4th iCDS

International *Clostridium difficile* Symposium

abstract book

September, 20th to 22nd 2012, Bled, Slovenia

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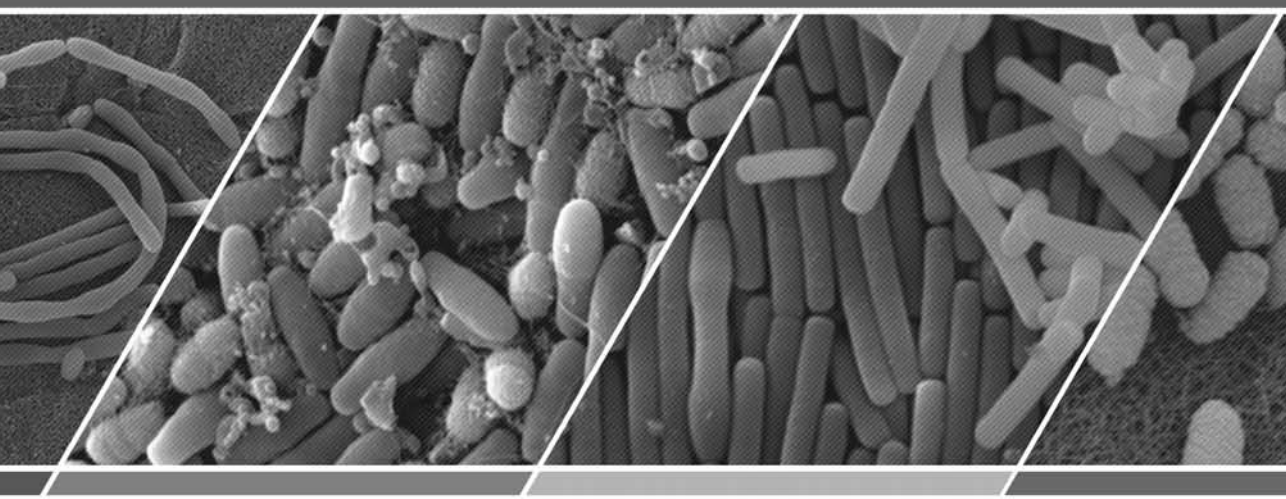


abstract book

with support of ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

4th International Clostridium difficile Symposium
Abstract book

Zbrali in uredili: Maja Rupnik
Sandra Janežič
Oblikovanje in prelom: Uroš Zupančič
Založil: Zavod za zdravstveno varstvo Maribor
Naklada: 300 kos

CIP - Kataložni zapis o publikaciji
Narodna in univerzitetna knjižnica, Ljubljana

579.852.13(082)
616.98:579.852.13(082)

INTERNATIONAL Clostridium Difficile Symposium (4 ; 2012 ; Bled)
Abstract book / 4th ICDS - International Clostridium Difficile Symposium, September, 20th to 22nd 2012, Bled, Slovenia ; [zbrali in uredili Maja Rupnik, Sandra Janežič]. - Maribor : Zavod za zdravstveno varstvo, 2012

ISBN 978-961-90895-6-9
1. Rupnik, Maja, 1967-
263192832

ICDS would like to acnowledge the contributions of

CONTENTS

WELCOME

Dear friends and colleagues,

It is a great pleasure to welcome you to the Fourth International Clostridium difficile Symposium.

Clostridium difficile continues to be one of the most important human health-care associated pathogens, and its role in community-associated infections and in animal disease is being more widely recognised. Following some success in preventing the infection in parts of the world where Clostridium difficile infection (CDI) was unacceptably high, it has begun to decline. However, in other parts of the world it is still increasing, or just being recognised as a problem.

We hope that the programme of the 4th ICDS reflects the range of research that is currently addressing the main biological, clinical and veterinary issues of CDIs. The major targets remain the prevention, epidemiology, control and diagnosis of disease, especially of recurrent infections and of outbreaks. The importance of the host has long been recognised as crucial in the susceptibility to infection and research emphasis on pathogenesis and immunology are increasing. Similarly, spores, the infectious agent in CDI are also being investigated in much more detail. Major advances in the genetics of C. difficile have been made, with genetic manipulation becoming generally available and many genome sequences have now been published. We hope, by the end of the meeting participants will be fully updated in all important areas of C. difficile research.

ICDS is established as the traditional C. difficile meeting internationally and the venue is also traditionally located in Slovenia. Following the popularity of Bled in 2010, we are here again (Kranjska Gora in 2004; Maribor 2007). The Organizing Committee hopes that you will enjoy the meeting and that it will stimulate new ideas and new collaborations.

On behalf of the Organizing Committee

Maja Rupnik

GENERAL INFORMATION

Congress venue

Hotel Golf, Bled, Slovenia

Registration and information desk opening hours

Wedneday 19th September	17.00 to 18.30
Thursday 20th September	10.00 to 14.30
Friday 21st September	8.00 to 8.30 and during lunch breaks and coffee breaks
Saturday 22nd September	8.00 to 8.30 and during lunch breaks and coffee breaks

Meals and Social Events

Lunch on Friday, 21st September, and Saturday, 22nd September and **Dinner** Friday, 21st September are included in the Registration. Meals will be served in the Restaurant of the Hotel Golf. **Coffee Breaks** will be served in front of the lecture hall and after the lunch in the poster area. **Welcome reception**, Thursday, 20th September, is included in Registration and will be held in the area in front of Hall Jupiter. **Congress dinner**, Saturday, 22nd September, is included in Registration and will be held in Grand Hotel Toplice.

Concert

On Wednesday, 19th September a half hour concert of Zither duo Fantasia is organized in Hotel Golf, Hall Jupiter at 18.30. Admission is free.

Badges

Name badges are requested during all congress scientific sessions as well as during social events.

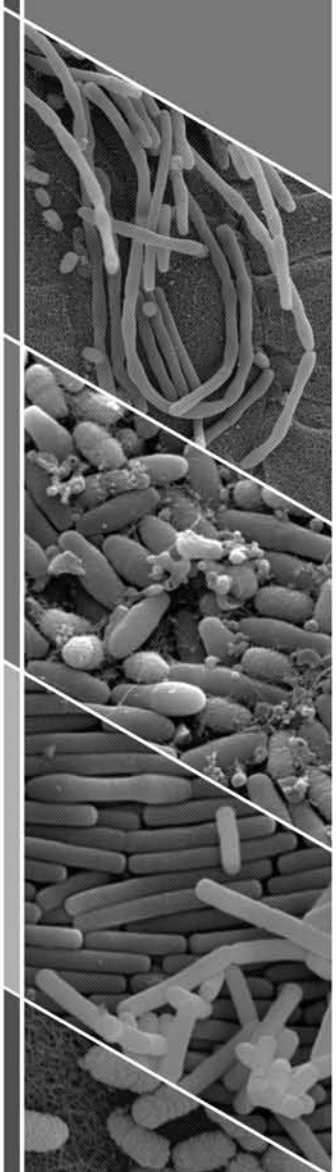
For Speakers

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

For poster presenters

Posters will be displayed in two sessions. Distribution into the sessions is given in the Poster overview table. Posters for Session I can be displayed from Thursday, 20th September morning and should be removed on Friday 21st September afternoon coffee break (16.30 to 17.00). Posters for Session II should be displayed from Friday 21st September afternoon coffee break (16.30 to 17.00) and removed after the last session on Saturday 22nd September.

4th International *Clostridium difficile* Symposium



Invited Speakers

INVITED SPEAKERS

Carroll Karen *(Johns Hopkins University School of Medicine, Baltimore, USA)*

Dingle Kate *(Oxford University, Oxford, United Kingdom)*

Donskey Curtis *(Cleveland VA Medical Center, Cleveland, USA)*

Henriques Adriano *(Instituto de Tecnologia Química e Biológica, Oeiras, Portugal)*

Indra Alexander *(AGES-IMED, Vienna, Austria)*

Kuijper Ed *(Leiden University Centre, Leiden, The Netherlands)*

Lacy Borden *(Vanderbilt University School of Medicine, Nashville, USA)*

Mulvey Michael *(Public Health Agency of Canada, Winnipeg, Canada)*

Ross Paul *(Teagasc, Fermoy, Ireland)*

Sansonetti Philippe *(Institut Pasteur / College de France, Paris, France)*

Sonenshein Linc *(Tufts University School of Medicine, Boston, USA)*

Wilcox Mark *(Leeds Teaching Hosptials/University of Leeds, Leeds, United Kingdom)*

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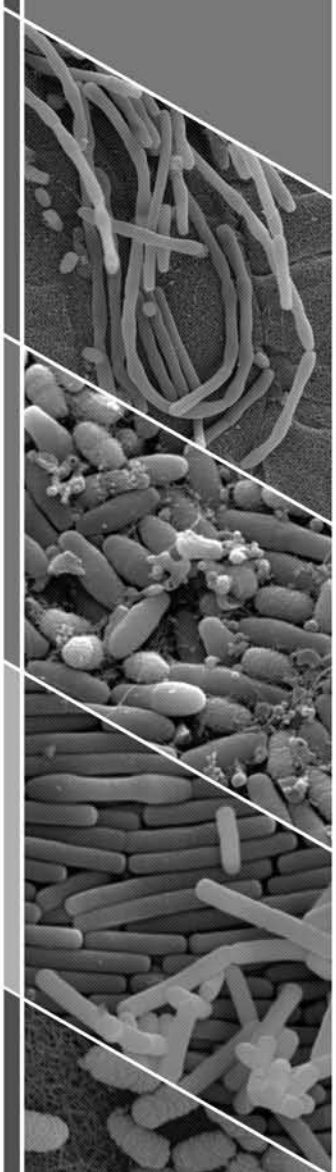

Attendance Grants

The ESCMID Attendance grants

Chilton Caroline *(University of Leeds, Leeds, United Kingdom)*

Crowther Grace *(University of Leeds, Leeds, United Kingdom)*

Di Bella Stefano *(National Institute for Infectious Diseases "L. Spallanzani", Roma, Italy)*

Donahue Elizabeth *(London School of Hygiene and Tropical Medicine, London, United Kingdom)*

El Meouche Imane *(GRAM Laboratory, University of Rouen, Rouen, France)*

Khanafer Nagham *(Lyon 1 University- Edouard Herriot Hospital, Lyon, France)*

Miyajima Fabio *(University of Liverpool, Liverpool, United Kingdom)*

Saujet Laure *(Pasteur Institute, Paris, France)*

Seager Zoe *(Imperial College London, London, United Kingdom)*

Shaw Helen Alexandra *(Imperial College London, London, United Kingdom)*

Wasels François *(Istituto Superiore di Sanità, Rome, Italy)*

Williams Rachel *(UCL Eastman Dental Institute, London, United Kingdom)*

Willing Stephanie *(Imperial College London, London, United Kingdom)*

The ESCMID Study Group for *Clostridium difficile*/ICDS grants

Cafardi Valeria *(University of Rome "La Sapienza", Siena, Italy)*

Dobreva Elina *(National Centre Of Infectious and Parasitic Diseases, Sofia, Bulgaria)*

Jovanovic Milica *(Clinic for Infectious and Tropical Diseases of Clinical Center of Serbia, Belgrade, Serbia)*

Mentula Silja *(National Institute for Health and Welfare (THL), Helsinki, Finland)*

Orfanidou Maria *(General Hospital of Athens G. Gennimatas, Ekali , Greece)*

Papatheodorou Panagiotis *(Albert-Ludwigs-Universität Freiburg, Freiburg, Germany)*

Pereira Fatima *(ITQB-UNL, Lisboa, Portugal)*

Romano Vincenza *(Parthenope University of Naples, Naples, Italy)*

van Eijk Erika *(Leiden University Medical Center, Leiden, The Netherlands)*

The ICDS grants

Ammam Fariza *(Paris Sud 11, Chatenay Malabry, France)*

Bahadur Tej *(All India Institute of Medical Sciences, New Delhi, India)*

Ghose-Paul Chandrabali *(Aaron Diamond AIDS Research Center, New York, USA)*

Moura Ines *(Istituto Superiore di Sanità, Rome, Italy)*

Pecavar Verena *(AGES-IMED, Vienna, Austria)*

Rodriguez Diaz Cristina *(University of Liège Faculty of Veterinary Medicine, Liège, Belgium)*

Senoh Mitsutoshi *(National Institute of Infectious Diseases, Musashimurayama, Japan)*

Sijoria Richa *(Defense Research and Development Establishment, Gwalior, India)*

Tagashira Yasuaki *(Asahi General Hospital, Fuchu-city, Japan)*

Taori Surabhi *(University of Edinburgh, Edinburgh, United Kingdom)*

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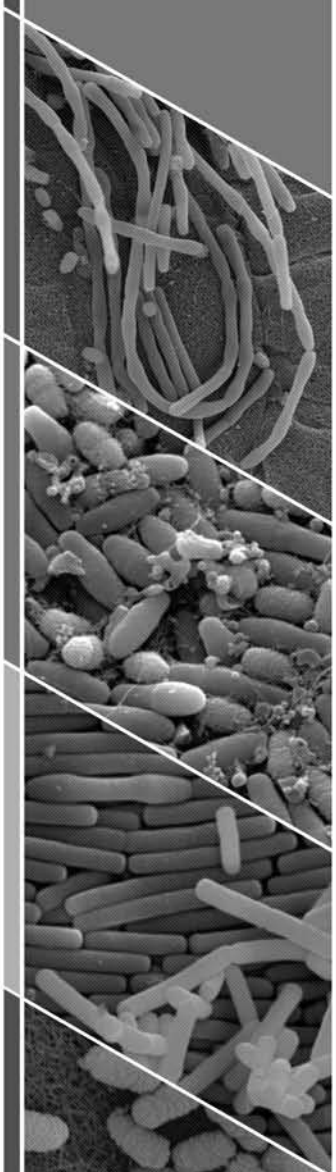

ICDS Programme

Thursday, 20th September

14:45-16:30			**SESSION I: IMMUNOLOGY I** **Chair: P. Mastrantonio**
14:45		Rupnik, M.	OPENING
15:00	INV1	Sansonetti, P.	LEARNING FROM PATHOGENS ORIGINAL STRATEGIES OF IMMUNOMANIPULATION
15:30	O1	Heinrichs, J. H.	DESIGN, PRODUCTION AND PRE-CLINICAL EVALUATION OF A NOVEL TOXIN-BASED VACCINE FOR THE PREVENTION OF Clostridium difficile DISEASE
15:50	O2	Jansen, K. U.	A NOVEL APPROACH TO A C. difficile TOXOID VACCINE: IMMUNOGENICITY AND PRECLINICAL EFFICACY
16:10	O3	Anosova, N.	TOXIN A AND TOXIN B SPECIFIC SYSTEMIC ANTIBODY LEVELS CORRELATE WITH PROTECTION AGAINST C. difficile ASSOCIATED DISEASE IN HAMSTERS
16:30-17:00			COFFEE BREAK

17:00-18:30			**SESSION II: SPORULATION AND GERMINATION** **Chair: N. Minton**
17:00	INV2	Henriques, A. O.	SPORE DEVELOPMENT IN RELATION TO PATHOGENESIS IN Clostridium difficile
17:30	O4	Dembek, M.	TRANSCRIPTIONAL ANALYSIS OF GERMINATON IN Clostridium difficile 630 ENDOSPORES
17:50	O5	Sorg, J. A.	IDENTIFYING FACTORS THAT INFLUENCE Clostridium difficile SPORE GERMINATION
18:10	O6	Cutting, S. M.	THE SPORE COATS OF C. difficile
19:00			WELCOME RECEPTION (Hotel Golf)

Friday, 21st September

8:30-10:00			**SESSION III: PATHOGENESIS AND VIRULENCE FACTORS** **Chair: I. Poxton**
8:30	INV3	Lacy, B.	STRUCTURES AND FUNCTIONS OF Clostridium difficile TcdA and TcdB
9:00	O7	Michell, S.	THE ROLE OF LIPOPROTEINS IN Clostridium difficile ADHERENCE
9:20	O8	Papatheodorou, P.	IDENTIFICATION OF THE HOST CELL RECEPTOR OF Clostridium difficile BINARY TOXIN CDT
9:40	O9	Pich, A.	IMPACT OF CLOSTRIDIAL GLUCOSYLATING TOXINS ON THE PROTEOME OF COLONOCYTES
10:00-10:30			COFFEE BREAK

10:30-11:50			**SESSION IV: IMMUNOLOGY II** **Chair: D. Gerding**
10:30	O10	Staelens, D.	IN VITRO AND IN VIVO CHARACTERIZATION OF NEUTRALIZING MONOCLONAL ANTIBODIES AGAINST Clostridium difficile TOXINS A AND B
10:50	O11	Songane, M.	MECHANISM(S) INVOLVED IN Clostridium difficile-MEDIATED INFLAMMASOME ACTIVATION
11:10	O12	Solomon, K.	HOST INFLAMMATORY AND IMMUNE RESPONSE TO Clostridium difficile INFECTION: ROLE OF BACTERIAL CYTOTOXIN TITRE AND RELATIONSHIP WITH MORTALITY
11:30	O13	Kansau, I.	FLAGELLA AND FLAGELLIN OF C. difficile 027 HYPERVIRULENT STRAIN ACTIVATE NF-κB AND ELICIT IL-8 PRODUCTION IN EPITHELIAL TLR-5-EXPRESSING CELL LINE

11:50-13:00			**POSTER SESSION I (P1-P62)**
13:00-14:00			LUNCH (Hotel Golf)
14:00-15:00			**POSTER SESSION I (P1-P62) and COFFEE BREAK**

15:00-16:30			**SESSION V: COMPARATIVE GENOMICS AND GENETICS** **Chair: S. Weese**
15:00	INV4	Dingle, K.	NOVEL INSIGHTS INTO C. difficile BIOLOGY AND EVOLUTION REVEALED BY WHOLE GENOME SEQUENCING
15:30	O14	Minton, N. P.	WHAT'S A SNP BETWEEN FRIENDS?
15:50	O15	Buckley, A.	WHAT'S LOV GOT TO DO WITH IT?
16:10	O16	Soutourina, O.	EXPLORING sRNAs IN Clostridium difficile BY BIOINFORMATICS AND DEEP-SEQUENCING
16:30-17:00			COFFEE BREAK

17:00-18:30			**SESSION VI: PHYSIOLOGY** **Chair: B. Dupuy**
17:00	INV5	Sonenshein, A. L.	METABOLIC CONTROL OF VIRULENCE IN Clostridium difficile
17:30	017	Williams, R.	THE CONJUGATIVE TRANSPOSON TN5397 INSERTS INTO SPECIFIC SITES IN THE Clostridium difficile R20291 GENOME RESULTING IN SEVERE ATTENUATION OF VIRULENCE IN THE HAMSTER MODEL
17:50	O18	Cartman, S. T.	IS TcdC REALLY A NEGATIVE REGULATOR OF TOXIN PRODUCTION IN Clostridium difficile?
18:10	O19	Shaw, H. A.	INSIGHTS INTO SORTASE IN Clostridium difficile
19:00			DINNER (Hotel Golf)

Saturday, 22nd September

8:30-10:00			**SESSION VII: DIAGNOSTICS/CLINICAL ASPECT/INFECTION CONTROL I Chair: T. Louie**
8:30	INV6	Donskey, C.	INFECTION CONTROL OF Clostridium difficile: WHY ARE WE FAILING AND WHICH SPECIAL APPROACHES COULD HELP US SUCCEED?
9:00	O20	Gupta, S. B.	A LARGE PROSPECTIVE NORTH AMERICAN EPIDEMIOLOGIC STUDY OF HOSPITAL-ASSOCIATED Clostridium difficile COLONIZATION & INFECTION
9:20	O21	Tenover, F. C.	STRAIN TYPES AND RESISTANCE PATTERNS OF Clostridium difficile ISOLATES FROM UNITED STATES HOSPITALS
9:40	O22	Khanafer, N.	HIGH MORTALITY RATE IN ONE YEAR COHORT STUDY AMONG PATIENTS WITH Clostridium difficile INFECTION (CDI) IN A LARGE TERTIARY HOSPITAL
10:00-10:30			COFFEE BREAK

10:30-11:50			**SESSION VIII: DIAGNOSTICS/CLINICAL ASPECT/INFECTION CONTROL II Chair: A. Collignon**
10:30	INV7	Carroll, K.	CLINICAL IMPACT OF MOLECULAR TESTING FOR C. difficile DIAGNOSIS: THERE'S GOOD NEWS AND BAD!
11:00	INV8	Ross, P.	PROBIOTICS, BACTERIOCINS AND BACTERIOPHAGE FOR LIMITING C. difficile INFECTION
11:30	O23	Crowther, G. S.	VALIDATION OF A TRIPLE STAGE IN VITRO HUMAN GUT MODEL TO STUDY THE BIOFILM MODEL OF GROWTH OF C. difficile AND THE INDIGENOUS GUT

11:50-13:00	**POSTER SESSION II (P63-P120)**
13:00-14:00	LUNCH (Hotel Golf) (coffee served in the Poster area)

14:00-15:30			**SESSION IX: ROUND TABLE ON C. difficile TYPING** **Chair: T. Riley and M. Rupnik**
14:00	INV9	Kuijper, E.	DIAGNOSTICS AND TYPING OF Clostridium difficile INFECTIONS IN EUROPE
14:10	INV10	Wilcox, M.	INCREASE USE OF RIBOTYPING - BUT WHERE NEXT?
14:20	INV11	Indra, A.	ADVANTAGES AND DISADVANTAGES OF WEB BASED SYSTEMS TO IDENTIFY DNA PROFILES
14:30	INV12	Mulvey, M.	THE CANADIAN EXPERIENCE WITH MOLECULAR TYPING METHODS FOR C. difficile: PFGE, PCR RIBOTYPING AND GENOME SEQUENCING
14:40			ROUND TABLE DISCUSSION

15:30-17:00			**POSTER SESSION II (P63-P120) AND COFFEE BREAK**

17:00-18:20			**SESSION X: VETERINARY** **Chair: M. Rupnik**
17:00	O24	Riley, T. V.	AN OUTBREAK OF COMMUNITY-ACQUIRED Clostridium difficile INFECTION IN AUSTRALIA, 2011-12
17:20	O25	Knight, D. R.	Clostridium difficile FROM AUSTRALIAN CATTLE - ALL WILL BE RE(VEAL)ED!
17:40	O26	Keessen, E. C.	Clostridium 078 IN PIGS, A THREAT FOR FARMERS, THEIR RELATIVES AND EMPLOYEES
18:00	O27	Weese, J. S.	EFFECT OF OXYTETRACYCLINE ON Clostridium difficile COLONIZATION AND THE FECAL MICROBIOME OF VEAL CALVES
19:30			CONGRESS DINNER (Grand Hotel Toplice)

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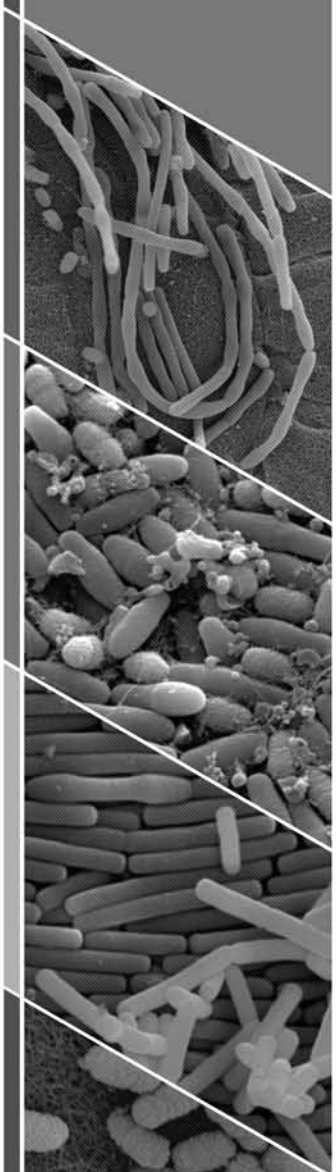

Abstracts of oral presentations

INV1

LEARNING FROM PATHOGENS ORIGINAL STRATEGIES OF IMMUNOMANIPULATION

Sansonetti Philippe and collaborators

Unité de Pathogénie Microbienne Moléculaire, Unité INSERM 78[6], Institut Pasteur, and Collège de France, Paris, France

Shigella, a gram-negative enteropathogenic bacteria causes the rupture, invasion and inflammatory destruction of the human colonic epithelium. It is a major cause of mortality and morbidity among pediatric populations in the most impoverished areas of the planet. The invasive phenotype of Shigella is encoded by a large plasmid encoding a Type III Secretory System (TTSS) and cognate effector molecules that are injected into the membrane and cytoplasm of eukaryotic cell targets. The invasive phenotype is linked to the triggering of a strong inflammatory response elicited in part by activation of the Nod intracellular sensors by bacterial muropeptides. Injected effectors can be subdivided into two major categories (i) Ipa proteins that are mainly involved in the formation of the translocating structure of the TTSS, and triggering of the massive actin cytoskeletal rearrangements that carry out the entry process via macropinocytosis. (ii) Osp and IpaH proteins whose genes are transcribed when the TTSS is functional. These proteins are major regulators of the innate and adaptive immune responses of the mucosa to the invading pathogen. They act very specifically upon key steps of major signalling pathways such as NF-kB and MAPK, through specific enzymatic activities. OspG is a kinase that binds a subgroup of E2 ubiquitine-ligases, thereby blocking ubiquitination of I-kB thus activation of the pro-inflammatory genes, that are under the control of this pathway. OspF reaches the cell nucleus where it dephosphorylates active Erk1/2 and P38, thereby regulating histone phosphorylation, chromatin compaction and the transcription of a set of important pro-inflammatory genes including IL-8. IpaH molecules form a new family of 10 proteins that share a common of E3 ligase activity. We will show how these effectors collectively « carve » a particular profile of immune genes expression in their target cells.

