, environmental and food isolates and 1 CG contained pig, human and environmental isolates. Quinolone and tetracycline AMR determinants were detected in 100% and 81% of genomes, respectively.

Conclusions: *C. difficile* RT078/ST11 has emerged as a major pathogen in both pigs and humans. Close genomic relationships between pig, environmental, food and human *C. difficile* isolates is supportive of interspecies transmission and necessitates a One Health approach to *C. difficile* control. Selective pressure derived from tetracycline and quinolone use in food-producing animals may facilitate the emergence and dissemination of *C. difficile* ST11 [2].

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OP18

SLOVENIAN FARMED MUSSELS HARBOR A DIVERSE POPULATION OF *CLOSTRIDIOIDES DIFFICILE*

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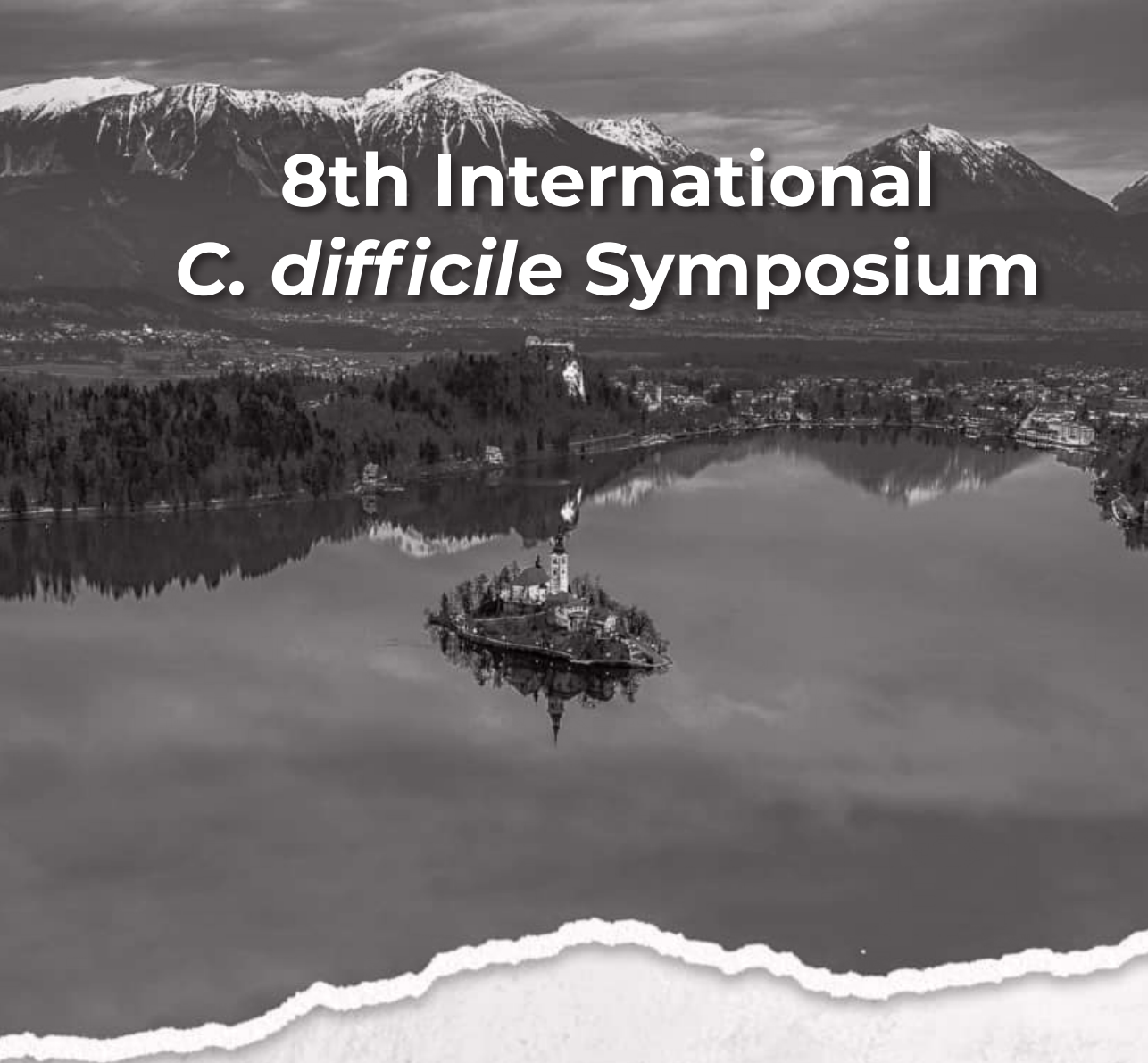
Mussels, due to their filter-feeding activity, can accumulate not only nutrients but also microorganisms and toxins from the environment. EU legislation on bivalve molluscs sets criteria only for *Salmonella* spp., *Escherichia coli*, and marine biotoxins, but not for other bacteria. In light of the increasing incidence of *Clostridioides difficile* infections in humans, the aim of our study was to determine the genetic characteristics of *C. difficile* isolates from mussels (*Mytilus galloprovincialis*) in the Slovenian sea.

C. difficile isolates (n = 76) from three Slovenian mussel farms, sampled in 2014, 2015 and 2021, were typed using whole genome sequencing (WGS) to determine their population structure and genetic characteristics (genes encoding the antimicrobial resistance and toxins). Sequencing was performed using Illumina technology (2×150 bp).

C. difficile isolates were classified into five different clades (1, 4, 5 and cryptic clades C-II and C-III) and into 31 sequence types (ST), of which two were newly identified. Eleven isolates had at least one resistance gene; we found genes encoding resistance to aminoglycosides (*aac(6')-aph(2''), ant(6)-Ia, aph(2'')-If and aph(3')-III*), tetracycline (*tet* (A), *tet*(B), *tet*(O) and *tet*(M)), linezolid (*cfr*(C)) and macrolides (*erm*(B)). Of the 76 isolates, 56 were toxigenic (73.7 %); of these, all three toxin genes were present in five isolates from clade 5 (ST11). Four clusters of closely related isolates (< 7 allele differences) were observed: cluster 1 belonged to ST42 (clade 1), cluster 2 to ST58 (clade 1) and clusters 3 and 4 to ST11 (clade 5). In three clusters, there were isolates that were obtained from different mussel farms, and in two clusters isolates from the same farm but collected several months apart.

The results showed that the population of *C. difficile* in Slovenian mussel farms is genetically diverse. As mussels are an indicator of environmental pollution, the diversity of strains in the sea was expected. On the other hand, the presence of genetically closely related *C. difficile* strains in all three mussel farms over a longer period of time indicates the persistence or continuous introduction of certain strains into the sea. As the mussels can be a source of bacteria causing infection in humans, monitoring of *C. difficile* contamination of mussels and comparison with clinical isolates from humans is crucial.



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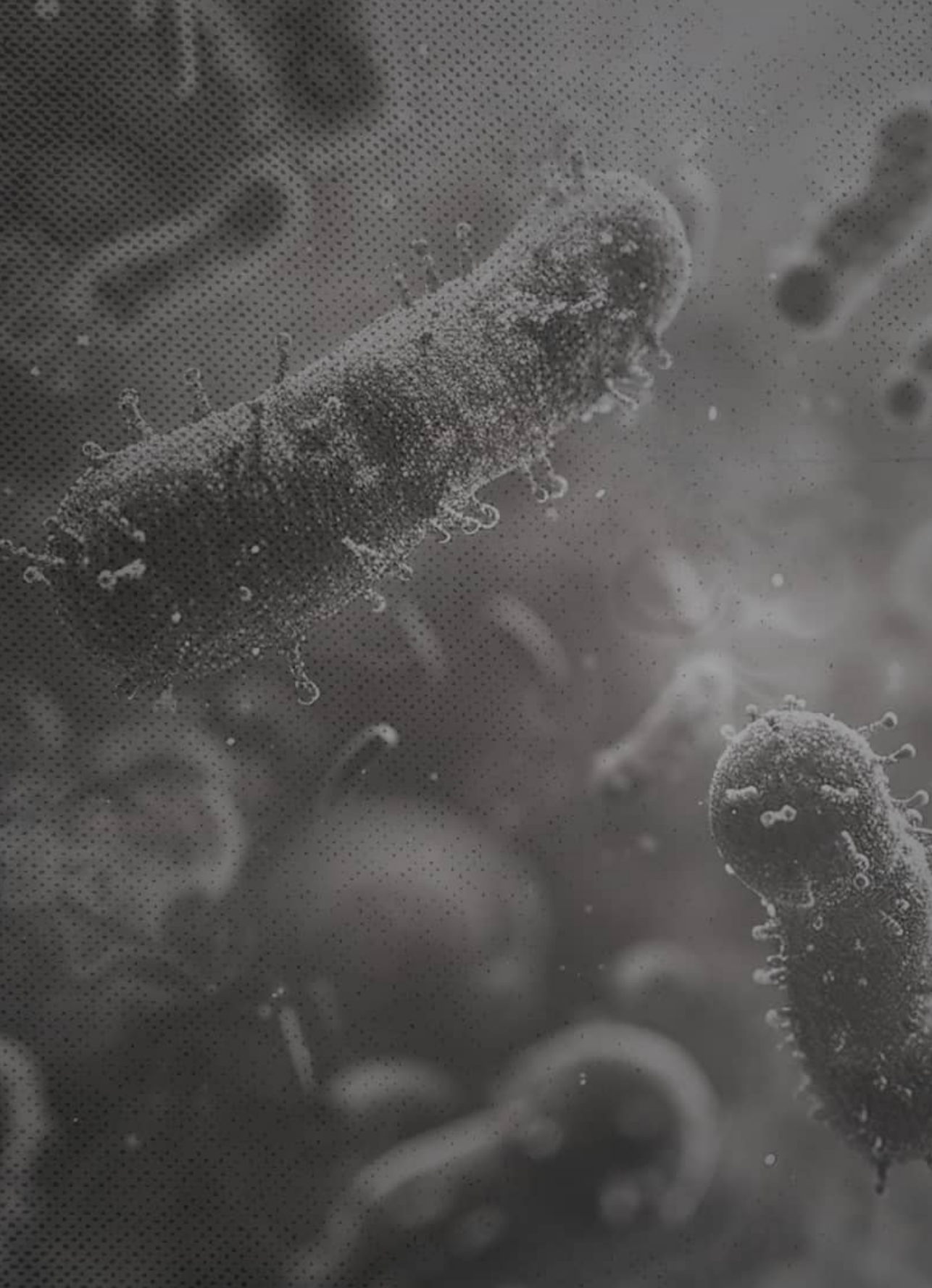
POSTERS OVERVIEW



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P2	Skinner, Andrew	MUTATION IN PENICILLIN BINDING PROTEIN 3 RESULT IN INCREASED CEPHALOSPORIN MINIMUM INHIBITORY CONCENTRATION FOR CLOSTRIDIOIDES DIFFICILE
P3	Barbut, Frédéric	SIMULTANEOUS EVALUATION OF 12 IMMUNO-ENZYMATIC ASSAYS FOR THE DETECTION OF GLUTAMATE DEHYDROGENASE (GDH) AND TOXINS OF CLOSTRIDIOIDES DIFFICILE.
P4	Barbut, Frédéric	PHENOTYPIC AND GENOMIC CHARACTERIZATION OF CLOSTRIDIOIDES DIFFICILE STRAINS INVOLVED IN MULTIPLE RECURRENCES.
P5	Kobeissy, Philippe Hussein	MICRORNA MIR-27A-5P REDUCES INTESTINAL INFLAMMATION INDUCED BY CLOSTRIDIOIDES DIFFICILE FLAGELLA BY REGULATING THE NF-κB SIGNALING PATHWAY
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P7	Le Monnier, Alban	SEROPREVALENCE OF ANTIBODIES TO CLOSTRIDIOIDES DIFFICILE TOXINS. PRELIMINARY RESULTS OF THE PREVADIFF STUDY
P8	Le Monnier, Alban	IMPACT OF ANTIBIOTICS ON THE DYNAMICS OF DIGESTIVE COLONIZATION BY CLOSTRIDIOIDES DIFFICILE AND ON THE ADAPTIVE IMMUNE RESPONSE
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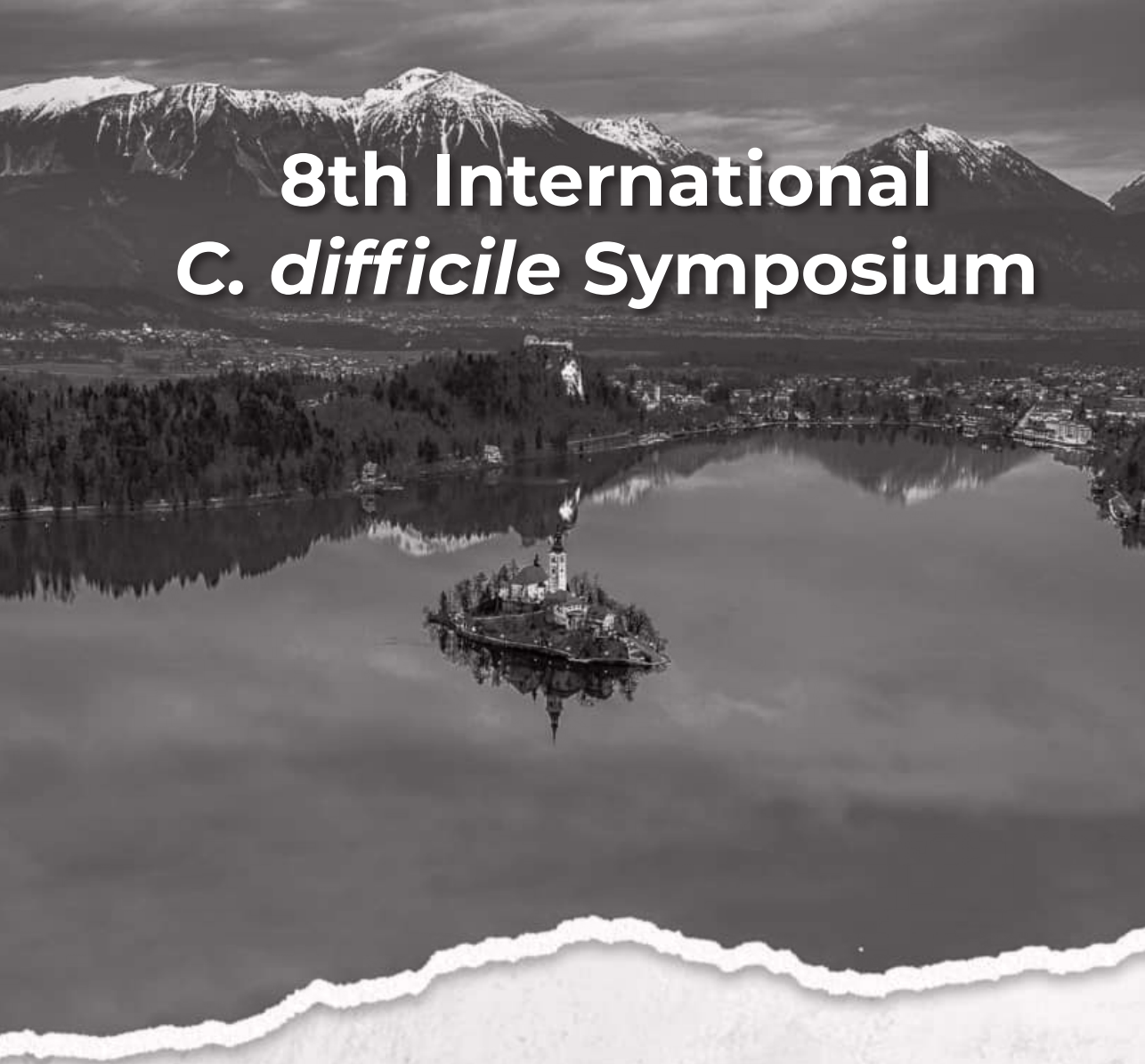

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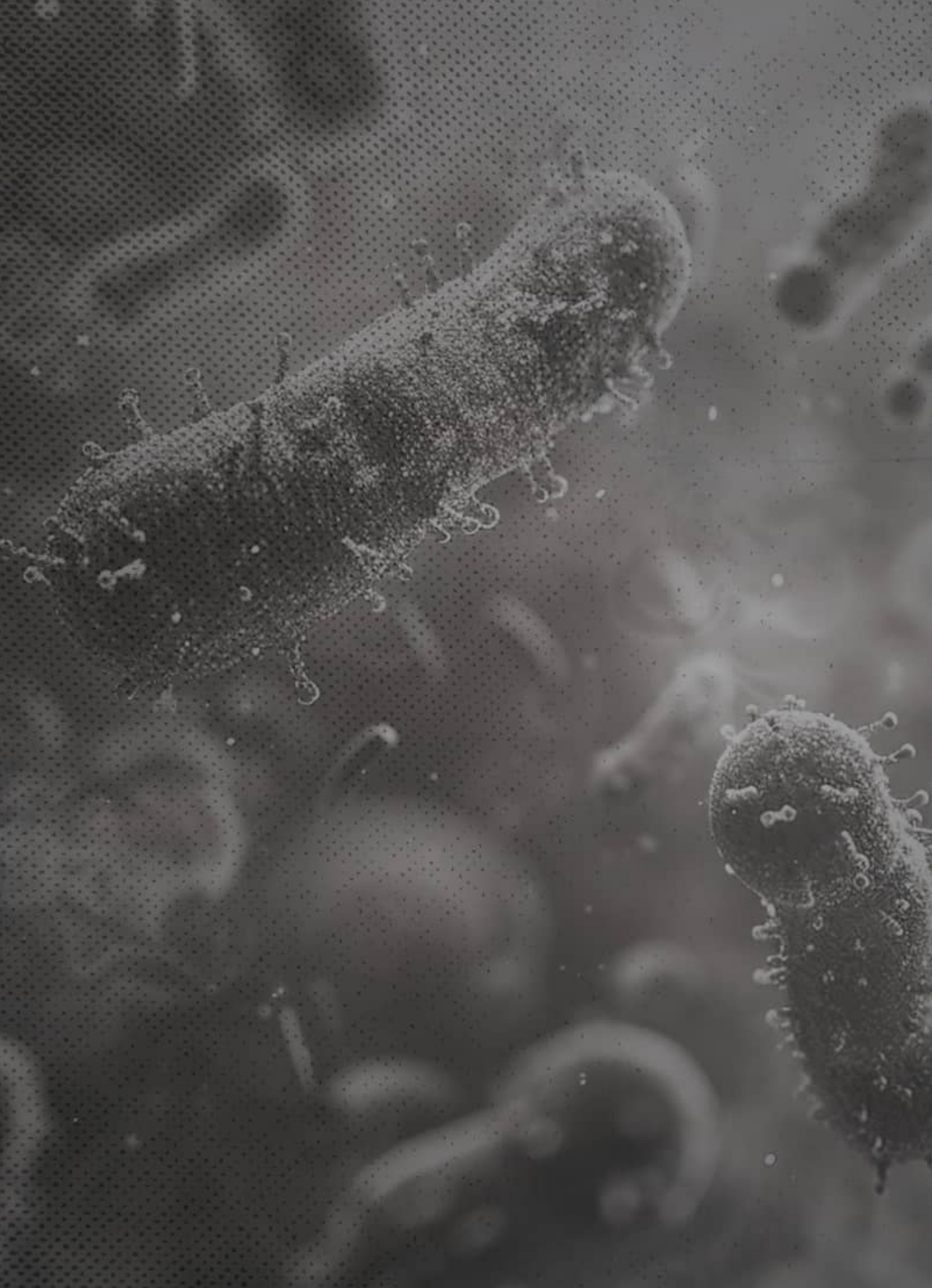
ABSTRACTS OF POSTER PRESENTATIONS



P1

THE L,D-TRANSPEPTIDATION PATHWAY IS INHIBITED BY ANTIBIOTICS OF THE β-LACTAM CLASS IN *CLOSTRIDIOIDES DIFFICILE*

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The resistance of the human enteropathogen *Clostridioides difficile* to the large group of β-lactam antibiotic cephalosporins is a leading contributor to the development of *C. difficile* infections. β-lactams are broad-spectrum antibiotics that target the cell wall peptidoglycan (PG) assembly. *C. difficile* has an original PG structure with a predominance of PG cross-links of the 3→3 type generated by L,D-transpeptidases (LDTs) that are insensitive to cephalosporins. This is a distinctive feature from most bacteria in which the peptide cross-links are primarily of the 4→3 type, created by D,D-transpeptidases belonging to the family of penicillin-binding proteins (PBPs) and that are the primary target of β-lactams. *C. difficile* is a sporulating bacterium and we show here that the spore cortex PG contains exclusively 3→3 cross-links. *C. difficile* encodes three predicted LDTS with an YkuD domain but a strain lacking the corresponding enzymes displayed only minor changes in the PG of the vegetative cells and no change in the spore cortex PG, revealing the presence of a new family of LDTS important for the polymerisation of both the PG of cells and spores. Growth of *C. difficile* cells in the presence of cephalosporins or carbapenems, resulted in drastic PG structure remodeling revealing the indirect inhibition of LDT activity due to reduced substrate availability. The decrease of 3→3 cross-links was balanced by an increase of 4→3 cross-links, resulting in an unmodified cross-linking degree. These data imply that cephalosporin resistance is not primarily mediated by LDTs in *C. difficile* and highlight the role of cephalosporin-resistant PBPs in PG biosynthesis. Altogether, our findings illustrate the plasticity of the PG biosynthesis machinery in *C. difficile*.