O1

DESIGN, PRODUCTION AND PRE-CLINICAL EVALUATION OF A NOVEL TOXIN-BASED VACCINE FOR THE PREVENTION OF *Clostridium difficile* DISEASE

Jon H. Heinrichs[1], *Su Wang*[1], *Matt Miezeiewski*[2], *Susan Secore*[1], *Andy Xie*[1], *Julie Zorman*[1], *Rachel Xoconostle*[1], *David Thiriot*[1], *Marie-Pierre Gentile*[1], *Adam Kristopeit*[1] *and Aaron R. Goerke*[1]

[1]*Merck Research Laboratories, MSD, West Point, PA and* [2]*Lancaster Laboratories, Lancaster, PA, USA*

The prevention of disease associated with *Clostridium difficile* infection in at risk individuals is a priority as the organism causes substantial morbidity and mortality, particularly in the elderly. The emergence of highly virulent and antibiotic-resistant epidemic strains has resulted in increased disease prevalence, particularly in hospital settings. The production of binary toxin by these epidemic strains has been proposed to contribute to their virulence. We characterized the impact of binary toxin production in a hamster lethal challenge model and demonstrated that immunization of animals with a vaccine containing detoxified TcdA and TcdB was not sufficient to protect these animals from a lethal infection following a spore challenge with an epidemic strain. Therefore, we have developed a four-component vaccine, consisting of TcdA and TcdB as well as CDTa and CDTb, and demonstrated that this vaccine is fully capable of protecting hamsters from a lethal challenge with an epidemic strain. To reduce residual toxicity, we have generated a series of mutations in TcdA, TcdB and CDTa which dramatically reduce reactogenicity in animal models and cell cytotoxicity which is measured in a novel, high-throughput assay. To facilitate production of this vaccine, we have successfully expressed all four components in insect cells using baculovirus transfection. The vaccine is immunogenic in multiple animal species and antisera raised following immunization is capable of neutralizing TcdA, TcdB and binary toxin killing of Vero cells. These data suggest a role for targeting binary toxin in addition to the large clostridial toxins and warrant further evaluation of this vaccine in the clinic.

O2

A NOVEL APPROACH TO A *C. difficile* TOXOID VACCINE: IMMUNOGENICITY AND PRECLINICAL EFFICACY

Johnson J.Erik, S. Megati, V.S. Roopchand, C.S. Kotash, J.H. Obregon, D.M. Illenberger, S.E. Witko, I. Yurgelonis, N.K. Kalyan, L. Chang, K. Xu, J.K. Moran, R.G.K. Donald, A.S.Anderson, M.W. Pride, K.U. Jansen, M.K.Sidhu,

Pfizer Vaccines Research and Early Development, Pearl River, New York, USA

Clostridium difficile (*C. difficile*) toxins, TcdA and TcdB, are the principal virulence factors for *C. difficile*-associated disease (CDAD) and can cause severe inflammation in the bowel. A vaccine that elicits potent antitoxin A and B neutralizing antibodies against a broad spectrum of clinically relevant *C. difficile* isolates is expected to prevent primary CDAD and associated potential recurrence of disease. In this study, we have evaluated the immunogenicity and efficacy of a novel *C. difficile* toxoid vaccine candidate in hamster and cynomolgus macaque (NHP) models. Genetically modified toxin genes were expressed under the control of a Clostridium sporogenes promoter in a *C. difficile* production strain that was engineered to be asporogenic . Toxoids were highly purified and chemically treated to abolish residual cytotoxicity of the genetically modified toxoids. Immunization with a combination of Toxoid A and B formulated with different adjuvants protected up to100% of hamsters from developing severe CDAD after challenge with *C. difficile* spores. Immunized hamsters developed potent and durable antitoxin (A and B) neutralizing and protective antibodies. Vaccine induced antibodies present in the pooled hamster sera efficiently neutralized toxins from a broad collection of clinically diverse strains circulating in the US and Europe. Non-human primates (NHPs) immunized with adjuvanted toxoids generated robust antitoxin A and B neutralizing antibodies that were maintained for up to 53 weeks after the start of the study. Overall, the long-term protection data in hamsters, long lasting neutralizing antibody responses in NHPs, and the neutralizing capabilities of vaccine induced sera against clinically relevant *C. difficile* strains indicate that Pfizer's vaccine candidate is capable of inducing broad, robust, and durable immune responses that are likely to afford protection from CDAD.

O3

TOXIN A AND TOXIN B SPECIFIC SYSTEMIC ANTIBODY LEVELS CORRELATE WITH PROTECTION AGAINST *C. difficile* ASSOCIATED DISEASE IN HAMSTERS

Natalie Anosova, A. Moralez Brown, N. Liu, L. Li, L. Cole, J. Zhang, H. Mehta, H. Kleanthous

Sanofi Pasteur (SP), R&D, Discovery, US, Cambridge, USA

We tested the ability of adjuvanted purified inactivated toxins A and B (toxoids) delivered via intramuscular administration, to protect against live toxinogenic *C. difficile* challenge. Hamsters were vaccinated twice with increasing concentrations of toxoided A and B combinations, pre-treated with clindamycin, and challenged with live *C. difficile* culture. Confirming previous studies, animals who received placebo died within 4 days post-challenge. The animals actively immunized with the adjuvanted toxoid preparations were protected from mortality and symptoms of disease in a dose-dependent manner. Moreover, passive immunization of naïve hamsters with immune antisera from protected animals afforded protection against morbidity and mortality associated with *C. difficile* challenge, demonstrating the principal role of circulating anti-toxin antibodies in immunity against disease. Pre-challenge serum of each individual animal vaccinated with the toxoid was tested for toxin A and toxin B specific IgG in ELISA using purified native toxins as capture antigens. Median anti-toxin A and anti-toxin B IgG titers in the group of surviving animals were statistically significantly higher compared to the group of animals that died. Ninety (90) percent of unprotected animals had anti-toxin A and anti-toxin B titers ≤5.4 log10 EU/mL and ≤5.3 log10 EU/mL, respectively. The ability of serum antibody to neutralize toxin A and B mediated cytotoxicity was also measured in an IMR-90 cell-based assay in vitro. Immune sera exhibited neutralizing activity against both toxins. Moreover, a statistically significant difference was observed between the levels of both toxin A and toxin B neutralizing titers in protected vs. unprotected animals. The animals that succumbed to challenge infection generated very low or non-quantifiable levels of anti-toxin B neutralizing antibody, but measurable anti-toxin A neutralizing titers. Median anti-toxin A and anti-toxin B neutralizing titers in the group of surviving animals were statistically significantly higher compared to the group of animals that died. Ninety (90) percent of unprotected animals had anti-toxin A and anti-toxin B titers equal or below 1:3000 and 1:240, respectively. Correlation between the toxin-specific titers measured by indirect ELISA or toxin neutralizing titers measured by the IMR-90 cell-based assay in actively-immunized animals was analyzed using the nonparametric Spearman test. Statistically significant correlations between the two methods were seen for both toxin A and toxin B responses. Toxin-specific antibody levels measured by ELISA and/or the IMR-90 cell-based neutralization assay can serve as a correlate of protection in the hamster challenge model of *C. difficile*.

INV2

SPORE DEVELOPMENT IN RELATION TO PATHOGENESIS IN *Clostridium difficile*

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Spores produced by *C. difficile* are highly resistant, infective and are the primary cause of transmission in health care institutions. Despite the central importance of spores in the pathogenesis of *C. difficile*, a detailed description of the cell differentiation process leading to spore formation in this organism is lacking. Evidence suggests that the main morphological stages of sporulation are conserved in the Clostridia. Moreover, the four cell type-specific RNA polymerase sigma factors that govern developmental gene expression in the well-studied model organism Bacillus subtilis are present in the *C. difficile* genome. In B. subtilis, SigF in the forespore, and SigE the mother cell, control early stages of development, and are replaced by SigG and SigK respectively, which control the final stages of development. We have inactivated the sigF, sigE, sigG and sigK genes of *C. difficile*, and we have characterized the mutants in detail. Several important deviations to the B. subtilis paradigm were found. For instance, while disruption of sigF, sigE and sigG abolishes spore formation, we found that SigK is not essential for the formation of heat resistant spores. This is because synthesis of the spore cortex peptidoglycan, which correlates with the development of heat resistance, is not under the control of SigK. Nevertheless, SigK has two important functions in *C. difficile* sporulation: it is required for the assembly of the spore coat layers, and is needed for spore release from the mother cell. We demonstrate the critical function of a SigK-controlled gene in the assembly of the spore outer coat layer. This gene is also involved in colonization in a mouse model. Thus, our study establishes a direct link between the spore surface and colonization.

Cell-cell signaling pathways operate at critical morphological stages of spore development in B. subtilis, linking the forespore and mother cell genetic programs, and keeping gene expression in register with the course of morphogenesis. Our results suggest a looser coupling between gene expression and morphogenesis in *C. difficile* and we show that this less tight connection has implications for the morphogenesis of the spore structures.

O4

TRANSCRIPTIONAL ANALYSIS OF GERMINATON IN *Clostridium difficile* 630 ENDOSPORES

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Clostridium difficile is the leading cause of hospital acquired diarrhoea, placing considerable economic pressure on healthcare systems and resulting in significant morbidity and mortality. Spores are the primary infectious agent and must germinate to allow for vegetative cell growth and toxin production. While spore germination in Bacillus is well understood, little is known about *C. difficile* germination and outgrowth. Here we report on the initial phases of a project aimed at elucidating the role of specific genes in germination of *C. difficile* endospores, reestablishment of metabolic activity and establishment of the colonized state. We have optimized spore purification methods allowing us to obtain pure spore suspensions free of vegetative cells and cell debris. The growth characteristics and germination dynamics of these spores have been characterized confirming their viability and sensitivity to cholate-based germinants. A microarray analysis of gene expression in germinating spores was performed. To this end, RNA extraction techniques were optimized providing RNA of high quality. Total RNA was extracted from dormant as well as germinating and outgrowing spores. Gene expression was found to be highly dynamic. Few genes were active in dormant spores, consistent with their metabolically quiescent state. In contrast, a significant up-regulation of gene expression was observed immediately after induction of germination, peaking at 30 min. 499 significantly ($p \leq 0.01$) up- or down-regulated genes have been identified in germinating spores when compared to a vegetative cell, including genes encoding transcriptional regulators, ABC transporters, phosphotransferase system enzymes, two-component systems and enzymes involved in redox reactions. These results were validated via semi-quantitative RT-PCR and 'proof of principle' experiments on selected genes.

O5

IDENTIFYING FACTORS THAT INFLUENCE *Clostridium difficile* SPORE GERMINATION

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Clostridium difficile spore germination represents a significant hurdle in overcoming *C. difficile* infections. The actively growing vegetative cell cannot survive for extended periods of time outside a host and the toxins that are necessary for disease are not deposited in or on the spore during spore formation. Thus, to cause disease, spores must germinate in the host in response to appropriate signals. Work done by our lab, and others, has shown that bile acids are important for *C. difficile* spore germination. Some bile acids stimulate spore germination while others inhibit germination. The receptors on the spore to which bile acids bind are not known. In other sporulating bacteria (e.g. Bacillus subtilis, B. anthracis or C. perfringens), germination receptors have been identified that respond to their respective germinants. However, based on sequence homology, no homologues of known spore germination receptors have been identified in *C. difficile*. In an effort to identify *C. difficile* germination receptors, we performed a genetic screen which resulted in the identification of 6 genes whose products may have a role in *C. difficile* spore germination (a putative peptidase, a putative rubrerythrin, a putative transcriptional activator, a putative protease and a hypothetical membrane protein). We are currently testing whether these proteins are bona fide *C. difficile* germination receptors and, once confirmed, we will analyze the role of bile acid-mediated germination during *C. difficile* pathogenesis.

O6

THE SPORE COATS OF *C. difficile*

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Clostridium difficile is an important human pathogen and one where the primary cause of disease is due to the transmission of spores. We have investigated the structure and composition of the spore coat and identified a number of proteins found in the spore coat and exosporium. This includes six spore coat proteins, and three putative exosporial proteins. The function of each protein has been investigated using ClosTron mutagenesis of the structural gene. Of the six proteins found in the spore coat, CotCB and CotD are manganese catalases, CotE, a novel bifunctional protein with peroxiredoxin and chitinase activity and SodA a superoxide dismutase. Enzyme activity has also been demonstrated with purified proteins. These enzymes could play an important role in coat assembly by polymerizing protein monomers in the coat. CotE in addition to a role in macromolecular degradation could play an important role in inflammation and this may be of direct relevance to the development of the gastrointestinal symptoms accompanying *C. difficile* infection. In the exosporium the three bclA genes have been inactivated and TEM analysis showed clear abberations in the exosporial layer. We have used RT-qPCR to analysis both the timing and dependence of developmental gene expression for all of these genes and the parallels with that in other spore formers will be discussed.

INV3

STRUCTURES AND FUNCTIONS OF *Clostridium difficile* TcdA and TcdB

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Clostridium difficile secretes two homologous toxins, TcdA and TcdB, which are responsible for the symptoms of *C. difficile* associated disease. The intoxication mechanism of TcdA and TcdB involves four steps: binding to the target cell, pore formation and translocation of a catalytic domain across the host membrane, release of the catalytic domain by autoproteolysis, and the enzymatic glucosylation of host Rho proteins. These four functional activities correlate with discrete regions of the toxins' primary amino acid sequence. To gain insight into the organization and mechanism of these four functional domains, the structural approaches of electron microscopy and X-ray crystallography have been used to elucidate three-dimensional models of the toxins. Analysis of these models provides an opportunity to examine how TcdA and TcdB differ, how sequence variation impacts function, and a framework for the development of novel therapeutics.

07

THE ROLE OF LIPOPROTEINS IN *Clostridium difficile* ADHERENCE

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Lipoproteins constitute a subset of proteins that perform a vast array of functions in bacteria. These cell wall associated proteins have been implicated in the virulence of several pathogens and the enzymes responsible for their biosynthesis have been shown to be possible targets for novel antimicrobials. We have constructed strains of *Clostridium difficile* 630 that contain deletions in genes encoding key enzymes involved in the biosynthesis of lipoproteins, namely lgt and lspA. Characterisation of these strains has been conducted, including their assessment in a reproducible in vitro cellular adhesion assay. The lgt mutant of *C. difficile*, in addition to demonstrating an altered localisation of lipoproteins also shows a marked reduction in cellular adherence. Further studies identified a solute binding lipoprotein component of an ABC transporter as potentially important in this reduced adherence. A mutant strain of *C. difficile* defective for this lipoprotein did demonstrate a reduction in adherence in our in vitro assay. Furthermore, adherence of the wild-type strain could be inhibited in a dose-dependent manner via an antibody against this lipoprotein, supporting its putative function as an adhesin. Biochemical analysis of this lipoprotein demonstrated its ability to bind zinc in a dose dependent manner through differential scanning fluorimetry. Further characterisation of this lipoprotein and the corresponding *C. difficile* mutant will be described.

O8

IDENTIFICATION OF THE HOST CELL RECEPTOR OF *Clostridium difficile* BINARY TOXIN CDT

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The nosocomial pathogen *Clostridium difficile* is the most serious cause of antibiotic-associated diarrhea and pseudomembranous colitis. Hypervirulent strains of the pathogen, associated with more severe disease and increased death rates, produce the binary actin-ADP-ribosylating toxin CDT (*C. difficile* transferase) in addition to the Rho glucosylating toxins A and B. CDT induces depolymerization of the actin cytoskeleton and the formation of microtubule-based cell protrusions that increase adherence and colonization of the pathogen. A human, haploid cell line Hap1 with single-gene knockouts (Carette et al., 2011, Nature), generated by retroviral, insertional mutagenesis, was used to identify the host cell receptor of CDT. Following treatment of mutagenized Hap1 cells with CDT, toxin-resistant cell clones were collected to identify disrupted genes by parallel sequencing of retroviral insertion sites. Using this approach we identify the lipolysis-stimulated lipoprotein receptor (LSR) as the target molecule for entry of CDT into host cells. Identification of the toxin receptor provides a most valuable basis for antitoxin strategies.

O9

IMPACT OF CLOSTRIDIAL GLUCOSYLATING TOXINS ON THE PROTEOME OF COLONOCYTES

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The anaerobe *Clostridium difficile* is a leading cause of nosocomial gastrointestinal infections ranging from mild diarrhea to life threatening pseudomembranous colitis. Two major virulence factors TcdA and TcdB are produced by *C. difficile* that specifically inactivate small GTPases. The consequences are: reorganization of the cytoskeleton, loss of cell-cell contacts, and finally cell death.

A comprehensive proteome analysis was conducted using Caco-2 cells that were incubated for different time periods with wild type TcdA or mutant TcdA. Short (5 h) and long term (24 h) effects were analyzed using ICPL and SILAC approaches. Proteins were fractionated by SDS-PAGE, digested, and peptides were analyzed and quantified by LC-MS. Results were verified by western blot and MRM analysis. The activity of clostridial glycosylating toxins was evaluated in vivo by identification of their target GTPases and determination of their extent of glucosylation.

Wild type TcdA induced considerable changes in the protein profile of colon cells. More than 800 proteins of the identified >6000 proteins were altered in their abundance. Higher abundant proteins were involved in regulation, metabolic processes, respiratory chain complexes, endocytosis, and organelle function. Less abundant proteins participate in cell-cycle, translation, cytoskeleton organisation, and RNA binding. Only weak changes after short incubation periods were observed by treatment with glucosyltransferase deficient TcdA. Regulation of several proteins was confirmed by western blot and MRM analysis. Besides known targets of TcdA such as RhoA, RhoC, and RhoG, glucosylation was also identified in Rap1(A/B), Rap2(A/B/C), Ral(A/B), and (H/K/N)Ras which had not been considered as TcdA targets before. The glucosylation spectrum of TcdB, the variant TcdBF, and TcsL from C. sordellii were also determined.

This proteome analysis demonstrates that TcdA affects several cellular processes that have not been considered before to be affected by clostridial glycosylating toxins.

O10

IN VITRO AND IN VIVO CHARACTERIZATION OF NEUTRALIZING MONOCLONAL ANTIBODIES AGAINST *Clostridium difficile* TOXINS A AND B

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Introduction - *Clostridium difficile* associated disease (CDAD) is increasing in incidence and severity with significant morbidity and mortality. Antibiotic treatment is not invariably successful with a relapse rate around 30%. Since only toxin A and/or B (TcdA, TcdB) producing strains are proven to be pathogenic, we aim to develop a novel treatment modality based on targeted mucosal delivery of anti- *Clostridium difficile* toxin-antigen binding fragments.

Methods - SJL/J mice were immunized with *Clostridium difficile* toxoid by a standard protocol for hybridoma generation. Monoclonal antibody (MA) production was evaluated on coated TcdA and TcdB. Selected MAs were evaluated for their in vitro neutralization capacities using CCL-186 fibroblasts and TcdA or TcdB at 570 pM or 56 pM, respectively. Affinities were determined on a Biacore 3000. Hemagglutination experiments were performed on rabbit red blood cells (1%, 16 nM TcdA) and relative epitope-mapping was based on pair-wise combination of MAs in sandwich-type ELISAs. In vivo neutralization capacities of the MAs were evaluated by mouse ileal loops in C57BL/6 mice (65 nM TcdA, 4 hours incubation, 2 cm loops either with or without 72 – 113 uM MA). Gene expression was analyzed by standard RNA extraction and RealTime-PCR.

Results - Out of 99 monoclonal antibodies that were reactive against TcdA and/or TcdB, 4 neutralized TcdA in vitro (average EC50: 15 nM), 6 neutralized TcdB in vitro (average EC50: 3,02 nM) and 1 antibody neutralized both toxins (EC50-TcdA: 13 nM, EC50-TcdB: 140 nM) in vitro. Hemagglutination against TcdA showed that 4 anti-TcdA MAs were targeting the receptor binding domain and 1 was not. Biacore affinity studies on 4 MAs against TcdA demonstrated high affinity against TcdA and moreover, very slow dissociation rates. Relative epitope mapping resulted in the selection of 6 neutralizing MAs representing 3 distinct epitopes at each toxin. In vivo neutralization activitity was confirmed for three selected MAs by a decrease of the expression at mRNA level of IL6, CXCL1 and TNFα after injection in the ileal loop of TcdA and the MA, compared to injection with TcdA alone.

Discussion - This study identifies several monoclonal antibodies with high affinity and bioactivity in vitro as well as in vivo. We will further seek in vivo confirmation in the well-established Syrian gold hamster model of CDAD.

O11

MECHANISM(S) INVOLVED IN *Clostridium difficile*-MEDIATED INFLAMMASOME ACTIVATION

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Background: Emerging epidemic *Clostridium difficile* (CD) strains are responsible for the recent increase in antibiotic-associated hospital and community acquired diarrhoea. CD can express up to three toxins: Toxin A (TcdA), Toxin B (TcdB) and a Binary Toxin (CTD). TcdA and TcdB are known potent activators of the 'Infammasome' complex that mediates caspase-1 activation leading to cytokine (IL-1β/IL-18) production and pyroptosis. Previous studies utilising recombinant toxins have implicated a role for NOD-like receptor (NLR) NALP3 in CD toxin-mediated Inflammasome activation, mechanism(s) involved however remain ill-defined. Furthermore the potential contribution, if any, of toxin-independent mechanism(s) to CD mediated Inflammasome activation is currently unknown.

Methods: Phorbol myristate acetate (PMA)-differentiated THP-1 macrophages and human blood monocyte-derived macrophages (MDM) were infected with two virulent wild-type (WT) R20291 and 630 Δerm CD strains and their respective toxin-deficient isogenic mutants, in the presence of inhibitors targeting Inflammasome function. Inflammasome activation was assessed by gene and protein expression, flow cytometry and confocal microscopy.

Results: R20291 and 630Δerm WT strains induced marked increase in bioactive IL-1β in a caspase 1-dependent manner. Significant reduction in IL-1β levels was observed during infection with isogenic mutants lacking TcdA or TcdB. IL-1β secretion was preceded by ASC speck formation accompanied by NALP3 cellular re-localisation. Inhibition of NALP3 through high intracellular K+ or blocking K+ channel activity led to diminished cytokine secretion.

Conclusion: Our study is the first to report the effect of CD infection on Inflammasome function. Preliminary data indicates that both TcdA and TcdB are equipotent in activating the inflammasome leading to IL-1β secretion. CD modulation of NALP3 function was partly dependent on low intracellular K+. We are currently investigating the implications of CD-mediated Inflammasome activation.

O12

HOST INFLAMMATORY AND IMMUNE RESPONSE TO *Clostridium difficile* INFECTION: ROLE OF BACTERIAL CYTOTOXIN TITRE AND RELATIONSHIP WITH MORTALITY

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Background: Reports of severe *Clostridium difficile* infection (CDI) have increased in the past decade. Numerous severity scores have been proposed that identify patients with CDI at risk of mortality and the majority of these scoring systems rely on patient clinical risk markers. The role of the infecting bacterial strain in severe CDI is less well understood. Recent studies have failed to demonstrate a relationship between bacterial genotypic markers or strain type in predicting CDI severity or outcome and the role of other bacterial factors are not yet known.

Aim: We aimed to investigate the relationship between patient clinical risk markers (laboratory markers of severe disease and serum immunoglobulin levels), infecting bacterial strain type (ribotype and toxinotype) and in vitro bacterial cytotoxin production in influencing CDI severity, host immune response and risk of mortality.

Methods: We conducted a prospective cohort study of 150 inpatient CDI cases. Clinical laboratory markers of inflammation, co-morbidity scores and CDI outcomes were recorded for each patient. Anti-toxin A and B IgG levels were measured by ELISA in serum collected at 1, 3 and 12 days post CDI diagnosis and peak titre ≤day 12 calculated. *C. difficile* was isolated and typed by ribotyping and toxinotyping and in vitro cytotoxin production assessed by Vero cell cytotoxicity assay.

Results: Ribotypes 027, 014 and 001 were the highest cytotoxin producers. We found a significant positive correlation between the cytotoxin titre of the infecting strain and markers for severe inflammatory disease; C-reactive protein concentration ($r = 0.28$; $P = 0.02$), peak white cell count ($r = 0.183$; $P = 0.047$) and minimum serum albumin concentration ($r = 0.214$; $P = 0.027$). CDI patients infected with highly toxinogenic isolates elicited significantly higher anti-toxin A ($P = 0.03$) and B ($P = 0.02$) IgG responses and did not have a greater risk of recurrence or death.

Risk of mortality in our study was not associated with either bacterial strain type or cytotoxin titre, but was strongly associated with high peak white cell count (>20 x109/L) (OR 6.3; 95% CI 1.85 – 21.47; $P = 0.003$) and high creatinine concentration (>133µmol/L) (OR 6.05; 95% CI 1.79 – 20.43; $P = 0.004$). Other risk factors for mortality were high Horn's index (> 3) (OR 4.4; 95% CI 1.05 – 18.37;

P = 0.03) and low serum anti-toxin A IgG response (4.23 vs 25.19 ELISA Units; P = 0.01) and a trend for low anti-toxin B IgG response (7.8 vs 18.01 ELISA Units; P = 0.07).

Conclusions: Host pro-inflammatory and humoral responses are related to the cytotoxin titre of the infecting strain and an effective anti-toxin immune response is associated with protection from a fatal outcome of severe CDI and reduced risk of mortality.

O13

FLAGELLA AND FLAGELLIN OF *C. difficile* 027 HYPERVIRULENT STRAIN ACTIVATE NF-κB AND ELICIT IL-8 PRODUCTION IN EPITHELIAL TLR-5-EXPRESSING CELL LINE

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Clostridium difficile is an enteropathogenic bacterium responsible for post-antibiotic nosocomial diarrhoea and pseudomembranous colitis. The outbreak and spread of a "hypervirulent" clone since 2002 responsible for severe epidemics in North America and Europe have given a new dimension to *C. difficile* infections. *C. difficile* pathogenicity is mainly due to the production of two toxins TcdA and TcdB. However, *C. difficile* adherence to host tissues and gastrointestinal tract colonization are considered as a prerequisite to infection due to disruption of the barrier effect of the intestinal microbiota. The role of the flagella of *C. difficile* in eliciting an inflammatory immune response that could be deleterious to the host has not been established yet. We previously constructed a TLR5-expressing Madin-Derby canin kidney (MDCK) cell line (MDCK-TLR5) and showed that flagellated *C. difficile* 027 (R20291) strain and the recombinant flagellin activate the Extracellular signal-Regulated kinases 1 and 2 (ERK1/2) MAPK through the Toll-Like Receptor-5 (TLR-5), suggesting that the flagellin of *C. difficile* might play a role in the initiation of an innate immune response and contribute to the pathogenesis of this bacterium. The objective of the present work is to pursue the characterization of the signaling pathways involved in the interaction between the *C. difficile* flagella and the epithelial cell via the receptor of the innate immune response, TLR5. MDCK (control) and MDCK-TLR5 cell lines were infected at different times by the *C. difficile* 027 wild type strain, an unflagellated isogenic mutant ΔFliC, the fliC complemented ΔFliC mutant and a non toxigenic strain A-B- (027 strain mutated in the tcdA and tcdB genes) or incubated with the recombinant flagellin FliC. The activation of NF-κB (degradation of IκB-α) was analyzed in cell lysates by western blot and interleukin 8 (IL-8) was quantified in culture supernatants by an ELISA kit. The MDCK-TLR5 infection by the flagellated *C. difficile* strains but no by the unflagellated ΔFliC strain triggered a time-dependent degradation of IκB-α thus indicating a NF-κB activation. The MDCK-TLR5 infection by a non toxigenic but flagellated strain also elicited a time-dependent IκB-α degradation thus suggesting that TcdA and TcdB are not responsible for this NF-κB activation. The recombinant flagellin also activated NF-κB in MDCK-TLR5 cells but no in MDCK cells. Furthermore, in this same cell model, flagellated bacteria including non toxigenic strain triggered the IL-8 synthesis, whereas no IL-8 production was observed when cells were infected by the unflagellated ΔFliC mutant. These results indicate that flagellated bacteria and the recombinant flagellin activate NF-κB over time and could lead to the synthesis of IL-8 by epithelial cells, suggesting a role for flagellin and *C. difficile* flagella in the induction of a pro-inflammatory host response.

INV4

NOVEL INSIGHTS INTO *C. difficile* BIOLOGY AND EVOLUTION REVEALED BY WHOLE GENOME SEQUENCING

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The availability of large numbers of microbial genomes determined by high throughput DNA sequencing is enhancing understanding of pathogen biology and evolution. Loci encoding essential and putative virulence determinants can now be studied in their entirety using long contiguous sequences determined without prior knowledge of gene content or sequence. Careful choice of isolates representing the population structure of a species and different clinical phenotypes enhances this approach.

Two clinically important regions of the *C. difficile* genome were investigated. Firstly, the 36.6kb cell wall protein (cwp) cluster which contains the slpA gene encoding the paracrystalline S-layer. This proteinaceous layer surrounds the cell and represents an immunodominant antigen. Secondly, the 19kb Pathogenicity locus (PaLoc) encoding toxins TcdA and TcdB which are responsible for the symptoms of *C. difficile* infection. A large collection of clinical isolates (n>2000) from Oxford and Leeds, UK, and Australia was used.

Genetic diversity across the cwp cluster peaked within slpA, cwp66 (adhesin), and secA2 (secretory translocase). These genes formed a 10kb cassette, of which 12 divergent variants were found. Homologous recombination (or "switching") involving the intact 10kb cassette caused it to associate randomly with genotype. *C. difficile* genotype is therefore not reliably predictive of antigenic type. One cassette contained a novel ~24kb insertion resembling S-layer glycosylation gene clusters.

A novel insertion of 9kb was also identified within the PaLoc of a subset of strains. This contained bacteriophage-like sequences encoding a putative endolysin and a 320nt predicted stem loop structure representing a possible origin of replication. The putative integrase, excisase and topoisomerase of the insertion showed sequence identity and synteny with a family of mobile

genetic elements, commonly found in other chromosomal locations within the isolate collection. Intriguingly, the 3′ half of the 320nt stem loop defined the 5′ boundary of the mobile elements. It is unclear whether this PaLoc insertion was acquired from, or gave rise to a family of mobile genetic elements.

These two examples illustrate the evolutionary insights to be gained, and further questions raised by the exploitation of newly available technologies for whole genome sequence determination.

O14

WHAT'S A SNP BETWEEN FRIENDS?

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With the advent of methods for the directed mutation of specific *Clostridium difficile* genes it is now relatively straight forward to generate isogenic mutants of selected strains and thereafter compare their virulence properties with the progenitor strain. Moreover, the differential effects of mutating distinct genes involved in the same process can be compared in studies undertaken in disparate laboratories. But can they? This assumes that the strains used by independent research laboratories are the same. The problem is that bacterial genomes are not static, and are capable of undergoing all manner of insertions, deletions, rearrangements and point mutations. The likelihood of this happening increases proportional to the number of times the chromosome is replicated, a reflection of the number of sub-cultures a strain is subjected to. The archetypal example is that of the two erythromycin sensitive derivatives of strain 630, strains 630E and 630Δerm. Both were isolated in two independent laboratories (Monash and UCL, respectively) following repeated sub-culture. Genome re-sequencing of these strains has revealed the presence of substantive numbers of SNPs compared to the progenitor, several of which account for the clear phenotypic differences that exist between these isolates. Their presence may explain the differences in virulence observed in the tcdB mutants made in the two strains (Lyras et al, 2009; Kuehne et al, 2010), a hypothesis that is currently being tested. In the meantime a similar story is beginning to emerge in terms of isolates of the PCR-Ribotype 027 strain, R20291. Some of the observed SNPs have arisen because a strain has been deliberately subjected to subculture. However, in other cases the subculture has been inadvertent. It is clear that if researchers wish to work on the same strain of *C. difficile* that more effective means of strain curation need to be implemented. Moreover, genome re-sequencing of mutant strains should become a routine undertaking.

O15

WHAT'S LOV GOT TO DO WITH IT?

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Clostridium difficile is an important cause of antibiotic associated disease, which is of high socio-economical importance that has recently been compounded by the emergence of epidemiological strains of different toxinotypes. Efforts to non-invasively visualise *C. difficile* using chromophores have been hampered by their essential requirement for oxygen. Plant phototropins are light activated protein kinases that contain two N-terminal Light, Oxygen or Voltage (LOV) domains. These small LOV domains (~110 animo acids) bind flavin mononucleotide as a blue-light-absorbing chromophore that upon excitation undergo a reversible photocycle, emitting a strong green fluorescence. Using LOV domains as fluorescent reporters has three main advantages over their GFP counterparts: 1, LOV reporter domains are small, only ~11 kDa, 2, fluorescence is independent of molecular oxygen and 3, is stable over a wide pH range.

The LOV domain of the Arabidopsis thaliana blue-light receptor phototropin 2 was engineered for increased photostability (LOV2.0) and its DNA sequence codon optimised for expression in *C. difficile*. LOV2.0 was cloned using two plasmids differing in promoter regions; constitutive expression (pRPF144) and inducible expression (pRPF185), and conjugated in to *C. difficile* strain R20291. This strain was isolated during the first incursion of ribotype 027 in U.K. hospitals and has previously been shown to be virulent in the hamster model of infection.

C. difficile R20291 harbouring pLOV were confirmed by PCR and shown to fluoresce above *C. difficile* natural background levels. Growth of LOV expressing strains was comparable to R20291 wild-type over 72 hrs and stability assays show ~90% plasmid stability. Data will be shown on the in vivo infection of hamsters challenged with LOV expressing strains, comparing survival rate, bacteriological recoveries, toxin production and histological analysis.

For the first time we show the use of LOV domains as fluorescent reporters in *C. difficile* and unveil their use for the emerging field of *C. difficile* optogenetics.

O16

EXPLORING sRNAs IN *Clostridium difficile* BY BIOINFORMATICS AND DEEP-SEQUENCING

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Clostridium difficile-associated diarrhoea is currently the most frequently occurring nosocomial diarrhoea in Europe. Many questions remain unanswered concerning the mechanisms controlling infection cycle of this human pathogen. We wonder whether in addition to several regulatory proteins small non coding RNAs (sRNAs) may contribute to regulatory networks governing *C. difficile* physiology and pathogenesis. Our recent data strongly suggest the importance of RNA-based mechanisms for the control of gene expression in *C. difficile*. To initiate the search for regulatory RNAs in *C. difficile*, we have combined several genomics approaches including in silico prediction and experimental detection using high-throughput sequencing. A recently developed in silico approach allowed us to identify a great number of potential sRNA candidates in both intergenic and coding regions. In addition, RNA-seq and differential RNA-seq approaches were used for global identification of transcribed sRNAs and their transcriptional start site mapping under three different growth conditions (exponential growth phase, stationary phase and starvation). The expression of a number of these sRNAs was confirmed by independent experimental approaches (Northern blot, qRT-PCR). This includes potential trans riboregulators located in intergenic regions, cis-antisense RNAs, CRISPR (clustered regularly interspaced short palindromic repeats) RNAs and a variety of riboswitches. All together, these results represent a first genome-wide identification and validation of sRNAs in *C. difficile* and bring important insights into the role of regulatory RNAs in this emergent enteropathogen. This study provides an essential basis for the complete and functional determination of the RNome of this bacterium in the future.

INV5

METABOLIC CONTROL OF VIRULENCE IN *Clostridium difficile*

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When grown in rich medium, *C. difficile* produces the TcdA and TcdB toxins primarily in stationary phase, because the genes that encode the toxins are not expressed during rapid exponential growth phase. Components of the medium known to restrict toxin synthesis include glucose, branched-chain amino acids and proline. Antunes and Dupuy showed that the glucose effect is mediated by the global regulator CcpA. Our studies of the role of a second global regulator, CodY, have revealed that CodY, which is activated by the combination of branched-chain amino acids and GTP, is a major repressor of toxin synthesis. CodY regulates the toxin genes indirectly by repressing tcdR, the gene that encodes the RNA polymerase sigma factor essential for toxin gene expression. The impact of CodY in vivo has not previously been examined. We have created stable codY null mutations in strains UK-1 (a NAP1/027 strain) and 630E by Targetron mutagenesis. The UK-1 codY mutant greatly overproduced TcdA and TcdB and was more virulent than its parent strain in a mouse model of infection, suggesting that, in UK-1, repression of virulence factors by CodY limits the severity of infection. The 630E codY mutant also overproduced the toxins, but there was little if any increase in virulence in the mouse model. The effect of proline on toxin production was shown to be related to the utilization of proline as a substrate for Stickland metabolism. That is, a mutant defective in D-proline reductase accumulated proline intracellularly and produced much less toxin than the parent strain. The proline reductase genes proved to be regulated by PrdR, a protein similar to activators of sigma-54-dependent transcription. The proline reductase gene cluster is preceded by a sigma-54-type promoter.

O17

THE CONJUGATIVE TRANSPOSON TN5397 INSERTS INTO SPECIFIC SITES IN THE *Clostridium difficile* R20291 GENOME RESULTING IN SEVERE ATTENUATION OF VIRULENCE IN THE HAMSTER MODEL

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Tn5397 is a conjugative transposon which is related to the prototype of these types of elements, Tn916. However, integration and excision of Tn5397 is mediated by a large serine recombinase, TndX, which is not found in Tn916. In *C. difficile* R20291, Tn5397 has two highly preferred integration sites. These are both within genes containing a fic domain and predicted to encode proteins involved in filamentation induced by cAMP.

As well as being involved in cell shape, other bacterial proteins containing the fic domain have been shown to modulate the activity of eukaryotic proteins by a process called ampylation. This involves the transfer of an adenosine monophosphate (AMP) onto a target protein, such as the Rho-GTPases which results in collapse of the actin cytoskeleton and cell death.

In order to investigate the biological relevance of this phenomenon, we investigated the ability of an R20291 strain carrying Tn5397inserted into two of the fic genes to colonise and cause disease in the hamster model of *C. difficile* infection. The double insertion mutant had a marked decrease in its ability to cause disease. Importantly, near full virulence could be restored by providing one of the fic genes in trans.

In conclusion this work shows that, at least in *C. difficile* R20291, expression of the fic genes is required for full virulence.

O18

IS TcdC REALLY A NEGATIVE REGULATOR OF TOXIN PRODUCTION IN *Clostridium difficile*?

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TcdC is widely considered to be a negative regulator of toxin production that acts by destabilizing the interaction between TcdR (the PaLoc associated group-5 extra-cytoplasmic-function sigma-factor) and the RNA polymerase core-enzyme. Speculation that TcdC was a negative regulator of toxin expression was first prompted by the inverted expression pattern of tcdC compared to that of tcdR and the toxin genes, tcdA and tcdB. Further weight was added to the idea when aberrations were observed in the tcdC open-reading-frame of epidemic *C. difficile* strains, coupled with reports that these strains are 'high-level' toxin producers. Subsequent genetic studies, detailed in vitro biochemical analyses and in vivo work have produced some compelling experimental evidence to support the role of TcdC as a negative regulator of toxin production. However, strain characterisation studies have repeatedly failed to find an association between tcdC genotype and toxin production by *C. difficile*. In addition, the type-strain, *C. difficile* VPI10463, has a completely intact tcdC gene yet produces substantially more toxin in the laboratory than even epidemic isolates with an aberrant tcdC gene. These findings suggest that tcdC genotype has limited scope as a marker of *C. difficile* virulence. Most recently, work was carried out in our laboratory to systematically restore the Δ117 frame-shift mutation and the 18-nucleotide deletion that occur naturally in the tcdC gene of *C. difficile* R20291 (PCR ribotype 027). In addition, we deleted the naturally intact tcdC gene in *C. difficile* 630 (PCR ribotype 012). Intriguingly, we did not observe any association between tcdC genotype and toxin production in either *C. difficile* R20291 or *C. difficile* 630. That is to say, toxin production remained unchanged, regardless of tcdC genotype, in otherwise genetically identical strains of *C. difficile*. This raises the question; is TcdC really a negative regulator of toxin production in *C. difficile*? Here we will present an objective appraisal of both experimental and circumstantial evidence for and against the role of TcdC as a negative regulator of toxin production.

O19

INSIGHTS INTO SORTASE IN *Clostridium difficile*

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The surface of *Clostridium difficile* has been implicated in colonization and infection of the host. The well characterized surface proteins of *C. difficile* are all found within the S-layer. However, little is known of proteins associated with the thick peptidoglycan cell wall which sits between the cell membrane and the S-layer. Sortases are bacterial enzymes that covalently attach specific proteins to the Gram-positive cell wall. In other Gram-positive bacteria such as Staphylococcus aureus, much is known of sortase, and sortase substrates have been shown to have a role in virulence. Although a sortase enzyme has been identified in *C. difficile*, there is no description of its substrates, or indeed whether the sortase enzyme is active.

Using bioinformatics analysis, we have identified potential sortase substrates in *C. difficile* strains 630 and R20291. Based on the requirements for an N-terminal signal sequence, a C-terminal sorting sequence containing an "LPxTG"-like motif, a hydrophobic domain and a charged tail, we identified 7 potential sortase substrates in the 630 genome, 4 of which are also found in R20291. These sortase substrates have variable C-terminal sorting sequences, which may have implications for processing and localization.

We have shown using RT-PCR that these sortase substrates are transcribed in *C. difficile* strains 630 and R20291. Knockouts have been produced and to investigate secretion, processing and surface localisation of these sortase substrates, we have developed experimental procedures based on manipulation of the "LPxTG"-like motifs. Our results demonstrate surface localisation of at least one sortase substrate, indicating that this family of proteins may represent an important family of cell wall anchored proteins in *C. difficile*.

INV6

INFECTION CONTROL OF *Clostridium difficile*: WHY ARE WE FAILING AND WHICH SPECIAL APPROACHES COULD HELP US SUCCEED?

Curtis Donskey, MD

Use of contact precautions and environmental cleaning and disinfection are evidence-based practices recommended for control of *Clostridium difficile* infection (CDI). Despite implementation of these basic practices, many facilities have struggled with unacceptably high CDI rates. A variety of special approaches may be considered when basic practices have failed. These special approaches include: 1) placement of patients with suspected CDI in contact precautions while test results are pending, 2) prolonging the duration of contact precautions after symptoms resolve, 3) screening for and isolating asymptomatic carriers, 4) "source control" (daily disinfection of high-touch surfaces in CDI rooms and daily bathing), and 5) use of environmental cultures to optimize environmental disinfection. Presently, there is considerable uncertainty regarding which of these approaches are likely to be beneficial. In this presentation, the potential impact of these special approaches will be evaluated based on factors including: 1) assessment of transmission risk based on studies of skin contamination and environmental shedding of spores, 2) numbers of patients involved, and 3) the practicality of the interventions required. The goal of this work is to develop evidence-based practices that infection control programs can use to prioritize interventions for successful control of CDI.

O20

A LARGE PROSPECTIVE NORTH AMERICAN EPIDEMIOLOGIC STUDY OF HOSPITAL-ASSOCIATED *Clostridium difficile* COLONIZATION & INFECTION

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BACKGROUND: *Clostridium difficile* (CD) infection (CDI) is the most common cause of infectious diarrhea in hospital patients in developed countries. Increases in CDI incidence and severity in some centers have been linked to epidemic CD strains, aging populations, and increased use of certain antibiotics. Although CD is an emerging pathogen, there are little data on natural history of CD acquisition, colonization and infection in hospital settings.
METHODS: A multi-hospital prospective study was conducted from February 2009 to June 2011 in the U.S. and Canada. Patients admitted to general medical and surgical units, on antibiotics, with no CDI diagnosis in the past 6 months, and over the age of 60 years were eligible. Patients were assessed for CD colonization status at enrollment and followed for 30 days post discharge or 60 days in the hospital, whichever came first, to examine the incidence rates of asymptomatic carriage and CDI. Stool or rectal swabs were collected every 3 days until discharge. Stool or swabs from patients with diarrhea were tested for CD toxins A and B. CDI was defined as toxin-positive diarrhea with a positive toxigenic CD stool culture. Asymptomatic carriage was defined as a positive culture for CD without CDI symptoms. CD isolates were typed via HindIII restriction endonuclease analysis (REA).
RESULTS: Among 1,256 patients enrolled, 1,099 were eligible for analyses. Among 1,008 patients non-colonized at enrollment, 25 asymptomatic carriers (2.5%) and 11 CDI cases (1.1%) were detected during follow-up with average age 74.3 years ± 7.9 s.d. and 79.2 years ± 7.0 s.d., respectively ($p = 0.09$). The proportion of REA type BI/Ribotype 027 strains was 73% for CDI cases and 20% for asymptomatic carriers ($p = 0.01$). CDI and colonization incidence rates were 13.7 and 32.5 per 10,000 patient-days, respectively. In multivariate logistic regression models of patients non-colonized at enrollment, higher albumin level and white blood cell (WBC) count at baseline were significantly associated with a patient becoming a CDI case vs. remaining a carrier (Albumin: OR = 4.8, 95% CI = 1.0-21.9, $p = 0.04$; WBC (> 16 vs. <= 16 (103/uL)): OR = 16.2, 95% CI = 1.2-214.5, $p = 0.03$). Among 91 patients who were colonized with CD but without CDI at enrollment, 9 (9.9%) developed CDI and 82 (90.1%) remained asymptomatic. Thus, a total of 20 CDI cases and 107 asymptomatic carriers were identified and assessed in this study.
CONCLUSION: Data from this large epidemiologic study furthers our understanding of the timing of healthcare-associated CD colonization and CDI, risk factors for development of CDI and planning of clinical trials to evaluate preventive and therapeutic CDI agents.

O21

STRAIN TYPES AND RESISTANCE PATTERNS OF *Clostridium difficile* ISOLATES FROM UNITED STATES HOSPITALS

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Introduction: *Clostridium difficile* continues to be a major cause of infectious diarrhea worldwide. Strain types of *C. difficile* often vary by geographic location as do antimicrobial resistance patterns. However, there are little data available on how *C. difficile* strain types and antibiograms change over time, especially in the United States (US). The goal of this study was to compare PCR ribotypes and antimicrobial resistance patterns of *C. difficile* isolates from the US from 2011-2012 to those from 2009.

Methods: A total of 302 toxigenic *C. difficile* isolates recovered from 302 unique patients from 19 US hospitals representing the Northeast, South, Midwest, and Western US during 2011-2012 were tested. Isolates were grown on prereduced Brucella blood agar plates (Anaerobe Systems, Morgan Hill, CA) at 35°C for 18-24 h in an anaerobe chamber and identified using colonial morphology, fluorescence, and standard biochemical methods. PCR-ribotyping was performed as previously described by Svenungsson et al. with minor modifications. Susceptibility testing was performed using Etest strips on prereduced Brucella blood agar as previously described.

Results: Ribotype 027 was the predominant strain type observed across the US in both time periods. In 2011-2012, ribotype 027 represented 31.8% of isolates overall, followed by ribotypes 014/020 (9.3%), 106 (7.3%), 053 (5.3%), 001 (5.0%), and 046 (3.0%). Ninety-four percent of 027 strains were resistant to moxifloxacin, 38.5% were clindamycin resistant, and 27.1% were rifampin resistant. Only 20% were resistant to all three drugs. Ribotype 027 represented 33.3% of isolates from the Northeast, 43.2% from the South, 22.2% from the Midwest, but only 14.3% from the Western US. When compared to 2009 ribotype results, there was an increase in ribotypes 014/020 (especially in the Western US), 001, and 046 during 2011-2012, and a dramatic decrease in ribotype 002 all across the US. The proportions of resistance among isolates within each ribotype were similar in the two time periods.

Conclusions: Ribotype 027 remains the major *C. difficile* strain type in the US and these isolates are typically moxifloxacin resistant; however, multidrug resistance is less common in this convenience sample than in what was observed in 2009. The percentage of non-027 ribotypes varied by geographic region, although the resistance patterns of various ribotypes appeared stable over time. The occurrence of several strain types shifted in 2011-2012 compared with those observed in 2009, but that may simply reflect the change in several of the hospitals from which the isolates were collected in the latter period. Nonetheless, the decrease in ribotypes 002 and 078 are notable and may indeed signal a shift in the epidemiology of *C. difficile* strain types in the US.

O22

HIGH MORTALITY RATE IN ONE YEAR COHORT STUDY AMONG PATIENTS WITH *Clostridium difficile* INFECTION (CDI) IN A LARGE TERTIARY HOSPITAL

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Context: *C. difficile* has emerged as an important health-care associated pathogen. Many studies reported that CDI severity, complications, and related deaths had substantially increased throughout the current decade. In France, the CDI surveillance is based on the mandatory notification of severe cases or outbreaks of CDI but additional data on mortality related to CDI are needed.

Objective: To describe the prognosis of CDI (relapses, cure or death). Design, Setting, and Patients: A one year cohort study was conducted in a University Hospital at Lyon, France. All hospitalized patients (>18 years) for more than 48 hours, suffering from CDI and accepted to participate were included. Patients were followed up to 60 days after CDI diagnosis. CDI case was defined by a positive stool EIA-toxins assay result or with a positive toxigenic culture result. Collected data included patients' demographics, hospitalization information, exposure to known risk factors of CDI, clinical symptoms, results of *C. difficile* toxin assay and toxigenic culture, and prognosis of CDI.

Results: Between January 2011 and January 2012, 1000 stool cultures were requested and 66 episodes (62 patients) of CDI were identified (Toxins &/or culture were positive). Among them, 54 patients were included. The mean of age was 66.8 years and there were more men than women (55.5% vs 44.5%). Before the infection, 69%patients had previous hospitalization, 65.5% and 76.4% were exposed to PPIs and antibiotics respectively. Most of episodes were hospital-acquired (n=45, 81.8%). The remaining cases were community-acquired (n=5, 9.1%) and unknown origin (n=5, 9.1%). Death was occurred in 10 patients (18.5%) during hospitalization and in 5 (9.3) after discharge. Two patients were transferred to ICU for complications: one presented an ischemic jejunitis and ileus; the second one for ileus and diffuse colitis. CDI was the primary cause of death for one patient (5 days after the onset of symptoms) and a contributing cause in 3 deceased patients from complications. Death occurred in 10, 27 and 40 days respectively after the onset of diarrhea. The survival analysis of patients who were at the end of follow-up (60 days) showed that the death rate was 22% (11 over 50 patients). In 50% of cases death occurred the first 6 days.

Conclusions: We reported a high mortality rate among patients suffering from CDI. A comprehensive *C. difficile* infection control management, an efficient communication network and an appropriate training are recommended within each health care facility. New approaches of diagnosis, therapy and prevention will improve the outcome of CDI.

INV7

CLINICAL IMPACT OF MOLECULAR TESTING FOR *C. difficile* DIAGNOSIS THERE'S GOOD NEWS AND BAD!

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Clostridium difficile is the most common cause of healthcare associated diarrhea. Associated morbidity and mortality have increased dramatically since the turn of the century related to organism factors, such as enhanced virulence and multiple antibiotic resistance, as well as increased host vulnerability. These factors have refocused attention on laboratory diagnosis. Multiple methods are available for the laboratory diagnosis of C difficile and range from relatively insensitive toxin enzyme immunoassays (EIAs) to sensitive nucleic acid amplification methods that detect the genes that encode toxins A and B. Cell culture cytotoxin neutralization assays are no longer considered by many to be acceptable "gold standards" and toxigenic culture, while sensitive, is labor intensive and difficult to perform well. Some laboratories have developed multiple step algorithms predicated upon detection of glutamate dehydrogenase (GDH), the common C difficile antigen, followed by an assay for toxin detection or a molecular test. However, variability in GDH performance has been reported and may limit this approach.

Currently in the USA there are six nucleic acid amplification assays that have been approved by the Food and Drug Administration for the detection of C difficile in fecal specimens. The majority of these assays detect conserved regions of tcdB and at least one assay also detects the tcdC deletion at nucleotide 117 and cdt, surrogates for C difficile ribotype 027. All of these assays have advantages and disadvantages with respect to the mechanics of assay performance. Published sensitivity and specificity of these platforms range from 77-100% and 93-100%, respectively. The good news is that molecular tests provide sensitive and rapid same- day results and emerging literature suggests that they may be cost-effective to healthcare systems by eliminating the need for retesting and reducing unnecessary treatment. Positive effects on infection control include reducing transmission and removing patients with negative tests from isolation more quickly. The bad news is that all of these molecular tests are 2-3 times more expensive than toxin EIAs, have poor specificity and positive predictive value if not used appropriately, and may increase rates of C difficile when making the switch from an EIA to nucleic acid amplification. In spite of advances in molecular technology, the optimum method for C difficile diagnostic testing remains elusive.