P2

MUTATION IN PENICILLIN BINDING PROTEIN 3 RESULT IN INCREASED CEPHALOSPORIN MINIMUM INHIBITORY CONCENTRATION FOR *CLOSTRIDIOIDES DIFFICILE*

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Background and Aims: Cephalosporins are a common cause of *Clostridioides difficile* infections (CDI). However, despite this increased risk for precipitating a CDI, there is a paucity of data exploring the mechanisms which mediate cephalosporin resistance in *C. difficile*. We sought to explore the genomic differences within the *C. difficile* penicillin binding proteins (PBP) of clinically relevant *C. difficile* in association with cephalosporin susceptibility.

Methods: We determined the minimum inhibitory concentration (MIC) of cefazolin, ceftriaxone, and cefepime by agar gel dilution for 75 clinically relevant *C. difficile* isolates that had previously been identified as REA group BI (n=15) [BI/RT027] or REA group Y (n=60) [Y/RT014/020] collected from 1984 – 2015. We subsequently performed whole genomic sequencing and multilocus sequence typing (MLST) evaluating for mutations within PBP 1,2,3 and 4 which could confer an increase in the respective cephalosporin MICs. Wilcoxon rank sum was used to compare isolates with and without PBP mutations.

Results: All 15 BI/RT027 isolates were identified as ST1. Among Y/RT014/020 isolates, STs 2, 14, and 110 accounted for 53% (32/60), 22% (13/60), and 12% (7/60), respectively. BI/RT027 isolates had no notable mutations in PBPs 1,2, and 4, but a valine to leucine mutation at position 497 (V497L) was noted in PBP3 in 73.3% (11/15) of the BI/RT027 isolates. The V497L mutation was detected in 16.7% (10/60) of Y/RT014/020 isolates. When compared to isolates that lacked the PBP3 V497L mutation, all isolates with the mutation had a higher cefazolin MIC (38.05 µg/ml vs. 18.08 µg/ml, $p<0.01$), ceftriaxone MIC (35.51 µg/ml vs 25.06 µg/ml, $p<0.01$) and cefepime MIC (187.40 µg/ml vs 135.15 µg/ml, $p<0.01$). BI/RT027 isolates with a V497L mutation had a higher cefazolin MIC when compared to Y/RT014/020 isolates with the mutation (56.42 µg/ml vs 25.99 µg/ml, $p<0.01$) but this difference was not present for ceftriaxone or cefepime.

Conclusions: Cephalosporin resistance in *C. difficile* is poorly understood and there is variable susceptibility between the cephalosporin generations. A PBP3 V497L mutation results in an increased 1st, 3rd, and 4th generation cephalosporin MIC. However, further study is required to determine why this varies between different strain groups as there could be a non-PBP3 mediated mechanism contributing to resistance.

P3

SIMULTANEOUS EVALUATION OF 12 IMMUNO-ENZYMATIC ASSAYS FOR THE DETECTION OF GLUTAMATE DEHYDROGENASE (GDH) AND TOXINS OF *CLOSTRIDIOIDES DIFFICILE*

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Background: *Clostridioides difficile* is the major cause of healthcare-associated diarrhea. Guidelines for the diagnosis of *C. difficile* infections (CDI) have been updated in 2016 by the ESCMID and recommend the use of a two-step algorithm and the detection of free toxins in stools. The objectives of this study were to assess the performances (sensitivity and specificity) of (i) 6 immuno-enzymatic assays (Techlab Quik Chek [Sobioda], Immunocard [Launch Diagnostics], JusCheck [Elitech Microbio], Certest [Theradiag], Monlab [Keyoflab], GDH sign [Servibio]) for the detection of GDH alone, and ii) 6 immuno-enzymatic assays for the combined detection of GDH and toxins (same companies).

Methods: 99 stool samples stored at -80°C were used for this evaluation: 84 were positive in culture on selective media (ChromId, Biomérieux) including 47 stools positive for free toxins (stool cytotoxicity assay on MRC-5 cell culture), and 15 were negative for both culture and cytotoxicity assay. The twelve tests were performed simultaneously according to manufacturer's instructions. Interpretation of the tests was done by two independent technicians.

Results: The sensitivity and specificity of each test are described in the table below

	GDH assay			Combined assay for GDH and Toxin				
	GDH			GDH		Toxins A or B		
	Sensitivity	Specificity	Invalid	Sensitivity	Specificity	Sensitivity	Specificity	Invalid
Quik Chek	99%	100%	2%	95%	100%	81%	100%	0%
Immunocard	96%	93%	2%	90%	100%	50%	100%	0%
JusCheck	79%	100%	0%	82%	100%	19%	100%	0%
Certest	95%	100%	0%	95%	100%	67%	100%	0%
Monlab	96%	100%	0%	96%	100%	71%	96%	0%
Servibio	93%	100%	0%	95%	100%	73%	98%	0%

Conclusion: The different assays under evaluation displayed a high sensitivity and specificity for detecting GDH. However, detection of toxins is highly variable across the different assays. In our hands, Quik Chek had the best performances whereas JusCheck lacks sensitivity for toxin detection.

P4

PHENOTYPIC AND GENOMIC CHARACTERIZATION OF *CLOSTRIDIOIDES DIFFICILE* STRAINS INVOLVED IN MULTIPLE RECURRENCES

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Background: Multiples relapses of *Clostridioides difficile* infection (CDI) are frequent, could impact patient's quality of life and are difficult to treat. They are linked either to patient's condition (immunity, intestinal dysbiosis), or potentially to the strain's characteristics.

Aim: To determine the genomic and phenotypic characteristics of *C. difficile* strains that have caused recurrent CDIs (rCDI).

Materials and methods: Between 2019 and 2022, 10 CD strains responsible for relapse were selected and matched on their PCR-ribotype to 10 CD control strains. The 20 genomes were sequenced and analysed (resistome, virulome, mobile genetic elements). The phenotypic characteristics (stress resistance (H2O2), motility, hydrophobicity, sporulation, germination, biofilm, susceptibility to bile acids) of relapse strains and controls were compared.

Results: We did not show any significant genomic or phenotypic difference between the strains involved in multiple relapses and control strains, apart from the susceptibility to deoxycholate 0,03%, which was difficult to link to relapses of CDI. However, all these tests indicate an important phenotypic and genomic variability across the strains.

Conclusion: The origin of rCDI does not seem to be directly linked to characteristics of the CD strain, suggesting that these relapses are more related to host factors such as intestinal dysbiosis or patient's immune status.

P5

MICRORNA MIR-27A-5P REDUCES INTESTINAL INFLAMMATION INDUCED BY *CLOSTRIDIOIDES DIFFICILE* FLAGELLA BY REGULATING THE NF-κB SIGNALING PATHWAY

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Background: *Clostridioides difficile* is a major cause of nosocomial post-antibiotic infections, often resulting in severe inflammation and watery diarrhea. Previous studies have highlighted the role of *C. difficile* flagellin FliC in activating the TLR5 receptor and triggering NF-κB cell signaling, leading to the release of pro-inflammatory cytokines. However, the microRNAs (miRNAs) mediated regulatory mechanisms underlying the FliC-induced inflammatory response remain unclear. This study aims to identify specific miRNA involved in modulating the inflammatory response induced by *C. difficile* flagellin and to elucidate its role in this process.

Methods: miRNA expression levels were analyzed in Caco-2 intestinal epithelial cells following FliC stimulation, infection with the epidemic *C. difficile* R20291 strain, or its unflagellated mutant by RT-qPCR. Chemical inhibitors were used to block NF-κB signaling, and their impact on miR-27a-5p expression was assessed. Knockdown and overexpression experiments with miRNA inhibitor and mimic were conducted to elucidate miR-27a-5p's functional role in FliC-induced inflammatory responses. Additionally, a mouse model of *C. difficile* infection was treated with miR-27a-5p to evaluate its therapeutic potential in vivo.

Results: miR-27a-5p showed significant FliC-dependent overexpression in Caco-2 cells. Inhibition of NF-κB signaling suppressed miR-27a-5p overexpression. Knockdown of miR-27a-5p increased NF-κB activation and TNF-α and IL-8 cytokine production, while its overexpression had the opposite effect. Moreover, miR-27a-5p was overexpressed in the caeca of *C. difficile*-infected mice, correlating with intestinal IL-8 levels. Treatment of infected mice with miR-27a-5p mimic reduced disease severity and intestinal inflammation.

Conclusion: miR-27a-5p plays a crucial role in regulating *C. difficile*-induced inflammation, suggesting its potential as a therapeutic target for controlling severe infection. These findings offer valuable insights into potential therapeutic strategies for managing *C. difficile* infection and associated inflammatory complications.

P6

PERCEPTION OF THE LEBANESE POPULATION TOWARDS FECAL MICROBIOTA TRANSPLANTATION

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Background and Aims: *Clostridioides difficile* infection (CDI) poses significant challenges, often involving severe intestinal inflammation and disruption of the gut microbiota. Fecal Microbiota Transplantation (FMT) has emerged as a potential therapy to replenish the microbiome and alleviate symptoms associated with CDI as well as conditions such as Inflammatory bowel disease (IBD). This study aims to investigate the perception of FMT among Lebanese individuals, including the general public, patients, and medical professionals, to gain insights into its acceptance and potential implementation in clinical practice.

Methods: A structured questionnaire is utilized to collect data from the general population, patients diagnosed with IBD or CDI, and medical professionals. The questionnaire covered various aspects, including sociodemographic characteristics, attitudes, concerns, and willingness to consider FMT as a treatment option. Data analysis involved assessing participant awareness, perceptions, and identifying key factors influencing their views on FMT.

Results: The study gathered data from 800 out of 1000 participants, resulting in an 80% response rate. Among the respondents, 20% were physicians, 15% were patients diagnosed with CDI or IBD, and 65% represented the general population. Analysis of the responses revealed a generally positive perception of FMT, with the majority considering it a viable treatment option. However, concerns were raised regarding the risk of infectious disease transmission, noted by 30.2% of respondents, along with apprehension about the process's perceived unpleasantness, mentioned by 25.2%. Interestingly, 62.3% of physicians expressed willingness to recommend FMT to patients if it demonstrated a remission rate exceeding 75%. The overall positive perception toward FMT across categories was 65.1% at the end of the survey.

Conclusions: This study provides valuable insights into the perception of FMT among Lebanese individuals, highlighting both opportunities and challenges associated with its implementation. Understanding public, patient, and healthcare professional perspectives is essential for the successful integration of FMT into clinical practice, potentially offering novel therapeutic options for individuals affected by CDI and IBD.

P7

SEROPREVALENCE OF ANTIBODIES TO *CLOSTRIDIOIDES DIFFICILE* TOXINS. PRELIMINARY RESULTS OF THE PREVADIFF STUDY.

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Background and Aims: Data on the seroprevalence of antibodies to *Clostridioides difficile* (Cd) surface proteins and toxins are scarce. In 1983, Viscidi et al. were the first to show that antibodies to Cd toxins A and B were widely detected in adult population (60-70%). However, these results were only qualitative and did not reveal inter-individual variations. Several studies have since shown that not all antibodies detected have a neutralizing capacity on the action of toxins and were therefore not all protective.

The aim of this study was to determine seroprevalence of antibodies directed to both TcdA and TcdB in a large population of adults and to stratify it according to age group, gender, and the main risk factors for *Clostridioides difficile* infection. This study also aims characterize the antibodies detected and in particular their neutralizing capacity and identify categories of patients at greater risk of not developing protective antibodies.

Methods: PREVADIFF is a prospective, multicenter study conducted in three regions of France. All adult patients (≥ 18 years) hospitalized in selected short-stay and long-term care wards for each of the five hospitals were eligible to be included in the study. Moreover, volunteers from the Blood Donors Bank (EFS) of the same three regions were also included for the community section of this study. Sera and clinico-demographic data were collected from hospitalized patients and volunteers. IgG concentrations against TcdA and TcdB were determined by quantitative ELISA.

Results: To date all the community section was enrolled and a total of 642 patients were included. We presented the preliminary data from the analysis of the 191 first cases.
16/35 (45.7%) of hospitalized patients and 88/156 (56.4%) of EFS donors had mean anti-TcdA IgG concentrations above five times the detection limit, with significant inter-individual variability at 0.7 mg/L (IQR: 0.4-1) and 1.2 mg/l (IQR: 0.5-1.5), respectively.

Conclusions: These preliminary results, based on the analysis of the first samples from one center and on antibodies directed against TcdA, show significant seroprevalence in both the hospitalized population and in the healthy volunteers of blood donors. These initial results confirm those already published and suggest that we are regularly exposed to *Clostridioides difficile* throughout life and in particular to its toxins.

P8

IMPACT OF ANTIBIOTICS ON THE DYNAMICS OF DIGESTIVE COLONIZATION BY *CLOSTRIDIOIDES DIFFICILE* AND ON THE ADAPTIVE IMMUNE RESPONSE

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Background and Aims: The current standard treatment for the first episode of *Clostridioides difficile* infection (CDI) involves the use of fidaxomicin (FDX) or vancomycin (VAN), and metronidazole (MTZ) in some case. However, some patients experience recurrence of CDI suggesting that these treatments do not achieve complete clearance of *C. difficile* (Cd). We studied the respective impact of these antibiotics on the dynamics of digestive Cd clearance and the modulation of the host's adaptive immune response which plays a central role in the evolution of CDI.

Methods: C57BL/6 mice were infected with Cd strain 630Δerm after antibiotic-induced dysbiosis. 48 hours after infection, mice were exposed two times a day with VAN, FDX, or MTZ for only two consecutive days. Mice were monitored for 28 days then re-infected with the same strain after a new dysbiosis. Fecal samples were collected at selected time points to follow-up the kinetics of Cd colonization and clearance. To assess the adaptive immune response, a quantitative ELISA was performed to measure the levels of IgG and IgA against TcdA and TcdB in both serum, and cecal contents.

Results: Short exposition to VAN and FDX led to a rapid clearance of Cd, then an early recolonization after discontinuation of VAN and FDX therapy associated to new signs of CDI. In contrast, MTZ had less to no effect on early rate of colonization and time to clearance was similar to that observed in the control group not exposed to antibiotic. In mice exposed to VAN and FDX, Cd persistence in the digestive tract was prolonged compared to the control group and appeared to shorten and enhance the development of an adaptive immune response.

Upon reinfection, no or less clinical signs of CDI were observed despite important digestive colonization. Our findings suggest that antibodies produced after the first infection neutralize the effects of the toxins and confer protection against reinfection. Furthermore, we demonstrated in a multiple reinfection model, that prolonged and repeated contact with *C. difficile* led to higher and earlier serum IgG anti-toxins.

Conclusions: In a mouse model of CDI, we showed that short exposure to antibiotics disrupts the dynamics of digestive colonization, leading in some cases to early recurrence of CDI. These preliminary results highlight that prolonged and repeated contact with Cd optimized the immune response.

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DECIPHERING HOST INFLAMMATORY RESPSONSES IN *C. DIFFICILE* INFECTION: ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR

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Background and Aims: Although intensity of host inflammation is a better predictor of *Clostridioides difficile* infection (CDI) outcomes than pathogen burden, most current CDI therapies are pathogen-directed. While targeting *C. difficile* does control the bacterium, due to concurrent impact of antibiotics on gut microbiota, there is an increased risk of recurrent CDI. Thus, novel host-targeted, microbiota-sparing therapies are needed for CDI treatment. We have previously demonstrated a key role for a pro-inflammatory cytokine, Macrophage migration inhibitory factor (MIF), and a common genetic SNP in leptin receptor (LEPR) are important regulators of CDI pathogenesis and clinical outcomes. Blocking MIF before CDI reduced colonic damage and tissue neutrophils and improved survival. Thus, our data raise the possibility that targeting MIF has potential to reduce CDI severity. In this study, we examined the association between MIF and LEPR SNP and determined the timing of peak MIF production in CDI patients.

Methods: We utilized pre-clinical murine model of CDI and samples from CDI patients. We performed ELISA assays on plasma samples of patients and mice, and spectral flow cytometry on colonic tissue of infected mice.

Results: In CDI patients, we found highest circulating MIF on days -2 and -1 from diagnosis (i.e., during CDI incubation period) with a subsequent decline in the days after diagnosis. Individuals with mutant LEPR SNP had significantly higher plasma MIF compared to those with WT LEPR. During a similar timeframe (i.e., on days 1, 2, and 3 after CDI), we found intracellular MIF in both immune and non-immune cells of colonic tissue from infected mice. Among these, the highest percentage of MIF was in cells of innate immune system on day 2 after infection, whereas in intestinal epithelial cells (IECs), intracellular MIF peaked on day 3 after CDI. The intensity of MIF expression was more in immune cells (i.e., eosinophils, neutrophils and macrophages), compared to IECs.

Conclusion: Our data provide novel insights into MIF kinetics during CDI and reveal that immune cells are a key producer of MIF during the early phase of infection. Further these findings suggest that early phase of CDI is the most appropriate window of opportunity, where anti-MIF intervention (potentially in combination with host SNP type), could be most impactful.

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CLOSTRIDIOIDES DIFFICILE SPORES TOLERATE DISINFECTION WITH SODIUM HYPOCHLORITE DISINFECTANT AND REMAIN VIABLE WITHIN SURGICAL SCRUBS AND GOWN FABRICS

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Clostridioides difficile is the most common cause of antibiotic-associated diarrhoea globally. Its spores have been implicated in the prevalence of *C. difficile* infection due to their resistance and transmission ability between surfaces. Currently, disinfectants such as chlorine-releasing agents (CRAs) and hydrogen peroxide are used to decontaminate and reduce the incidence of infections in clinical environments. Our previous research demonstrated the ability of *C. difficile* spores to survive exposure to recommended concentrations of sodium dichloroisocyanurate in liquid form and within personal protective fabrics such as surgical gowns; however, the present study examined the spore response to clinical in-use concentrations of sodium hypochlorite. Spores were exposed to a 10 min contact time of 1000, 5000 and 10 000 p.p.m. sodium hypochlorite, and spore recovery was determined. To understand whether biocide-exposed spores transmitted across clinical surfaces in vitro, biocide-exposed spores were spiked onto surgical scrubs and patient gowns and recovery was determined by a plate transfer assay. Scanning electron microscopy was used to establish if there were any morphological changes to the outer spore coat. The results revealed that viable biocide-exposed *C. difficile* spores can be recovered from surgical scrubs and patient gowns, with no observable changes to spore morphology, highlighting the potential of these fabrics as vectors of spore transmission. This study demonstrates that alternative strategies should be urgently sought to disinfect *C. difficile* spores to break the chain of transmission in clinical environments.

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IS *CLOSTRIDIOIDES (CLOSTRIDIUM) DIFFICILE* INFECTION (CDI) A THREAT TO AUSTRALIA'S BIOSECURITY?

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Background: *C. difficile* is recognised increasingly as a cause of serious gastrointestinal infection in human and non-human animals throughout the world. Human disease, once thought exclusively a hospital problem, is now occurring commonly in the community, at the same time as infections in animals, particularly production animals, are increasing. The value of meat produced from Australian livestock (cattle and calves, sheep and lambs, pigs, and poultry) in the last year was approximately AUD 6 billion. Apart from damage to the Australian livestock industry per se, contaminated manure from production animals may be driving human community infection.

Aims: The overarching aim of this project is to determine whether CDI is a risk to Australia's biosecurity? To answer this question requires:

1) Determining the current level of understanding of CDI amongst stakeholders and the community, ie: agriculture, food production, veterinary.
2) Developing a sampling model to find *C. difficile* in key environments, ie: agriculture, food production, veterinary.
3) Evaluating techniques for finding *C. difficile* in animals to determine common modes of transmission.
4) Creating a deeper understanding of the potential for *C. difficile* to be a risk to Australia's veterinary and agriculture biosecurity.
5) Developing biosecurity risk mitigation methods/models to reduce the threat to Australia and other countries.

Methods:

1) Survey of awareness of *C. difficile* in human, animal, plant and food environments.
2) Sampling of food production facilities to detect *C. difficile.*
3) Sampling of locally-produced agricultural products for *C. difficile.*
4) Whole genome sequencing to compare human strains of *C. difficile* with strains from food/agriculture and look for evidence of transmission.
5) Developing interventions to reduce/eliminate *C. difficile* in various populations.

Results: Ongoing.

Conclusion: The aims and data compiled from this research will provide a deeper understanding of the potential for *C. difficile* to be a risk to Australia's veterinary and agriculture biosecurity. The results will lead to a better understanding of epidemiology of CDI in the community and help develop mitigation strategies to reduce infections, and the health and economic effects on Australia.

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EFFICACY OF UV-C DEVICES IN *CLOSTRIDIOIDES DIFFICILE* ENVIRONMENTAL DECONTAMINATION, A REAL-LIFE PROSPECTIVE STUDY

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Background and Aim: *Clostridioides difficile* (*C. difficile*) is the most widespread healthcare-associated infection in the USA. Effective room decontamination to eliminate resilient *C. difficile* spores is vital for preventing this infection—Ultraviolet C (UV-C) devices are increasingly incorporated into hospital cleaning protocols to address this issue. However, evidence regarding the efficacy of these devices in reducing *C. difficile* spores remains inadequate, particularly in real-world environmental studies. This study aims to evaluate the effectiveness of UV-C devices in reducing environmental contamination of *C. difficile* in rooms previously occupied by *C. difficile* symptomatic patients and asymptomatic carriers.

Methods: We conducted an interventional prospective study, sampling ten "high-touch" locations within 33 rooms in internal medicine wards, occupied by patients with either symptomatic *C. difficile*-infection (n=5) or asymptomatic carriers (n=28), before terminal cleaning. Samples were obtained using environmental sponge wipes before and after UV-C disinfection, these were cultured, and the number of colony-forming units (CFU) was measured from each location. Contamination levels were evaluated using a Scale of zero to four, ranging from clean to heavy contamination, accounting for both the number of affected areas and the CFU counts found in each room.

Results: Before the application of the UVC device 9/33 (27 %) rooms were considered heavily contaminated and 4/33 (12%) had medium contamination. After the use of the UV-C device, the proportion of rooms that were heavily contaminated was 7/33 (21%) and 6/33 (18%) had medium levels of contamination (p=0.464). The average scale score of the rooms before cleaning was (1.94±1.56) and it was reduced to (1.58±1.64) after the use of a UV-C device (p=0.08). Additionally, when assessed separately, no substantial reduction was observed in CFU counts (before 29.61±61.07 after 17.97±42.536 p=0.1) or the number of contaminated sites (before 2.24±2.264 after 1.91±2.185 p=0.07).

Conclusions: Our findings indicate that UV-C devices offer, at best, only a limited additional benefit over standard cleaning protocols. Further cost-benefit analysis is required before widespread adoption in hospital settings can be considered.

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MODELING OF HEALTHCARE-ASSOCIATED *C. DIFFICILE* INFECTION AND QUANTIFICATION OF ACQUISITION ROUTES IN ONCOLOGICAL UNITS

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Background and Aims: Leukemic and hematopoietic cell transplant patients are severely immunocompromised, have near ubiquitous antimicrobial exposures, and are hospitalized for prolonged periods of time. For those reasons, this population has one of the highest incidences of *C. difficile* infection (CDI). There are four routes to new CDI cases: importation of asymptomatic carriers, who subsequently progress to CDI, importation of symptomatic patients, or in-ward transmission from asymptomatic carriers or symptomatic CDI patients. We aimed to quantify the relative contribution of these routes to new colorizations and CDI cases in oncological units.

Methods: We developed and parameterized a stochastic CDI transmission network model using data from a prospective cohort study of patients admitted to two oncological units. Stool/rectal swabs were collected upon admission and weekly until discharge for *C. difficile* culture and nucleic acid amplification test (NAAT). Toxin enzyme immunoassay was used to diagnose CDI as clinically indicated. Data on healthcare workers' room assignments was used to characterize the network connectivity across rooms. The sensitivity (Se) and specificity (Sp) of *C. difficile* culture test and NAAT for asymptomatic carriers were estimated using the Bayesian Hui-Walter method. We use particle-filtering methods to infer the unknown parameters (colonization rate, incubation period, infectious period, and effectiveness of room cleaning) from the patient testing data.

Results: Stool culture had higher Se and Sp than NAAT to detect shedding. Se for culture was 0.84 (0.66,0.99), and for NAAT, it was 0.76 (0.56,0.99). The estimated median for the incubation period from time to colonization to clinical disease was 4 days. The estimated median for the shedding duration of asymptomatically colonized and CDI patients was 2.7 days and 3 days, respectively. Asymptomatic carriers contributed more to new colorizations than CDI patients.

Conclusions: Asymptomatic carriers were identified as an important source of CDI through transmission and disease progression.

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ISOLATION AND CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* IN CATTLE BY TOXIGENIC CULTURE AND PCR ASSAY IN IRAN

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Clostridium difficile is an anaerobic, spore-forming, rod-shaped bacterium causes severe colitis in people and is a significant enteric pathogen in many species of animals, including swine, horses, and potentially cattle. The aim of this study was to isolate Clostridium difficile from cattle feces and identify it by molecular and toxigenic culture assay.

The diagnosis of C difficile diarrhea is suggested by a history of recent treatment with antimicrobials and is supported by the detection of the presence of C difficile toxin A and/or B in a freshly passed or frozen fecal sample submitted to a laboratory using toxigenic culture involving the isolation of the bacterium from faeces and further in vitro demonstration of toxins with a cytotoxicity test. The toxin gene was identified by PCR ribotyping. Sampling was carried out between September 2023 and March 2024, and a total of 58 fecal samples from cattle were collected.

Clostridium difficile was isolated by microbial culture techniques and in the following stage, The culture method involved heat shock treatment (in water at 60C), after which samples were inoculated onto selective media, Clostridium difficile Selective Medium (Oxoid PB5054A; Oxoid, Basingstoke, UK) and Brazier's Clostridium difficile selective agar (Oxoid PB5191A). Plates were incubated anaerobically in jars (Mart; Anoxomat) for 7 days at 37C. Enrichment of heat shock-treated samples was performed for 7 days (1 g in 9 mL BHI broth supplemented with cycloserine-cefoxitin.*C. difficile* was identified based on characteristic colony morphology (yellow, ground glass appearance) and odor (horse dung smell). The identity of doubtful isolates was confirmed by Gram stain and a latex agglutination test kit (Oxoid). Confirmation of *C. difficile* by detection of genes for toxin B, and binary toxin (cdtA) was performed according to a specific multiplex PCR.genomes of bacteria were isolated by the DNA extraction kit then tcdA, tcdB, cdtA and cdtB were identified by multiplex PCR method and in the final stage PFGE analysis was used to determine the ribotypes.

In the next stage, toxigenic culture was performed for isolated *C. difficile* . Vero cells were grown in a flask containing Dulbecco's modified Eagle's medium (DMEM; Gibco), 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (FBS Gibco), and incubated at 37°C and 5% CO2 for 3-5 days. The cells were trypsinized and counted. About 10,000 cells were added to the wells of the microtiter plate and were incubated at 37°C and 5% CO2 for 24 h to reach about 80% confluency.

C. difficile colonies were cultured in BHI broth for 5-7 days at 37°C, then the culture medium was centrifuged (10 min at 1500 g). The obtaining supernatants were filtered (0.22 µm pore size), and 200 µl of the filtrate was added to Vero cell culture. The microtiter plate was

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incubated for 24-48 h at 37°C and 5% CO2. *C. difficile* strains which produce toxin (positive result), cause cytopathic effects in more than 50% of the cell monolayer. Supernatant obtained from a toxigenic *C. difficile* strain, which was previously isolated from a diarrheal patient, was used as a positive control in the toxigenic culture test. Results: A total of 57 samples were collected from cattle, Out of those isolates 2 isolates, were toxigenic. The toxigenic isolates carried both tcdA, and tcdB (A+B+) the ribotypes of cattle isolate were (014 and ACD 010). PFGE analysis could not distinguish similar ribotypes/toxin types of toxigenic isolates. Both the toxigenic isolates had cytopathic effects on Vero cell monolayers at 1:100 dilutions of cell-free culture supernatants duration the time of inoculation. Conclusion: According to the results, the overall prevalence of clostridium difficile with toxigenic genes in cattle accounted for 3.50%. Although this amount seems negligible, this bacterium is one of the main reasons for calves' deaths, so it can cause financial damage to the agricultural industry. Furthermore, as this bacterium has detrimental effects, the healthcare system will be forced to pay more costs, so control and prevention measures should be taken into consideration.

P15

PROTEOLYTIC ACTIVITY OF SURFACE-EXPOSED HtrA DETERMINES ITS EXPRESSION LEVEL AND IS NEEDED TO SURVIVE ACIDIC CONDITIONS IN *CLOSTRIDIOIDES DIFFICILE*

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To survive in the host, pathogenic bacteria need to be able to react to the unfavourable conditions that they encounter, like low pH, elevated temperatures, antimicrobial peptides and many more. These conditions can lead to unfolding of envelope proteins and this may be lethal. One of the mechanisms through which bacteria are able to survive these conditions is through the protease/foldase activity of the high temperature requirement A (HtrA) protein. The gut pathogen *Clostridioides difficile* encodes one HtrA homolog that is predicted to contain a membrane anchor and a single PDZ domain. The function of HtrA in *C. difficile* is hitherto unknown but previous work has shown that an insertional mutant of htrA displayed elevated toxin levels, less sporulation and decreased binding to target cells.

Here, we characterize the molecular and biological details of HtrA in *C. difficile* strain 630Δerm. Using diverse molecular biology and proteomics tools, we show that HtrA is membrane associated and localized on the surface of *C. difficile.* Furthermore, we characterize the requirements for proteolytic activity of recombinant soluble HtrA.

In addition, we show that inactivation of HtrA proteolytic activity in the bacteria results in a dramatic elevation of HtrA and several other proteins of unknown function. Finally, we show that proteolytic activity of HtrA is required for survival under acidic conditions.

In conclusion, we show that *C. difficile* HtrA is a surface-exposed protease that is needed to survive acidic conditions and regulates its own level through its proteolytic activity. .

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CHARACTERIZATION OF REGULATORY NON-CODING RNAS IN THE PATHOGENICITY OF *CLOSTRIDIOIDES DIFFICILE* AND THEIR ROLE IN HOST-PATHOGEN INTERACTIONS

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Clostridioides difficile (CD) is an anaerobic spore-forming bacterium and the major cause of nosocomial infections associated with antibiotic therapy. Due to the intensification of severe forms of CD infections, alternative treatments are required. During infection, bacteria reprogram their gene expression in response to environmental constraints. In this context, combining studies of genome-wide in silico, high-throughput sequencing and Transcriptional start site (TSS) mapping in CD, allowed the identification of more than 250 of non-coding RNAs (ncRNAs), playing a crucial role in the regulation of adaptive responses and pathogenic processes in this bacterium. In particular, dual RNA-seq experiment performed by our team revealed several ncRNAs that were highly induced in-vivo in a mouse model of infection.

Two specific ncRNAs have been selected among the most expressed during infection. The general aim of this study is to decipher the direct contribution of ncRNAs in the CD pathogenicity and host-pathogen interactions. To characterize two selected ncRNAs, their expression under pathogenesis-relevant conditions and their roles in CD have been evaluated, followed by in silico and experimental analyses for the identification of the ncRNAs targets and their regulatory networks. Moreover, the roles of ncRNAs during infection in the host will be assessed. The achievement of finding of key players in RNA-dependent network controlling CD pathogenicity together with the characterization of original molecular mechanisms in CD will lead to the discovery of new markers of virulence and new promising therapeutic targets.

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SUSCEPTIBILITY TO PHAGE INFECTION IN *C. DIFFICILE* IS DRIVEN BY SPECIFIC INTERACTION BETWEEN THE S-LAYER PROTEIN SlpA AND PHAGE RECEPTOR BINDING PROTEINS

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The first line treatment for *Clostridioides difficile* (Cd) infections is antibiotherapy, which promotes microbiota dysbiosis and recurrence of the infections. Hence, targeted treatments with minimal impact on the microbiota are needed, like phage therapy. To develop an effective phage therapy, phages that are strictly lytic and that target a large panel of strains is required. Unfortunately, all Cd phages are temperate and most have a relatively narrow host range. Recent studies identified the S-layer protein SlpA as a major receptor used by many phages for infection, which opens the way to genetic engineering of phages to improve their host range. However, in-depth characterization of RBP-slpA interactions will be necessary to fully harness the potential of phage genetic engineering. To further investigate phage-SlpA interactions, we created various SlpA mutant and chimeric isoforms that we expressed in the FM2.5 slpA mutant strain. Our results show that the D2 domain of the low molecular weight fragment of SlpA is crucial for infection by certain phages but not for others. Notably, we observed that certain modified SlpA isoforms led to strong phage adsorption without infection, suggesting complex interactions between phage tails and the host cell surface. To gain further insight into phage binding, we identified the receptor binding protein (RBP) in our collection of phage genomes using in sillico predictions. Next, as a proof-of-concept, we used the endogenous CRISPR-Cas system of Cd and successfully exchanged the RBP gene in the conserved phi027 prophage with its homolog from phage ΦCD508. This recombinant phi027RBP508 phage now targets the same strains as ΦCD508, demonstrating that the host range can be modified through RBP engineering. Finally, we developed a functional assay to identify the gene encoding the CI repressor in phi027, which is predicted to participate to lysogeny control. Using CRISPR-Cas engineering, we successfully deleted the cI gene from the phi027 prophage. The resulting phi027ΔcI recombinant phage became strictly lytic and no lysogens were recovered after an overnight infection in broth culture. Altogether, these results show that it is possible to genetically engineer phages to make them strictly lytic and to modify their host range by engineering their tail proteins.

P18

THE IMPACT ANALYSIS OF THE NEWLY DISCOVERED PHAGE ΦCDKH02 ON THE VIRULENCE OF THE BACTERIAL HOST *CLOSTRIDIOIDES DIFFICILE*

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Background and Aims: Within the last decade relatively few bacteriophages infecting an important human pathogen *Clostridioides difficile* have been described with all of them characterized as temperate. In the course of our research, we have detected and isolated bacteriophage phiCDKH02 infecting a hyper-virulent clinical strain of *C. difficile* 500/12 with RT 176. Because the genome of the newly discovered phage contains genes encoding putative transcription factors and virulence-associated products, we wanted to investigate whether its presence in the bacterial genome as a prophage indeed influences the host's virulence.

Methods: We performed a curing of prophage from *C. difficile* host genome using the CRISPR technology. Subsequently, we conducted an in vitro analysis of the effect of the tested phage on the bacterial host, comparing the cytotoxicity effect on HT-29 cell line cultures treated with supernatants obtained from lysogenic and phage-free strains. As a measure of cytotoxic activity, we adopted the IC50 concentration of supernatants, which corresponds to 50% inhibition of the growth of the tested cell population.

Results: Cytotoxicity tests on the human colon adenocarcinoma cell line HT-29 showed that the supernatant obtained from the prophage-free strain exhibits cytotoxic activity two orders of magnitude lower compared to the supernatant from the lysogenic strain.

Conclusions: The presence of a prophage in the genome of the hypervirulent strain of *C. difficile* with RT 176 increases its virulence. At this stage of research, we are unable to determine whether it is the effect of transcriptional factors encoded by the prophage that can increase the production of toxins, or genes whose virulence-associated products can increase the cytotoxicity of *C. difficile* extracts.

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WHOLE GENOME SEQUENCING-BASED CHARACTERISATION OF *CLOSTRIDIOIDES DIFFICILE* INFECTION CASES IN UNIVERSITY HOSPITAL CENTRE ZAGREB

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Background and Aims: The European Centre for Disease Prevention and Control has been conducting surveillance on *Clostridioides difficile* infections (CDI) since 2016, with the latest report covering the 2018-2020 period. Molecular characterisation was performed and reported by ribotyping. We investigated the intra-hospital distribution of *C. difficile* strains by whole genome sequencing (WGS) isolates collected in 2022 at University Hospital Centre (UHC) Zagreb.

Methods: In total, 103 patients with the first episode of CDI in 2022 at UHC Zagreb were included in the study based on the screening stool antigen test for GDH (RidaQuick CD GDH; R-Biopharm AG, Germany), confirmed by Eazyplex *C. difficile* assay (Eazyplex CD assay; AmplexDiagnostics GmbH, Germany) specific for A, B and binary toxins. Demographic and clinical data were prospectively analysed from medical records. All samples included in the study were subjected to WGS analysis. Genetic clusters were formed from isolates with ≤6 allelic differences by core genome MLST.

Results: We identified six clusters, containing 2-59 isolates with 15 singletons. Most of the isolates belonged to ST3 (60%), followed by ST1 (26%), ST13 (3%), ST15 (2%), ST8 (2%), ST2 (2%), ST12 (2%), ST35 (1%), ST48 (1%) and ST110 (1%).

Conclusions: WGS analysis proved as a useful tool in identifying clusters of isolates connecting various patient wards with possible transmission routes in the hospital setting. It could be used to support local and national surveillance of CDI infections and their transmission pathways.

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DECIPHERING THE REGULATION MECHANISM OF A POTENTIAL ANTI-PHAGE SYSTEM IN *CLOSTRIDIOIDES DIFFICILE*

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Abortive infection (Abi) is a bacterial defense mechanism against bacteriophages, which induces bacterial cell death (or dormancy) before the infecting phage can complete its replication cycle, thus protecting the bacterial population. So far, no Abi system has been described in *Clostridioides difficile*, a human pathogen classified as a main cause of antibiotic-associated diarrhea. Recently, an abi-like gene has been identified within the phi027 prophage, conserved in most epidemic ribotype 027 strains. Using RNA sequencing and Northern blotting on the R20291 strain, our group revealed a non-coding RNA (ncRNA) called RCd22 in the intergenic region upstream of the abi gene. Our goal is to study the function and regulation of this predicted Abi system. In *C. difficile*, overexpression of the abi gene results in a slower growth in both liquid and solid medium. RT-qPCR experiments on a deletion mutant lacking RCd22 showed increased expression of the downstream abi gene, indicating a negative regulatory effect of RCd22 on abi gene expression. Moreover, transcriptional fusions with the alkaline phosphatase gene showed a decrease in reporter gene expression in the presence of RCd22. Interestingly, co-expression of RCd22 in cis but also in trans showed a restoration of normal growth in liquid and solid medium. Expression of variants carrying mutations in conserved motifs of RCd22 could not restore the normal growth, implying an important role of these motifs in the regulation of this system. A MS2-Affinity Purification technique coupled with mass spectrometry allowed us to identify in vivo the interaction between the Abi-like protein and the MS2-tagged RCd22, suggesting a mechanism of regulation similar to type III Toxin-Antitoxin systems where the ncRNA interacts with the toxin protein to inhibit its toxicity. The function of the Abi-like protein as a defense mechanism against phages, and its role in stress response or prophage maintenance are currently under investigation.

P21

EXPLORATION OF CORE GENOME PREDICTORS OF *C. DIFFICILE* INFECTION SEVERITY IN RT014/020

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Background and Aims: In The Netherlands, PCR ribotyping for *C. difficile* surveillance is being replaced by core genome multilocus sequence typing (cgMLST). The *C. difficile* infection (CDI) characteristics of many cgMLST sequence types are unknown, and currently inferred from their corresponding PCR ribotypes. We aimed to explore sequence types (STs) and their associated clinical characteristics within the highly prevalent ribotype014/020, and to identify cgMLST target alleles predictive of CDI severity.

Methods: Fifty-four isolates collected between 2017 and 2022 through Dutch sentinel surveillance were sequenced on the Illumina NovaSeq 6000platform. We selected samples of clinically mild (n=27) and severe (n=27) CDI cases in a 2:1 ratio of RT014:RT020. A phylogenetic tree was constructed to identify STs within RT014/020. Clinical characteristics of STs were assessed through Chi-square tests and logistic regression. A modified version of the Hogwash algorithm [Saund & Snitkin 2020, PMID 33206035] was used to explore the existence of alleles predicting CDI severity.

Results: Seven STs were identified within RT014/020 (ST2, ST13, ST14, ST49, ST110). ST2 contained both RT014 and RT020, with other STs being limited to a single RT. No significant relationship between STs and clinical severity was observed. Four genes (CD630_01980, CD630_11480, CD630_18250, CD630_21130) were associated with clinical severity.

Conclusions: Identifying alleles related to specific clinical outcomes may help to identify new bacterial pathogenicity markers, and can possibly replace exhaustive epidemiological analysis in assessing the risk of novel strains. Further investigation is required as our explorative study is limited by sample size and conversion of cgMLST allele numbers to binary allele numbers to fit the Hogwash algorithm, thereby limiting sensitivity.