INV8

PROBIOTICS, BACTERIOCINS AND BACTERIOPHAGE FOR LIMITING *C. difficile* INFECTION

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Broad spectrum antibiotics bring about severe perturbation of the gut microbiota primarily in the form of dysbiosis and as such can create opportunities for over-growth of bacteria which are normally restricted by microbial competition in the undisrupted flora. Nowhere is this more obvious than in cases of *C. difficile* infection whereby it is universally accepted that major perturbation of the gut flora post antibiotic treatment is a major risk factor for *C. difficile* proliferation. The antibiotics of choice for the treatment of *C. difficile* infection are the broad spectrum antibiotics vancomycin and metronidazole and while the development of resistance to these antibiotics is infrequent there is a trend towards decreased susceptibility, which is often manifested as increased recurrence of symptoms. Therefore there is an interest in developing therapeutics which specifically target *C. difficile* such as narrow spectrum bacteriocins and bacteriophage therapy. The bacteriocins nisin and lacticin 3147 have been shown to have anti-*C. difficile* activity, however, their broad spectrum of activity probably reduces their usefulness due to their impact on the wider gut flora. However, thuricin CD, a newly discovered two component bacteriocin, has been shown to have a narrow spectrum of activity with limited impact on the wider gut flora. Compositional sequencing, using 454 pyrosequencing, has shown little or no collateral damage to the wider gut flora when thuricin was tested in a laboratory model of the distal colon. In addition in vivo studies have shown that thuricin CD is capable of eliminating *C. difficile* from the mouse colon when administered rectally thereby overcoming the barrier posed by oral administration and the negative effects of transit through the upper gastrointestinal tract. A second possible approach to *C. difficile* therapeutics is the exploitation of bacteriophage which infect and kill the organism: an example is bacteriophage ΦCD6356, a latent bacteriophage of the Siphoviridae family of viruses recently isolated in our laboratory. In addition, there has been increased interest in the use of probiotics to limit *C. difficile* infection. All three of these approaches will be discussed in this presentation.

023

VALIDATION OF A TRIPLE STAGE IN VITRO HUMAN GUT MODEL TO STUDY THE BIOFILM MODEL OF GROWTH OF *C. difficile* AND THE INDIGENOUS GUT

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Background: The human gastrointestinal tract harbours a complex microbial community, some of which exists as biofilms on the mucosal surface. These sessile bacteria exhibit distinct characteristics from their planktonic counterparts, and therefore there is a fundamental need to understand the biofilm mode of growth of *C. difficile* (CD) and the gut microbiota. CD is known to adhere to colonic cells and mucus, but its interaction with gut biofilms remains unexplored. Baines et al have validated an in vitro triple stage chemostat gut model as a representative model of CDI; however, this model is unable to reflect the complex bacterial communities that colonise intestinal mucosal surfaces within biofilm structures. We describe here adaptations to this model to allow monitoring of biofilm composition at the different stages of CDI.

Methods: A triple stage chemostat gut model was inoculated with a fresh faecal emulsion and maintained at steady state for 3 weeks before an inoculum of CD 027 spores were added to the system. Vessel 3 of the model incorporated 18 glass rods to facilitate the formation and subsequent sampling of biofilm at 3 different time points (prior to CD inoculation, and 6 and 21 days post CD inoculation). The planktonic populations of the indigenous gut microbiota were also monitored using classical culture methods. Six rods were sampled at each time point and the standard errors between bacterial populations present on each rod were determined.

Results: All rods within the system successfully facilitated the formation of biofilm. All indigenous gut microbiota populations enumerated were present within the biofilm including CD spores (post-CD inoculation). The main concern for validation of the system was to ensure that the composition of biofilm on all the rods sampled at one time point was consistent. Whilst some variation was evident between individual rods this was limited to a standard error of ~0.3 log10cfu/g per species group.

Conclusions: The biofilm human gut model successfully monitors the biofilm mode of growth of CD and the indigenous gut microbiota. Further investigational studies utilising this model will allow researchers to monitor the presence of CD spores, vegetative cells, cytotoxin and antimicrobial agents both within biofilm structures and in the planktonic phase. Such approaches may provide a greater understanding of the role of biofilms in the infection process in vivo, potentially including factors that influence the risk of recurrent CDI.

INV9

DIAGNOSTICS AND TYPING OF *Clostridium difficile* INFECTIONS IN EUROPE

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Objectives *Clostridium difficile* infections (CDI) are an important cause of nosocomial diarrhoea. A pan-European initiative by the European Centre of Disease Prevention and Control (ECDC) to enhance awareness and diagnostic capacity of CDI was launched in 2010. We assessed the current laboratory diagnostic capacity and burden of CDI at an institutional level.

Methods National coordinators of 32 European countries were requested to select approximately 10% of microbiological laboratories in their country for participation in this survey.

Results Of 31 countries, 126 laboratories (61%) completed a questionnaire. The estimated CDI incidence was 17,9 per 10,000 admissions and 3,7 per 10,000 patient-days. Of the laboratories, 93% performed CDI testing on request of a physician, whereas testing for antibiotic-associated and nosocomial diarrhoea was performed in 51% and 32%, respectively. Approximately 55% applied a two-step testing as recommended by ESCMID; EIA GDH detection with a second confirmation test was used by 17% of the laboratories. In 74% of the countries, at least one laboratory could perform *C. difficile* typing. Polymerase chain reaction (PCR) ribotyping was most frequently (n=49, 89%) performed method to type isolates at the laboratories; 36 laboratories reported performance of only conventional agarose gel-based ribotyping on their samples compared to 10 for capillary gel based ribotyping, and 3 laboratories did not specify the technique of PCR ribotyping.

Conclusion Across Europe CDI is diagnosed approximately once every 500 admissions. Nearly all European laboratories who participated in the survey had diagnostic tests available for CDI, but reasons for testing varied widely. The majority of the laboratories were capable to culture, but half had capacity for typing. This mixed picture emphasises the uncertainty of the true levels of CDI across Europe.

INV10

INCREASE USE OF RIBOTYPING - BUT WHERE NEXT?

Mark Wilcox

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The original (Cardiff-based) ribotyping database was constructed using gel based ribotyping. It is clear that capillary-based ribotyping offers better resolution and consistency of banding patterns. There is a need to create an accessible ribotyping database; demand for this is increasing as more countries and laboratories are basing *C. difficile* typing surveillance on ribotyping. A collection of 25 common ribotype strains has been circulated to aid local laboratories. 50 further strains are currently being assessed to ensure consistency of ribotype patterns. The next steps towards increasing access to ribotype database information will be discussed.

INV11

ADVANTAGES AND DISADVANTAGES OF WEB BASED SYSTEMS TO IDENTIFY DNA PROFILES

Alexander Indra

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Although PCR-ribotyping is widely used for *C. difficile* typing the method lacks an interlaboratory interchangeable format. In order to overcome this problem capillary-sequencer-based PCR-ribotyping and AGES-WEBRIBO (webribo.ages.at) – a free to use internet platform – was developed.

AGES-WEBRIBO enables every user to automatically analyse their capillary-sequencer-based PCR-ribotyping data by uploading their fsa-File from the most common Applied-Biosystems sequencers-systems. AGES-WEBRIBO is able to analyse data without the need of any additional software whatsoever and overcome capillary-sequencer-based PCR-ribotyping result variations of different primers (e.g. Bidet, Stubbs or Janezic) or size standards by applying a newly developed deterministic identification algorithm.

Currently, users from 27 countries have successfully submitted approximately 11000 samples leading to a total of over 400 different PCR-Ribotype patterns. Of these only 100 PCR-ribotyps in the database are concordance with the nomenclature of the Cardiff strain collection.

A recent study showed that more than 98% of the submitted samples were correctly identified regardless the primer, size-standard or sequencing platform used.

We consider the AGES-WEBRIBO in combination with capillary gel electrophoresis based PCR ribotyping to be a universal tool for inter-laboratory comparability of *C. difficile* ribotyping.

INV12

THE CANADIAN EXPERIENCE WITH MOLECULAR TYPING METHODS FOR *C. difficile*: PFGE, PCR RIBOTYPING AND GENOME SEQUENCING

M. R. Mulvey

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In Canada the current method used for typing *C. difficile* is pulsed-field gel electrophoresis (PFGE). This allows us to compare PFGE types across Canada and with the Centers for Disease Control and Prevention in the USA with some limitations. It is also the standard method used for outbreak investigations in Canada. Unfortunately, this methodology does not allow comparisons to the standard typing methods in Europe and other areas of the world that use PCR-ribotyping. Efforts are underway to try to standardize PCR-ribotyping to allow global comparisons of isolates. With that said, we are entering an exciting new world of molecular typing of *C. difficile* using whole genome sequencing. The technology has advanced so quickly that it is now feasible for real-time outbreak investigations using whole genome sequencing. I will discuss our experience with these three methods and try to give a prediction of where we are headed in terms of molecular typing of *C. difficile* in the future.

O24

AN OUTBREAK OF COMMUNITY-ACQUIRED *Clostridium difficile* INFECTION IN AUSTRALIA, 2011-12.

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Australia has seen a significant increase in rates of *C. difficile* infection (CDI) in the 12 months from July 2011. Data from Western Australia (WA) are representative of all Australian States: the CDI rate during the 1st quarter of 2012 was over 8 cases/10,000 bed days at tertiary hospitals versus 4 cases/10,000 bed days for the 1st quarter in 2011. There was a suggestion of seasonality in 2010-11 that became obvious in 2011-12, with a dramatic increase in spring 2011 and a decline in autumn 2012. Notably, the proportion of CDI cases defined as community-acquired was high during this period, accounting for 45% of CDI at community hospitals in WA, and 25% of CDI at tertiary hospitals.

Late in 2011, we identified approximately 30 cases of infection with an unusual ribotype (RT) (244) in New South Wales. About 30 cases of infection with the same RT were also detected in Victoria, with an attributable mortality of 30%. Interestingly, the main reason the latter strains were detected is that they signalled in the Cepheid Xpert system as putative RT 027 strains. About 20 of these strains were also identified in our WA database during the same period. The vast majority of RT 244 cases were community-acquired with almost all presenting at hospital emergency departments. Based on whole genome sequencing (WGS) undertaken in Oxford, UK, the strains from various States were clonal, suggesting a common source, possibly food.

We have been looking at *C. difficile* carriage/infection in Australian production animals for several years. In adult pigs and cattle the rate of carriage is low and we have not found *C. difficile* in Australian poultry. Rates of carriage and infection in neonatal piglets are very high but neonatal piglets are not slaughtered for food. We have recently sampled veal calves (aged 7 d) from two abattoirs in different States. There was a 72% prevalence of carriage of *C. difficile* overall but we did not find the RT 244 strain. There were 3 predominant RTs: RTs 033 (A-B-CDT+), 126 (A+B+CDT+) and 127 (A+B+CDT+) and several isolates of these RTs are in our human isolate database. These may represent sporadic or endemic infection that has been occurring over a number of years in the community. Why RT 244 seems to have become predominant recently is unclear. Interestingly, the WGS data place this strain into clade 2, which is not traditionally associated with animal strains

but does contain RT 027. The decline in CDI cases in the 1st quarter of 2012 is possibly because the susceptible population in the community has declined. The peak incidence of CDI was just after antibiotic use peaked last spring following the peak in viral RTIs. So, 6 months later, the gut flora in those susceptible, ie those that got antibiotics, has normalised and they are no longer susceptible. If the reservoir is still there then another rise should occur after the next winter influenza season in Australia.

O25

Clostridium difficile FROM AUSTRALIAN CATTLE – ALL WILL BE RE(VEAL)ED!

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Recent reports in North America and Europe of *Clostridium difficile* being isolated from livestock and retail meats has raised concerns of a potential public health risk. Although zoonotic transmission has yet to be proven, there is clear evidence that toxigenic strains of *C. difficile* are present in neonatal livestock and thus present a threat to public health and biosecurity. To date no studies have looked at the situation in Australia. We therefore investigated the prevalence and genetic diversity of *C. difficile* in Australian cattle and calves at slaughter.

Carcass washings, gastrointestinal contents and faeces were collected from abattoirs across five states in Australia. Selective culture was performed and isolates characterised by PCR for toxin A, toxin B and binary toxin genes, and PCR ribotyping.

C. difficile prevalence was 72% (63/88) in faeces from 7 day old calves, 3.8% (1/26) in 2-6 month old calves, and 2.2% (5/225) in adult cattle. The observed decline in prevalence with increasing age supports studies reported elsewhere and in other animals. No *C. difficile* was found in samples of adult cattle carcass washings or gastrointestinal contents. All the calf strains isolated were positive for one or more toxin genes, and 11 different ribotypes were identified. Of those able to be matched with reference strains, three ribotypes, 126 ($A^+B^+CDT^+$), 127 ($A^+B^+CDT^+$) and 033 ($A^-B^-CDT^+$) were identified in 95% (61/64) of calf strains, the latter two ribotypes being from geographically distinct locations. All three identified ribotypes belong to Multi Locus Sequence Type (MLST) 11 and are genetically related to PCR ribotype 078. Ribotype 078 is the most commonly encountered strain in livestock in Europe and North America, and has emerged recently as a significant cause of human infection in Europe with high morbidity and mortality.

Strains belonging to ribotypes 033, 126 and 127 have all been isolated from humans with disease in Australia, however, it remains to be seen as to whether the same ribotypes in calves are indistinguishable by a more discriminatory typing method such as MLVA or whole genome sequencing. Nonetheless, the prevalence of these ribotypes in Australian calves at slaughter is a worrying development, and suggests transmission from calves to humans may be occurring by a yet to be established mechanism, possibly meat contamination. Clearly, *C. difficile* has become a threat to Australia's biosecurity.

O26

Clostridium 078 IN PIGS, A THREAT FOR FARMERS, THEIR RELATIVES AND EMPLOYEES

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Clostridium difficile Type 078 is emerging in humans and animals and is currently the third most frequently found type at the National Reference Laboratory in The Netherlands. The finding of identical *Clostridium difficile* PCR ribotype 078 isolates in piglets with diarrhea and in humans with *Clostridium difficile* infection (CDI) led to the suggestion that interspecies transmission could occur. The aim of this study was to investigate intestinal colonization with *C. difficile* of pig farmers, employees, their relatives, and their pigs.

Farmers and employees (55), partners (31), and children (41) living on 32 pig farms participated in the study. Participants submitted a stool sample, and veterinarians collected pooled fecal samples of 10 different wards at each farm. Fecal samples were cultured using enrichment strategies. Suspected colonies for *C. difficile* were further identified and characterized by PCR ribotyping. Antimicrobial susceptibility was exmanined by E-tesing. Multiple-locus-variable-number-tandem-repeat analysis (MLVA) was used to investigate the genetic similarity of selected human and porcine isolates.

C. difficile was isolated from fecal samples of pigs at 31 of the 32 farms. Type 078 was the predominant ribotype at 30 of the farms positive for *C. difficile*, at 1 farm only type 045 was present. In total 14 (25%) of the farmers and employees were positive, 4 (13%) of the partners and none of the children. All 4 partners reported regular contact with the pigs. The Odd's ratio for colonization and daily contact with pigs versus no contact with pigs was >2. All positive farmers, employees and partners worked on positive farms and corresponding ribotypes were found in the pigs and the humans. This was type 078 in the humans and pigs on 13 farms and type 045 in the farmer and the pigs on 1 farm. Application of MLVA on C.difficile type 045 and 078 isolates from 3 different farms, revealed genetical related complexes encompassing human and pig isolates. Human and pig isolates did not differ in susceptibility to imipenem, co-trimoxazole, erythromycin, clindamycin , tetracycline and moxifloxacin.

The intestinal carriage rate in the population of people with direct contact with pigs positive tested for *C. difficile*, is 25%. The finding of identical isolates from humans and pigs from the same farms with MLVA, indicates that transmission, either via direct contact or the environment, likely occurs. Prospective studies are needed to determine the risk for development of CDI in this population.

O27

EFFECT OF OXYTETRACYCLINE ON *Clostridium difficile* COLONIZATION AND THE FECAL MICROBIOME OF VEAL CALVES

MC Costa[1], *H Staempfli*[1], *LG Arroyo*[1], *FLF Feitosa*[2], *JS Weese*[1]

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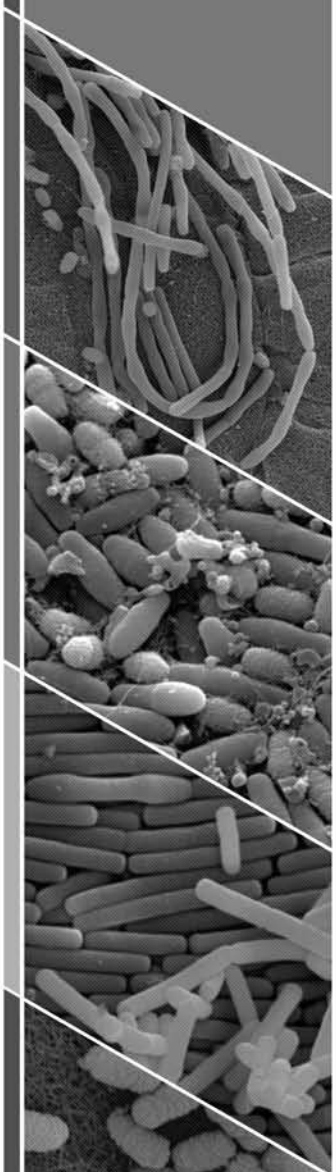
The intestinal microbiome plays an important role in both the pathophysiology and protection from disease. Many factors can influence this complex bacterial population, particularly antimicrobials. *C. difficile* can be found commonly in calves and previous work has suggested that oxytetracycline administration may influence the prevalence of *C. difficile* colonization as well as the strain distribution and prevalence of resistance genes. The objectives of this study were to evaluate the influence of oxytetracycline administration on *C. difficile* shedding and the fecal microbiome in veal calves.

Feces were collected from 22 calves (2-7 days of age) within 24h of arrival on a veal farm. 12 were treated with oxytetracycline as per normal practices while 10 were left untreated as controls. Feces were collected on day 3 of treatment and 2, 10 and 27 days after treatment. *C. difficile* culture, ribotyping and tetM and tetO PCR were performed. DNA was extracted from samples from arrival and 2 days after treatment for 16s rRNA gene PCR and next generation sequencing. The MOTHUR package of algorithms was used for data cleaning and quality control, and subsequent analysis. Overall, *C. difficile* was isolated from 47/110 (43%) of samples; 24/60 (40%) treatment and 23/50 (46%) control samples (P=0.57), with a peak prevalence 2d after cessation of treatment. 21/22 (95%) calves were positive at least once. There was a significant difference between prevalence amongst different sampling days for both the treatment and control groups (both P=0.04) but there was no difference between treatment and control groups, either overall or at any single timepoint. Ribotype 078 was the most prevalent (94% of toxigenic isolates) and was the only type found after day 2. tet(M) was identified in 97% of isolates, with tetO in 11%.

110,274 high quality sequences were examined, with 747 unique operational taxon units overall and 46 per calf. Bacteroidetes was the main Phylum among both groups before oxytetracycline (66 and 47%, respectively), with Firmicutes being the 2nd most prevalent (33 and 29%, respectively). There was a significant decrease in abundance of Bacteroidetes and a concurrent increase in Firmicutes and Actinobacteria in both groups between arrival and day 2. Parsimony test and analysis of molecular variance both identified a significant difference between age but not treatment groups, with phylogenetic clustering by age, not treatment group. Bacterial diversity increased greatly between the two sampling times. Age, not antimicrobial therapy, appears to be a major determinant of changes in the prevalence of *C. difficile* and the fecal microbiome. Natural changes in *C. difficile* and the microbiome must be considered when designing and interpreting studies.

4th International *Clostridium difficile* Symposium



Abstracts of poster presentations

(section 1 P1 to P62, Friday)

First author	#	Title
Gebhart, D.	P1	Diffocins: novel, high-molecular weight bacteriocins highly specific for Clostridium difficile
Ivarsson, M.	P2	Alternatives to antibiotics for the treatment of Clostridium difficile infection
Leuzzi, R.	P3	Functional screening of TcdA and TcdB recombinant fragments as vaccine candidates against Clostridium difficile
Miyajima, F.	P4	The IL8 -251 SNP (rs4073) and other common candidate polymorphisms are not associated with Clostridium difficile Infection (CDI) in a prospective cohort
Péchiné, S.	P5	Impact of the immunization with the heat shock protein GroEL on Clostridium difficile intestinal colonization
Pedneault, L.	P6	Clostridium difficile anti-toxin A and B neutralization antibody titers and carriage in healthy adult volunteers aged 18 to 85 years
Quemeneur, L.	P7	Aluminum hydroxide adjuvant significantly increases immunogenicity and efficacy of a candidate bivalent toxoid vaccine in preclinical hamster challenge model
Spencer, J.	P8	Evaluation of the protective immunity induced by recombinant fragments from toxin A and B from Clostridium difficile in the hamster model
Spencer, J.	P9	Protective efficacy induced by recombinant Clostridium difficile toxin fragments
Sun, X.	P10	Essential involvement of TNF-α in Clostridium difficile infection
Cafardi, V.	P11	Identification and functional characterization of surface and secreted proteins of Clostridium difficile
Dawson, L. F.	P12	Production of biofilms by Clostridium difficile is affected by Spo0A
Faulds-Pain, A.	P13	Investigating the post-translational modification of the Clostridium difficile flagellin
Gerhard, R.	P14	Experimental strategies to combat cytoxic effects of TcdA and TcdB
Kuehne, S. A.	P15	Are both toxins A and B important in Clostridium difficile infection?

First author	#	Title
Lima, B. B.	P16	Clostridium difficile toxin A attenuates Wnt/beta-catenin signaling in intestinal epithelial cells
Olling, A.	P17	Cellular uptake of Clostridium difficile toxin A
Pereira, F.	P18	A protein required for efficient host colonization by Clostridium difficile is an abundant component of the spore surface layers
Salgado, P. S.	P19	Structural studies of the Clostridium difficile S-layer
Seager, Z.	P20	Investigation of potential colonisation factors in a mouse model of Clostridium difficile infection
Spigaglia, P.	P21	Clostridium difficile S layer variability and role in adherence to epithelial cells and intestinal colonization
Tulli, L.	P22	Characterization of a novel collagen binding protein of Clostridium difficile
Walter, B.	P23	Atypical cytophatic activity caused by Clostridium difficile A- B- CDT- strains
Williams, R.	P24	Characterisation of the prophage of Clostridium difficile 12727
Heeg, D.	P25	Spores of Clostridium difficile clinical isolates exhibit heterogeneous germination properties
Kõljalg, S.	P26	Population dynamics and sporulation of quinolone resistant and sensitive Clostridium difficile strains in different environmental conditions
Saujet, L.	P27	The regulatory cascade of sporulation in Clostridium difficile
Ammam, F.	P28	Clostridium difficile is surprisingly not resistant to vancomycin
Ammam, F.	P29	Functional analysis of the putative vancomycin resistance gene cluster (vanG-like) of Clostridium difficile
Biazzo, M.	P30	Diversity of cwp loci in clinical isolates of Clostridium difficile
Bollard, N.	P31	Characterization of Phosphotransferase Systems (PTS) in Clostridium difficile

First author	#	Title
Chapetón Montes, D.		P32Expression and maturation process of the Clostridium difficile protease Cwp84 could be influenced by environmental conditions
Chong, P.	P33	Proteomic analysis of a NAP1 Clostridium difficile clinical isolate with reduced susceptibility to metronidazole
Collery, M. M.	P34	Splitting the difference: the role of SNPs in the virulence of different strains of Clostridium difficile
Dembek, M.	P35	Clostridium difficile cell wall protein CwpV undergoes enzyme-independent intramolecular autoproteolysis
Donahue, E.H.	P36	Investigating the roles of the cell wall anchoring sortase enzyme and sorted proteins in Clostridium difficile
Jabbari, S.	P37	Mathematical modelling of the gene regulation network governing toxin production by Clostridium difficile
Kirby, J. M.	P38	A Clostridium difficile surface-located cysteine protease Cwp84 mutant displays decreased cell integrity and alteration of autolysin transcription
Kuehne, S. A.	P39	Analysing the growth characteristics, spore germination and outgrowth of Clostridium difficile clinical isolates challenged with fidaxomicin
Mac Aogáin, M.	P40	Investigation of QRDR-independent fluoroquinolone resistance in Irish Clostridium difficile isolates
Mascio, C.	P41	CB-183,315 demonstrates low in vitro frequency of spontaneous or multistep resistance in Clostridium difficile, Enterococcus faecalis and E. faecium
Metcalf, D.	P42	The effect of antimicrobials on expression of Clostridium difficile toxin B and binary toxin genes in vitro
Moura, I.	P43	In vitro susceptibility to metronidazole in different C. difficile PCR-ribotypes
Negri, A.	P44	Expression and display of FliD of Clostridium difficile on the surface of Bacillus subtilis spores
Nimitray, J.	P45	Genome-wide identification of small RNAs in Clostridium difficile

First author	#	Title
Pecavar, V.	P46	Clustered regularly interspaced short palindromic repeats (CRISPR) in Clostridium difficile
Peltier, J.	P47	Characterization of two major autolysins in Clostridium difficile
Peltier, J.	P48	Clostridium difficile has an original peptidoglycan structure with high level of N-acetylglucosamine deacetylation and mainly 3-3 cross-links
Rosenbusch, K.	P49	DNA binding by the master regulator of sporulation, Spo0A, of Clostridium difficile 630Δerm
St-Pierre, É.	P50	Inactivation of chemotaxis-associated genes increases flagellar motility in Clostridium difficile
Van Eijk, E.	P51	Identification and characterization of the replication machinery of Clostridium difficile
Willing, S.	P52	C. difficile cell wall protein anchoring
Elliott, B.	P53	Australia — home of Clostridium difficile clade 5?
El Meouche, I.	P54	The SigmaD factor controls the expression of flagella and toxins A and B in Clostridium difficile
Kelly, M. L.	P55	Toxin expression by Clostridium difficile is not entirely dependent on TcdR
Van Leeuwen, H. C.		P56Clostridium difficile TcdC binds quadruplex DNA structures
Wasels, F.	P57	Behaviour of CTnCD11-like elements in Clostridium difficile
Wasels, F.	P58	Tn5398 from Clostridium difficile 630: new insights in mechanism of transfer, biological burden and expression of erm(B)
Chilton, C.H.	P59	Lactobacillus casei affects Clostridium difficile toxin detection in-vitro
Daigneault, M.	P60	A novel ecosystem therapeutic for the treatment of Clostridium difficile infections
Miezeiewski, M.	P61	Analysis of the microbiota of Syrian hamsters following clindamycin treatment and impact on Clostridium difficile susceptibility
Rousseau, C.	P62	Gut microbiota associated with Clostridium difficile colonization in infants investigated by pyrosequencing

P1

DIFFOCINS: NOVEL, HIGH-MOLECULAR WEIGHT BACTERIOCINS HIGHLY SPECIFIC FOR *Clostridium difficile*

Dana Gebhart[1], *Dean Scholl*[1], *Chuck Vacin*[1], *Louis-Charles Fortier*[2], *Steve Williams*[1], *and* Gregory Govoni[1]

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Clostridium difficile infections (CDIs) are a major cause of antibiotic-associated colitis in and out of healthcare settings. Novel therapeutic and preventative treatments are desperately needed to help combat this disease. We have isolated from *Clostridium difficile* novel, high molecular weight bacteriocins, termed "Diffocins", with unique properties that make them potential anti-*C. difficile* agents. Diffocins are phage tail-like particle structures similar to R-type pyocins from Pseudomonas aeruginosa. Based on the contractile structure, we believe Diffocins kill bacteria in a manner analogous to R-type pyocins, whereby the contractile tail punches a small hole in the target membrane to dissipate the membrane potential without leaking larger molecules such as DNA or toxins. Our further investigations of Diffocins have found: 1. Diffocins exhibit a narrow bactericidal spectra, highly selective for *C. difficile*. 2. Diffocins can be manipulated and heterologously expressed in Bacillus subtilis. 3. Diffocins are targetable; we have identified the receptor binding protein (RBP) responsible for killing specificity and can switch specificity by swapping RBPs. 4. Diffocin killing spectra can be expanded by exchanging endogenous Diffocin RBPs for RBPs from *C. difficile* phages/prophages. 5. Orally administered Diffocins remain active after passage through the mouse GI tract. Taken together, these properties indicate that Diffocins are excellent candidates for therapeutic and, especially, prophylactic management of CDIs without causing unintended collateral damage to the gut microbiota.