P22

SURVEILLANCE OF *C. DIFFICILE* IN CANADIAN RETAIL MEAT FROM 2016 - 2018

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Background and Aims: *Clostridioides difficile* infections (CDI) continue to be a concern in Canada, with 28.0% of CDI reported in 2020 being identified as community-associated (CA). Previous Canadian studies have identified toxigenic *C. difficile* from retail meat, suggesting that it may be a source of exposure for CA-CDI. This study aims to identify the prevalence of *C. difficile* in Canadian retail pork and beef, and to compare these isolates to human CA-CDI within the Canadian farm-to-fork continuum.

Methods: Canadian retail pork (n = 208) and beef (n = 85) were purchased between 2016-2018 in select provinces through the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). Retail meat was rinsed in PBS, frozen, and sent to the National Microbiology Laboratory for *C. difficile* isolation. C .difficile isolates were characterized by antimicrobial susceptibility testing (AST) via Etest (Biomérieux), PCR-ribotyping, and whole genome sequencing. Illumina reads were assembled using Shovill and sequence types were determined using PubMLST. Human clinical CA-CDI isolates collected through the Canadian Nosocomial Infection Surveillance Program (CNISP) were used for comparison with retail meat isolates.

Results: *C. difficile* was isolated from 1.4% (3/208) of pork rinsates and was not isolated from beef rinsates (0/85). Two isolates (ST1/RT027, associated with NAP1; ST10/RT015, associated with NAP12) were from pork purchased in Western Canada in 2016 and Central Canada in 2018, respectively. The third isolate (ST8/RT002, associated with NAP6) was from retail pork purchased in Western Canada in 2018. All three retail pork isolates were susceptible to metronidazole, clindamycin, vancomycin, rifampin, moxifloxacin, and tigecycline, with the exception of ST8/RT002, which showed intermediate resistance to clindamycin. All three NAP/ST/RTs from retail pork have been identified in human clinical CA-CDI collected through CNISP.

Conclusions: Epidemic *C. difficile* strain types found in human clinical CA-CDI cases have been identified in retail meat samples collected in this study, suggesting that retail meat may be a source of exposure for CA-CDI in Canada. Additional sampling and genomic analysis are ongoing to further delineate relatedness and possible transmission across the Canadian farm-to-fork continuum.

P23

DISSECTING THE BIOSYNTHETIC PATHWAY OF THE FLAGELLIN TYPE A GLYCAN IN *CLOSTRIDIOIDES DIFFICILE* STRAIN 630ΔERM

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In *Clostridioides difficile* (*C. difficile*), flagellin, forming the filament of flagella, is modified with exceptional glycan structures. One of these, i.e. Type A, consists of an O-linked β-N-acetylglucosamine (GlcNAc), which is coupled to an N-methyl-L-threonine through a phosphodiester linkage at C-3. The Type A modification is attached to a number of threonine and serine residues of flagellin and is important for proper flagellar function (1). In *C. difficile* strain 630Δerm, a cluster of five genes (encoding CD0240-CD0244) is linked to the biosynthesis of Type A and a model for its biosynthesis was previously proposed (1). However, in this model no role for CD0244 was predicted and the individual biosynthetic steps, including intermediates, were poorly defined.

We recently performed quantitative proteomics experiments to analyze the Type A glycan structure, and variations thereof, in individual cd0241-cd0244 mutant strains. Contrary to previous data, we found an essential role for CD0244 for full Type A biosynthesis and based on these data, we have postulated a revised model for the Type A biosynthesis (2), including hypotheses about the activity of the enzymes involved. The aim of our current studies is to dissect the biosynthetic pathway of the Type A glycan in *C. difficile*.

Fundamental to the revised model is the prediction of a novel biosynthetic intermediate, i.e. CDP-threonine. We hypothesize that CD0244 is a phosphotransferase that can transfer phospho-threonine (pThr) from CDP-threonine to the GlcNAc, thereby releasing CMP. To test this hypothesis, we chemically synthesized the labile CDP-threonine using established workflows in the lab. Next, we incubated recombinant CD0244 (expressed in and purified from E. coli) with CDP-threonine and a synthetic GlcNAc-containing acceptor peptide, to reveal product formation using mass spectrometry. Based on these experiments, we could demonstrate that CD0244 possesses the predicted activity, thereby establishing it as an authentic CDP-threonine:GlcNAc threoninephosphotransferase.

In conclusion, we provide new groundbreaking insights in the biosynthetic pathway of the Type A glycan of *C. difficile*. Having established the activity of one of the key enzymes involved, i.e. CD0244, we aim to develop inhibitors to block its activity, e.g. by using CDP-threonine analogs.

1. A. Faulds-Pain et al, Mol Microbiol 94, 272-289 (2014)
2. B. Claushuis et al., ACS Infect Dis 9, 2665-2674 (2023)

P24

STRUCTURAL INSIGHT INTO THE BIOSYNTHESIS OF THE S-LAYER-ANCHORING POLYSACCHARIDE FROM *CLOSTRIDIOIDES DIFFICILE*

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Background and aims: The surface of *Clostridioides difficile* is covered with a 2D protein array known as the S-layer. S-layer is attached to the bacterial cell through non-covalent interaction with the secondary polysaccharides (PSII) that protrude from the peptidoglycan. Disruption of enzymes involved in the PSII biosynthesis leads to severe defects in cell growth and to alterations in peptidoglycan, PSII, S-layer and biofilm formation, and ultimately, virulence. Therefore, our objective was to validate these enzymes as novel targets for specific treatment of *C. difficile* infection.

Methods: We used X-ray crystallography to determine the structure of one representative of each major group of enzymes involved in the PSII biosynthesis: glycosyltransferases, mannose converting enzymes and enzymes anchoring the PSII to the peptidoglycan. Chemical synthesis was performed to obtain variations of predicted ligands (building blocks of PSII assembly). Co-crystallization and/or crystal soaking was used for determination of structure of complexes with the ligands. Molecular simulations were employed to investigate the enzymatic mechanisms of the enzymes and to aid in inhibitor design.

Results: All three structures (glycosyltransferase, alpha-D-phosphohexamutase, phosphotransferase) revealed domain organization similarities to their homologues and conserved cation/ligand binding regions. Structural analyses and effects of bound ligands on the flexible loops in the active site regions enabled us to identify the structural characteristics specific for PSII building block binding and assembly. Molecular simulations indicated important differences in predicted enzymatic activity mechanisms of some of the enzymes. In molecular docking experiments, potential inhibitors were identified.

Conclusion: Our results illuminate possible ways of specifically treating *C. difficile* infections, and are applicable to other Gram-positive bacteria that are in possession of homologous proteins.

P25

PHENOTYPIC CHARACTERIZATION OF *CLOSTRIDIOIDES DIFFICILE* PCR RIBOTYPE 046, REVEILING HIGH TOXIN PRODUCTION AND MULTI DRUG RESISTANCE AS VIRULENCE FACTORS

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Background and aims: An outbreak of *Clostridioides difficile* ribotype (RT) 046 at Högland Hospital, Sweden was associated with high mortality (1). We compared toxin production, sporulation capacity and multidrug resistance of RT 046 to other RTs in the outbreak.

Methods: For each RT (046, 001, 012 and 014), one isolate from a patient who died and one from a patient that survived was included. A RT 027 (NCTC13366) was included for comparison (not present in outbreak). Each *C. difficile* isolate was inoculated and cultured anaerobically in individual BHI broths. Sporulation was measured as colony forming units/ml after heating and counted in microscope before heating every 24h (0h-120h). *C. difficile* toxin A and B quantitative detection was done using ELISA (tgcBIOMICS GmbH, Bingen, Germany), every 24h (48h-120h). Antimicrobial susceptibility testing for vancomycin, metronidazole, clindamycin, moxifloxacin, and rifampicin was done using Etest™ (Bio-Merieux, Solna, Sweden).

Results: Maximum spore count was reached earlier in the microscope compared to colonies seen on plates after heating. Colonies developed from heat-resistant spores could be seen first after 48 h and reached a maximum after 96-120 h. The sporulation characteristics between different RTs did not differ. This indicates that spores seen early in the microscope not necessarily had completed sporulation while the number of CFU after heating represent spores that have completed both sporulation and germination. Both strains of RT 046 and RT 001, and RT 014mortal continually produced more toxin, especially toxin A. All strains, except for RT 027, produced more toxin A than toxin B. RT 027 continuously produced more toxin A and B compared to the other strains. No statistically significant differences in toxin production were found comparing strains from patients who survived and died. RT 046 and RT 012 were only susceptible to metronidazole and vancomycin, and RT027 was only susceptible to vancomycin.

Conclusions: *C. difficile* RT 046 produced the same proportion of spores compared to other outbreak strains but produced more toxin. RT 046 were multidrug-resistant, also seen in other outbreak strains (2, 3). High toxin production and multidrug-resistance could have contributed to the higher mortality of the outbreak.

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P26

DIFFERENTIAL *CLOSTRIDIOIDES DIFFICILE* TOXIN PRODUCTION AND SPORULATION IN REA GROUP Z CLOSELY RELATED TO THE HIGH TOXIN PRODUCING STRAIN, VPI10463

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Background: The pathogenicity of *Clostridioides difficile* is multifaceted and relies on sporulation and toxin production to both infect and produce symptoms in humans. VPI10463, recognized as restriction endonuclease analysis (REA) group Z3 (RT087/ST46), was first reported in 1980. The strain was notable for high toxin production in vitro but was rarely seen in historic clinical isolate collections. More recently, we identified several REA group Z isolates from contemporary clinical collections since 2008.

Methods: We compared VPI10463 to the more contemporary REA group Z isolates (n=4) by determining the in vitro toxin A/B production and sporulation capacity. For control, an isolate identified as REA group BI (RT027/ST1) and a non-toxigenic *C. difficile* strain were utilized. All REA group Z isolates underwent whole genomic sequencing (WGS) for multilocus sequence typing (MLST) and in silico analysis of key sporulation and toxin genes.

Results: In vitro sporulation assay revealed that VPI10463 had a 96-hour spore count of 0.11 x 106 CFU/ml. In comparison, 3 of the 4 contemporary REA group Z isolates had a median spore count of 140 x 106 CFU/ml (IQR, 120 – 275) and one isolate (MRL5038) had a spore count of 0.93 x 106 CFU/ml. The isolates with a low spore count, VPI10463 and MRL5038, had in vitro toxin A/B concentrations of 956 ng/ml and 996 ng/ml, respectively. Whereas an isolate with a high spore count, MRL5032, had an in vitro toxin concentration of 113 ng/ml. WGS revealed that all Z isolates were identified as ST46. WGS of these new group Z isolates compared to VPI10463 showed no obvious changes in potential promoter SNPS within the 500 bp region upstream of the tcdR, codY, ccpA, recV, and spo0A genes associated with sporulation or toxin production. Several cryptic mobile genomic elements were, however, identified.

Conclusions: Unlike VPI 10463, 3 of 4 contemporary Z isolates demonstrated high sporulation and relatively low in vitro toxin production. Further research is underway to understand the disconnect between toxin production and sporulation in REA group Z strains and how this switch from high toxin production to high sporulation might influence transmission and clinical prevalence of this strain group.

P27

A NOVEL PEPTIDOGLYCAN HYDROLASE PLAYS PLEIOTROPIC ROLES IN *CLOSTRIDIOIDES DIFFICILE* R20291

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Background and Aims: *Clostridioides difficile* is a Gram-positive, spore-forming, toxin-producing anaerobe known to cause nosocomial antibiotic-associated intestinal disease. While the production of toxin A (TcdA) and toxin B (TcdB) is central to the pathogenesis of *C. difficile*, the mechanism of TcdA and TcdB release from cells remains unclear. In this study, we aimed to identify and characteriz a novel cell wall hydrolase, Cwl0971 (CDR20291_0971), from *C. difficile* R20291, which plays a role in bacterial autolysis.

Methods and Results: Using CRISPR-AsCpfI, we generated a gene deletion mutant (R20291Δ0971), demonstrating significantly delayed cell autolysis and enhanced cell viability compared to R20291. Furthermore, purified Cwl0971 exhibited hydrolase activity against Bacillus subtilis cell walls. Notably, deletion of gene 0971 impaired TcdA and TcdB release due to reduced cell autolysis in the stationary/late phase of growth. Additionally, sporulation in the mutant strain was significantly reduced compared to the wild type. In vivo experiments using a mouse infection model revealed decreased fitness of the Cwl0971-deficient strain compared to the parent strain.

Conclusions: Overall, Cwl0971 plays a crucial role in cell wall lysis, impacting cell viability, toxin release, sporulation, germination, and pathogenicity of R20291. These findings suggest that Cwl0971 could serve as a promising target for therapeutics and prophylactics against *C. difficile* infections.

P28

LOW-TOXIN *CLOSTRIDIOIDES DIFFICILE* RT027 STRAINS EXHIBIT ROBUST VIRULENCE

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Background and Aims: *Clostridioides difficile* is a leading cause of healthcare-associated infections worldwide. Currently, there is a lack of consensus for an optimal diagnostic method for *C. difficile* infection (CDI). Multi-step diagnostic algorithms use enzyme immunosorbent analysis (EIA)-based detection of *C. difficile* toxins TcdA/TcdB in stool, premised on the rationale that EIA toxin-negative (Tox-) patients have less severe disease and shorter diarrhoea duration. The aim of this study is to characterize toxigenic (i.e. tcdA/tcdB-positive) *C. difficile* strains isolated from diarrheic patient stool with an EIA Tox- (i.e. "discrepant") CDI diagnostic test result by assessing their toxin production and virulence potential.

Methods: To-be-discarded stool samples were collected from Southern Arizona hospitals and *C. difficile* isolated using TCCFA. These strains were assessed for toxin production using commercially-available TcdA/TcdB EIA kits and cytotoxicity assays. Low-toxin producing strains were further assessed for virulence in the Golden Syrian hamster model and colonization in C57BL/6 mice. These strains were also subjected to genome and proteome evaluations.

Results: Overall, of 1243 *C. difficile*-positive patient stool specimens from Southern Arizona hospitals, 31% were discrepant. For RT027 (the most prevalent ribotype)-containing specimens, 34% were discrepant; the corresponding RT027 isolates were cytotoxic to cultured fibroblasts, but their total toxin levels were comparable to, or lower than, the historic low-toxin-producing *C. difficile* strain CD630. Nevertheless, these low-toxin RT027 strains (LT-027) exhibited similar lethality to a clade-matched high-toxin RT027 strain in Golden Syrian hamsters, and heightened colonization and persistence in C57BL/6 mice. Genomics and proteomics analyses of LT-027 strains identified unique genes and altered protein abundances, respectively, relative to high-toxin RT027 strains.

Conclusions: Collectively, our data highlight the robust virulence of LT-027 *C. difficile*, provide a strong argument for reconsidering the clinical significance of a Tox- EIA result, and underscore the potential limitations of current diagnostic protocols.

P29

A PRECISION ANTI-INFECTIVE TARGETING *CLOSTRIDIODES DIFFICILE*

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Background and Aims: Currently, the only US FDA-approved primary ("first episode") CDI treatments are conventional antibiotics (vancomycin and fidaxomicin), and an intravenously administered monoclonal antibody (Bezlotoxumab). There are no CDI vaccines. Despite the development of a narrow-spectrum antibiotic (fidaxomicin), disease recurrence rates of 15-25% after the first CDI are common and are associated with morbidity, mortality, and the increased utilization of healthcare resources. Thus, the lack of a microbiota-sparing CDI anti-infective represents an important treatment gap and an urgent unmet medical.

We have leveraged cationic bola-amphiphiles (CABs) as anti-infective platforms to deliver antisense oligonucleotides (ASOs) cargo intracellularly. The aim of this study is to create and characterize a microbiota-sparing anti-*C. difficile* treatment utilizing our facile anti-infective platform.

Methods: We successfully refined CAB-ASO engineering to generate SRPNT ("serpent") which is a specific nanocomplex of the dimethylpyridinium compound CAB-964 and an ASO targeting the highly conserved, genetically-essential, *C. difficile*-specific gene dnaE. We have characterized SRPNT's activity against *C. difficile* infection.

Results: SRPNT exhibits favorable properties compared to all previous nanocomplexes: it (1) is water-soluble; (2) kills *C. difficile* at low micromolar MIC; (3) does not kill key mammalian gastrointestinal tract bacteria, (4) is well-tolerated by mice and hamsters; (5) ablates *C. difficile* colonization in mice; (6) protects the murine GI tract from CDI damage; (7) delays lethal hamster CDI even at low doses, and (8) allows for post-CDI microbiota restitution.

Conclusions: SRPNT exhibits potent activity against *C. difficile*, and is microbiota-sparing; therefore, it is a precision anti-microbial *C. difficile* infection. The bola-amphiphile platform, SRPNT, can be readily repurposed to target other *C. difficile* genes or diverse bacterial pathogens.

P30

INVESTIGATING THE ROLE OF SMALL, ACID-SOLUBLE SPORE PROTEINS IN *C. DIFFICILE* SPORE OUTGROWTH

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Background and Aims: *C. difficile* spores are resistant to many insults, partly due to the presence of small, acid-soluble spore proteins (SASPs), a class of DNA-binding proteins which are highly abundant in spores. Upon germination, these proteins are quickly degraded into free amino acids.

We aim to investigate whether these amino acids, specifically glycine and leucine, act as a substrate reservoir for reductive Stickland metabolism, a pathway by which *C. difficile* cells generate ATP and recycle NADH into NAD+. Metabolism of these amino acids could give outgrowing *C. difficile* cells an advantage in colonizing the competitive gut microbiome.

Methods: Microscopic analysis of the length / width ratio of individual spores allows us to track outgrowth at a single cell level. Outgrowing spores are imaged via natural autofluorescence at the GFP excitation / emission wavelengths of 488 nm / 510 nm. Cells are then detected and measured via MatLab script, given length / width ratios which are then compared to spore and vegetative cell control measurements. We generated mutant strains of *C. difficile* R20291 including single, double, and triple deletions of Stickland pathway genes as well as knockouts and scrambled replacement insertions of three SASP genes that were introduced at their native loci using our new theophylline-controlled allelic exchange system.

Results: Preliminary results show accurate detection and differentiation of spores, vegetative cells, and outgrowing spores. We are continuing to test media conditions and reproducibility as well as finishing strain construction.

Conclusions: We have developed a novel microscopy-based approach to investigate *C. difficile* spore outgrowth and are constructing strains in order to conduct more experiments.

P31

FROM THE BENCH TO THE PRODUCT EFFICACY EVALUATION: EUROPEAN STANDARD REVIEW & TECHNICAL CHALLENGES

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It is well established that both symptomatic patients with active disease and asymptomatic patients shed *C. difficile* spores into their environment, which results in transmission risk for other patients. Therefore, the environment acts as a reservoir for bacterial spores and suitable disinfectants with proven efficacy are key in eradicating spores from hard surfaces, patient equipment, and floors. There are currently 2 laboratory methods to use to test the activity of a surface product against spores of *C. difficile*: EN 17126 (quantitative suspension test also call Phase 2, step 1 standard) & EN 17846 (carrier tests or surfaces tests known as Phase 2 step 2 standards). Results of those 2 standards are needed to support the sporicidal efficacy claim against *C. difficile*. European standards are developed by experts of the CEN (European Commission of Standardization) TC/216, a legal association constituted of national Standards bodies, aiming to ensure availability of standardized methodologies to assess products antimicrobial efficacy. Testing criteria of those standards, as initial spore concentration, required logarithmic reduction of spore load, specific range of contact time & strain collection in the presence or absence of soil materials are based on the current scientific state of the art and may differ from realistic situations. However, the extent to which these standards reflect the current practices in healthcare facilities may be challenge. Only limited knowledge regarding the relationship between the activity of products as determined by suspension as compared with surface tests, and the relevance of the results of both tests to condition of use is known.

On top of that, when we deep dive into the spore culture and germination steps of those European standard protocols in comparison of the one available in the scientific community, we observe some discrepancy. Repeatability and reproducibility are crucial points in the design of standards that are here highly dependent on spore preparation and susceptibility.

The aim of this work is to share knowledge and laboratory challenges when testing product sporicidal efficacy according to European standard with the scientific community experts to increase accuracy and representativeness of European methodologies with infection prevention.

P32

PHAGE-DERIVED PARTICLES MODULATE MICROBIOME VIA KILLING OR EDITING OF GUT BACTERIA

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Depending on their gene content, bacteria can either provoke disease, be neutral or be beneficial to their host. Antibiotics represent the primary class of drugs utilized to treat bacterial associated disease, yet they exhibit a lack of specificity, leading to microbiome disruption and potentially aggravating disease. As an example, in the treatment of *C. difficile* infections (CDI), broad spectrum antibiotics create an environment where C difficile has a fitness advantage. Eligo Bioscience is developing phage-derived particles that deliver synthetic DNA payloads into bacteria in situ. These DNA payloads can be programmed to express different therapeutic proteins. By encoding CRISPR-Cas systems on such particles, we demonstrated efficient DNA sequence-based killing of E. coli in the mouse gut. Alternatively, by replacing the CRISPR-Cas system with a base editor, we demonstrated the efficient gene editing of E. coli with a median editing efficiency of 93% with a single dose. By enabling the precise killing or edition of *C. difficile* in the gut, our approach would offer unique opportunities for the treatment of recurrent CDI.

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CLOSTRIDIOIDES DIFFICILE AGGRAVATES DEXTRAN SULFATE SOLUTION (DSS)-INDUCED COLITIS BY SHAPING THE GUT MICROBIOTA AND PROMOTING NEUTROPHIL RECRUITMENT

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Background and Aims: *Clostridioides difficile* (*C. difficile*) is a pathogen contributing to increased morbidity and mortality of patients with inflammatory bowel disease (IBD). This study aimed to investigate how *C. difficile* affects the severity of dextran sulfate solution (DSS)-induced colitis in mice.

Methods: A DSS-induced colitis model challenged with *C. difficile* was constructed without antibiotic administration and monitored for symptoms and burden of *C. difficile*. Profiling of gut microbiota and transcriptome alterations in colonic tissue was conducted through 16s rRNA and RNA sequencing, respectively. Lamina propria cells were isolated and evaluated via flow cytometric analysis. Fecal microbiota transfer assay was performed to validate the role of microbiota in disease exacerbation. Neutrophils were collected from mouse bone marrow for transmigration assay. The selective inhibitor SB225002 and MCC950 were used to perform CXCR2 and NLRP3 inhibition in the in vivo model.

Results: *C. difficile* led to transient colonization in mice with colitis, but still significantly enhanced disease severity as assessed by weight loss, histopathological damages, and inflammatory cytokine concentrations. Because this effect is independent of toxin production as validated by infection with a non-toxigenic strain, we focused on changes in the gut microbiota. The microbiota altered by C.difficile, featured with reduced proportions of g_Prevotellaceae_UCG-001 and g_Muribaculaceae, were confirmed to contribute to disease severity in colitis mice. The inflamed colon showed neutrophil accumulation and was enrichment of upregulated genes in leukocyte chemotaxis or migration. The isolated neutrophils from *C. difficile*-infected mice with colitis showed a robust migratory ability. We observed a detrimental role of neutrophils by hindering neutrophil recruitment with SB225002. Furthermore, neutrophil recruitment appeared to be regulated by interleukin(IL)-1β, as inhibition of IL-1β production by MCC950 markedly ameliorated inflammation with decreased neutrophil accumulation.

Conclusions: Our study provides information on the complicated interaction between microbiota and immune responses in *C. difficile*-induced inflammation in mice with colitis, which could help determine potential therapeutic targets for patients with IBD concurrent with *C. difficile* infection.

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IMPROVED HEALTH-RELATED QUALITY OF LIFE IN PATIENTS WITH RECURRENT *C. DIFFICILE* INFECTION AFTER TREATMENT WITH FECAL MICROBIOTA TRANSPLANTATION

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Backgrounds and aims: *Clostridioides difficile* infection (CDI) is a common cause of nosocomial diarrhoea, especially in elderly patients who have received treatment with broad-spectrum antibiotics. Recurrences are common, among 15-30 %, and causes, in addition to increased costs for the healthcare, patient suffering in the form of increased morbidity, mortality and anxiety (1). Fecal microbiota transplantation (FMT) is a safe and effective treatment recommended after recurrences of CDI (2, 3). In addition to a treatment being effective, it is also important to find out how the treatment affects the patient (4).

Methods: The study design was a prospective observational cohort study, in which essentially all patients, who were offered FMT in Jönköping and Östergötland Counties, Sweden (369 000 and 472 000 inhabitants respectively) between October 2015- July 2021, were invited to participate. A questionnaire about the patient's health related quality of life (HrQoL) was completed before and at least once two weeks and/or two months after FMT. The questionnaire consisted of three parts: SHS (Short health scale), a second part which was excluded because it was sparsely filled in, and EQ-5D including EQ-VAS.

Results: A total of 64 patients were included, of which 52 patients were cured. HrQoL measured via the SHS improved significantly, between 1-2 steps for all four dimensions that the questions covered (symptoms, function, worry and well-being). Patients who did not heal CDI showed increased quality of life, although with lower significance. The dimension around functionality improved the most. The results from the EQ-5D parts were not as clear and not always significantly improved, although the same trend as for SHS was noted. The EQ-VAS part improved significantly after FMT.

Conclusion: This study shows that patients with recurrent CDI have a low quality of life that improves significantly after FMT.

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MADECASSOSIDE ALLEVIATES *CLOSTRIDIOIDES DIFFICILE* INFECTION BY TARGETING ASC TO INHIBIT INFLAMMASOME ACTIVATION

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Background and Aims: *C. difficile* infection (CDI) is a major cause of healthcare-associated diarrhea. Large clostridial toxins can result in inflammation. Madecassoside (MA), a pentacyclic triterpene extracted from Centella asiatica, has broad anti-inflammatory effects. The therapeutic impact and associated mechanisms of MA in CDI remain unexplored.

Methods: We performed a CDI mouse model to analyze the improvement of symptoms by MA. Additionally, we constructed an inflammation model stimulated by *C. difficile* isolates or toxins in macrophage, and analyzed the effects of MA.

Results: The administration of low and high concentrations of MA via continuous intragastric administration was found to alleviate the symptoms of mice infected with C.difficile, including improvements in body weight, colon length, histopathological damage. ELISA and immunohistochemical staining demonstrated that MA reduced the secretion of IL-1β in a dose-dependent manner in vivo and in vitro. Furthermore, the hyperactive state of inflammasome-related signaling pathway from the stimulated by C.difficile and toxin B was inhibited . Co-immunoprecipitation and confocal microscopy results indicated that MA inhibited the formation of the inflammasome complex and ASC speck. Molecular docking revealed good binding ability between MA and ASC.

Conclusions: Our study demonstrated that MA can alleviate the activation of inflammasome initiated by C.difficile toxins. This effect may be attributed to its specific binding affinity with ASC. These findings may offer novel avenues for developing treatment strategies against *C. difficile*.

MEMBRANE VESICLE AND PHAGE INTERACTIONS IN *CLOSTRIDIOIDES DIFFICILE*

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Background/Aims: *C. difficile* zoonotic transmission prompts investigation into mechanisms of bacterial evolution (i.e., horizontal gene transfer), aided by bacterial viruses (phages) and membrane vesicles (MVs). Both phages and MVs can confer exogenous genetic traits to its bacterial host cells, and MVs were reported to transfer phage receptors between bacterial cells in Bacillus subtilis, but little is known about MVs and MV-phage interactions in *C. difficile*. This work aims to isolate and characterise *C. difficile* MVs and investigate their ability to sensitize bacteria to phage infection.

Methods: *C. difficile* MVs purified in an iodixanol density gradient, were characterised in morphology, particle count, and protein concentration. To investigate MV-phage interactions, a *C. difficile* phage-resistant MV-recipient strain (R), and a phage-sensitive MV-donor strain (S) were grown in brain heart infusion broth separately and in a 1:5 R :S ratio mixture. *C. difficile* phage Φ027, was introduced to cultures at logarithmic growth phase. To monitor killing, optical density (600nm) and viable counts were monitored hourly for 5 h post Φ027 infection. To determine phage dormancy (lysogeny) within bacterial cells, phage infected colonies were screened by PCR using phage specific primers.

Results: *C. difficile* R and S strains produced MVs ranging from 29-520 nm from log phase cultures , with high accumulation at stationary phase. Yield of MVs from 100 mL of *C. difficile* stationary phase culture was 3.8 x1012 particles/mL, and purified MVs contained 24 µg/mL of protein. The S strain decreased in viable count when infected with Φ027 as expected, and (4.5%) were PCR positive for lysogeny at 5 h. The R strain did not decrease in viable count when infected with Φ027 unless when grown together with the S strain. R strain lysogens (1.7%) were positive by PCR in mixed culture with S, indicating Φ027 infection.

Conclusion: *C. difficile* MVs are heterogeneous in size, released at different stages of growth, contain protein, and could be purified in an iodixanol density gradient. Detection of R lysogen only in mixed culture with S suggests presence of the latter enabled phage infection of the former possibly mediated by S strain MVs carrying phage receptors, as described in Bacillus subtilis.

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EPIDEMIOLOGY OF CLINICAL *CLOSTRIDIOIDES DIFFICILE* ISOLATES IN BELGIUM: NRC SURVEILLANCE DATA 2013-2023

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Background and Aims: In response to a rise in *Clostridioides difficile* infections (CDI) incidence and the emergence of hypervirulent strains, national surveillance of CDI in Belgian hospitals was initiated in 2007. Each acute care hospital is encouraged to submit at least five consecutive isolates per semester to the National Reference Center (NRC). Participation was initially mandatory but became optional from 2014. The aim of this study was to analyze the epidemiology of *C. difficile* clinical isolates circulating in Belgium over a decade-long period from 2013 to 2023.

Methods: All *C. difficile* isolates received at the NRC from 2013 to 2023 (n=10440) were typed by capillary gel-based electrophoresis PCR-ribotyping and further characterized by multiplex PCR, targeting virulence factor genes (tcdA, tcdB, cdtA cdtB and deletions in tcdC). Since 2019, susceptibility to antibiotics for CDI treatment (metronidazole, vancomycin and fidaxomicin) was monitored by agar dilution methods on a hundred strains, representing the 20 most common ribotypes (RTs).

Results: Although the participation rate of hospitals in the CDI national surveillance has remained stable over the years, the number of strains sent has decreased since 2020 (985 strains in 2019 vs 718 in 2023). A wide variety of RTs (over 80 every year) has been observed among the strains collected with 15 of these RTs accounting for 70% of all strains. Since 2013, the four most prevalent RTs nationwide (one third of the isolates) have been 014, 020, 002 and 078. The RT106 (2.8% in 2013 vs 9.4% in 2023), RT154 (0.2% in 2013 vs 5.2% in 2023) and RT023 (2.8% in 2013 vs 5.0% in 2023) have emerged in recent years. The spread of RT027 has been effectively controlled in Belgium, with only 2 strains reported in 2023 (55 in 2016). In 2023, 15% of *C. difficile* isolates tested positive for the binary toxin, and all isolates demonstrated susceptibility to the three antibiotics.

Conclusion: CDI has evolved into an endemic challenge in Belgium, highlighting a large diversity of ribotypes. Notably, certain RTs exhibit the ability to disseminate nationally and persist over extended periods. A continuous surveillance system is imperative for monitoring the ever-evolving epidemiology of CDI and the emergence of both virulent and resistant clones.

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PERSISTENCE AND GENETIC DIVERSITY OF *CLOSTRIDIOIDES DIFFICILE* IN PIG FARMS

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Clostridioides difficile is an important pig pathogen that particularly affects newborn piglets. This bacterium produces toxins that cause severe diarrhea and intestinal inflammation, usually in piglets up to seven days of age. One of the most prevalent PCR ribotypes in Slovenian pig farms is 045, which possess all three major toxins (toxin A, toxin B and binary toxin). The aim of this study was to determine the genetic diversity of *C. difficile* 045 isolates in two large pig farms.

C. difficile isolates were collected between 2006 and 2015 in two large pig farms. A total of 20 isolates underwent whole-genome sequencing (WGS). The sequence type (ST) was determined and cgMLST was performed to gain insight into the relatedness of the isolates.

All WGS-typed isolates belonged to ST11 (clade 5). The cgMLST analysis revealed two clusters (< 7 allele differences) comprising isolates from a single farm. Interestingly, the isolates from farm B (n = 3) from 2015 differed from the isolates in the cluster, indicating the introduction of novel 045 strains on the farm.

Persistence of a single clone over several years was observed on both farms, although both farms were managed by the same owner. Further studies will include WGS typing of additional isolates to gain better insight into the transmission of *C. difficile* clones between pig farms. Preventive measures focusing on strict hygiene and biosecurity are necessary to minimize the impact of this disease.

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PREVALENCE AND MOLECULAR TYPES OF *CLOSTRIDIUM DIFFICILE* ON AUSTRALIAN RETAIL VEGETABLES AND HOUSEHOLD SURFACES

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Background: Community-associated Clostridium difficile infection (CA-CDI) is rising in Australia. Increasing evidence suggests that the use of animal manure and human biosolids and effluents as fertilizer contributes to high prevalence of *C. difficile* spores in many community sources including retail root vegetables, gardens and parks. In turn, household surfaces may become contaminated with *C. difficile* due to storage and handling of contaminated vegetables. This study aimed to determine the prevalence and molecular types of *C. difficile* present on retail vegetables and household surface contamination in Queensland (QLD) and Western Australia (WA).

Methods: From April 2023 to March 2024, unwashed potatoes (n=255) and onions (n=305) were purchased at major retail outlets, independent grocers and farmers' markets. Randomly selected householders in Brisbane (QLD, n=105) and Perth (WA, n=124) were contacted and asked whether they regularly purchased unwashed potatoes. If so, consent was requested to collect environmental samples in their home. Ethical approval was received from the University of Western Australia Human Research Ethics Committee. Moistened sterile cellulose sponges (Medical Wire & Equipment, England) were used to collect one swab each from their countertop/chopping board, their vegetable storage area and an unwashed potato. Potato peels, onion roots and household swab samples were subjected to enrichment culture for *C. difficile*. Isolates underwent PCR ribotyping and PCR for toxin genes.

Results: The overall prevalence of *C. difficile* was 35.3% on potatoes and 20.3% on onions. Among household samples, 2.2% of countertops/chopping boards, 5.8% of vegetable storage areas and 8.8% of potatoes were positive. Ribotypes (RTs) varied between the two states with RT 056, 286, 101 and 125 predominating on vegetables in WA and RTs 101, QX 098, RT 014/020 and QX 601 most common on QLD vegetables. RT 056 was present in households in both states, and QX 690 predominated in WA households in particular.

Conclusions: A wide variety of toxigenic strains of *C. difficile* was identified on retail vegetables and within households, highlighting potential for CA-CDI cases to be acquired within households. Populations at high risk of CDI e.g. inflammatory bowel disease and cancer patients should be educated about safe handling and cleaning of potential sources of *C. difficile* in their homes.

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CLOSTRIDIOIDES DIFFICILE INFECTIONS IN GERMANY, 2017-2023 – THE CALM BEFORE THE STORM?

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Background: *Clostridioides difficile* infections (CDI) are one of the most common causes of healthcare-associated infections. Several countries have reported a decrease in CDI incidence in recent years. We describe the epidemiology of CDI in Germany from 2017-2023 to identify the prevailing trends.

Methods: Severe CDI (sCDI) in Germany are notifiable by physicians to local public health authorities and further communicated to federal state and national level. Using national surveillance of sCDI for 2017–2023 we describe annual and mean incidences per 100,000 population by sex and federal state, as well as median age with interquartile range (IQR). We describe annual and mean incidence of hospitalised CDI using hospital surveillance from the national reference centre of nosocomial infections (CDI-KISS) and federal hospital discharge diagnoses registry (GBE-Bund hospital registry) as well as annual and mean incidence (MI) of deaths from CDI using federal death cause registry (GBE-Bund death cause registry). Data from CDI-KISS, GBE-Bund hospital registry and GBE-Bund death cause registry were only available from 2017–2022, at the time this study was conducted.

Results: Between 2017–2023, 14,050 cases of sCDI were reported through national surveillance with a MI of 2.4/100,000. The median age of notified sCDI cases was 79 (IQR=67–85). Annual sCDI incidences were higher among females (MI = 2.7/100,000 female population) compared to male cases (MI = 2.1/100,000 male population). Hospitalised CDI incidences were MI CDI-KISS = 71/100,000 and MI GBE-Bund hospital registry = 81/100,000, respectively, while MI of CDI related deaths was 1.3/100,000. Across all four databases, a decline in CDI incidence was observed between 2017 and 2022. However, national surveillance data revealed an increase of sCDI cases again in 2023 (27%) across all federal states of Germany, which continued in the first quarter of 2024.

Conclusion: Preventive measures, improvements in Antibiotic Stewardship, and the COVID-19 pandemic appear to have led to a decline in CDI incidences up to 2022. However, we are seeing a resurgence in cases since 2023 alongside high incidences of hospitalisations, demonstrating the high disease burden. Close monitoring of the emerging trend and prevention efforts must continue.

P41 TRENDS OF *CLOSTRIDIOIDES DIFFICILE* INFECTIONS IN BELGIAN HOSPITALS BETWEEN 2010 AND 2022

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Clostridioides difficile infections (CDI) remain one of the more important gastro-intestinal infections in European hospitals, being responsible for 4.9% of all healthcare-associated infections. For this study, we present the latest trends on incidence of CDI in Belgium.

Data for period 2010-2022 was obtained through the national surveillance of CDI in Belgian acute hospitals. Hospitals voluntarily participating should report at least for one semester. Data are collected, validated, and reported through the secured environment of the Healthdata system.

Participation of eligible hospitals has decreased from 82% to 77% between 2010 and 2022, throughout this period the percentage of type and region of the participating hospitals remained comparable. In 2010, 62% of all reported CDI could be categorized 'hospital-associated' (HA-CDI), meaning symptoms occurring two days or more after admission. Twelve years later this accounts for 58% of all CDI. Over the study period, the reported CDI labelled as 'recurrent' remained around 10%. Between 2015 and 2021, a decrease of annual HA-CDI incidence was observed. In 2022, a significant increase in HA-CDI incidence was reported of 1.31 (95%CI 1.23 – 1.39) to 1.62 (95%CI 1.53 – 1.71), and for all CDI this was from 2.41 (95%CI 2.30 – 2.51) to 2.78 (95%CI 2.66 – 2.89).

HA-CDI are still a burden on hospitals compared to twelve years ago, although recent literature suggest that the importance of community-associated CDI should not be ignored. The COVID-19 pandemic did not seem to increase the burden of CDI in Belgian hospitals, possibly through the focus on infection, prevention and control measures, change in care seeking behaviour and lower use of some antimicrobials. The 2022 increase in incidence might be related to a post-pandemic change in the behaviour mentioned above and should be analysed further.

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AI4CDIFF: INTRODUCING A NOVEL MACHINE LEARNING APPROACH FOR EARLY IDENTIFICATION OF AT-RISK POPULATIONS FOR *CLOSTRIDIOIDES DIFFICILE* INFECTIONS

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Introduction: *Clostridioides difficile* infection (CDI) is an urgent public health threat causing considerable global illness and death. Risk factors may be short-term (e.g., antibiotic use, recent hospitalization) or long-term (e.g., advanced age, comorbidities). Potential *C. difficile* vaccines have been explored to be administered in 3 doses over 6 months1. We developed a machine learning model to identify patients at high risk for primary CDI 6-12 months before disease onset.

Methods: A retrospective administrative claims analysis was performed using subjects aged 18 years and over in the United States diagnosed with CDI (2017-2019) in Optum de-identified Market Clarity data. Cases were identified via ICD-10 code with a positive stool test for *C. difficile* toxin A/B and were matched 1:1 with non-CDI controls using a propensity scoring algorithm based on age, sex, and patient history. A gradient boosted tree algorithm model was developed based on >900 features derived from demographics, diagnoses, healthcare visits, medications, clinical observations and comorbidities. Data six months prior to index dates were hidden to the model to account for hypothetical vaccine administration timing. Features were removed for co-linearity and final model training:testing using 80:20 data subsets. Model performance was evaluated and optimized for sensitivity and a bias assessment was performed.

Results: 4736 CDI cases and 4732 controls were identified. The comprehensive model (AUC-ROC: 0.801) was able to predict CDI cases versus controls with a sensitivity of 74.4% and specificity of 73.3%. Hospitalization days (10·0%), elevated white blood cell count (9.6%), and emergency interactions (7.4%) were the top three differentiating features between patients in the case and control cohorts.

Conclusions: We demonstrate the utility of machine learning as a novel approach to identify features capable of predicting primary CDI cases in adult populations. Our model showed acceptable performance in identifying cohorts at risk for CDI 6-12 months prior to disease onset to inform risk-based recommendations for a potential *C. difficile* vaccine. Future work aimed at bias mitigation, external validation and prospective testing could improve model generalizability and applicability in clinical care.

Reference: 1. Toth et al. Vaccine 2020 Aug 18; 38(37):5927-5932

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HIGH PREVALENCE OF *CLOSTRIDIOIDES DIFFICILE* IN RETAIL GARDEN SOIL CONDITIONERS, MIXES AND TURF IN AUSTRALIA

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Background: The incidence of community-associated *Clostridioides difficile* infection (CA-CDI) has been increasing in Australia, with healthcare-associated infection now accounting for less than 20% of all diagnosed cases. Previous findings of a high prevalence of *C. difficile* in public parks, root vegetables and gardens have been largely attributed to the broad use of animal manures as fertilisers. Similarly, the use of animal manure in commercial garden products could provide a potential *C. difficile* source. This study aimed to determine the prevalence and molecular characteristics of *C. difficile* present in commercially available garden supplies in two Australian states, Western Australia (WA) and Queensland (Qld).