P2

ALTERNATIVES TO ANTIBIOTICS FOR THE TREATMENT OF *Clostridium difficile* INFECTION

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The current use of antibiotics to treat *Clostridium difficile* infection is suboptimal due to the persistence of relatively high rates of mortality and recurrence, as well as the threat of rising pathogen resistance. New therapies to combat the infection are therefore urgently needed. Toxins A and B, secreted by the bacteria in the gastro-intestinal tract are the agents that cause disease symptoms and it has been shown that neutralizing these toxins could be a viable therapeutic approach.

Polymeric binders are one example of a toxin-targeted therapeutic approach being developed in our lab. Random copolymers of N-2-hydroxypropyl methacrylamide and azidotriethyleneglycol methacrylamide containing varying proportions of each monomer were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. The trisaccharide α-Gal-(1,3)-β-Gal-(1,4)-β-Glc that is known to bind the receptor-binding domain of toxin A was conjugated to a linker with an alkyne group at the extremity, which was used to functionalize the polymers via Cu(I)-catalyzed alkyne-azide "click" cycloaddition. The polymers were characterized by GPC, NMR, and FTIR spectroscopies before being tested for their ability to protect HT-29 cells from toxin-mediated cytotoxicity.

Polymers with controlled proportions of azide groups were successfully synthesized and functionalized by "click" cycloaddition. The functionalized polymers showed a significant reduction in cytotoxic effects caused by toxin A compared to control polymers without the toxin-specific ligand. We believe that the approach presented here has properties that are potentially more favorable than previous efforts to sequester *C. difficile* toxins, namely Tolevamer and Synsorb-90. These properties include (i) specificity through the trisaccharide ligand (which Synsorb-90 also contained) combined with flexibility of the backbone for high binding affinity; (ii) relatively small molecular weight (compared to previously tested binders) and lack of charge to favor diffusion through the mucus layer to the location of toxin secretion and (iii) the tunable nature of the system with regard to size and ligand density allowing systematic optimization of binding properties.

This work was supported by an ETH Zurich Research Grant (ETH-08-10-3).

P3

FUNCTIONAL SCREENING OF TcdA AND TcdB RECOMBINANT FRAGMENTS AS VACCINE CANDIDATES AGAINST *Clostridium difficile*

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Clostridium difficile associated disease (CDAD) is a rising concern worldwide. Many of the clinical symptoms can be directly attributed to the secretion of the glusosyl-transferase toxins TcdA and TcdB, which are extensively recognized as attractive targets for vaccine development.

Currently several vaccine formulations have been proposed, including chemically or genetically inactivated toxoids. As an alternative approach, we expressed a panel of recombinant fragments from both toxins in Escherichia coli and explored their ability to generate neutralizing antibodies to both TcdA and TcdB.

The cross-neutralizing activity of single fragments and their combinations was also investigated, leading to the identification of effective combinations that generate neutralizing antisera against both toxins.

P4

"THE IL8 -251 SNP (rs4073) AND OTHER COMMON CANDIDATE POLYMORPHISMS ARE NOT ASSOCIATED WITH *Clostridium difficile* INFECTION (CDI) IN A PROSPECTIVE COHORT"

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Clostridium difficile has been blamed for several outbreaks and fatalities worldwide, accounting for increased bed utilisation and significant economic burden on the healthcare systems. Exposure to antibiotics and advanced age are clearly risk factors to developing *Clostridium difficile* infection (CDI). However, it remains elusive as to the extent to which host factors contribute to the susceptibility and progression of the disease. The -251 T>A polymorphism of the interleukin-8 gene (rs4073) has been associated with both susceptibility and recurrence of CDI but this has not been replicated by independent research groups.

Methods: We prospectively recruited 421 inpatients (286 cases and 135 controls), who were monitored for twelve weeks (discovery cohort). IL-8 levels in stools were measured in a subset of individuals to confirm previous claims that faecal IL-8 levels were: a) elevated in CDI compared to controls; and b) predicted by genotypes of rs4073. Subsequently, Case-Control genetic association analysis was performed to calculate the odds of developing CDI and recurrent disease using the following candidate gene polymorphisms: Interleukin-8 (-251 T>A), Lactoferrin (632L632 T>C), interleukin receptor antagonist (-1018 T>C) and tumour necrosis factor-α (-238 G>A and -308 G>A). A replication cohort comprising 270 individuals (100 cases and 170 controls) was employed to further confirm results with the risk of developing CDI.

Results: Faecal IL-8 was significantly more elevated in Cases compared to Controls ($p<0.001$). We observed that the minor allele frequencies for all markers above were also consistent with data reported by reference genome sources. Even though this is the largest genetic study on CDI carried out to date, no associations were found with any of the tests performed. Our results suggest that previous genetic studies on CDI would have been underpowered to detect genetic factors of modest effect. CDI is likely to feature very complex interactions between the host, pathogen and the microbiome, thus requiring more powerful and sophisticated approaches in order to elucidate its molecular basis.

P5

IMPACT OF THE IMMUNIZATION WITH THE HEAT SHOCK PROTEIN GroEL ON *Clostridium difficile* INTESTINAL COLONIZATION

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Clostridium difficile is responsible for various intestinal diseases, particularly after treatment with antibiotics. The main virulence factors are the two toxins TcdA and TcdB. Bacterial surface proteins involved in adhesion to the gut and in the colonization process can also be considered as virulence factors. Blocking the primary stages of infection as bacterial attachment to host cells and colonization of the mucosal surface, may be an effective strategy to prevent *C. difficile* infection.
In this study, using the hamster model of *C. difficile* infection, we assessed the protective effect of the cell wall extract of a non toxigenic *C. difficile* strain used as vaccine antigen. After three rectal immunizations with the cell wall extract combined with Cholera toxin as adjuvant, hamsters received Clindamycine. Then, they were challenged by a toxigenic strain of *C. difficile*. Animal sera were sampled before and after immunization and analyzed in detail by two-dimensional electrophoresis coupled with an immunoblot and mass spectrometry in order to reveal the most immunogenic proteins.

In the group immunized with cell wall extract, survival was prolonged in comparison with the control group. Moreover, *C. difficile* fecal enumeration showed that survival animals were later and less colonized by *C. difficile* than the control. For the group immunized by cell wall extract, three immunogenic proteins were detected by immunoblot in the animal sera. Among these, the heat shock protein GroEL was identified.

To confirm the role of the specific GroEL antibodies in the delayed *C. difficile* colonization of hamsters, we performed an immunization assay in a conventional mouse model. After intranasal immunization with the recombinant protein GroEL, we observed a significant difference of *C. difficile* intestinal colonization as compared to the control group.

These results suggest that mucosal immunization with surface proteins more precisely GroEL could protect against *C. difficile* intestinal colonization.Email address of corresponsing author: louise.pedneault@pfizer.com

P6

Clostridium difficile ANTI-TOXIN A AND B NEUTRALIZATION ANTIBODY TITERS AND CARRIAGE IN HEALTHY ADULT VOLUNTEERS AGED 18 TO 85 YEARS

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Background: *Clostridium difficile (C. difficile)* is the main cause of nosocomial infectious diarrhea in industrialized countries. The incidence of *C. difficile*-associated disease (CDAD) has increased during the last decade, mainly due to the emergence of hypervirulent pathogenic strains such as BI/NAP1/027. Several clinical studies suggest a correlation between high serum concentrations of antitoxin A antibodies (as measured by ELISA) and protection from CDAD or recurrence after primary CDAD. Considering the recent changes in the epidemiology of *C. difficile* infection and the lack of recent seroprevalence and carriage data, a better understanding of *C. difficile* carriage and of the immune response against *C. difficile* toxins using more relevant assays is needed.

Methods: A single blood specimen and a rectal swab were collected from healthy individuals aged 18 to 85 years living in the US. Information on baseline demographics and underlying medical conditions was also collected. The rectal swab was cultured in anaerobic conditions for the detection of *C. difficile*. The BD ColorPAC rapid chromatographic assay and Cepheid Xpert PCR assay were used for the detection of toxin A and toxin B on all *C. difficile* isolates, respectively. Neutralizing antibody (Nt Ab) responses against *C. difficile* toxins A and B were measured using an assay developed at Pfizer. Seropositivity was determined as Nt Ab titers > lower limit of quantification of the assay.

Results: A total of 212 subjects were enrolled, with approximately 26 to 34 per age stratum (18-25 years old [yo], followed by 10 year individual age strata). The mean/median age at entry was 52 years (range 18 – 85 yo). The majority (81%) were white and 57% were males. Five of 212 (2.4%) of individuals were *C. difficile* carriers (all strains were toxins A and B negative), and 3 of the 5 subjects were > 70 yo. A total of 47 (22%) subjects had detectable Nt Ab against toxin A and/or toxin B, 9 (4%) and 43 (20%) of them with Nt Ab against toxin A and toxin B, respectively. No difference in seropositivity was observed by age group (p=NS). A trend was observed for higher anti-toxin B Nt Ab titers amongst carriers when compared to non-carriers. Anti-toxin A Nt Ab titers were similarly low amongst carriers and non-carriers. Discussion and Conclusions: A low *C. difficile* carriage rate in a healthy non-hospitalized adult population was confirmed. However, these data suggest that when antibodies are measured by a functional assay, a smaller proportion of individuals have antibodies against *C. difficile* toxins A or B than previously reported. These findings can have implications for the development of a *C. difficile* vaccine.

P7

ALUMINUM HYDROXIDE ADJUVANT SIGNIFICANTLY INCREASES IMMUNOGENICITY AND EFFICACY OF A CANDIDATE BIVALENT TOXOID VACCINE IN PRECLINICAL HAMSTER CHALLENGE MODEL

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SANOFI PASTEUR is currently developing a *Clostridium difficile* toxoid vaccine for the prevention of symptomatic CDI. The vaccine contains formalin-inactivated toxins A and B purified from anaerobic cultures of *Clostridium difficile*. The candidate vaccine is formulated with aluminum hydroxide adjuvant and is currently undergoing Phase 2 clinical testing. In order To understand the role of the adjuvant in the immunogenicity and efficacy of the vaccine formulation, a challenge protection study has been conducted in hamsters with both the adjuvanted and undajuvanted formulations. The hamster is a natural *C. difficile* host and one of the best recognized preclinical models of *C. difficile*-associated diarrhea. Challenge with spore-enriched bacterial suspension induces clinically relevant symptoms after antibiotic pretreatment which reproduces to some extent human disease. Following a 3-dose regime vaccination with different doses of the formulations, Serum IgG antibodies to both toxin A and B were measured by ELISA and toxin-neutralizing antibodies measured using an IMR90 cell-based assay. Protection against morbidity and mortality induced by lethal challenge with live toxinogenic spore-enriched bacterial preparation was also evaluated in vaccinated hamsters. In hamsters vaccinated with adjuvanted formulation, high serum antibody titres to both toxin A and B were induced in a dose-dependent manner. Anti-toxin A seroneutralizing titres were detectable as early as after the first immunization and increased significantly after each boost. Anti-toxin B seroneutralizing titres were detectable only after the second immunization and increased significantly after the third immunization. In hamsters vaccinated with unadjuvanted formulation, anti-toxin A and B seroneutralizing titres were induced only by the highest dose of Ag and detectable only after the second and third immunization. After three immunizations, both anti-toxin A and B serum IgG titres and seroneutralizing titres induced by the adjuvanted formulation were significantly superior to the unadjuvanted formulation. The adjuvanted formulation conferred strong protection at all tested doses whereas the unadjuvanted formulation was only partially protective at the highest dose. Statistical analysis showed that there was a 450-fold reduction in estimated ED50 (efficacy dose inducing 50% protection) with the adjuvanted formulation as compared to the unadjuvanted formulation. Therefore the adjuvant significantly increased immunogenicity and efficacy of this bivalent toxoid vaccine, confirming that the candidate *C. difficile* vaccine is strongly immunogenic and protective in hamsters, when administered in combination with aluminum hydroxide adjuvant, as per intended clinical use.

P8

EVALUATION OF THE PROTECTIVE IMMUNITY INDUCED BY RECOMBINANT FRAGMENTS FROM TOXIN A AND B FROM *Clostridium difficile* IN THE HAMSTER MODEL

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Clostridium difficile associated disease causes major morbidity and mortality especially in the elderly receiving antibiotic therapy in a nosocomial setting. Manifestations of *C. difficile* disease including antibiotic associated diarrhoea (AAD) is largely attributed to the production of two exotoxins, Toxin A and B (TcdA and TcdB). Previously, it has been shown that vaccination with recombinant fragments from the binding region of these proteins provides protection against severe disease in the hamster model of infection. This model is an acute model with clinical signs being observed 25-48h post challenge of clindamycin treated animals with *C. difficile* spores.

Other recombinant fragments from the toxins which have high neutralising antibody titres in mice (Leuzzi et al) have been tested in the hamster model to investigate their efficacy. Results have shown that combinations of such fragments can protect and reduce clinical signs in the hamsters challenged with either *C. difficile* 630 or B1. Vaccination at a lower dose of antigen using such combinations was also successful in protecting the animals against lethal challenge with *C. difficile* B1. In contrast, combination of antigens delivered in a chimeric form reduced the efficacy.

Whilst animals could be protected from death by vaccination, most suffered from short bouts of diarrhoea. These coincided with an elevation of approximately 1°C in body temperature, which also occurred in control animals. The body temperature of the vaccinated animals returned to normal whereas the temperatures of the control animals subsequently dropped below the endpoint of 35°C.

Bacteriology taken during the acute phase shows equivalent levels of bacteria and spores in the caecum and colon of vaccinated and non vaccinated animals. Similarly toxin levels measured in filtered gut material was comparable. However, damage to tissue was minimal suggesting neutralization of the toxins.

Ribosomal 16S sequencing of faecal samples taken throughout the infection process showed gross microbiota changes occurred following antibiotic treatment. In non-vaccinated animals, the flora failed to recover to its pre-antibiotic composition. In contrast, the majority of the flora was restored 6 days post challenge although some minor changes were still observed at the end of the experiment.

P9

PROTECTIVE EFFICAICY INDUCED BY RECOMBINANT *Clostridium difficile* TOXIN FRAGMENTS

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Clostridium difficile associated disease (CDAD) is largely considered to be a toxin mediated disease, with many of the clinical symptoms directly attributed to the secretion of the two large glusosyl-transferase toxins, A and B. The potential to develop vaccines based on these toxins is attractive; indeed vaccines based on inactivated toxoids are currently being evaluated clinically. As an alternative approach, we have rationally designed and tested a panel of recombinant toxin fragments for their ability to protect against acute and fatal infection in the hamster model. Initial screening identified several fragments that generated high levels of neutralizing antibodies in mice. When tested in hamsters, three combinations protected 100% animals from fatal disease when challenged with strains 630 and B1, and using one combination full protection was observed at lower antigen doses. High levels of toxin were detected in gut washes (both vaccinated and controls) at 48 hours from infection. However in contrast to controls, tissue from vaccinated animals showed limited inflammatory influx and damage. Fourteen days after challenge, protected animals showed specific anti-toxin IgGs in the gut and their mucosal tissue was relatively normal except for the appearance of additional goblet cells. On the basis of these data we propose a combination of fragments from toxin A and toxin B that may have potential for use as a vaccine.

P10

ESSENTIAL INVOLVEMENT OF TNF-α IN *Clostridium difficile* INFECTION

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Release of proinflammatory cytokines or other mediators from immune cells is a critical activation event in *C. difficile* toxin-mediated intestinal inflammation. TNF-α is one of the central mediators of inflammation and plays a critical role in host response to infection and cell injury. We found that the direct exposure of *C. difficile* toxins induced mouse dendritic cells (DCs) and peritoneal macrophages to produce TNF-α. More interestingly, the *C. difficile* toxin intoxicated-CT26 cells also stimulated DCs to produce TNF-α. In mouse and piglet models of *C. difficile* infection (CDI) it was found that TNF-α significantly increased in intestine tissues and sera. Subsequent experiments revealed that TNF-α synergized with *C. difficile* toxins on the induction of apoptosis of CT-26 cells. We further examined the roles of TNF-α in toxin A-induced enteritis using TNF receptor (TNFR) knockout mice in a well-established ileal loop model. Injection of TcdA did not induce significant fluid accumulation in TNFR knockout mice, whereas the same dose of the toxin induced massive fluid accumulation in age- and genetic background- matched mice. Histological studies showed that TcdA induced a complete destruction of villi and massive infiltration of immune cells in wild type mice, whereas TNFR KO mice exhibited mild damage of intestinal villi and moderate infiltration of immune cells in response to TcdA. Finally, we demonstrated that TNFR knockout mice were more resistant to *C. difficile* associated diseases than wild type mice in a mouse model of CDI. These results demonstrate that TNF-α plays a crucial role in *C. difficile* toxin-induced intestinal inflammation.

P11

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF SURFACE AND SECRETED PROTEINS OF *Clostridium difficile*

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The emergence of new hypervirulent strains and spread of antibiotic resistance, has resulted in an increase in *C. difficile* associated infections in the last few years. Despite the importance of processes such as gut colonization, persistence and transmission in *C. difficile* infections, molecular events involved in these are poorly characterized. The most widely studied surface components are the proteins that constitute the S-layer, a paracrystalline array surrounding the cell. Several cell wall proteins (CWPs) have been characterized, however biological functions of many of these remain unclear. Very few clostridial surface proteins have been demonstrated to have a role in clostridial infections.

In an attempt to identify and characterize surface and secreted proteins of *C. difficile*, we analyzed culture supernatants prepared from two clinical strains, 630 and Stoke-Mandeville, under various growth conditions using mass spectrometry. Analysis of culture media led to the identification of 60 proteins. While there was no detectable cell lysis, interestingly almost all the proteins identified were predicted to be surface-exposed by bioinformatic analysis and belong to the families of flagellar proteins, CWPs, cell wall hydrolases and ABC transporters. Two proteins of unknown function were selected for further analysis: a first protein, believed to be a peptidase, was tested in vitro for proteolytic activity on several extracellular matrix elements. A second protein, annotated only as a putative lipoprotein, was analyzed in silico and characterized by the generation and analysis of deletion mutants.

Using a combination of proteomic and molecular approaches we have investigated extracellular factors to provide new insights into the role of two Clostridial proteins of previously unknown function.

P12

PRODUCTION OF BIOFILMS BY *Clostridium difficile* IS AFFECTED BY Spo0A

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The formation of biofilms is an integral survival mechanism in many clinically important pathogens such as Staphylococcus aureus and Pseudomonas aeruginosa, to colonise a particular niche, and provide resistance to external influences such as antimicrobial. The important nosocomial pathogen *Clostridium difficle* has been shown to form in-vivo mats in mouse infections, and aggregates in the presence of c-di-GMP, a known inducer of biofilm formation in B. subtilis. We demonstrate that *C. difficile* aggregates and forms biofilms in vitro on abiotic surfaces. These polymicrobial aggregates are attached to each other and to an abiotic surface by an extracellular polymeric substance (EPS), which provides the scaffold bonding together vegetative cells and spores. The EPS matrix provides a protective barrier for vegetative cells against oxygen stress. The master regulator of sporulation Spo0A, may be involved in biofilm formation, as insertional inactivation of spo0A in strain R20291 exhibits significantly decreased biofilm formation. Our findings highlight an important attribute of *C. difficile*, which may have significant implications for *C. difficile* infection, treatment and relapse.

P13

INVESTIGATING THE POST-TRANSLATIONAL MODIFICATION OF THE *Clostridium difficile* FLAGELLIN

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Clostridium difficile is the major cause of health care associated diarrhoea, generally affecting those with disrupted gut microflora. The major virulence factors produced by *C. difficile* are the two toxins, TcdA and TcdB, which cause the more severe symptoms of disease. It is likely however that other properties of these bacteria contribute to their success as a human pathogen.

Flagella-mediated motility is essential to the ability of some pathogens to colonise a human host, such as Helicobacter pylori and Pseudomonas aeruginosa. *Clostridium difficile* also utilises flagella-mediated motility and produces multiple flagella distributed in a peritrichous arrangement across the surface of the cell. The flagella are a major feature of the cell surface of many bacteria and flagellin is the main structural protein that makes up this organelle, with up to 30000 subunits per flagellum. It is this abundance that makes it highly immunogenic and it may elicit innate and/or adaptive immune responses in an animal host. The exposed regions of the flagellin are highly variable between bacteria and in some can be modified; this presumably provides a competitive advantage to the organism.

The *C. difficile* genome encodes a single flagellin gene (fliC) and the protein that it produces is post-translationally modified. Interestingly different modification structures have been reported on the flagellins of strain 630 (ribotype 012) and ribotype 027 strains isolated from recent outbreaks. These differences have been attributed to two predicted alternative modification loci located downstream of fliC. It has been demonstrated in 630Δerm that a glycosyltransferase (GT), encoded immediately downstream of fliC, is both essential for the modification of the 630 flagellin and the organisms' motility. We have determined that the four previously uncharacterised coding sequences (CDSs) downstream of the GT have a role in flagellin modification and motility. Phenotypic and biochemical characterisation have lead us to further elucidate the molecular details for these phenotypes and to investigate their biological significance. The current results of our study will be presented.

P14

EXPERIMENTAL STRATEGIES TO COMBAT CYTOXIC EFFECTS OF TcdA AND TcdB

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Recently, the pathogenic impact of *C. difficile* Toxin A (TcdA, enterotoxin) and B (TcdB, more potent cytotoxin) was intensively discussed. Both toxins, however, share the same molecular mode of action and also more or less identical substrate specificity. Whereas the prevalence of TcdA or TcdB might play a role in diagnostic of pathogenic strains, it is dispensable for therapeutical purpose to know which toxin does represent the main pathogenic factor. Current strategies focus on antibiotic treatment, neglect absorption to polymers, and envisage developing antibody based therapies. Recent findings about functional domains of TcdA and TcdB give reason to explore new ways in toxin inhibition. The present study summarizes experimental strategies to reduce toxin-mediated effects by affecting the toxins itself beside antibody based neutralization or by modulating cellular processes to make host cells less susceptible.

The most promising target domain or function to substantially reduce cytotoxic potency of TcdA and TcdB is the cysteine protease domain (CPD) accompanied by the autoproteolytic cleavage site. Chemical inactivation as well as point mutations of catalytically essential amino acids of the CPD leads to an almost complete loss of cytotoxicity of TcdB. In line with this, mutation of the cleavage site reduces cytotoxic potency of TcdA about 100fold. Substantial reduction of cytotoxic effects can also be achieved by induction of extracellular toxin cleavage of Toxins. Since only the glucosyltransferase domain of the single chain toxins can be translocated from endosomes into the cytosol, pre-cleaved toxins lack their cytotoxicity. First attempts to develop specific glucosyltransferase inhibitors have also been made and will be discussed.

Host cells can be protected against toxins by disturbing the endosomal translocation process of toxin domains from endosomes into the cytosol. The reference substance Bafilomycin A1 inhibits acidification of endosomal lumen thereby completely inhibiting effects of TcdA and TcdB. Modulation of endosomal H^+-ATPase is therefore a pontential target structure to desensitize cells against toxins. In addition, manipulation of the intracellular target structures, the Rho GTPases, was shown to reduce cytopathic effects of TcdA and TcdB, as well. This can be achieved by either activation or phosphorylation of RhoA and Rac1, respectively. Both approaches render Rho GTPases as poor substrate for glucosylation and significantly reduce cytopathic effects of both toxins. The degree of the protective effects as well as their pro and contras will be discussed.

P15

ARE BOTH TOXINS A AND B IMPORTANT IN *Clostridium difficile* INFECTION?