Methods: In 2023, 465 samples consisting of soil conditioners (including manures, compost, soil improvers and fertilisers), soil mixes and turf were collected from retail garden stores and suppliers in two Australian states. Ten-gram aliquots were inoculated into a selective enrichment broth and incubated for 7 days at 35°C, followed by alcohol shock and culture on ChromID *C. difficile* agar. Isolates were characterised by PCR ribotyping and toxin gene PCR.

Results: Overall, *C. difficile* was isolated from 55.5% (258/465) of retail garden samples, including 67.9% (53/78) of turf, 59.8% (155/259) of soil mixes and 39.1% (50/128) of soil conditioners. Notably, prevalence was significantly higher in Qld (71.1%, 175/246) than in WA (37.9%, 83/219) (Fisher's exact test, $p < 0.00001$). *C. difficile* ribotypes (RTs) QX 686, 014/020, 010 and QX 145 predominated, although RTs were highly variable between the two states with 123 distinct RTs identified, including 36 novel types. Of the 295 isolates, 81 (27.6%) were toxigenic strains, including RTs associated with both human and animal CDI such as RTs 014/020, 056, 297/310 and 070.

Conclusions: A high prevalence of *C. difficile* was identified in commercially available soils and turf in Australia, including toxigenic RTs associated with both human and animal CDI. This highlights the potential risk of acquiring CA-CDI faced by home gardeners and can inform heightened hygiene practices for those at greater risk, such as those taking antimicrobials or immunosuppressive drugs, and cancer patients, or just old. Furthermore, these findings emphasise the importance of a One Health approach to controlling CDI.

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MOLECULAR AND WHOLE GENOME SEQUENCING ANALYSIS OF PEDIATRIC AND ADULT HEALTHCARE AND COMMUNITY-ASSOCIATED *CLOSTRIDIOIDES DIFFICILE* INFECTIONS 2022, CANADA

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Background and Aims: *Clostridioides difficile* is the leading cause of healthcare-associated diarrhea in high income countries. Capillary electrophoresis PCR ribotyping (CE-PCR RT) is currently the gold standard for *C. difficile* typing; however it lacks the discriminatory power for investigating transmission and outbreak events. We describe a multicenter study consisting of 84 hospitals (47 adult, 24 mixed, 13 pediatric) to evaluate CE-PCR RT, multi-locus sequence typing (MLST), core genome multi-locus sequence typing (cgMLST), and single nucleotide variant (SNV) analysis to characterize genetic diversity and clustering of *C. difficile* infection (CDI) isolates identified from participating hospitals in the Canadian Nosocomial Infection Surveillance Program (CNISP) for the study year 2022.

Methods: Using standardized case definitions to identify hospitalized inpatients with CDI (Adults: two months targeted; March-April, Pediatric: year round), we found 527 cases which met inclusion criteria. Eligible samples were ribotyped using CE- PCR RT and further characterized by WGS to determine MLST and cgMLST using Ridom SeqSphere+. Possible transmission or outbreak events as identified by SeqSphere clustering data were further investigated by looking at SNV differences using SNVPhyl.

Results: Analysis of 527 cases (396 adult, 131 pediatric), revealed 100 total unique RTs. The five most common adult RTs were 106 (15.4%), 014 (9.9%), 020 (7.8%), 002 (6.1%), and 056 (3.8%). The five most common pediatric RTs were 106 (10.7%), 014 (9.9%), 015 (6.9%), 020 (6.1%), and 056 (5.3%).

MLST typing revealed 69 unique sequence types (ST), nine of which were novel. SeqSphere+ minimum spanning tree analysis utilising cgMLST data revealed 21 putative clusters (2-10 samples). All samples within an individual cluster had identical RT and STs. SNV analysis of these individual clusters did not suggest any outbreak or transmission events; however there

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were two samples, each within a cluster of three, that were observed to have less than 3 SNV differences.

Conclusions: We report the first genomic analysis of Canadian CDI isolates using CE-PCR RT and Ridom SeqSphere+. SeqSphere's cgMLST scheme consisting of 2147 core genes offers enhanced resolution over CE-PCR RT that can be augmented with epidemiological information to improve outbreak and transmission investigations.

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CLOSTRIDIUM DIFFICILE IN CAMBODIA

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Introduction: Clostridium difficile ribotype (RT) 017 is endemic in Asia, mainly reported in non-outbreak circumstances and frequently the most predominant RT reported in Asia. Recent *C. difficile* studies in 2020-2022 in Vietnam found RT012 the most common RT in humans, while studies in Thailand indicated RT017 and RT014/020 were the most predominant. There is limited data on *C. difficile* prevalence in Cambodia. We aimed to determine the distribution of *C. difficile* RTs in this country.

Methods: *C. difficile* isolates were collected in cross-sectional studies of *C. difficile* infection (CDI) in adults and children in Cambodia between 2020-2022. Toxigenic culture of stool samples was done, and PCR ribotyping used for typing all isolates. Previously published collections of 769 *C. difficile* isolates from humans in Thailand (2005-2018) and 199 from Vietnam (2014-2022) were used to compare with isolates from Cambodia. The Chi-squared test was used to identify significant associations between RT and country.

Results: *C. difficile* RT017 (15/192, 8%) was the most predominant toxigenic strain in Cambodia, followed by RT012 (14/192, 7%) and RT014/020 (7/192, 4%). High numbers of novel strains and strains with QX internal nomenclature (128/192, 66%) were recovered in Cambodia. A similar order of predominant RTs was seen in Vietnam, however, in Thailand the second most common RT was RT014/020. *C. difficile* RTs 017 and 014/020 were more commonly found in Thailand ($p<0.0001$) and *C. difficile* RT012 more commonly found in Cambodia and Vietnam ($p<0.0001$). All known Cambodian non-QX strains were commonly found in Thailand, while some known non-QX strains from Thailand and Vietnam were found only in those countries.

Conclusions: Similar strains in the three countries suggest plausible common sources of *C. difficile* in these countries and/or cross-border transmission. The significant number of *C. difficile* RT017 and RT014/020 in Thailand and RT012 in Cambodia/Vietnam may indicate different local risk factors between Thailand and Cambodia/Vietnam. Further investigation into *C. difficile* isolates from Asian countries and clinical outcomes of infection with these strains could contribute to CDI management in Asia. The presence of many diverse QX and novel strains of *C. difficile*, all likely clade 4 strains, adds further evidence to the theory that Asia is the ancestral home of clade 4.

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DETECTION AND CHARACTERIZATION OF *CLOSTRIDIOIDES DIFFICILE* IN BROILER SLAUGHTERHOUSES

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The raise of community-acquired *Clostridioides difficile* infection over the past decade in humans has resulted in the study of the potential reservoirs of *C. difficile* strains and risks associated with identified sources. Attention has been paid to the contamination of food as this can be an important route of transmission. Studies currently available in the literature have revealed that a wide range of foods are contaminated by *C. difficile*, including poultry meat. No data is available regarding the contamination of the poultry meat in France, however a previous study has shown that *C. difficile* is frequently detected at farm level in broilers. The objective of this study, part of the ClostAbat project funded by the French National Research Agency (CE21-21-007), was to evaluate the contamination by *C. difficile* at the broiler slaughterhouse level all along the process from animal reception to cutting and to characterize the isolated strains.

We performed 12 sampling campaigns in three broiler slaughterhouses with 2 flocks per campaign and collected 29 samples per flock (broiler caeca, meat, surface and ambient air samples) along the slaughter line and in the meat-cutting plan. *C. difficile* was detected using a culture method (enrichment in supplemented BHI during 7 days at 37°c under anaerobic conditions followed by plating on ChromID) and further characterized (PCR-ribotype, main virulence genes using PCR and antibiotic susceptibility).

C. difficile was detected in 42 samples out of the 695 that were collected: 8/24 in transport crates, 14/24 in scalding tank, 11/24 in plucking machine, 1/24 on apron and gloves, and 3/24 in viscera conveyor at the evisceration stage, 1/24 in carcass conveyor at the chilling stage, 1/24 on the floor in the cutting area and in 3 pool of cecal contents/120. Up to 5 isolates were collected per positive sample and further characterized. Among the 198 collected strains, 21 different PCR-Ribotypes were detected, RT002/2 and 014/0 being the most prevalent ones. Genes encoding Toxin A and B were detected in 182 isolates but none of the isolates harboured binary toxin genes. All strains were susceptible to vancomycin, metronidazole, rifampicin and moxifloxacin; 97% were resistant to clindamycin, 2.5% to erythromycin, 3.5% to tetracycline and 0,5% to tigecycline.

While *C. difficile* was detected at the slaughterhouse during the process, neither neckskins nor drumsticks were positive suggesting a low or absence of contamination of the final products in the poultry sector.

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MOLECULAR CHARACTERIZATION OF *CLOSTRIDIOIDES DIFFICILE* ISOLATED FROM HOSPITALIZED OLDER ADULTS DURING PROSPECTIVE POPULATION-BASED ACTIVE SURVEILLANCE AT NINE HOSPITALS IN TOKYO, JAPAN, 2022

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Background and Aims: To further characterize circulating strains of toxigenic *Clostridioides difficile* in Japan, *C. difficile* isolates cultured from stool specimens from hospitalized CDI patients identified during a prospective multi-hospital population-based study were characterized.

Methods: Surveillance identified inpatients ≥50 years-of-age with new onset diarrhea in nine Tokyo hospitals from December 17, 2018, to March 30, 2020. Stool specimens were screened by Quik Chek Complete and GDH positive stools tested by PCR to determine toxin gene (toxin B) status. PCR positive stools were tested by cytotoxicity neutralization assay (CCNA) to determine presence of functional toxin. GDH positive stools were also anaerobically cultured and whole genome sequence acquired. A primary CDI case was defined as a hospitalized patient with diarrhea (≥3 episodes in 24 hours) that was PCR+/CCNA+ or from which a toxigenic *C. difficile* isolate was cultured. Ribotypes (RT) of the *C. difficile* isolates were inferred by whole genome multi locus sequence typing (wgMLST) using the WGS data.

Results: Sixty-five primary CDI cases were identified; 64 cases yielded *C. difficile* isolates, 11 had pseudomembranous colitis, eight recurrent CDI, and nine died. Of the patients that died, CDI was listed as the primary cause of death in one. Of the 64 isolates, 22 were RT018/356, 9 were RT369, 4 each were RT106 and RT002, and 10 had an unknown RT. Four isolates were positive for binary toxin: one isolate each of RT027, RT078/126, RT080, and one unknown. The patient whose death was attributed to CDI was culture positive for an RT078/126 isolate. Of the 64 cases with isolates, 49 stools tested positive for toxin by Quik Chek; of the 15 that tested negative, 12 were CCNA+. Each of the three CCNA- stools yielded toxigenic *C. difficile*. In terms of the pathogenicity locus (PaLoc) profile, 54 were tcdA+/tcdB+, 9 were tcdA-/tcdB+, and one was tcdA-/tcdB-.

Conclusions: CDI resulted in severe clinical consequences. Isolates of *C. difficile* from patients with CDI were a diverse collection of ribotypes with one third of the isolates RT018/356, 14% RT369 and only a single isolate each of RT027 and RT078/126, which is consistent with prior studies of contemporary *C. difficile* isolates in Japan. Public health interventions are needed to reduce the CDI burden in Japan.

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LONGITUDINAL TRENDS IN ANTIBIOTIC RESISTANCE AND RIBOTYPE DISTRIBUTION OF *C. DIFFICILE* IN SOUTH KOREA: INSIGHT FROM A SIX-YEAR SURVEILLANCE STUDY IN KorGLASS

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Background and Aims: Since 2018, monitoring the genotype and antimicrobial resistance of *C. difficile* isolated from two hospitals in Seoul and Gyeonggi Province has been conducting as part of the national antimicrobial resistance surveillance with KorGLASS. This study aims to study the genotype variations and antimicrobial resistance patterns of *C. difficile* strains isolated from hospital-onset *C. difficile* infection (HO-CDI) patients over a six-year period until 2023.

Methods: All *C. difficile* strains isolated from diarrheal samples of patients suspected of CDI in the two hospitals were analyzed. Identification of *C. difficile* was conducted using MALDI-TOF, followed by PCR ribotyping for strain typing. The minimum inhibitory concentration (MIC) for ampicillin, cefotetan, clindamycin, imipenem, chloramphenicol, tetracycline, moxifloxacin, vancomycin, metronidazole, and rifaximin was determined using agar dilution method. Electronic charts of patients with isolated *C. difficile* were reviewed.

Results: Between 2018 and 2023, a total of 1738 unduplicated toxic *C. difficile* strains were isolated, of which 1215 strains (70%, ranging from 62.3% to 76.6% annually) were HO. In 2018, the predominant ribotype in hospitals A and B was RT018, but its prevalence gradually declined, with RT014/020 becoming the most predominant ribotype by 2023. Antimicrobial resistance exhibited a continuous decrease over the six-year period, notably in cefotetan non-susceptibility from 51.3% to 25.9%, moxifloxacin non-susceptibility from 48.4% to 26.4%, and rifaximin non-susceptibility from 22.0% to 7.9%. None of the strains exhibited MIC > 2 μg/mL for metronidazole and vancomycin.

Conclusions: The decline in the prevalence of RT018, which exhibited higher antibiotic resistance compared to other ribotypes, and the increase in the prevalence of RT014/020, which displayed lower antibiotic resistance compared to other ribotypes, are believed to be responsible for the reduction in antibiotic resistance to cefotetan, moxifloxacin, and rifaximin. Further research is warranted to elucidate the underlying reasons for this ribotype shift.

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CLOSTRIDIOIDES DIFFICILE STRAINS BELONGING TO RT 955, ISOLATED DURING THE COVID-19 PANDEMIC IN SOUTHERN SERBIA, ARE GENOMICALLY DISTINCT FROM RT 955 OUTBREAK ISOLATES FROM THE UK

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Introduction: After the emergence of new hypervirulent *Clostridioides difficile* types early in 2000, ECDC started ECDIS-net to develop a European surveillance programme. In 2018, a special program was developed for EU candidate and potential candidate countries, including Serbia. The CDI Expertise Centers at Leiden and Leeds University were requested to support Serbia with molecular characterization of the isolates. End 2023, UK Health Security Agency sent an alert of a new hypervirulent RT 955 causing slowly progressing outbreaks in hospitals in Midlands. Leiden searched its European database and found RT 955 strains isolated in southern Serbia dating back to 2018. Here we report on the clinical findings of RT 955 in southern Serbia during the COVID-19 period with characterisation of strains.

Methods: A prospective CDI surveillance was performed in southern Serbia with participation of Community Health Center Niš, various hospitals, military hospitals and one clinical center in city Niš. CDI was defined as the occurrence of diarrhea with a positive test for free toxin in feces or the presence of toxin-producing *C. difficile*. The severity of CDI was assessed as mild, moderate or severe using clinical characteristics as previously described. Positive tested stool samples were cultured for the presence of *C. difficile* and isolates were sent to Leiden for further characterization by PCR ribotyping, cgMLST and WGS with SNP typing.

Results: In total, 383 patients with CDI were included in the surveillance of which the majority (n=318) derived from the clinical center Niš (284 patients) and Community Health Center Niš (93 patients), providing medical services for approximately 750,000 inhabitants. *C. difficile* RT 955 was identified in 27 (7%) of 383 patients. 60% of 27 patients were older than 60 years and 70% were male. CDI had a community onset in 15% and was always associated with previous antibiotic therapy, mostly containing a cephalosporin (74%). Of 27 patients, 16 (60%) had COVID-19 as concomitant disease. CDI was categorised as severe in 10% of the patients. At 90 days follow up, no CDI related mortality was found and 11% developed a presumed recurrence. Of 13 patients treated with metronidazole, 9 patients switched to vancomycin. All sequenced strains belonged to ST1, CT 5259 and were very much related with 0-1 alleles differences, except one (>15 alleles). The strains differed clearly from the UK RT 955 reference strains, contained gyrA_T82I encoding fluoroquinolones resistance and also had a Pnim promoter mutation, suggestive for metronidazole resistance.

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Conclusion: Since 2018, *C. difficile* RT 955 is present in Serbia, without large outbreaks. The Serbian strain differed clearly from the UK reference strain, but shared its presumed metronidazole resistance.

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PREDOMINANCE OF PREVIOUSLY UNCOMMON *CLOSTRIDIOIDES DIFFICILE* PCR RIBOTYPE 695 (CLADE 5, SEQUENCE TYPE 11) IN CATTLE FROM DUTCH DAIRY FARMS

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Background: *Clostridioides difficile* is a leading cause of infectious diarrhea in both humans and production animals. In particular, *C. difficile* strains belonging to sequence type 11 are common as zoonotic enteropathogens. The aim.of this study was to determine the presence and types of *C. difficile* in dairy cattle and calves and to compare the isolates for genetic relatedness.

Methods: Dutch dairy farms were selected and visited between February and December 2021. Feces was collected from adult dairy cattle and calves. Feces samples were also requested from dairy farmers, family members and employees. Fecal samples were cultured in an enrichment medium for 10 days and subcultured on solid media for capillary PCR ribotyping and whole genome sequencing.

Results: Of a total of 157 farms, n=31 (19.8%) were positive for *C. difficile*. The highest prevalence rates were found amongst calves < 4 weeks (17.5%). None of the 99 human samples taken during the study was positive. Thirty-seven cultured isolates belonged to 11 different PCR ribotypes of which PCR ribotype (RT) 695 (56.8%) and RT078/126 (16.2% were most abundant. Sequence analysis of 21 *C. difficile* RT695 from cattle revealed that all isolates belonged to clade 5, ST 11 and contained genes encoding both toxin A, toxin B and binary toxin. All RT695 strains carried S366V/S416A mutations in the gyrB gene and one additionally carried the gyrA-pT81I mutation. RT695 carried less antimicrobial resistance genes than other ST11 isolates. Further, the tetracyclin resistance gene tet(M) was present in 10 RT695 isolates (47.6%). Clusters of genetically related RT695 isolates were found across farms. Interestingly, The ermB gene, associated with macrolide-lincosamide-streptogramin antibiotic resistance, was not found in any of the isolates and a single RT078 isolate carried a cfr(B) gene, which may be associated with resistance to PhLOPSA antibiotics.

Conclusions: *C. difficile* was found in ~20% of the dairy farms with a predominance of the relative unknown RT 695. Cultured isolates of RT695 belonged to the same clade and sequence type as RT 078/126, but carried less antimicrobial resistance genes.

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A MICROSIMULATION MODEL FOR EVALUATING THE LONG-TERM EFFECTIVENESS OF *C. DIFFICILE* VACCINATION IN ADULTS AGED 50 AND OLDER

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Background and Aims: *Clostridioides difficile* is the leading cause of infectious diarrhea and a significant cause of healthcare-associated infections. Vaccines based on toxin-related antigens have been tested in clinical trials. However, clinical studies follow participants for a limited amount of time. In our study, we developed a microsimulation model, an approach in which individuals in a population are explicitly simulated during their lifetime, to evaluate the potential effectiveness of different vaccination scenarios in decreasing the lifetime incidence of *C. difficile* infection (CDI) in the US.

Methods: We developed a microsimulation of the natural history of CDI. Individuals can be in the following three health states: non-diseased, CDI, and death, initialized at age 50. The timeframe of the model is the lifetime of the individuals. Transition rates stratified by age and sex were obtained from two administrative databases: MarketScan® Commercial Claims and Encounters Database for individuals under 65 years old and Medicare 5% sample for 65 years old and older from 2012 to 2017. We evaluated vaccination scenarios that differ in the vaccination schedules and the vaccine characteristics, including 1) age at vaccination (50 vs. 60 years old), 2) vaccine efficacy (0.31 and 1), 3) uptake (0.6), 4) duration of protection (5 or 10 years), 5) booster application (none or every ten years). Ten thousand individuals per scenario were evaluated.

Results: Vaccine scenarios were compared against a no-vaccination scenario. The no-vaccination scenario's baseline CDI incidence was 499 per 100,000 person-years (PY). The most effective scenarios in reducing the CDI incidence were those with booster applications and vaccine efficacy of 1, with a 60 to 50% reduction compared to the baseline scenario. Reduction in CDI incidence in scenarios with a vaccine efficacy of 0.31 ranges from 2 to 27%. The age at which vaccination was initiated (50 vs 60) had a marginal effect on CDI incidence (less than a 5 % difference among otherwise identical scenarios).

Conclusions: Vaccines can significantly reduce the lifetime burden of CDI, particularly if vaccine strategies that confer immunity during the life stages with the highest risk of CDI are implemented.

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THE ROLE OF ONE HEALTH IN UNDERSTANDING THE TRANSMISSION OF *CLOSTRIDIOIDES DIFFICILE*

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Background: *Clostridioides difficile* infection (CDI) epidemiology in Australia is currently largely community-driven with >80% of cases having symptoms before hospitalisation. Increasing evidence indicates that CDI has a One Health aetiology, with animals and the environment playing key roles in the amplification and long-range dissemination of *C. difficile*.

Aims: Here, we consolidated past and current molecular and genomic data from Australia to highlight possible transmission routes of *C. difficile* in a One Health setting.

Methods: Standardised enrichment culture, toxin gene profiling and PCR ribotyping were performed. Whole-genome sequencing and core genome single nucleotide polymorphism (cgSNP) analysis were carried out on a subset of *C. difficile* ribotype (RT) 014, the most common cause of CDI in Australia.

Results: A high prevalence of *C. difficile* was found in diverse sources, including neonatal pigs (67%), horses (32%), root vegetables (30%), compost (23%), gardens (60%), shoe soles (32%), lake/pond water (47%), lawn (59%), effluent (48%) and human biosolids (94%), with *C. difficile* RT014 as one of the most predominant RTs. cgSNP analysis showed clonal clustering of human RT014 strains (separated by ≤2 cgSNPs) with one or more animal and environmental strains, consistent with recent transmission. Clones were spread over a vast geographic area, suggesting persistent community reservoirs with nationwide dissemination, possibly due to agricultural recycling of human biosolids and animal effluent.

Conclusions: This work provides novel insights on the strain-relatedness of *C. difficile* RT014, a lineage of emerging One Health importance that appears to spread via community sources. Ongoing molecular and genomic surveillance of strains in humans, animals, and the environment is imperative to identify opportunities to reduce the overall CDI burden.

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CLOSTRIDIOIDES DIFFICILE CONTAMINATION IN DAIRY FARMS

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Background: Community-associated *Clostridioides difficile* infection (CA-CDI) has been rising in Australia. There is increasing evidence that suggests inter-species transmission of *C. difficile* between animals and humans. Neonatal animals have a much higher prevalence of *C. difficile* compared to adult animals, and bobby (male) calves from dairy farms (~5 days old) are the only neonatal animals destined for the slaughterhouse in Australia, representing a potential source of contamination in the community. Yet, the ecology of *C. difficile* in Australian dairy calves is still largely unknown.

Aims: To determine the prevalence and molecular epidemiology of *C. difficile* in dairy calves and their immediate environment in Queensland (QLD) and Western Australia (WA).

Methods: During August 2022 and September 2023, manure, soil, and effluent samples were collected from three dairy farms in WA. In March 2024, rectal swabs and effluent samples were collected from four dairy farms in QLD. All samples were subjected to enrichment culture for *C. difficile*. All isolates underwent toxin gene profiling and PCR ribotyping.

Results: In WA, the overall prevalence of *C. difficile* was 64.5% (40/62) in calf manure, 95.8% in soil and 42.9% in effluent. Ninety-five per cent of the isolates from WA were toxigenic. Three *C. difficile* ribotypes (RTs), RT127 (A+B+CDT+), RT033 (A-B-CDT+) and RT014/020 (A+B+CDT-), predominated in WA and comprised 79.7% (63/79) of the isolates. In QLD, the prevalence of *C. difficile* was 12.5% (5/40) on rectal swabs and 75.0% in effluent. Almost half of the isolates in QLD were non-toxigenic (10/21). Fourteen different *C. difficile* RTs were identified in QLD, with RT127 (28.6%, 6/21) being the most common.

Conclusions: *C. difficile* RT127 is the dominant strain in Australian dairy calves. The high rate of carriage/colonisation suggests that dairy calves are potential sources/reservoirs of *C. difficile*, including toxigenic strains that cause CDI in humans. There is a risk of contaminating (i) retail veal products during the slaughter process, and (ii) crops if manure and effluent from dairy farms is recycled onto land as fertiliser. Further investigations are needed to determine the prevalence and concentration of *C. difficile* in the downstream supply chain.

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HIGH PREVALENCE OF *CLOSTRIDIOIDES DIFFICILE* IN SOILS FROM WESTERN AUSTRALIAN (WA) PUBLIC PARKS

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Background: Previously rare, community-associated *Clostridioides difficile* infection (CA-CDI) now accounts for >80% of CDI cases in Australia. Strains of *C. difficile* related to those causing human CDI have been isolated from a variety of food, animal, and environmental sources in Australia, but the presence of *C. difficile* within public parks is understudied. As such, knowledge of how these spaces may mediate transmission of toxigenic *C. difficile* within the community is limited. Herein we evaluate the prevalence and molecular characteristics of *C. difficile* within the soils of WA public parks and provide insight into how these spaces may serve as a source of CA infection.

Methods: During 2023, 100 soil samples were collected from five public parks in the Perth, the capital of WA. Samples were cultured in selective-enrichment media and on CCFA and ChromID agars. Isolates were characterized by PCR ribotyping and toxin-gene profiling. A Vero cell cytotoxicity assay was performed on five esculin-hydrolysis negative isolates, and one isolate with an uncommon toxin-gene profile was whole genome sequenced (WGS).

Results: *C. difficile* was isolated from 73% (73/100) of samples. Nearly 18% (14/79) of isolates were toxigenic. The most common ribotypes (RTs) isolated were nontoxigenic strains QX 518 (15/79) and RT 010 (10/79). *C. difficile* RT 106 (A+B+CDT-), an epidemic strain in Europe and North America, was the most prevalent toxigenic strain isolated (5/79). None of the five esculin-hydrolysis negative isolates induced a cytopathic effect in a Vero cell culture, but one of these isolates possessed the atypical toxin profile A+B-CDT-, as confirmed by WGS.

Conclusions: The presence of toxigenic *C. difficile* common to both human and animals, such as RT 014/020 found here, suggest public park soils may serve as a point of exchange between animal, human, and environmental *C. difficile* sources. Additionally, the presence of unusual esculin-hydrolysis negative strains, which may challenge laboratory diagnostics, is of concern and such strains should be further investigated. Further studies are required to enhance understanding of human and non-human *C. difficile* dissemination within the environment. Altogether, we have demonstrated that *C. difficile* is present in the soils of WA public parks, and these spaces may play a role in the transmission of CA-CDI in WA.

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PREVALENCE BINARY TOXIN PRODUCING STRAINS OF *CLOSTRIDIOIDES DIFFICILE* - VARIED RIBOTYPES, INCLUDING RT955, IN THE SILESIA REGION OF SOUTHERN POLAND

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Background and aim: *Clostridioides difficile* is currently the main factor responsible for bacterial outbreaks in Polish hospitals. The epidemiology of strains in Central-Eastern Europe, for many years, has indicated the dominance of RT027, which is capable of producing binary toxin, in addition to toxin A and B. This study is aimed to analyze the occurrence of *C. difficile* strains of different ribotypes (including RT955, an emerging PCR ribotype) capable of producing binary toxins, based on materials obtained from patients hospitalized in three hospitals in Silesia in Southern Poland.

Material and methods: 183 stool samples were selected for this study. Samples were obtained from patients with clinical symptoms of antibiotic-associated diarrhea. Patients were hospitalized in three multi-specialist hospital. The samples were diagnosed in hospital laboratories by detection of GDH and toxins. They were cultured on selective media and then incubated anaerobically (Whitley A35 Workstation, UK). Multiplex PCR (mPCR) was used for the detection of genes encoding GDH (gluD), toxins A (tcdA) and B(tcdB) and binary toxin (cdtA/cdtB) in *C. difficile* isolates. Ribotyping of *C. difficile* was performed at Leiden University Medical Center, Charles University, and Medical University of Warsaw.

Results: Among the studied *C. difficile* strains, 126 (68.9%) had binary toxin, of which 98 (77.8%) were classified as RT027, 16 (12.7%) as RT955, 6 (4.8%) as RT023 and 2 (1.6%) as RT078. The remaining four strains were unclassified (3.1%). The emerging ribotype RT955 appeared in three different hospitals: 7/120 in the multi-specialist hospital (5.8%), 8/39 in the pulmonology hospital (20,5%), and 1/24 in the municipal hospital (4.2%).

Conclusions: RT027 still dominates in the Silesia region, but new ribotypes producing binary toxins, such RT955, is evident. This situation requires increased efforts from teams dealing with hospital-acquired infections (HAI) and the implementation of all corrective procedures, including an increased intensity of epidemiological surveillance and procedures for controlling the hospital environment.

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CLOSTRIDIOIDES DIFFICILE IN GB PIGS AT FARM AND AT SLAUGHTER

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Background and Aims: *Clostridioides difficile* is an emerging One Health pathogen found in the environment, in both healthy and diarrhoeic pigs and may pose a risk to people via the food chain. There is limited information on *C. difficile* in pigs and pork in Great Britain (GB), where approximately 40% of commercial pig farrowing herds are outdoors. This study aims to determine the prevalence of *C. difficile* in GB pig farms and abattoirs, to examine the diversity of isolates by ribotyping and whole genome sequencing, and ultimately improve our understanding of pathogen transmission.

Methods: Since Autumn 2023, pens with sows and piglets <1 week old in indoor and outdoor farms, and abattoirs have been sampled. Farm samples collected were indoor pen floor faeces, outdoor pen straw bedding, and soil and water. Abattoir samples collected were scald tank water, caecal contents and, carcass swabs. Isolation of *C. difficile* from samples was by direct and enrichment culture, and confirmation by MALDI-TOF mass spectrometry. Toxin genes were detected by multiplex PCR in selected isolates and then ribotyped.

Results: Nine abattoirs and nine farms have been sampled. For farms, 72.8% (67/92) indoor faecal, 82.7% (48/58) outdoor straw bedding, 84.7% (50/59) soil and 11.1% (6/54) water samples were positive for *C. difficile*, with 38/40 selected isolates being toxigenic. In abattoirs, 28% (6/21) scald tank water, 2.5% (9/360) caecal, and 1.6% (6/362) carcass swab samples were positive for *C. difficile* with 9/9 selected *C. difficile* isolates being toxigenic. The main ribotype was 078, followed by 002, 023, 087, 081, 015 and 014.

Conclusions: Preliminary farm prevalence, abattoir prevalence and pig prevalence from abattoirs are similar to reports from Australia, United States, and European countries. RT 078 was the most prevalent, similar to other reports of *C. difficile* in pigs and pork. RT 014, which was abundant in pigs and pork elsewhere, is less prevalent here. In GB, *C. difficile* appears to pose a low risk to food safety,but its prevalence on pig farms and the surrounding environment is worthy of further investigation. .

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THE RECOGNITION AND CHARACTERISATION OF *C. DIFFICILE* RIBOTYPE 955 IN POLAND

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Background: The epidemiology of *Clostridioides difficile* infections (CDI) has changed dramatically after the emergence of hypervirulent strains belonging to ST1, Clade 2, such as PCR ribotype (RT) 027. On January 29th 2024, an EpiPulse notification on a *C. difficile* outbreak of new RT955 in England was distributed by ECDC. Because this RT is suggested to be genetically related to RT027, we initiated a retrospective surveillance study to search for this ribotype in the *C. difficile* collection held by Motol University Hospital Prague that includes Czech (n=>8000), Polish (n=>378) and Slovak (n=>400) isolates.

Material and Methods: The ribotyping profiles with "unknown ribotype" and the presence of binary toxin genes were re-analyzed in the WEBRIBO database where the profile of RT955 was recently added. Identified RT955 isolates were tested for antimicrobial susceptibility to 15 antimicrobials (E-test, Brucella agar, fidaxomicin by agar dilution using Wilkins Chalgren) and sequenced (Illumina NextSeq2000 (all), GridION (n=1).

Results: Thirteen RT955 CDI cases (between 2021 and 2022) from three different Polish hospitals (cities) were found. The average age of patients was 74 years (ranged 35 – 98), eight were male. Clinical data were available from 10 patients (77%); five cases were a healthcare-associated origin, five patients had recurrent CDI, and two patients died.

All isolates belonged to ST1, Clade 2. One clonal complex (0-3 allele differences) consisting of 10 isolates including UK strain ERR12670107, was identified by wgMLST (Bionumerics, 3279 loci). All tested isolates were phenotypically resistant to ciprofloxacin/moxifloxacin with the presence of T82I in the GyrA, to rifampicin with R505K and V134I in the RpoB and to erythromycin/clindamycin mediated by the ermB gene. Two isolates were phenotypically resistant to metronidazole, but the recently described PnimBG mutation, plus Y130S, L155I in NimB, were found in all isolates and the UK reference strain. Plasmid pCD-METRO was not detected.

Conclusion: The recently recognised *C. difficile* ribotype 955 was found in three hospitals in Poland. The isolates were clonally related to the UK strain. Because the details of the UK strain are unknown, we cannot conclude whether the origin of RT955 is in the UK or Poland.
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IMPACT OF EARLY ANTIBIOTIC TREATMENT AND PROBIOTICS ON *C. DIFFICILE* COLONIZATION IN NEONATES

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Background: *Clostridioides difficile* is often found in asymptomatic children (≤ 2 years). Our study examined the gut microbiome and *C. difficile* prevalence in neonates treated with antibiotics within the first 3 weeks of life.

Methods: Samples of 76 neonates treated with antibiotics within the first 3 weeks of life and receiving the probiotics were included in the study. No confirmed cases of *C. difficile* infection were reported, subsequently we consider all positive samples in this study to be asymptomatic colonization. *C. difficile* concentration was obtained with species-specific ddPCR, bacterial community structure was assessed by 16S amplicon sequencing of the V3V4 variable region.

Results: Stool samples collected approximately one year post-treatment revealed a 36.8% colonization rate (28/76) with *C. difficile.* Microbiome analysis showed no significant differences in alpha or beta diversity between *C. difficile* colonized and non-colonized samples. However, strong associations were found between microbiota composition and *C. difficile* concentration. Higher *C. difficile* concentrations correlated with increased distance from group average based on Bray-Curtis dissimilarity (Pearson's $r = 0.761$, $p < 0.001$), which was predominantly linked to alterations in alpha diversity. Decreases in richness and diversity were observed with increasing *C. difficile* concentration, most significantly with Faith's phylogenetic diversity (Pearson's $r = -0.625$, $p < 0.001$). Additionally, *C. difficile* concentration correlated positively with Escherichia coli relative abundance (Pearson's $r = 0.811$, $p < 0.001$). Combination of probiotics and antibiotic treatments (in addition to primary treatment) significantly impacted microbiota composition ($p = 0.049$) and lead to elevated *C. difficile* concentrations, however the increase was not significant. Extensive metadata analysis revealed no significant risk factors for *C. difficile* colonization.

Conclusions: While *C. difficile* colonization did not significantly alter microbiome structure, its concentration correlated with microbiome composition and diversity. Probiotics combined with antibiotics also influenced the microbiome, warranting further investigation into their role in *C. difficile* colonization in neonates.

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MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIOIDES DIFFICILE* IN SLOVENIA, 2022 AND 2023

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In Slovenia, reported infections caused by *C. difficile* (CDI) have been stable over the last years, with the 5-year average incidence of 29.8 per 100.000 population. Alongside the CDI surveillance coordinated by the ECDC, we have implemented molecular surveillance of circulating *C. difficile* strains. For this, diagnostic laboratories are requested to submit all *C. difficile* isolates or positive stool samples obtained over a three-month period to the National Laboratory for Health, Environment, and Food. All isolates undergo PCR ribotyping (RT) and toxinotyping, selected isolates are also subjected to whole genome sequencing.

Here we present data of the molecular *C. difficile* strain surveillance for years 2022 and 2023 (September – November).

A total of 330 isolates from 279 patients (median age 69, >1 to 97 years, 51 % female) were submitted. Of these, 292 isolates (only the first isolate per RT per patient and study year) were included in the analysis, with 131 isolates from 2022 and 161 from 2023.

A variety of RTs (n=68) were identified, with 34 represented by a single isolate. The six most common RTs, accounting for over half of all isolates (52%) in both years, were 014/020 (19.5%), 255/258 (7.9%), 011/072 (7.9%), 002 (7.5%), 011/049 (5.1%), and 070 (4.1%). No significant change in RT prevalence was observed between the two years. Forty-nine RTs were toxigenic, belonging to nine different toxinotypes (CDT-negative toxinotypes 0, I and XII, and CDT-positive 0/v, III, IV, V, VI and XIV). Additionally, 19 RTs were non-toxigenic, with two belonging to cryptic clades C-I (RT SLO106/MLST-ST1188) and C-III (SLO204/ST344).

Selected RTs, represented by multiple isolates, were subjected to whole genome sequencing to determine clonality based on clustering with cgMLST. Twenty-eight clusters (cluster distance threshold < 7 loci) were identified among 222 isolates, 115 isolates were singletons. The largest MST cluster included 18 of the 22 sequenced RT255/258 isolates, originating from eight different healthcare facilities.

The stable incidence of CDI in Slovenia is accompanied by diverse strain circulation, a significant decline in RT027 prevalence (from 17 % in 2016-19 to 1 % in 2022-23), and a notable increase in RT255/258 (2% in 2016-19 to 8% in 2022-23), highlighting the importance of ongoing surveillance and genomic analysis to manage and control potential transmissions.

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PRE-CLINICAL DEVELOPMENT OF AJ-024, A NOVEL CLASS OF ANTIBIOTICS AGAINST *C. DIFFICILE*

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Despite *C. difficile* being listed as the urgent threat by the US Centers for Disease Control and Prevention (CDC), current treatment options are limited to fidaxomicin and vancomycin, each with limitations that call for a more effective antibiotic. Vancomycin is associated with a high relapse (30%) due to its severe impact on gut microbiota. While fidaxomicin reduces recurrence down to 20%, it exhibits diminished efficacy against emerging hypervirulent *C. difficile* strains, such as ribotype 027 and 078. We have identified AJ-024, a novel class of antibiotics, that showcases elevated in vitro activity compared to vancomycin and fidaxomicin against clinical isolates collected and extensively classified in South Korea. Moreover, AJ-024 is rapidly bactericidal against hypervirulent *C. difficile* ribotype 027 and exhibits a prolonged post-antibiotic effect (PAE) compared to fidaxomicin. AJ-024 shows no recurrence and a superior overall survival rate in a mouse in vivo model. These results have been corroborated by extensive 16s rRNA and shotgun sequencing data. Therefore, AJ-024 possesses distinctive advantages that could potentially change the landscape of the current CDI market. Currently, AJ-024 is undergoing GLP-level toxicology studies and we expect to initiate Phase I clinical study in 2025.

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S-LAYER MEDIATED *C. DIFFICILE*-HUMAN EPITHELIEUM INTERACTIONS

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Background: Many bacteria are coated with a proteinaceous surface layer - S-layer - formed by the self-assembly of monomeric proteins into a regularly spaced, two-dimensional array. S-layers are implicated in growth and survival, cell integrity, enzyme display and, in pathogens and commensals, interaction with the host and its immune system.

In *C. difficile*, the principal S-layer protein, SlpA, displays considerable sequence diversity between strains, with 13 antigenically distinct types distributed across clades and associated with variable disease profiles1. We recently determined the structure of SlpA, the main S-layer protein in *C. difficile*, and an S-layer assembly model characterised by a tightly packed array2. Importantly, SlpA-deficient strains fail to cause disease in hamsters3 and spontaneous variants with a restored slpA gene quickly outcompete an SlpA-deficient strain in a mouse model4.

Methods: Despite its critical role in pathogenicity, including in colonisation and activation of inflammatory, immune responses, the details of S-layer-host interactions are still poorly understood. We have recently adapted a co-culture system that recapitulates the oxygen gradient across the epithelium5, allowing anaerobic conditions for *C. difficile* growth and aerobic media for the human-derived intestinal organoid (HIO) monolayer. In this pilot study, HIOs were co-cultured with CD630 and R20291 strains carrying two different SlpA types, (SLCT7 and SLCT4, respectively), and an SlpA-deficient mutant (FM2.53).

Results: Contact with HIOs increases sporulation, particularly in the SlpA-deficient strain. Conversely, proinflammatory cytokine IL-18 is only induced in the presence of *C. difficile* cells with a functional S-layer. Dual RNA sequencing data indicates that the presence of SlpA induced a differential response from the HIO and has considerable implications in bacterial transcription when in contact with host cells.

Conclusions: SlpA mediates the interactions between *C. difficile* and the host epithelium and further work is planned to elucidate the molecular details of these responses.