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The two large toxins, TcdA and TcdB, are undoubtedly the main virulence factors in *Clostridium difficile* infection (CDI). However, their respective roles in disease still remain controversial. Notably, whilst two recent studies using isogenic toxin mutants of *C. difficile* both showed the importance of toxin B, one study demonstrated that toxin A alone could not cause disease in hamsters (Lyras et al, 2009) whereas the other showed that an equivalent mutant in the same animal model was virulent (Kuehne et al, 2010). The mutants used in both studies were generated in erythromycin sensitive derivatives of strains 630, which had been isolated independently after repeated serial passage. Therefore, the emergence of ancillary mutations that impact on virulence cannot be discounted. This is currently being investigated through genome re-sequencing and further experiments.

In the meantime, we have extended our analysis to another strain, R20291. This is a PCR ribotype 027/NAP1/B1 (epidemic) strain. Strains of this ribotype are linked to more severe CDI, and produce a third toxin, which is a binary toxin called *Clostridium difficile* Transferase (CDT). We have created stable toxin mutants in strain R20291 using ClosTron technology, to allow examination of each individual toxin and combinations of the three toxins during the disease process. In common with our early study using strain 630 (Kuehne et al, 2010), experiments have shown that R20291 derivatives producing either of the two main toxins (A and B) alone can cause fatal disease in the hamster infection model. Furthermore we started to examine a possible synergistic effect of CDT with toxin A and B. These findings re-establish the importance of TcdA and TcdB in CDI and emphasize a need to consider both when developing effective countermeasures.

P16

Clostridium difficile TOXIN A ATTENUATES WNT//BETA-CATENIN SIGNALING IN INTESTINAL EPITHELIAL CELLS

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Clostridium difficile's main virulence factors are the toxins A and B (TcdA and TcdB), which are homologous glycosyltransferases that inhibit a group of small GTPases (Rho, Rac1 and Cdc42) within the host cells. However, several mechanisms underlying their pathogenic activity still remain unclear. Here, we evaluated the effects of TcdA on the Wnt/BETA-catenin pathway, the major driving force behind the proliferation of epithelial cells in colonic crypts. To accomplish that, we firstly used IEC-6 and RKO cells that were transfected with TOPFlash reporter plasmid and stimulated with Wnt3a-enriched conditioned medium (CM). Then, we treated those cells with different concentrations of TcdA (10, 50 and 100 ng/mL) and saw a dose-dependent inhibition of the Wnt signaling by the TOPFlash luciferase assay for both cell lines. This result was confirmed by immunofluorescence staining for nuclear localization of BETA-catenin and western blotting for BETA-catenin and c-MYC, a Wnt target gene. Moreover, there was a decrease in the BETA-catenin protein levels also noted by western blot analysis. Further evaluation demonstrated that this finding was consistent with caspase activation, since it was attenuated by z-VAD-fmk, a pan-caspase inhibitor, but not reverted by LiCl, a GSK3BETA inhibitor. Nonetheless, even in the presence of z-VAD-fmk, TcdA was still able to attenuate the Wnt/BETA-catenin pathway as demonstrated by TOPFlash luciferase assay. Taken together, these findings suggest that, albeit the activation caspases by toxin A is promoting BETA-catenin downregulation, there are other mechanisms contributing to the inhibition of the Wnt/BETA-catenin signaling by TcdA.

P17

CELLULAR UPTAKE OF *Clostridium difficile* TOXIN A

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The major pathogenicity factors of *Clostridium difficile*, the toxins TcdA and TcdB, reach their target substrates, the Rho GTPases, after entering host cells via receptor-mediated endocytosis. Binding of the C-terminally located combined repetitive oligopeptides (CROPs) of the toxins to so far unknown receptors is acknowledged as prerequisite for toxin uptake. Inconsistent with this view, we recently demonstrated that truncated toxin forms lacking the CROPs are still capable of a specific cellular uptake though exhibiting 5-10 fold reduced cytopathic potency compared to the full length toxins. With respect to these findings we assume that the CROP deletion mutants TcdA1-1874 and TcdB1-1852 are endocytosed by an alternative, however, less efficient uptake mechanism compared to full length TcdA and TcdB, respectively. This might be based either on the recognition of different receptor structures or on the use of various endocytotic routes. In fact, competition experiments revealed that TcdA and TcdA1 1874 predominantly use different receptor structures corroborating the notion of alternative internalization processes utilized by TcdA. In addition, potency of full length and CROP-truncated TcdA towards polarized CaCo-2 cells depended on the site of application. Cells were hardly susceptible towards apically applied TcdA1-1874 whereas basolateral treatment caused a pronounced cytopathic effect. In contrast, susceptibility towards full length TcdA was almost identical and independent on the side of application. This indicates that TcdA and TcdA1-1874 differ in their repertoire of proteins and structures primary used during endocytosis. We therefore examine the proteins essentially involved in endocytosis and discriminate between the uptake processes of full length and truncated toxin A. The use of different internalization routes might enable the *C. difficile* toxins to enter a broader spectrum of cell types. Thus, identification of new target molecules involved in endocytosis could be the basis for the development of toxin intervention strategies.

P18

A PROTEIN REQUIRED FOR EFFICIENT HOST COLONIZATION BY *Clostridium difficile* IS AN ABUNDANT COMPONENT OF THE SPORE SURFACE LAYERS

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Spores produced by *Clostridium difficile* are highly infective and difficult to eradicate, being the primary cause of transmission in health care institutions. Despite the central importance of spores in the pathogenesis of *C. difficile*, our knowledge of the spore-related mechanisms involved in host colonization and infection is still incomplete. Here, we show that a cysteine-rich protein, termed Sp17, unique to *C. difficile*, which is important for colonization in a mice axenic model, is an abundant component of the spore coat. Sp17 is surface exposed, as shown by immunofluorescence microscopy and trypsin accessibility assays, and undergoes extensive multimerization both in vitro and in vivo at the spore surface. Spores of an sp17 mutant fail to assemble an electrondense outer layer of the spore coat, and Sp17 appears to be the main component of this layer. Spores of the sp17 mutant are also susceptible to lysozyme. Importantly, Sp17 also serves a role as a suppressor of spore germination in response to the bile salt taurocholate. Consistent with a role in the assembly of the spore coat, Sp17 is produced during spore development, under the control of the mother cell-specific regulatory proteins SigE and SigK. Our work establishes a direct link between the spore surface and infection. We suggest that both the altered surface of sp17 spores, their increased sensitivity to lysozyme and germination rate contribute to the reduced colonization ability of the mutant.

P19

STRUCTURAL STUDIES OF THE *Clostridium difficile* S-LAYER

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Clostridium difficile expresses a surface layer (S-layer) which coats the surface of the bacterium and is proposed to facilitate interaction of the bacterium with host enteric cells. In *C. difficile*, the S-layer is composed of two SLPs which are derived from post-translational cleavage of a single precursor, SlpA. The SlpA pre-protein contains a signal peptide directing translocation across the cell membrane, after which cleavage occurs producing the mature SLPs. The mature SLPs are a high-molecular-weight protein (~40kDa, HMW SLP) and a low-molecular-weight protein (~35kDa, LMW SLP). These proteins form a tightly associated non-covalent complex, the H/L complex, and the regions of both proteins responsible for complex formation have been identified. Although the structure of the LMW SLP has been determined previously (Fagan et al., 2009), structure of the interacting complex or the HMW SLP have remained elusive.

Recently, we have obtained diffracting crystals of the whole LMW/HMW complex for the native and seleno-methionine derived protein and X-ray structural determination is ongoing. We have also been able to express and purify the two interacting domains, LID (residues 244-321 from LMW SLP) and HID (residues 1-44 from HMW SLP) in a very stable complex. Crystallisation and NMR studies have been carried out to allow the structural determination of these interacting regions of the S-layer proteins. We will report results of our structural efforts which contribute to the understanding of the assembly and structure of the mature S-layer in *C. difficile*.

P20

INVESTIGATION OF POTENTIAL COLONISATION FACTORS IN A MOUSE MODEL OF *Clostridium difficile* INFECTION

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The surface of *Clostridium difficile* is believed to be important for colonisation of the host and for infection. The S-layer consists mainly of two proteins termed the high-molecular weight and low-molecular weight S-layer proteins. 28 minor cell wall proteins (CWPs) are also present within the S-layer, of which only a few have been assigned a function. CwpV and Cwp66 are both members of the CWP family.

CwpV has been shown to be expressed in a phase variable manner and to be an auto-aggregation-promoting factor in *C. difficile* 630. Cwp66 is a putative adhesin, and anti-sera raised against Cwp66 has been shown to block adhesion of *C. difficile* to Vero cells in culture.

We have created insertional deletion mutants of strain 630 in cwp66 and cwpV and have characterised these strain in vitro. In order to investigate the contribution of these genes to colonisation, we have employed and extended a recently described mouse model of colonisation and transmission. We have analysed the behaviour of the cwpV mutant and the cwp66 mutant in mice pre-treated with different antibiotics, either clindamycin or cefoperazone, and we will describe the outcome of both single infection and co-infection studies.

P21

Clostridium difficile S LAYER VARIABILITY AND ROLE IN ADHERENCE TO EPITHELIAL CELLS AND INTESTINAL COLONIZATION

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The aim of this study was to characterize the SlpA of animal and human strains belonging to different PCR-ribotype (PR) and to compare the in vitro adherence and in vivo colonization properties of strains showing different SlpA variants. A close relationship between *Clostridium difficile* Surface layer protein A (SlpA) and PCR-ribotypes (PR) was observed. An identity of 99-100% was found among the SlpA amino acid sequences of isolates belonging to PR 012, 014/020, 066, 078. One exception was the SlpA of a poultry isolate PR 014/020, which showed 53% identity with the SlpA of the other strains 014/020 but 89% identity with that of strains PR 078. In particular, five different SlpA variants (V1-V5) were identified in strains PR 014/020.

In vitro adherence assays and in vivo colonization experiments were performed on four strains PR 014/020: poultry/SlpA V4, poultry/SplA V2, human/SlpA V2 and human/SlpA V5. In vitro adherence assays on Caco-2 cells was assessed at 3 (non confluent monolayers) and 15 days (post confluent monolayers), with or without EGTA. Colonization properties were assessed in vivo using a dixenic mouse model of colonization. The intestinal kinetics of implantation (faecal shedding) was followed during 7 days in this competition model. Caecal adherence was determined at day 7. Statistical analyses were performed using Mann and Whitney test with GraphPad Prism software. A p value< 0.05 was considered significant. In vitro adherence was significantly higher for the human /SlpA V2 compared to the others strains, except when it was compared to poultry/SlpA V4 at day 3. In vivo, according to antibiotic resistance profiles, we were able to compare the colonization properties of human/Slp type V2 to poultry/Slp V2, poultry/Slp V4 and human/Slp V5. The kinetics of implantation was similar when human/Slp V2 strain was compared to poultry/Slp V2 and human/Slp V5 strains. In contrast, poultry/SlpV4 strain outcompeted human/SlpV2 strain and its adherence to caeca at day 7 was significantly higher.

The results indicate that different SlpA variants can be found among strains of different origin belonging to PR 014/020 and that these strains can have a different behavior. In particular, in vitro adhesion results indicate the human strain/SlpA V2 as the most adhesive, followed by the poultry strain/SlpA V4. In contrast, in the dixenic mouse model, the poultry strain/SlpA V4 displayed an ecological advantage to colonize the intestinal animal niche. . Interestingly, the SlpA V4 shows 89% identity with the SlpA of strains PR 078, which are frequently isolated during in CDIs in both animals and humans from the community.

P22

CHARACTERIZATION OF A NOVEL COLLAGEN BINDING PROTEIN OF *Clostridium difficile*

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Clostridium difficile, a Gram-positive spore forming anaerobic bacterium, is a frequent cause of hospital-associated infections. *C. difficile* infection is primarily associated with the use of broad-spectrum antibiotics that suppress the normal gut flora allowing the bacterium to colonize the intestine. Pathogenicity of *C. difficile* is mediated by toxin A and B and extensive studies have defined their precise modality of action and their interferences in cellular pathways of the host, via disruption of the cell cytoskeleton by the glucosylation of the Rho family of GTPases.

Whereas it is assumed that colonization is a prerequisite to toxin pathogenicity, little is known yet about the role of nontoxin proteins in *C. difficile* virulence, either during colonization or in the pathogenic process of the bacterium.

The availability of the whole *C. difficile* genome allows the identification of putative surface proteins of *C. difficile* that could mediate the adherence to mucosal surfaces and the colonization of the intestine. By searching for sequence homologies to known virulence factors and bacterial protein motifs involved in interaction with the host, we have selected a protein with an LPXTG-like motif and characterized it by the following approaches: i) ability of the recombinant protein to bind to human cells, tissue sections and extracellular matrix components; ii) expression on the surface of Lactococcus lactis to evaluate its capacity to confer adhesiveness to extracellular matrix components and ECM-producing human fibroblasts and iii) contribution to bacterial adherence to human cell lines.

These studies have revealed that the antigen is exposed on the bacterial surface and has the ability to adhere both to extracellular matrix components and epithelial cells. These findings open new perspectives in the understanding of *C. difficile* mechanism of colonization.

P23

ATYPICAL CYTOPHATIC ACTIVITY CAUSED BY *Clostridium difficile* A- B- CDT- STRAINS

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C. difficile is known as a producer of toxin A, toxin B and binary toxin which contribute to the pathogenicity of the bacterium. Their activity in vitro can be seen as typical rounding of different cell lines. Several non-toxinogenic strains (A-B-CDT-) have been observed to exhibit atypical cytotoxicity even though they are lacking the genes encoding *C. difficile* toxins. The aim of this study was to investigate this new potentially toxic *C. difficile* molecule and characterize its effect on eukaryotic cells.

The biochemical analysis encompassed size determination, evaluation of heat stability along with susceptibility to proteinases and lipase treatments. The size of the *C. difficile* compound, established using commonly available centrifugal concentrators, was determined as less than 3KDa. The compound was found to be heat and proteinase stable. Interestingly, the lipase A treatment did not inhibit cytophatic activity either.

Moreover, the cytotoxic activity of studied *C. difficile* A-B-CDT- strains was observed in vitro on various cell lines such as HeLa, McCoy and Vero. The activity was primarily examined under a phase contrast microscope and revealed a decrease in the number of viable cells and also noticeable alteration to the cell morphology. Further analysis and confirmation of the toxic action on the eukaryotic cells was evaluated by flow cytometry. The analysis with propidium iodide and AnnexinV showed 20% higher numbers of early apoptotic cells for *C. difficile* compound treated samples in comparison to non-treated samples. The cell cycle analysis allowed observation of accumulation of cells in G2/M phase. Moreover, the extended cell synchronisation facilitated apoptosis showing significant number of dead cells in *C. difficile* treated samples.

Heat and proteinase stability significantly decreases the possibility of protein - like structure; however, it does not exclude it completely as small peptides can be resistant to the proteolytic activity. Moreover, the small size of the compound makes further purification particularly difficult. Nevertheless the microscopic evaluation of the *C. difficile* compound treated eukaryotic cells, confirmed by cytotoxic assays, established on flow cytometry provide strong evidences for the presence of yet another, toxin-like compound that might play a role in *C. difficile* pathogenicity.

P24

CHARACTERISATION OF THE PROPHAGE OF *Clostridium difficile* 12727

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The *Clostridium difficile* strains sequenced to date contain a number of mobile genetic elements, including temperate bacteriophages. We have characterized the prophage present in *C. difficile* 12727, including ϕCD27. This prophage is integrated in a gene encoding a putative ABC transporter, and at the same chromosomal location as prophage 2 in *C. difficile* 630. The integrated phage is flanked by 10bp almost perfect direct repeat sequences (1bp difference), and the attP site lies between ORFs 41 and 42 of ϕCD27. We have amplified both the circularized form of ϕCD27 and the empty genomic site from colonies of NC12727, indicating that this phage forms a circular intermediate upon excision from the chromosome, and that within a subset of cells ϕCD27 is not present at this chromosomal location. We have also identified a second prophage within NCTC12727which is integrated in an ORF encoding a putative flagellum-specific ATP synthase. This integrated phage is flanked by 7bp direct repeats. Similar to phage 27, we have amplified the empty genomic site from colonies of 12727. To investigate the regulation of the lytic and lysogenic cycles of ϕCD27 further, we have carried out RT-PCR experiments on lysogens of ϕCD27sensitive strains, and demonstrate that the putative phage lytic cycle repressor (orf44) is expressed in some of these lysogens.

Temperate bacteriophage have been shown to have an effect on toxin production in *C. difficile* and we have demonstrated that lysogeny with ΦCD27 into sensitive strains leads to a decrease in toxin production in some strains, but an increase in others.

P25

SPORES OF *Clostridium difficile* CLINICAL ISOLATES EXHIBIT HETEROGENEOUS GERMINATION PROPERTIES

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Spores are important factors in the spread of and infection with *Clostridium difficile*. Lying dormant in the environment, spores of *C. difficile* can resist a variety of industrial cleaning products, alcoholic hand gels and antibiotics. Once ingested by susceptible individuals, spores of *C. difficile* must abjure dormancy and return to vegetative cell growth through germination in order to cause disease. Previously, it has been shown that bile salts play an important role in the germination of *C. difficile* and although there is evidence for the presence of putative germinant receptors, such remain to be disclosed. Building on previous work, in which we had shown that spores of *C. difficile* clinical isolates differ significantly in their response to the known germinant taurocholate and that chenodeoxycholate cannot universally inhibit germination or outgrowth in *C. difficile* spores, we have further examined the spore germination properties of *C. difficile* clinical isolates of various PCR-ribotypes in response to different germinant solutions. Initiation of spore germination was assessed as loss in OD600 in combination with measurement of release of Ca- dipicolinic acid, a method predominantly described for other spore formers, which has been adapted for use in *C. difficile*. In addition, the ability to complete the germination process and return to vegetative cell growth was examined by measuring outgrowth and detection of colony forming units. Combining these assays we have further investigated the role of bile salts and other factors in the germination process, showing that dormancy and germination properties are highly heterogeneous among different isolates of *C. difficile*. Interestingly, we have observed an increased release of Ca- dipicolinic acid from spores of some isolates in response to a combination of the bile salts taurocholate and chenodeoxycholate as well as a return to vegetative cell growth in response to chenodeoxycholate alone, suggesting that chenodeoxycholate might play a role in spore germination in these isolates. Additionally, we have observed the ability of spores of some isolates to return to vegetative cell growth after boiling at 100°C for 2 hours. This alludes to an increased heat resistance compared to Bacillus spores and to the possibility of wet heat activation of spores of *C. difficile*.

P26

POPULATION DYNAMICS AND SPORULATION OF QUINOLONE RESISTANT AND SENSITIVE *Clostridium difficile* STRAINS IN DIFFERENT ENVIRONMENTAL CONDITIONS

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C. difficile (CD) is anaerobic spore forming Gram-positive bacillus responsible for hospital acquired diarrhoea. The sporulation is important for pathogen to survive in unfavourable conditions (e.g aerobic atmosphere or presence of intestinal indigenous bacteria such as lactobacilli [LB]). Quinolone (especially moxifloxacin [MF]) resistance has been associated to higher virulence of CD and used for respective screening. **The aim** of our study was to evaluate the differences between MF resistant (MFR) and sensitive (MFS) strains of CD concerning population dynamics and sporulation in different environmental conditions.

Methods. Dynamics in growth and sporulation of 30 CD non-027 ribotype strains (9 MFR, 21 MFS) were studied in brain-heart infusion broth using serial dilution and plating at 0, 10, 24 and 48 h (initial conc 5x10^5 CFU/ml). Environmental conditions compared were anaerobic at 37°C vs. ventilated tubes at 22°C and for 12 CD strains anaerobic at 37°C with or without presence of 5 LB of human origin in equal amounts (strains were tested separately).

Results. Median growth of CD at 48 h was 4.0 log10 CFU/ml higher (7.0 log10 CFU/ml vs. 3.0 log10 CFU/ml) and median sporulation 2.8 log10 CFU/ml higher (5.3 log10 CFU/ml vs. 2.5 log10 CFU/ml) at 37°C compared to 22°C. Growth at 22°C influenced more MFR strains compared to MFS ones (median -2,8 log10 CFU/ml vs. median -1.4 log10 CFU/ml, respectively, p=0.044, at 48 h). The spore amount at the starting point (0 h) was similar in MFR and MFS strains. After 48 h significantly less spores were detected in MFR compared to MFS strains (median 2.0 log10 CFU/ml vs. 3.0 log10 CFU/ml, respectively, p=0.024). The growth and sporulation activity of MFR and MFS strains was similar at 37°C. Co-culturing of CD strains with LB strains significantly lowered the growth rate (median 2.3 log10 CFU/ml vs. 0 log10 CFU/ml, respectively; p<0.001) and amount of detected spores (5.0 log10 CFU/ml vs. 2.0 log10 CFU/ml, respectively; p<0.001) at 48 h when compared to CD alone. The influence of LB to growth of CD was similar regardless of MF susceptibility, however the amount of detected spores was significantly lower in MFR compared to MFS strains (median 1.3 log10 CFU/ml vs. 2.3 log10 CFU/ml, respectively; p=0.012) at 48 h co-growth with LB.

Conclusion. The population dynamics and sporulation of moxifloxacin resistant CD strains is more influenced by different unfavourable environmental conditions.

P27

THE REGULATORY CASCADE OF SPORULATION IN *Clostridium difficile*

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The spore is the infective form of *Clostridium difficile* and is involved in the persistence of this bacterium in the environment. The regulatory cascade controlling spore formation is still poorly characterized in *C. difficile* compared to Bacillus subtilis. We first tested the role of SigH, an alternative sigma factor involved in the post-exponential phase and the initiation of sporulation in B. subtilis. We inactivated the sigH gene in *C. difficile* and we compared the expression profiles of the 630E strain and the sigH mutant at the onset of stationary phase. SigH positively controls genes required for sporulation. Accordingly, sigH inactivation results in an asporogeneous phenotype. The expression of tcdA and tcdB encoding the toxins and of tcdR encoding the sigma factor required for toxin production increased in a sigH mutant. So, the sigH mutant is unable to sporulate but still produces toxins demonstrating that toxin synthesis is a stationary phase event. We are now studying the regulatory cascade of the sporulation sigma factors, SigF, SigE, SigG and SigK. We compared the expression profiles of the wild type and the sigF and sigE mutants after 14 h of growth. 177 and 200 genes were down regulated in the sigF and the sigE mutants, respectively. As observed in B. subtilis, SigF positively controls the transcription of gpr, spoIIQ and spoIIR and SigE activates the transcription of spoIIID, spoIVA and the spoIIIAA operon. Even if many genes of the SigF and SigE regulons are conserved, we observed some significant differences between *C. difficile* and B. subtilis.

Finally, the genome wide determination of the transcriptional start sites of the 630E strain, recently performed in the laboratory, allowed us to map about 1000 transcriptional start sites. Among them we identified SigH, SigF, SigE, SigG and SigK dependent promoters and a consensus sequence for each sigma factor. We identified 26, 20 and 33 genes with a mapped SigH, SigF and SigE promoter, respectively and SigG and SigK direct target genes. We found SigE and SigK consensus sequences in the promoter region of genes encoding proteins of unknown function that are down regulated in the sigE mutant. These genes could encode proteins involved in cortex or coat formation in *C. difficile*.

P28

Clostridium difficile IS SURPRISINGLY NOT RESISTANT TO VANCOMYCIN

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Vancomycin is a major antibiotic for the treatment of *Clostridium difficile* diarrhea. Although vancomycin resistance remains unknown in this organism, a cryptic gene cluster homologous to the vanG operon of Enterococcus faecalis was detected in 85% of *C. difficile* strains. We observed by QRT-PCR that the vanG-like resistance operon in *C. difficile* 630 is not expressed but is nevertheless inducible by vancomycin. This is consistent with the functionality of the two-component vanRSG-like regulatory system. Analysis of the purified resistance proteins in vitro VanGG-like, VanXYG-like and VanTG-like: indicated ligase, dipeptidase and racemase activity, respectively. These functions led to peptidoglycan precursors ending by D-Ala-D-Ser and elimination of natural precursors ending by D-Ala-D-Ala, confering vancomycin resistance in various Gram-positive bacteria. The precursor composition of *C. difficile* was therefore analyzed. Growth of *C. difficile* with a sub lethal concentration of vancomycin led to production of D-Ala-D-Ser precursors in the cytoplasm. This result confirmed that the resistance operon is functional in *C. difficile*. vanG-like and VanC-type resistance operons are similarly organized. The main difference is that VanXYC protein displays both dipeptidase and carboxypeptidase activities whereas VanXYG-like only acts as a dipeptidase. Introduction of vanC-operon from Enterococcus gallinarum in *C. difficile* 630 increased weakly the minimal inhibition concentration (from 2 to up to 4). These results showed that vanG-like cluster of *C. difficile* is inducible by vancomycin and led to synthesis of D-Ala-D-Ser precursors, however, host factor likely prevent the emergence of vancomycin resistance.

P29

FUNCTIONAL ANALYSIS OF THE PUTATIVE VANCOMYCIN RESISTANCE GENE CLUSTER (VANG-LIKE) OF Clostridium difficile

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Analysis of *C. difficile* genome revealed presence in 85% strains of a gene cluster homologous to the vanG operon responsible for vancomycin resistance in Enterococcus faecalis. The vanG-like cluster is governed by a functional two component regulatory system VanR/SG-like. Until now, vancomycin resistance remains unknown in *C. difficile*, and presence of this cryptic operon raises the question of its capacity to lead to resistance emergence. To assess this possibility, VanG-like, VanXYG-like and VanTG-like proteins were purified for enzymatic assays. Kinetic characterization indicated that VanG–like ligase was 290 fold more efficient in synthesizing D-Ala-D-Ser than D-Ala-D-Ala. VanXYG-like exhibited a D,D-dipeptidase without D,D-carboxypeptidase activity. VanTG-like catalyzed both serine and alanine racemase activity with Kcat/Km of 70 min-1/mM and 120 min-1/mM, respectively. Comparison with VanG of E. faecalis and VanC of Enterococcus gallinarum indicated that vanG-like cluster encoded similar functional enzymes. Taken together, these results suggest that vancomycin susceptibility of *C. difficile* could result from intrinsic factors. We therefore analyzed MurF from *C. difficile*, since a defective transfer in peptidoglycan precursors could led to incapacity for resistance. MurF catalyzes the addition of the D-Ala-D-Ala to the UDP-MurNAc-tripeptide. In fact, specificity of MurF in *C. difficile* was found similar for both D-Ala-D-Ala and D-Ala-D-Ser excluding a role of MurF. In conclusion, the cryptic vanG-like cluster encoded functional enzymes indicating that the susceptibility of *C. difficile* to vancomycin is due in part to host factors, however specificity of MurF was not involved.

P30

DIVERSITY OF CWP LOCI IN CLINICAL ISOLATES OF *Clostridium difficile*

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An increased incidence of *Clostridium difficile* infection is associated with the emergence of hypervirulent strains characterised by high genetic diversity. In this study, we analyse the distribution and variability of 14 cwp genes in 40 *C. difficile* clinical isolates of the 6 prevailing PCR-ribotypes in Italy and, more generally, in Europe. To date, 29 cwp genes have been identified in *C. difficile* which code for a family of cell wall proteins (CWPs) involved in colonisation and pathogenesis. All of these CWPs contain two or three cell wall binding motifs in addition to a domain specifying a unique function. Several of the cwp genes are not conserved in all the *C. difficile* genomes characterised so far. In general, 12 of the 29 cwp genes are clustered in the same region of the genome, named after slpA (cwp1) the slpA locus, whereas the remaining 17 paralogs are distributed throughout the genome.

The sequence of 14 of these 17 cwp paralogs has been determined in the majority of the 40 *C. difficile* clinical isolates. Based on sequence conservation, these cwp genes could be divided into two groups, one comprising cwp loci having identical sequences in all isolates irrespective of the PCR-ribotype, and the other showing low conservation between isolates of the same PCR-ribotype as well as between different PCR-ribotypes.

Several of the cwp genes under study have been characterised further by Western blot analysis of total cell extracts or S-layer preparations of the *C. difficile* clinical isolates. Expression of Cwp18 and Cwp25, two highly conserved but uncharacterised Cwps, was detected in total extracts of all the clinical isolates analysed. In contrast, the non-conserved Cwp27 was found to be present in S-layer preparations of isolates of PCR-ribotypes 012, 014, 018 and 027 but absent in strains of PCR-ribotypes 001, 078 and 126. Similarly, expression of the variable Cwp26 was detected in most but not all isolates.

Knowledge of the conservation of these genes in clinical isolates will provide another tool for classifying newly emerging strains.

P31

CHARACTERIZATION OF PHOSPHOTRANSFERASE SYSTEMS (PTS) IN *Clostridium difficile*

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PhosphoTransferase Systems (PTSs) are one of the main methods for sugar uptake in bacteria and have been well described in the past. However research into PTSs within the genus Clostridium has been mainly restricted to the non-pathogens. Analysis of the genome of *Clostridium difficile* 630 revealed over 40 intact PTSs, a perhaps surprisingly large number; this is over three times as many as in other pathogenic Clostridia like C. perfringens and C. botulinum. Previously carbon catabolite repression has been shown to affect toxin production in *C. difficile*. Being capable of utilizing carbohydrates efficiently could be important for *C. difficile* to grow and survive in the human gut. So far very little work has been done to elucidate the role of individual systems in carbohydrate uptake, sensing of the environment and regulation of toxin expression. A deeper understanding of the PTSs of *C. difficile* and their importance in virulence could lead to the development of new drug targets.

The aim of this study is to characterize the main PTSs of *C. difficile*, determine their role in carbohydrate uptake and their effect on regulation of virulence. So far we have chosen what we believe are the main candidates involved in glucose, mannitol, and fructose uptake, and have inactivated these PTSs using the ClosTron. Firstly, we aim to prove their role in uptake of the relevant sugar, and secondly, to determine their role in toxin regulation.

P32

EXPRESSION AND MATURATION PROCESS OF THE *Clostridium difficile* PROTEASE CWP84 COULD BE INFLUENCED BY ENVIRONMENTAL CONDITIONS

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The two major surface proteins of *Clostridium difficile* are the HMW- and the LMW- S-layer, that are involved in the colonization step of the gut. These two proteins are derived from a common precursor SlpA after cleavage by a cysteine protease, Cwp84. Cwp84 is mainly surface-associated, but is also recovered from the extracellular medium. It displays proteolytic activity toward some extracellular matrix proteins (ECM). Previously, we have shown that in surface-associated protein extracts obtained in the stationary growth phase, Cwp84 expressed different protein profiles depending on the growth medium: in TY, Cwp84 was primarily recovered as a ~80 kDa form, while in TYG the protease was both recovered as the ~80 kDa form and as the 47 kDa-form. The ~80 kDa form is undoubtedly the form responsible for the cleavage of SlpA, and the 47 kDa form may correspond to the cleaved mature form of the protease, able to degrade ECM proteins. This difference suggests that glucose, or decrease of the pH medium due to the metabolism of glucose, could have a regulatory role in the maturation process of Cwp84 or in its subcellular localization. Therefore, we analyzed the potential modulation of Cwp84 expression and maturation in response to glucose and environmental acidification. We quantified the level of expression of cwp84 by qRT-PCR, from bacteria grown in TY, TYG and TYG buffered media. Our results suggested that expression of cwp84 was moderately up-regulated by decreasing pH due to glucose metabolism, and this was correlated to the level of expression of Cwp84 in the glycin extract, quantified by immunoblot analysis. Furthermore, in the cell surface-associated protein extracts, the Cwp84 proteolytic process observed in the stationary phase in TYG was not observed in TYG buffered media. This suggests that the acidic pH could also play a role in the modulation of the Cwp84 maturation process. To address this question, we purified the recombinant protein Cwp84 under pH 8.0 or 5.8 conditions. The recombinant protease purified in acidic conditions was converted into the mature form of 47 KDa during the purification. In contrast, this mature form was not observed in the elution fractions recovered from purification at pH 8.0. All these results suggest that growth of *C. difficile* at an acidic pH derived from sugar metabolism led to enhanced Cwp84 expression and maturation process. During the course of infection, after spore germination, *C. difficile* encounters a gradual decrease in pH along the digestive tract. In response to pH modification, *C. difficile* could both increase the production of the protease and initiate the release of the ~80 kDa form from its surface, followed by its cleavage in the mature 47 kDa form, that may degrade ECM host proteins. A differential partitioning of Cwp84 under different pH conditions could therefore participate in the adaptation of Cwp84 to the host microenvironment.

P33

PROTEOMIC ANALYSIS OF A NAP1 *Clostridium difficile* CLINICAL ISOLATE WITH REDUCED SUSCEPTIBILITY TO METRONIDAZOLE

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NAP1 *Clostridium difficile* strains have been implicated in the sharp increase of *C. difficile* infections (CDI). Metronidazole (MTZ) has long been prescribed as a frontline treatment for CDI, but recent reports describe clinical isolates of *C. difficile* that are hetero-resistant to MTZ. In 2009 our laboratory received a *C. difficile* clinical isolate (CD26A54) which displayed an MICMTZ of 32µg/ml by E-test. The isolate's MIC diminished to 2µg/ml (herein referred to as CD26A54_S) after a freeze/thaw at -80oC. However, a subpopulation of CD26A54, with a stable MIC of 16µg/ml (CD26A54_R), was isolated after passage on BAK containing 8µg/ml MTZ. A shotgun proteomics approach, using iTRAQ-labeling and two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS), was used to study changes to the proteomes of CD26A54_S, CD26A54_R, and MTZ-sensitive control strain VLOO13, to identify potential proteins that may be involved with MTZ resistance in *C. difficile*. The cultures were grown to mid-log phase (OD600=0.3) in BHI broth, and spiked with MTZ at concentrations 2 doubling dilutions below the MIC: CD26A54_R, 4µg/ml; CD26A54_S, 0.75µg/ml; and VLOO13, 0.19µg/ml. The cultures were incubated for an additional 30 minutes prior to cell harvest, followed by protein extraction for trypsin digestion, and iTRAQ-labeling. After combining the results of 5 independent 2D-LC-MS/MS experiments, 1983 proteins were identified, with a false discovery rate of 0.5%. Comparisons were made on all strains, with or without MTZ treatment. In the absence of MTZ, higher expression was observed in some proteins that may be involved with reduced susceptibility to MTZ in both CD26A54 strains, but not observed in VLOO13, including DNA repair proteins, such as the Uvr excinuclease, and a putative 5-nitroimidazole reductase, which is involved in MTZ resistance in other anaerobic bacteria. After treatment with MTZ, several stress response-related proteins were expressed in all strains, including the 10 kDa chaperonin, heat-inducible transcription regulator HrcA, and a heat shock protein. Previous work from our laboratory indicated the presence of a single nucleotide polymorphism in the fur gene of CD26A54_R, which encodes the ferric uptake regulator (Fur), a multifunctional regulator involved in iron homeostasis; expression of Fur was ~2-fold higher in CD26A54_R compared to CD26A54_S, and ~3-fold higher than in VLOO13. Interestingly, the expression of the DNA repair protein RecA was increased 2-fold in CD26A54_R after MTZ exposure, but not in CD26A54_S or VLOO13, suggesting a greater rate of repair of MTZ-damaged DNA in CD26A54_R, which may account for the higher MICMTZ observed with CD25A54_R. Our data suggests that a multi-factorial response may be associated with MTZ- resistance in *C. difficile*.

P34

SPLITTING THE DIFFERENCE: THE ROLE OF SNPS IN THE VIRULENCE OF DIFFERENT STRAINS OF Clostridium difficile

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The precise role in disease of the two major toxins of *Clostridium difficile* – TcdA and TcdB – has recently become the subject of controversy. In 2009, Lyras et al published a paper claiming that TcdB alone was responsible for *C. difficile* infection (CDI) and that an isogenic strain producing only TcdA was incapable of causing CDI in a hamster infection model. Eighteen months later, in the same journal and using a closely related strain, Kuehne et al showed that both TcdA and TcdB are independently capable of causing CDI in the hamster model. Both the strain used by Lyras et al (630E) and Kuehne at el (630Δerm) are erythromycin-sensitive strains derived by serial passage (Kuehne, n=30, Lyras, n = unknown) of strain CD630. It seems probable that the differences in virulence of the two strains are being caused by ancillary mutations which could have arisen during passage. Genome re-sequencing of both stains and comparison of these to each other and to the original CD630 genome revealed the presence of 12 single nucleotide polymorphisms (SNPs) that were common to 630E and 630Δerm. In addition to these, 630Δerm had a further nine unique SNPs compared to CD630 and 630E, while 630E also had 11 unique SNPs compared to 630Δerm. To test the hypothesis that these SNPs account for the reduced virulence of 630E, we began the process of correcting several of these SNPs in 630E back to the CD630/630Δerm genotype using allelic exchange technology, while simultaneously introducing the SNPs into the 630Δerm genome. We have also used the ClosTron system to knock out genes that have been found to be truncated by SNPs in 630E to assess their impact on the virulence of the strain. It is anticipated that this approach will resolve the controversy surrounding the roles of TcdA and TcdB in CDI by demonstrating categorically that SNPs in 630E are responsible for the loss of virulence and other observed phenotypic differences.

P35

Clostridium difficile CELL WALL PROTEIN CwpV UNDERGOES ENZYME-INDEPENDENT INTRAMOLECULAR AUTOPROTEOLYSIS

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Clostridium difficile infection is the leading cause of antibiotic associated diarrhoea, placing considerable economic pressure on healthcare systems and resulting in significant morbidity and mortality. Although toxin-mediated pathogenicity has been studied extensively in recent years, relatively little is known about the early stages of infection that are critical for colonization of the human gut. Factors expressed on the bacterial cell surface are likely to contribute to host colonization via interactions with host tissue, the immune system, and other bacterial cells. *C. difficile* produces a proteinaceous array on its cell surface known as the S-layer, consisting primarily of the major S-layer protein SlpA and a family of SlpA homologs. CwpV is the largest member of this family and is expressed in a phase-variable manner. The protein is post-translationally processed into two fragments that form a noncovalent, heterodimeric complex. To date, no specific proteases capable of cleaving CwpV have been identified. Using site-directed mutagenesis we show that CwpV undergoes intramolecular autoproteolysis, most likely facilitated by a N-O acyl shift, with Thr-413 acting as the source of a nucleophile driving this rearrangement. We demonstrate that neighboring residues are also important for correct processing of CwpV. Based on protein structural predictions and analogy to the glycosylasparaginase family of proteins, it appears likely that these residues play key roles in determining the correct protein fold and interact directly with Thr-413 to promote nucleophilic attack. Furthermore, using a cell-free protein synthesis assay we show that CwpV maturation requires neither cofactors nor auxiliary enzymes.

P36

INVESTIGATING THE ROLES OF THE CELL WALL ANCHORING SORTASE ENZYME AND SORTED PROTEINS IN *Clostridium difficile*

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Bacterial sortases are transpeptidases that anchor a variety of proteins to the Gram-positive cell wall. Sortase-anchored surface proteins are important virulence factors involved in bacterial adherence, immune system evasion, and nutrient acquisition. Protein anchoring is mediated by a conserved cell wall sorting signal on the anchored protein comprising of a C-terminal recognition sequence (LPxTG), followed by a hydrophobic transmembrane domain and a positively charged tail. The sortase cleaves the LPxTG motif of the polypeptide and covalently attaches the protein to the peptidoglycan layer. Gram-positive bacteria typically encode multiple classes of sortase, each catalysing similar transpeptidation reactions and recognising different motifs. Sortases and their substrates have been well described in human pathogens such as Staphylococcus aureus and Bacillus species, and are essential for virulence in animal models. Genome analysis of *C. difficile* strain 630 revealed that *C. difficile* encodes a single functional sortase that shares 31% amino acid identity with S. aureus sortase B, along with several predicted substrate proteins. However, little is known about their role in *C. difficile* pathogenesis.

This study aims to investigate the function of both the predicted sortase and its substrates in *C. difficile*. Gene inactivation mutants have been constructed in the predicted sorted proteins for functional and phenotypic characterisation. Attempts to inactivate the sortase in *C. difficile* have been unsuccessful, suggesting it may be essential for viability. As mutational analysis has not been possible for the *C. difficile* sortase, it has been expressed and purified from Escherichia coli for use in an in vitro fluorescence resonance energy transfer (FRET) assay to measure the activity of the protein. In addition, thirty-five predicted sortase inhibitors were tested for antimicrobial activity against a variety of bacteria, of which three compounds showed bacteriostatic activity selective against *C. difficile*. This is further evidence the sortase may be essential for viability in *C. difficile* and an appropriate target for antimicrobial development.

P37

MATHEMATICAL MODELLING OF THE GENE REGULATION NETWORK GOVERNING TOXIN PRODUCTION BY *Clostridium difficile*

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Mathematical modelling is increasingly being used to glean important information from gene regulation networks in both prokaryotic and eukaryotic systems. As additional components of such networks are uncovered through experimental work, it becomes increasingly difficult to predict the outcome of varying expression levels of relevant genes and signal inputs, or the effects of extended perturbations like medical treatments, without the aid of tools such as computational models which can describe and quantify multiple gene, protein and signal interactions. When extra components are discovered, it is relatively straightforward to develop the models to accommodate and analyse these. Furthermore, combined with the appropriate experimental data for model parameterisation to ensure that any predictions are reliable, mathematical models can be used to investigate problems which are difficult to access from a purely experimental approach, be that for practical, financial or even moral reasons.

Given the prevalence of antibiotic-resistant strains of *Clostridium difficile*, it is clear that an urgent need to develop novel therapies exists. However, a key obstacle to this is our incomplete understanding of the gene regulation networks governing *C. difficile* virulence. Before novel drugs can be developed, we need to understand as fully as possible the downstream consequences of disrupting a particular aspect of the network. Mathematical modelling is an extremely useful tool for making such predictions and should enable us to identify optimal drug targets. We present a mathematical model of the PaLoc genes (specifically a deterministic ordinary differential equation model) and illustrate how a mathematical approach can shed light on the roles of genes in this network and lead us to experimentally-testable hypotheses.

P38

A *Clostridium difficile* SURFACE-LOCATED CYSTEINE PROTEASE Cwp84 MUTANT DISPLAYS DECREASED CELL INTEGRITY AND ALTERATION OF AUTOLYSIN TRANSCRIPTION

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Clostridium difficile is surrounded by a heterodimeric para-crystalline S-layer which is derived from the post-translational cleavage of SlpA by a surface located cysteine protease, Cwp84. The inhibition or knockout of Cwp84 results in the presentation of immature SlpA at the cell surface. Here we demonstrate that cwp84 mutants suffer from an inability to maintain high culture density post log-phase i.e. maintain a typical stationary phase. Viable count data suggest this growth deficiency corresponds with an increase in cell lysis, which also results in the release of *C. difficile* toxins TcdA and TcdB as determined by ELISA. Microarray analysis on log-phase mRNA suggests that alterations in genes involved in cell wall processing particularly autolysins, including those containing a PFam04122 cell wall anchoring motif may be involved in the decrease in cell integrity. Given the results of this study, caution may need to be exercised when designing a therapeutic that targets the Cwp84-mediated cleavage of SlpA, as although it may reduce cell integrity it may result in the release of intracellular toxin(s), the main virulence factors of CDI.

P39

ANALYSING THE GROWTH CHARACTERISTICS, SPORE GERMINATION AND OUTGROWTH OF *Clostridium difficile* CLINICAL ISOLATES CHALLENGED WITH FIDAXOMICIN

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Fidaxomicin, a novel macrocyclic antibiotic, exhibits potent inhibitory activity at low concentrations against the Gram-positive bacterium *Clostridium difficile*. *C. difficile* is an increasingly important causative agent of nosocomial infection, having overtaken MRSA as the leading cause of healthcare associated infections worldwide. Seven of the most commonly isolated PCR-ribotypes in Europe were challenged with a range of fidaxomicin concentrations and growth characteristics assessed by minimum inhibitory concentration (MIC). In addition, the initiation of spore germination and vegetative cell outgrowth has been examined in six clinically relevant isolates of *C. difficile*. For germination assays, spores were incubated with fidaxomicin and taurocholate, a known germinant of *C. difficile* spores. Initiation of spore germination was measured experimentally by a decrease in OD at 600nm, which is due to a loss of birefringence. Outgrowth from the spore and successful return to vegetative cell growth was measured as an increase of OD at 600 nm in rich medium supplemented with fidaxomicin. The ability to return to vegetative cell growth through germination was also assessed by the ability of spores to form colonies on solid media supplemented with fidaxomicin. The conclusions drawn from these experiments are that fidaxomicin is able to inhibit growth of these seven strains of *C. difficile* at low concentrations, with MIC values ranging from 0.01µg/ml to 0.04µg/ml. Spore germination was able to proceed, as cultures supplemented with fidaxomicin showed a decrease in OD concurrent with those incubated without fidaxomicin, However, subsequent vegetative cell outgrowth following germination was halted by fidaxomicin. The inability of cells to return to vegetative cell growth, in the presence of fidaxomicin, has good therapeutic implications for lowering the rate of recurrence in individuals recovering from *C. difficile* infection.

P40

INVESTIGATION OF QRDR-INDEPENDENT FLUOROQUINOLONE RESISTANCE IN IRISH Clostridum difficile ISOLATES

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Clostridium difficile infection (CDI) is precipitated by the use of broad-spectrum antibiotics such as the cephalosporins and fluoroquinolones, which decrease microbial diversity in the gut with an associated increase in the risk of infection (Paterson 2004). The emergence of antibiotic resistance in *C. difficile* has the potential to promote the occurrence of CDI by affording *C. difficile* a selective advantage during antibiotic therapy. Ciprofloxacin therapy has been identified as an important risk factor for the development of *C. difficile* infection (Sundram, Guyot et al. 2009; Borgmann, Jakobiak et al. 2010). In correlation, high rates of ciprofloxacin resistance are observed among *C. difficile* clinical isolates (Spigaglia, Barbanti et al. 2011). Several studies have identified mutations in the quinolone resistance determining region (QRDR) of genes encoding the GyrA and GyrB subunits of the topoisomerase; a known target of the fluoroquinolones (Drudy, Quinn et al. 2006; Solomon, Fanning et al. 2011; Spigaglia, Barbanti et al. 2011). To date, such mutations represent the only known mechanism of resistance against the fluoroquinolones in *C. difficile*. Here we report QRDR-independent ciprofloxacin resistance in a collection of *C. difficile* strains isolated recently at St James's Hospital, Dublin, Ireland. The emergence of ciprofloxacin resistance among *C. difficile* isolates at this institution is thus explained in part by the emergence of a novel, QRDR-independent resistance mechanism with a preference for ciprofloxacin over moxifloxacin. This mechanism is a potential contributing factor to the heightened risk of CDI associated with ciprofloxacin therapy.

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P41

CB-183,315 DEMONSTRATES LOW IN VITRO FREQUENCY OF SPONTANEOUS OR MULTISTEP RESISTANCE IN *Clostridium difficile, Enterococcus faecalis* AND *E. faecium*

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Background: CB-183,315 is a novel oral lipopeptide antibiotic currently in phase 3 clinical development for the treatment of *Clostridium difficile* associated diarrhea. *C. difficile* infection is the most common cause of health care-related infectious diarrhea in developed countries, accounting for up to 20% of the cases of antibiotic-associated diarrhea and nearly all cases of antibiotic-associated colitis.

Methods: We evaluated the emergence of resistance for *C. difficile* (ATCC 700057 and 3 clinical isolates from the REA groups BI, BK and K), vancomycin susceptible (VS) *E. faecalis* (ATCC 49452), vancomycin resistant (VR) *E. faecalis* (ATCC 700802), *VSE. faecium* (ATCC 6569) and *VRE. faecium* (ATCC 51559) against CB-183,315, vancomycin and rifampicin. Spontaneous resistance incidence (RI) was determined under anaerobic conditions at 8, 16 and 32X MIC. Multistep resistance under selective pressure was evaluated under anaerobic conditions by serial passage over 15 days.

Results: The RI was below the limit of detection ($<10^{-8}$ to $<10^{-9}$) for CB-183,315 and vancomycin at 16 and 32X MIC for all isolates tested. Rifampicin, used as an assay control, had RI frequencies of 10^{-7} to 10^{-8} against *C. difficile* and 10^{-6} to 10^{-7} against E. faecalis. In the CB-183,315 serial passage study, strains grew in 1-4 µg/mL CB-183,315 (shifted 1-4 fold compared to day 1) by day 15, with the exception of the *C. difficile* BK isolate. Against the BK isolate, the concentration permitting growth remained low (1-4 µg/mL) until day 9 and shifted up to 16-32 µg/mL by day 15. After 3 daily drug-free transfers of the BK isolate, the MICs were 2-4 µg/mL (4-8 fold shift compared to naïve). In the vancomycin serial passage, strains grew in 1-8 µg/mL vancomycin by day 15 (shifted 2-8 fold compared to day 1) for the *C. difficile*, VSE. faecalis and VSE. faecium strains. After 3 daily drug free transfers, day 15 CB-183,315 and vancomycin isolates had MICs shifted 1-8 fold compared to naïve controls for the remaining strains tested. Rifampicin was included as a positive control and exposed strains were able to grow in significantly increased concentrations of rifampicin. After 3 daily drug-free transfers, day 15 rifampicin isolates exhibited MICs shifts of >128 fold for all strains tested.

Conclusions: CB-183,315 demonstrated a low frequency of spontaneous resistance in *C. difficile* and Enterococcci, including VRE. Similar to vancomycin, no stable resistant colonies were generated following serial passage with selective pressure for any of the strains tested. These in vitro data suggest the emergence of resistance to CB-183,315 against *C. difficile*, E. faecalis and E. faecium (VSE and VRE) is likely to be rare.

P42

THE EFFECT OF ANTIMICROBIALS ON EXPRESSION OF *Clostridium difficile* TOXIN B AND BINARY TOXIN GENES IN VITRO

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Introduction: In addition to antibacterial effects, some antimicrobials can have broader influences including alteration of gene expression. Sub-MIC antimicrobial exposure has been shown to up-regulate tcdAB expression, but study of CDT genes is lacking, as is an evaluation of the relationship between toxin B and CDT gene expression.

Methods: The effect of sub-MIC (1/8th) treatments of clindamycin (Clinda), levofloxacin (Levo) and enrofloxacin (Enro) on cdtR, cdtA and tcdB expression during exponential (EX) and stationary (ST) phase was assessed in ribotype 027 and 078 strains by quantitative real-time PCR.

Results and Discussion: Clinda had no effect except for repression of EX tcdB in 027 (1.8X, P = 0.02). This effect may confer an advantage related to early pathogenesis by allowing multiplication and colonization of the intestinal tract to progress unhindered by the immune response which would be triggered by toxin production. Levo generally had a repressive effect on gene expression with the exception of tcdB expression in ribotype 027, where a marked increase (3.8X increase, P = 0.016) occurred. Although tcdB expression in response to Levo has not been previously reported, it has previously been shown to induce higher yields of toxin B in feces. In contrast, significant repression of tcdB was observed in the ribotype 078 strain (3.6X, P = 0.038). Reasons for the differences between 027 and 078 are unclear, but may be associated with the diverse genetic background of these two strains or the need to use lower Levo concentrations for 078 because of the low MIC. Enro did not have a significant effect on expression of the genes with the exception of cdtR in ribotype 078 (increases of 3.6X in EX, P = 0.0001 and 2.5X in ST, P = 0.0016). This increase was not correlated with an increase in cdtA. It is not clear why a significant effect would be observed in cdtR expression in the one strain but sub-MIC treatments with Enro have induced numerous regulatory genes in E. coli. CdtR may regulate expression of other, currently unidentified genes.

Conclusion: Antimicrobials influence the expression of cdtA, cdtR and tcdB, although not consistently between ribotypes and increases and decreases in expression of different genes may occur concurrently in the same isolate. Expression patterns of tcdB and cdtA did not correlate. The increase in tcdB expression in response to Levo is consistent with studies implicating this drug as a risk factor for CDI. Further investigation into the role of antimicrobials in regulating virulence factor gene expression and protein production can contribute to our understanding of the pathogenesis of *C. difficile* infection.