1 Dingle et al. J. Infect. Dis. 207, 2013;2 Lanzoni-Mangutchi et al. Nat. Commun. 2022 131 13, 1–13, 2022;3 Kirk et al. Sci. Transl. Med. 9, eaah6813, 2017;4 Ormsby et al. PLOS Pathog. 19, e1011015, 2023;5 Stewart et al. Methods Mol. Biol. 2121, 185–198, 2020

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EVALUATION OF CLINICAL PERFORMANCE OF A NEW MOLECULAR POINT-OF-CARE ASSAY FOR DETECTING *C. DIFFICILE*

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Background: *Clostridioides difficile* (*C. difficile*) is a significant cause of healthcare-associated infections, leading to conditions ranging from mild diarrhea to severe colitis. Accurate and timely diagnosis of *C. difficile* infection (CDI) is critical for patient management and infection control. Traditional diagnostic methods face challenges, including delayed results and the need for specialized laboratory facilities. Point-of-care (POC) assays offer a promising alternative, potentially enabling rapid and accurate diagnosis

Methods: The clinical performance of a new molecular POC assay, Vivalytic *C. difficile*, was evaluated using 124 liquid or soft human stool samples from two study sites. At MVZ Labor Dr. Limbach & Kollegen GbR in Heidelberg, Germany, 44 samples (21 positive and 23 negative) were tested. At aprimeo diagnostics GmbH in Pfungstadt, Germany, 80 samples (39 positive and 41 negative) were tested. A total of 122 valid samples were included in the analysis, with the reference methods RIDA®GENE Clostridium difficile (R-Biopharm AG). Discrepant results were resolved with Allplex™ GI-Bacteria(I) assay (Seegene) and Xpert® *C. difficile* BT assay (Cepheid)

Results: The Vivalytic *C. difficile* assay showed 12 initial discrepant results compared to the reference tests. Upon retesting these samples with the reference methods, 5 discrepancies remained: 3 false positives and 2 false negatives. The assay demonstrated a Positive Percent Agreement (PPA) of 96.61% and a Negative Percent Agreement (NPA) of 95.24%.

Conclusion: The Vivalytic *C. difficile* assay exhibits high sensitivity and specificity, making it a viable option for rapid diagnosis of CDI. Its implementation could enhance clinical decision-making and infection control practices by providing timely results without the need for specialized laboratory infrastructure.

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CLOSTRIDIOIDES DIFFICILE INCREASES UNDECAPRENYL PYROPHOSPHATE RECYCLING AND DRUG EFFLUX IN RESPONSE TO IRON STARVATION

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Clostridioides difficile infection (CDI) is the leading nosocomial infection in the United States and an urgent threat to public health. CDI onset begins with *C. difficile* outcompeting both the host microbiota and the innate immune response for limited nutrients. A critical factor in the host immune response to CDI is the innate immune protein calprotectin (CP) that chelates essential nutrient metals from the pathogen through a process termed nutritional immunity. CP is essential for the host to combat CDI, yet how *C. difficile* overcomes CP to acquire nutrients is not well understood. To uncover how *C. difficile* responds to nutritional immunity, we evaluated the transcriptional changes that *C. difficile* undergoes when challenged with CP. We identified a putative two-component system (TCS), 2822 and 2823, to be transcriptionally increased in the presence of CP and iron chelators. We found 2822/2823 regulates three genes immediately downstream: 2821, 2820, and 2819. Based on bioinformatic predictions, 2820 and 2819 encode an ATP driven efflux pump, and 2821 encodes an undecaprenyl pyrophosphatase. Further experiments revealed that 2822/2823 is activated by the cell surface targeting antibiotic bacitracin, and mutants lacking the TCS are extremely sensitive to bacitracin in both in vitro and in vivo. Our results support a model in which *C. difficile* overcomes nutritional immunity by coordinating an increase in undecaprenyl pyrophosphate recycling and drug efflux to defend against external threats such as cell envelope targeting antimicrobials.

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FECAL MICROBIAL TRANSPLANTATION IN PROTECTION FROM RECURRENT *C. DIFFICILE* INFECTION

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Background and Aims: 20-30% of patients with a primary episode of *Clostridioides difficile* infection (CDI) experience recurrence. Fecal microbial transplantation (FMT) is currently the most effective therapy for recurrent CDI (rCDI). Previous work with a mouse model in our lab identified a role for FMT-induced IL-25 in protection from CDI. A pilot study on patients undergoing FMT for rCDI found that FMT induces IL-25 associated Type 2 immunity in the colon; our current study seeks to extend these findings and more completely define features of the protective mucosal immunity.

Methods: Colon biopsies, stool, and peripheral blood samples were collected from patients (N=19, 74% female) undergoing FMT for rCDI. Samples were also collected for 16 of the patients at a two-month follow-up. Bulk RNAseq of colon biopsies was carried out to determine broad changes in the intestinal tissue following FMT. Plasma antibodies to Toxin B (TcdB) were assessed by ELISA and toxin neutralization assays. Investigation of colon tissue by immunofluorescence microscopy and spatial transcriptomics, and of stool and peripheral blood for soluble and cellular biomarkers is ongoing.

Results: FMT was effective in preventing recurrence out to two months in all cases. PCA analysis of RNAseq results identified distinct colonic transcriptional profiles post-FMT with 3,677 differentially expressed genes between groups. Significantly up-regulated genes included those associated with Type 2 immune responses as well as genes associated with tissue remodeling and resolution of inflammation. Gene pathways associated with cell proliferation, including DNA replication, translation, and protein processing and export, were up-regulated post-FMT. No significant changes were observed in anti-TcdB antibody levels or neutralization capacity, indicating that antibodies did not provide sustained protection from rCDI.

Conclusions: These data suggest that FMT therapy works primarily by promoting regeneration of the intestinal barrier. Ongoing work seeks to characterize epithelial and immune-specific pathways that promote these recovery processes and operate in conjunction to protect from recurrence.

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IS GROWTH OF *CLOSTRIDIOIDES DIFFICILE* LIMITED TO THE HOST?

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Clostridioides difficile is predominantly recognized as a nosocomial pathogen, yet recent studies have demonstrated its prevalence in diverse environments, suggesting that these may act as reservoirs and potential sources of transmission to clinical settings. As a spore-forming obligate anaerobe, *C. difficile* can persist in the environment until encountering a suitable host with specific conditions conducive to its growth. Germination of *C. difficile* spores is known to be triggered by signals such as bile acids, amino acids like L-glycine and L-histidine, and optimal pH (6.5–8.5) and temperature (37–40°C). However, physiological studies to date have focused exclusively on clinically relevant strains. Environmental *C. difficile* strains, particularly those from cryptic clades, remain largely unexplored in terms of their physiological capabilities. Genomic analysis has shown that these cryptic strains are genotypically distinct from the main *C. difficile* clades, featuring clade-specific genetic markers and exhibiting relatively low average nucleotide identity. It remains unclear whether this genetic diversity and evolutionary divergence enable these strains to metabolize or proliferate under non-host conditions. To address this gap, we investigated whether spores from two *C. difficile* strains could germinate and grow in a non-host environment, specifically sterilized compost. Over a 20-day period, we monitored their growth using both culture-dependent and -independent methods. The two strains studied were isolated from non-host environments but were genetically aligned to clade 1 and cryptic clade I. Remarkably, both strains demonstrated significant growth, with a 100-fold increase in colony-forming units (CFU) and more than a 3-fold increase in 16S rRNA gene copy numbers. These findings suggest that non-host environments can indeed induce *C. difficile* spore germination and support subsequent growth, irrespective of their clade affiliation.

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CRS3123: A NARROW SPECTRUM AGENT FOR TREATMENT OF CDI

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CRS3123 is a methionyl-tRNA synthetase inhibitor in development for treatment of *Clostridioides difficile* Infection (CDI). It exhibits excellent biochemical potency (Kd = 20 pM vs *C. difficile* MetRS) and is highly selective for Type 1 MetRS. This target selectivity imparts an exceedingly narrow spectrum which spares commensal gut flora while markedly inhibiting *C. difficile* growth, toxin production and spore formation. Target selectivity furthermore contributes to its excellent safety profile in Phase 1 and Phase 2 human clinical studies. CRS3123 inhibits formation of methionyl-adenylate and demonstrates competitive inhibition with the methionine substrate and uncompetitive (co-operative) inhibition with the ATP substrate. We have solved the three-dimensional structure of CRS3123 and a non-hydrolyzable ATP analog bound to CDMetRS. The ternary complex elucidates the structural basis for the narrow spectrum of CRS3123 and features an induced fit spanning two adjacent hydrophobic binding pockets on MetRS. CRS3123 showed minimal perturbation of normal gut flora in healthy human subjects in Phase 1 studies. In recently completed Phase 2 studies, CRS3123 showed promising results with comparable clinical cure rates at the test of cure visit at day 12 in all three treatment groups in the Intent to Treat population, including 28/29 (97%) in patients receiving one of two dosage levels of CRS3123 versus 13/14 (93%) in those receiving vancomycin. In addition, CRS3123 exhibited exceptionally low rates of recurrence. We will present an update on this novel agent focusing on unpublished data ranging from the structural underpinnings of narrow spectrum to exciting topline data from Phase 2.

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FIT-FOR-PURPOSE BINDING PROTEINS FOR IN SITU BLOCKING OF *C. DIFFICILE* TOXINS

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Background and Aims: *Clostridioides difficile* infection (CDI) is a severe gastrointestinal infection affecting individuals with a dysbiotic gut microbiota, often following antibiotic treatment. Bacterial spores germinate and secrete cytotoxins A (TcdA) and B (TcdB), causing severe tissue damage. Since *C. difficile* colonizes the distal colon, oral delivery of antibody-based toxin inhibitors could be advantageous for early toxin neutralization, before disease onset, protecting gut barrier integrity until the healthy microbiota is restored. Through a developability selection process we have engineered BL5-6.2, a specific binding protein comprising two monomeric single-domain antibodies (V Hs). BL5-6.2 demonstrates high functional stability and effectively neutralizes TcdB cytotoxicity in simulated gut conditions. Furthermore, we investigate the neutralizing capacity of BL5-6.2 across various cell models to understand its capacity to protect cell barrier integrity. Additionally, we evaluated the protective effect a newly developed TcdA blocking V H constructs, BL7.1 and BL8.1 in various cell lines.

Methods: Mammalian cell lines show differential susceptibility to TcdA and TcdB cytotoxicity. Therefore, multiple cell models were employed to assess the blocking capacity of binding proteins against these toxins. Neutralization assays for TcdB were conducted using two human colon adenocarcinoma cell lines: HCA7 and differentiated Caco2 epithelial colon cells. Additionally, the anti-inflammatory effect BL5-6.2 by quantifying IL-8 released. Neutralization assay for TcdA were performed using a high susceptible TcdA non-human cell line (Vero) and compared with human colon cell Caco2.

Results: We observed that both the TcdA and TcdB blocking binding proteins efficiently neutralized cytotoxicity in vitro in differently across the colonic cell lines, ranging from 80-100% neutralization in a dose dependent manner. Further, BL5-6.2 construct reduces the release of inflammatory marker IL-8 to 110 pg/ml compared with the V H benchmark of 224 pg/ml in CACO2 cell.

Conclusions: We show that employing specific binding proteins, comprised of V H constructs, we can effectively block TcdA and TcdB cytotoxicity in vitro across several colonic cell lines and models emulating epithelial barrier function. The binding proteins are suitable for oral use and may show applicability for in situ toxin blocking to protect the colon in dysbiotic individuals exposed to *C. difficile.*

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CLOSTRIDIOIDES DIFFICILE THIOURACIL DESULFURASE MAINTAINS PATHOGEN RNA INTEGRITY PROVIDING A COMPETITIVE ADVANTAGE IN THE VERTEBRATE GUT

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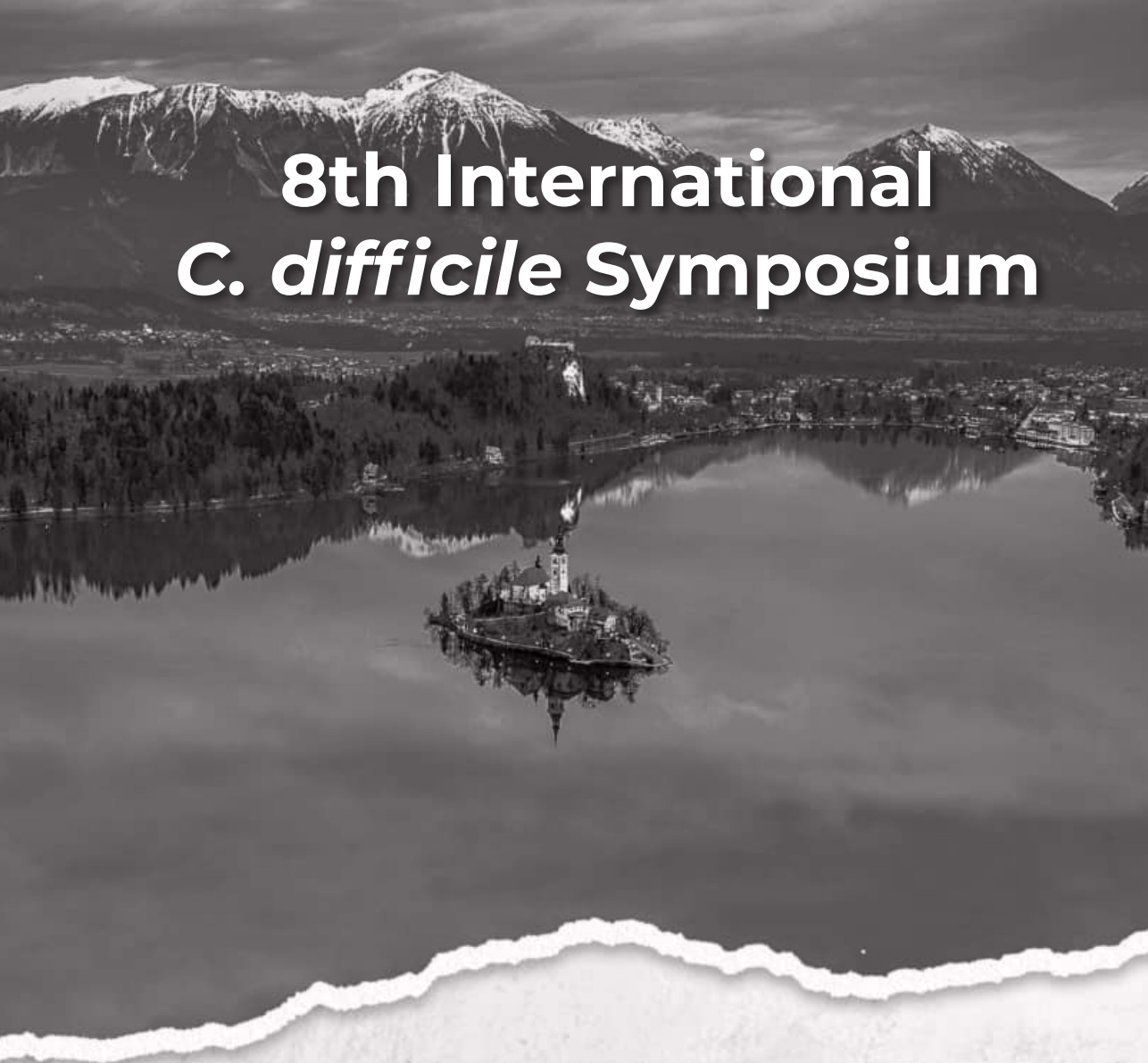
Clostridioides difficile is the leading cause of nosocomial infectious diarrhea, and faces competition for nutrients from both the microbiota and immune system during infection. Amongst these nutrients are nucleobases, and how nucleobase acquisition influences *C. difficile* infection is not well understood. Here, we show that pyrimidine nucleobase acquisition is critical for *C. difficile* pathogenesis, and that *C. difficile* can metabolize 4-thiouracil (4-TU), a uracil analog that is present in the human gut and is toxic to commensal bacteria. We report the discovery of a thiouracil desulfurase encoded by *C. difficile*, TudS, that protects *C. difficile* from 4-TU toxicity and enables utilization of 4-TU as a nutrient. Based on the structural similarity between 4-TU and uracil, we hypothesized that 4-TU is toxic due to misincorporation into RNA. Indeed, we discovered that 4-TU is incorporated into RNA in the absence of TudS. To investigate the mechanism of 4-TU incorporation into RNA, we conducted a genetic selection to identify mutations that confer resistance to 4-TU. We discovered that mutations in uracil phosphoribosyltransferase (Upp), a component of the uracil salvage pathway, and the regulator of pyrimidine biosynthesis (PyrR) confer resistance to 4-TU, and reduce 4-TU incorporation into RNA. Furthermore, recombinant Upp and PyrR can use 4-TU as a substrate, leading to incorporation into RNA. We show that TudS is critical for *C. difficile* fitness in the presence of 4-TU in minibioreactor arrays containing complex human microbial communities, and contributes to pathogenesis in the vertebrate host harboring 4-TU. In summary, our results reveal a molecular mechanism for the metabolism of a toxic uracil analog that influences host-pathogen-commensal interactions in the vertebrate gut.



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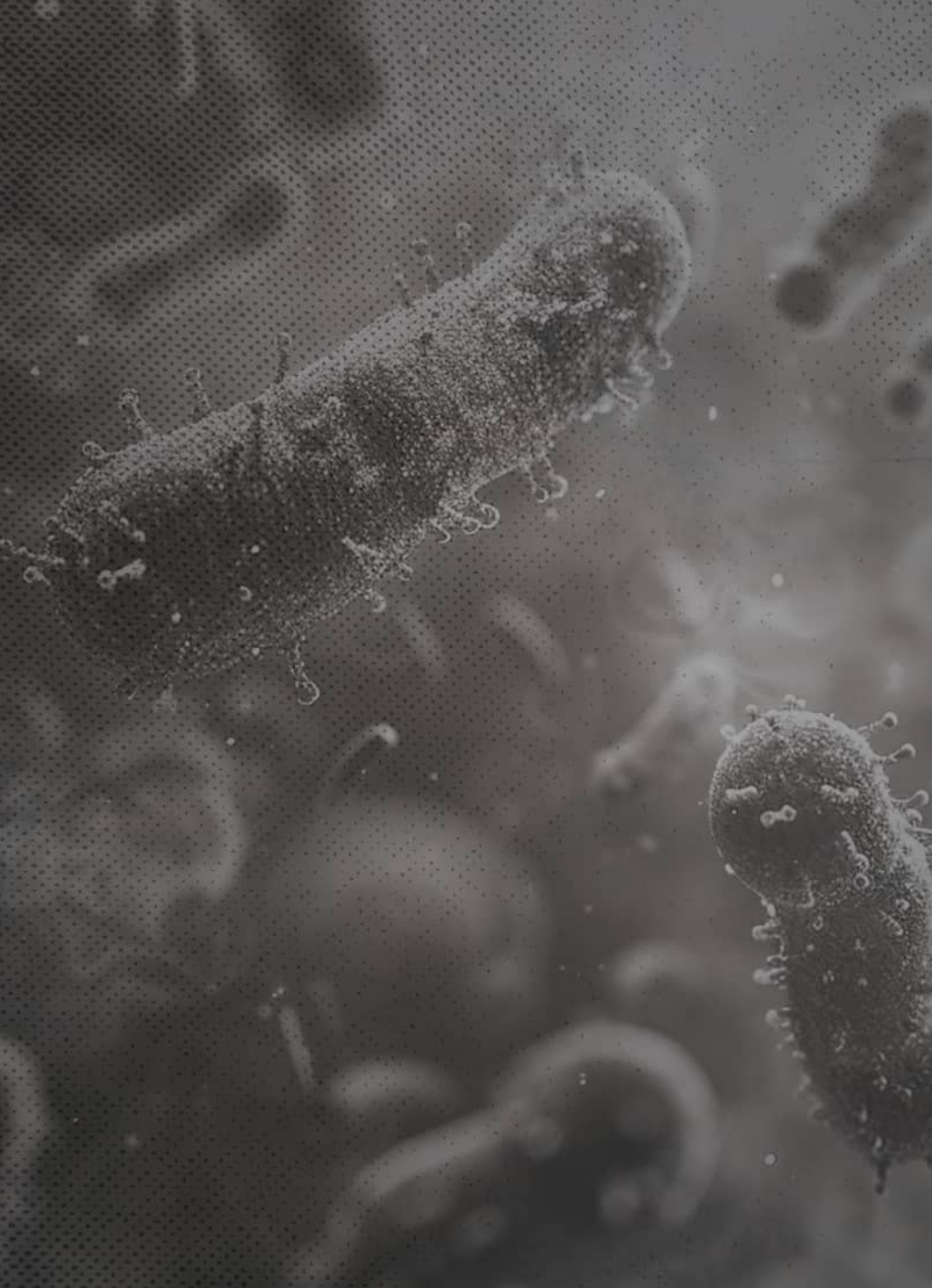

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