P43

IN VITRO SUSCEPTIBILITY TO METRONIDAZOLE IN DIFFERENT *C. difficile* PCR-RIBOTYPES

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Clostridium difficile infection (CDI), the main hospital-acquired intestinal disease in Europe nowadays, is frequently associated with strains belonging to PCR-ribotypes 014/020, 001, 078, 018 and 027. Metronidazole (MZ) is the drug of choice to the treatment of mild to moderate CDI cases; however, *C. difficile* strains in vitro susceptible to this antibiotic have been associated with cases of treatment failure. Since some studies reported discordant results on MICs to MZ depending of the method used, we compared three different methods, the Etest, agar dilution method (ADM) and agar incorporation method (AIM), to determine the susceptibility to MZ of 81 *C. difficile* strains, belonging to nine different PCR-ribotypes. Twenty selected strains were also subjected to sub-inhibitory concentrations of MZ and heterogeneity of MIC values was investigated by both ADM and AIM in strains showing an increase of MIC after exposure to the antibiotic.

Overall, the MICs obtained by Etest were lower compared to ADM and AIM. Reduced susceptibility was observed by both ADM (3.7%) and AIM (7.4%) in strains belonging to PCR-ribotypes 001 and 010, however, the same isolates resulted fully susceptible when analysed by Etest. Only one isolate, belonging to PCR-ribotype 010 showed a high MIC (≥ 16 mg/L) by all three methods.

After in vitro exposure to MZ, four strains belonging to PCR-ribotypes 001 and 010 showed MIC increase by Etest and ADM, but not by AIM. Since MICs of colonies randomly isolated from these strains were less heterogenic by AIM compared to ADM, both before and after exposure to MZ, it can be hypothesized a better ability of the first method in detecting strains with reduced susceptibility.

The results obtained indicate that *C. difficile* sub-populations with reduced susceptibility to MZ may be responsible for CDI treatment failures in vivo and underline the importance of the method chosen to not underestimate the MIC values.

This study was funded by the Marie Curie European Project number 237942 CLOSTNET.

P44

EXPRESSION AND DISPLAY OF FliD OF *Clostridium difficile* ON THE SURFACE OF *Bacillus subtilis* SPORES

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Two *Clostridium difficile* flagellar proteins, FliC, structural monomer, and FliD, the cap protein, have been reported to be involved in the attachment to host cells and the intestinal mucus layer.
We decided to use the spore surface display system to expose the most immunogenic fragment of FliD to develop eatable vaccine against infections with *C. difficile*. Spore coat assembly involves the deposition of at least 50 protein species into two major layers: an electron-dense outer layer, called the outer coat, and a less electron-dense inner layer with a lamellar appearance, called the inner coat. The locations of CotA, CotB, CotC, and CotG were shown to be externally exposed on the surface of the spore.

To obtain recombinant spores that efficiently express FliD fragment we used CotC coat protein as a carrier and tested the efficiency of display of obtained fusion protein.

Currently immunization of the laboratory animals is in progress to assess whether the surface display of FliD antigen is sufficient for inducing a protective immune response.

P45

GENOME-WIDE IDENTIFICATION OF SMALL RNAs IN *Clostridium difficile*

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Clostridium difficile is a major hospital acquired pathogen that can cause symptoms ranging from mild diarrhoea to more serious, sometimes life-threatening inflammation of the colon. Despite its medical importance, very little is known about the regulatory networks that are operating in this organism, and almost nothing about the roles played by small regulatory RNAs (sRNAs). sRNAs have been found in many other bacteria where they accomplish important functions in the regulation of cellular processes such as sporulation, quorum sensing, metabolism, and virulence (e.g. toxin production). Unravelling the sRNA regulatory networks in *C. difficile* would be an important step toward understanding how this pathogen survives in the gut environment and causes disease.

To identify and map *C. difficile* sRNAs on a genome-wide scale, total RNA was extracted from triplicate cultures at different time points during the growth cycle (mid exponential, transition to stationary phase, and stationary phase) and subjected to RNA-Seq analysis. After filtering out abundant tRNAs and rRNAs, data were normalised and analysed for regions of intergenic and antisense transcription. Numerous putative sRNAs were identified (~260) and the existence of several of these were independently confirmed by Northern blot analysis. The biological function and regulatory targets of selected sRNA candidates is currently being investigated. As the regulatory function of trans-encoded sRNAs is generally mediated by the chaperone protein Hfq, *C. difficile* hfq mutants were also generated by ClosTron mutagenesis and are currently being characterised.

P46

CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR) IN Clostridium difficile

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Clustered regularly interspaced short palindromic repeats (CRISPRs) are a common feature in the genomes of many bacteria and almost all archaea. Direct repeat (DR) sequences interspersed by unique spacer sequences derived from extrachromosomal elements like phages are the constituent parts of a CRISPR array building a CRISPR/cas system together with a group of CRISPR associated (cas) genes. Prokaryotic cells possessing a CRISPR/cas system are protected against extrachromosomal elements by this antiviral system if the spacer sequences show 100% sequence similarity to an invading phage or plasmid.

We investigated whole genome sequences (GenBank database) of nine different *Clostridium difficile* isolates of different PCR ribotypes for the presence of CRISPR arrays and CRISPR associated (cas) genes. The sequences were analyzed by using the online tools "CRISPRfinder" and "Crispi". Detected spacers were blasted (NCBI) to check up if they have an extrachromosomal origin. Additionally, Cas genes of more than 90 *C. difficile* strains of the AGES strain collection were verified using different PCR systems and the CRISPR array adjacent to the CAS operon was sequenced.

Aim of this study was to compare the gained CRISPR results with standard typing methods like PCR ribotyping and Multi-locus-sequence-typing (MLST) to find out if a CRISPR based typing method has the potential to complement classical typing methods in *Clostridium difficile*.

P47

CHARACTERIZATION OF TWO MAJOR AUTOLYSINS IN *Clostridium difficile*

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Peptidoglycan (PG) is constantly remodelled during bacterial growth, to allow for the incorporation of new PG and to accommodate changes in cell shape. This remodelling requires peptidoglycan hydrolases (PGH) activity capable of hydrolysing some covalent bonds in the bacterial cell wall PG. These PGH, also called autolysins, are implicated in many physiological processes such as PG turnover, cell separation, sporulation, or germination.

We previously identified a putative autolysin, named Acd, in *Clostridium difficile*, which displays N-acetylglucosaminidase activity. The study of acd expression during various growth phases of *C. difficile* showed that acd is significantly expressed during the vegetative growth to the both levels and that the Acd protein is accumulated during the late phase of growth. These results suggest Acd could be implicated in cell separation and maybe in sporulation. The ClosTron method of insertional gene inactivation was successfully performed on the acd gene. Acd is a major autolysin in *C. difficile* since an important lytic activity disappears, as shown in zymogram analysis, in the mutant strain. However, to date no particular phenotype could be detected in the acd mutant either in cell separation or in sporulation.

At least 28 putative autolysins encoding genes were identified on the genome of *C. difficile*. Therefore, the study of the physiological role of autolysins in *C. difficile* could be hampered by this great number and their functional redundancy. A second major lytic is still visible on zymogramm in the acd mutant. The corresponding protein Acdi was identified by LC-MS/MS. Acdi has a modular structure with a C-terminal domain containing repeated sequences and an N-terminal catalytic domain belonging to the DUF187 uncharacterized proteins family. The acdi transcript was expressed during mid-exponential growth phase and the expression declined during the late exponential growth phase. The insertional inactivation of acdi showed that Acdi is not implicated in cell separation, but is involved in triton X-100 induced autolysis. An acd-acdi double mutant strain displayed a slightly impaired cell separation, indicating functional synergy of Acd and Acdi for this function.

P48

Clostridium difficile HAS AN ORIGINAL PEPTIDOGLYCAN STRUCTURE WITH HIGH LEVEL OF N-ACETYLGLUCOSAMINE DEACETYLATION AND MAINLY 3-3 CROSS-LINKS

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Clostridium difficile is the major cause of intestinal diseases associated with antibiotic therapy, with clinical manifestations ranging from asymptomatic colonization or mild diarrhea to pseudomembranous colitis. Antibiotic treatment of *C. difficile*-associated disease requires metronidazole or vancomycin therapy. The cell wall peptidoglycan (PG) is the specific target of β-lactams and vancomycin. However, little is known about the PG structure and biosynthesis in *C. difficile*.

In this study, we determined the structure of the vegetative cell wall PG of *Clostridium difficile* by analysis of its constituent muropeptides, generated by digestion with mutanolysin. Analyses were performed with a combination of reverse-phase high-pressure liquid chromatography (RP-HPLC) separation of muropeptides, amino acid analysis, mass spectrometry (MS) and tandem mass spectrometry (MS-MS). The structures assigned to 36 muropeptides evidenced several original features in *C. difficile* vegetative cell PG. First, it is characterized by a strikingly high level of N-acetylglucosamine deacetylation, which is usually linked to lysozyme resistance. In addition, the majority of dimers (around 75 %) contains $A_2pm^3 \rightarrow A_2pm^3$ cross links and only a minority of the more classical $Ala^4 \rightarrow A_2pm^3$ cross-links. Moreover, a significant amount of muropeptides contains a modified tetrapeptide stem ending in Gly instead of D-Ala^4.

Two L,D-transpeptidases homologues encoding genes present in the genome of *C. difficile* 630 and named ldt_{cd1} and ldt_{cd2}, were inactivated. The inactivation of either ldtcd1 or ldt_{cd2} significantly decreased but not abolished the abundance of 3-3 cross-links, leading to a markedly decrease of PG reticulation and demonstrating that both ldtcd1 and ldt_{cd2} encoded proteins have a redundant L,D-transpeptidase activity. A double ldt_{cd1} ldt_{cd2}mutant strain was also obtained. Interestingly, no obvious differences were observed when compared its muropeptide profile to that of the ldtcd1 mutant strain, indicating the involvement of at least a third L,D-transpeptidase able to partially compensate for the lack of both Ldt_{cd1} and Ldt_{cd2}.

The contribution of 3-3 cross-links to peptidoglycan synthesis increased in the presence of subinhibitory concentrations of ampicillin, indicating that this drug does not inhibit the L,D-transpeptidation pathway in *C. difficile*.

P49

DNA BINDING BY THE MASTER REGULATOR OF SPORULATION, *Spo0A, OF Clostridium difficile* 630Δerm

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Endospore formation in Firmicutes, including *C. difficile*, is governed by the key regulator for sporulation, Spo0A. In Bacillus subtilis, the transcription factor is also directly or indirectly involved in various other cellular processes. In *C. difficile*, Spo0A is a transmission and persistence factor, but the targets of the protein are poorly defined.

We carried out an in vitro analysis of DNA binding by *C. difficile* Spo0A, and determined differences between wild type and a Clostron-derived spo0A mutant strain with respect to toxin production. Through a combination of bioinformatics and in vitro binding assays using the purified C-terminal DNA binding domain (DBD) of the *C. difficile* Spo0A, we show that the protein recognizes a motif that is highly similar to the B. subtilis 0A box. We demonstrate direct binding of Spo0A-DBD to DNA upstream of spo0A and sigH, several early sporulation genes and other putative targets not implicated in sporulation. Importantly, immunoblotting shows that the protein is present at early timepoints, prior to spore formation. We do not find a positive effect of Spo0A on toxin production, despite low affinity binding to PtcdB, in contrast to previous findings. These results identify for the first time putative targets of the Spo0A protein in *C. difficile* and make a positive effect of Spo0A on toxin production unlikely.

Current efforts are aimed at investigating the effects of Spo0A at the RNA and protein level in vivo and an update on our progress will be presented.

P50

INACTIVATION OF CHEMOTAXIS-ASSOCIATED GENES INCREASES FLAGELLAR MOTILITY IN *Clostridium difficile*

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Flagella are important for motility and chemotaxis in many bacterial species, but their contribution in the virulence of *Clostridium difficile* is unclear. The expression of chemotaxis-associated genes enables bacteria to sense their surrounding environment and control the rotation of their flagella to move along a chemical gradient. In model organisms like Escherichia coli and Bacillus subtilis, chemotaxis and flagella rotation is controlled by a cascade of regulation and the concerted action of several proteins, of which CheA and CheY play central roles. Upon binding of a specific chemical signal by a membrane chemoreceptor, the histidine kinase CheA eventually becomes phosphorylated. CheA-P then phosphorylates CheY, which acts directly on the flagellar motor, thus altering rotation of flagella. *C. difficile* 630 and R20291 strains encode a complete chemotaxis locus comprising at least 10 genes including cheA and cheY homologs, as well as a putative diguanylate cyclase (DGC) encoded by gene cd0537. This DGC is thought to be involved in the synthesis of cyclic-di-GMP, a second messenger controlling motility, biofilm formation and virulence in several pathogens. Our objective is to define the role of chemotaxis-associated genes in flagella-mediated motility, c-di-GMP signaling, as well as virulence of *C. difficile*.

The ClosTron gene inactivation system was used to inactivate cheA, cheY and cd0537 within the chemotaxis locus of *Clostridium difficile* strain R20291. We also inactivated fliC to generate an unflagellated mutant to be used as a control. In vitro motility assays were done on semi-solid BHI agar plates (0.3% agar) to assess the impact of these mutations on flagellar-mediated motility. Transmission electron microscopy (TEM) was used to observe flagella in the different mutants.

TEM analyses did not reveal differences in the size and amount of flagella between the wild type strain and the CheA, CheY and CD0537 mutants, but confirmed the absence of flagella on the surface of the fliC mutant. When grown on semi-solid agar, the CheA and CheY mutants were significantly more motile by a factor up to 2-fold relative to the wild type strain, whereas motility of the CD0537 mutant was relatively unaffected. As expected, the FliC mutant was non motile.

Until now, our results suggest that CheA and CheY are involved in the control of motility but mutating the putative DGC CD0537 had little effect in the conditions tested. Additional experiments will be required to fully understand how these processes interplay in *Clostridium difficile* . In vivo assays in a C57BL/6 mouse model of infection are currently underway to determine whether chemotaxis has a role to play in the virulence of *Clostridium difficile* .

P51

IDENTIFICATION AND CHARACTERIZATION OF THE REPLICATION MACHINERY OF Clostridium difficile

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DNA replication is an essential and conserved process, and may therefore serve as a target for the development of novel antimicrobials. However, knowledge of the molecular biology and genetics of *Clostridium difficile* replication is very limited, compared to its well characterized close relative Bacillus subtilis. The identification and subsequent characterization of proteins involved in the initiation of replication can contribute to fundamental understanding as well as future clinical applications.

In the initial phase of our project, we focus on the proteins required for helicase loading in *Clostridium difficile*. The genes coding for these initiation proteins were identified on the basis of homology to replication genes of Bacillus subtilis. The putative replication proteins are investigated through biochemical and molecular methods, including (but not limited to) assessment of their DNA-binding activity and their ability to load the replicative helicase in vitro. We have purified the putative initiation protein (CD0001), a putative primosomal protein (CD3653) and the putative helicase loader ATPase (CD3654), as well as the helicase (CD3657) itself. Antibodies have been raised against these purified proteins for the purpose of downstream experiments, which include an investigation of protein-protein interactions. At this symposium, we will present the results from our ongoing experiments.

P52

C. difficile CELL WALL PROTEIN ANCHORING

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The surface of *Clostridium difficile* is likely to play an important role in host colonization and infection. The major surface structure is the S-layer, comprised of a complex between the High Molecular Weight S-layer protein (HMW SLP) and Low Molecular Weight S-layer protein (LMW SLP). There are 28 paralogues of the HMW SLP in the *C. difficile* 630 genome, which together form the cell wall protein family. All *C. difficile* cell wall proteins contain three PFAM-04122 (PF04122) repeats at their N or C termini, which are believed to be responsible for anchoring the proteins to the underlying peptidoglycan or other cell wall polymer. PF04122 repeats are widespread throughout surface proteins of Gram positive bacteria and the Archaea but their ligand or anchoring mechanism are unknown. To gain insight into the role of the PF04122 repeats, we have used mutagenesis to systematically delete the repeats from two members of the cell wall protein family; Cwp2 and Cwp66. The affect of the deletions on the localization of the proteins will be described.

P53

AUSTRALIA - HOME OF *Clostridium difficile* CLADE 5?

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Although *C. difficile* is recognised as an important nosocomial pathogen and an emerging cause of infections in the community, very little data exist on its molecular epidemiology outside Europe and North America. Phylogenetic analysis of *C. difficile* isolates has revealed at least five clades, some containing subclades. Although clade 1 is the largest and most heterogeneous, all of the five clades contain toxigenic and clinically important strains. Clade 5 is the lineage containing PCR-ribotype (RT) 078, (multilocus sequence type [MLST] ST11) an important animal strain which recently emerged as a common human pathogen, particularly in Europe. This clade is genetically divergent from the other four clades and unusually homogeneous.

In this study, 34 Australian isolates were chosen to be representative of known genetic diversity in Australia and whole genome sequences were determined. The ST of each isolate was extracted in silico and 19/34 isolates (55.9%) belonged to clade 5. These were isolated in four Australian states (WA, NSW, QLD, VIC) from urban and rural areas, and from human and animal (porcine and equine) hosts.

Of the 19 clade 5 isolates, six (31.6%) were ST11, while the remaining 13 (68.4%) represented six newly identified STs; ST163 (n=2), ST164 (n=2), ST166 (n=2), ST167 (n=5), ST168 (n=1), and ST169 (n=1). All of the novel STs that were identified more than once represented more than one PCR-ribotype. Two (ST164 and ST167) also contained both toxigenic (A-B+) and non-toxigenic (PaLoc-negative) strains. The A-B+ strains of ST164 and ST167 represented toxinotypes XXX and XXXI. Neither of these toxinotypes has been reported outside Australia, nor have most of the clade 5 PCR-ribotypes to which the Australian clade 5 strains belonged.

The unusual diversity and widespread geographic distribution of clade 5 strains in Australia, coupled with the presence of types not reported elsewhere, suggests that this clade may originate on the Australian continent.

P54

THE SIGMAD FACTOR CONTROLS THE EXPRESSION OF FLAGELLA AND TOXINS A AND B IN *Clostridium difficile*

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C. difficile intestinal disease is mainly due to the production of toxins A and B, but requires an initial step of colonization in which different parietal or flagellar proteins are involved. We found in the *C. difficile* 630 genome a gene encoding a putative SigD factor homologous to SigD of B. subtilis (34% identity and 60% similarity), which controls flagellar synthesis, motility and vegetative autolysins LytC, LytD, and LytF in B. subtilis . We analyzed in this study the role of SigmaD factor in the control of motility, autolysis, and also in the regulation of toxins A and B, the main virulence factors of *C. difficile*.

A *C. difficile* sigD mutant, constructed using the Clostron® system, displayed a loss of motility and flagellin expression. Conversely, the inactivation of sigD did not affect autolytic activity, unlike in B. subtilis. A transcriptomic analysis of the sigD mutant was achieved to characterize the members of the SigD regulon: a total of 103 genes were differentially expressed in wild-type and mutant strains, most of which are involved in functions such as motility, metabolism, virulence, or protein regulation. The sigD mutant displayed a decreased expression of genes encoding toxin A, toxin B, and TcdR (the positive regulator of these toxin genes), and of flagellar genes, as revealed by both transcriptomic and qRT-PCR experiments. The complementation of the sigD mutant with the wild-type sigD gene restored the motility and toxin genes expression. Thus, SigD appears as an important positive regulator of virulence genes, especially toxin genes, of *C. difficile*.

P55

TOXIN EXPRESSION BY *Clostridium difficile* IS NOT ENTIRELY DEPENDENT ON TcdR

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TcdR is a group-5 extra-cytoplasmic-function sigma factor that governs toxin expression in *C. difficile* by directing the RNA-polymerase holoenzyme to promoter regions upstream of the toxin genes, tcdA and tcdB, and its own gene, tcdR. However, it has never been demonstrated in *C. difficile* itself, whether or not TcdR is an absolute requirement for toxin production. In this work, we made an in-frame deletion of tcdR in *C. difficile* R20291 (PCR ribotype 027) and measured toxin production in TY medium, over a time-course experiment. Although toxin production by the ΔtcdR mutant was reduced by an order of 100-fold, following 24 hours incubation, it was not completely abolished. Furthermore, it followed the same temporal pattern as the wild-type. Thus, while TcdR is clearly the principal governor of toxin production by *C. difficile*, it is interesting to note that toxin production is not entirely dependent on it. This finding suggests that one or more sigma factors, other than TcdR, may facilitate expression of the toxin genes, albeit to a much lesser extent than TcdR itself.

P56

Clostridium difficile TcdC BINDS QUADRUPLEX DNA STRUCTURES

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The main virulence factors of *Clostridium difficile* are the two large clostridial toxins TcdA and TcdB. Transcription of the toxin genes is positively regulated by the alternative sigma factor TcdR1. Negative regulation is believed to occur through TcdC, a proposed anti sigma factor2.

Bioinformatic analysis of the TcdC protein sequence predicted the presence of a hydrophobic stretch (amino acids 31-50), a potential dimerization domain (amino acids 89-130) and a C-terminal OB (oligonucleotide/oligosaccharide binding) fold domain (amino acids 130-232). The OB fold was predicted to belong to the group of single stranded DNA binding proteins.

Gel filtration chromatography of truncated recombinant TcdC, lacking the proposed dimerization domain (TcdCΔ1-130), showed that the domain is indeed involved in dimerization of the protein. Binding of soluble recombinant TcdC (truncated amino acids 1-89, TcdCΔ1-89) to single stranded DNA was studied using a single stranded SELEX approach. This involved specific selection of DNA sequences from a pool of random oligonucleotides binding to TcdC, as monitored by an electrophoretic mobility shift assay (EMSA). Multiple binding and amplification rounds yielded an enriched pool of TcdC binding sequences.

Sequence analysis of the selected oligonucleotides bound to TcdC showed that the OB fold domain of TcdC can bind specifically to DNA folded into quadruplex structures. A quadruplex requires the presence of multiple repetitive guanine nucleotides in the sequence, which enables it to form a four-stranded structure.

Deletion of the TcdC multimerization domain resulted in a lack of DNA binding, indicating that dimerization of TcdC is required for DNA binding. Based on our results and previously observed anti sigma activity of TcdC2, we are trying to create a model in which both transcription and quadruplex DNA binding can be combined.

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P57

BEHAVIOUR OF CTNCD11-LIKE ELEMENTS IN Clostridium difficile

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Elements carrying erm(B) genes, conferring resistance to erythromycin and/or clindamycin in *C. difficile* show an high heterogeneity in their genetic structure. Among them, only the mobilizable non-conjugative Tn5398 from strain 630 has been extensively studied. However, this element is not the most widespread among European clinical isolates of *C. difficile*, according to a recent surveillance study. Among the 14 different genetic organizations of elements carrying an erm(B) gene identified by PCR-mapping, the arrangement denominated E4 was the most frequently found.

In this work, the characteristics of these genetic elements were investigated. Two different clinical isolates containing an ErmB determinant E4 were used as donors in filter-mating assays with a recipient strain of *C. difficile*. Analysis of the transconjugants obtained from both donors indicated that, unlike Tn5398, these determinants integrate into the recipient's genome in different regions. Genome sequencing of one transconjugant was performed, revealing that in this case part of the element was inserted by homologous recombination involving a putative conjugative transposon already present in the recipient strain. Interestingly, this recombination led to the generation of a new composite element. The inserted sequence has 100% identity with part of the sequence of CTnCD11, a putative conjugative transposon recently detected in *C. difficile* 2007855, a PCR-ribotype 027 clinical isolate. The possibility that the elements examined in this study may also integrate into the recipient's genome by a conjugative-like transposition mechanism seems supported by preliminary molecular analysis of other transconjugants. Further studies will investigate in depth the mechanisms by which these elements transfer and integrate into the genome of the recipient strain.

P58

TN5398 FROM *Clostridium difficile* 630: NEW INSIGHTS IN MECHANISM OF TRANSFER, BIOLOGICAL BURDEN AND EXPRESSION OF ERM(B)

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In *C. difficile*, resistance to erythromycin and clindamycin is usually conferred by erm(B) genes. In strain 630, two copies of this gene are present on Tn5398, a mobile element that has been successfully transferred to recipient strains of *C. difficile* and Bacillus subtilis by filter-mating experiments. Despite the fact that this element has been extensively studied, its mechanism oftransfer is still unclear, since it does not contain any gene involved in its excision, integration or conjugation. Therefore, it has previously been designed as a mobilizable non-conjugativeelement. The 630Δerm derivative mutant contains only one copy of the erm(B) gene, which appears to be usually silent, since this strain is sensitive to erythromycin.In this study, filter-mating assays were performed to transfer Tn5398 from 630 to two *C. difficile* recipient strains, CD37 and CD13. Integration of the element within the unique site already described was confirmed in all transconjugants obtained. Analysis of the regions flanking theelement in transconjugants suggested that its integration occurred by a mechanism of homologous recombination involving sequences from the donor notably longer than the elementitself. In vitro growth and competition assays between transconjugants carrying Tn5398 and wild-typerecipient strains were performed and revealed that the acquisition of this element imposes asignificant cost on the fitness of the bacterium. Resistance to erythromycin was induced in the 630Δerm mutant by exposure to a sub-inhibiting concentration of the drug. Highly resistant colonies of this strain were obtained, in which theexpression of the only one copy of erm(B) present was demonstrated. Filter-mating assays weredone using these resistant colonies as donors and the partial element was successfully transferred to CD13. Both donor and transconjugants became progressively susceptible to erythromycin inthe absence of antibiotic pressure.These preliminary results provide new details either in the transfer mechanism of Tn5398 and in the regulation of the erm(B) genes expression. Moreover, these results suggest that the acquisition of this element imposes a fitness burden in vitro.

P59

Lactobacillus casei AFFECTS *Clostridium difficile* TOXIN DETECTION IN-VITRO

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Background. Toxins A and B are key virulence factors responsible for the pathogenicity of *Clostridium difficile*. In order to cause disease, *C. difficile* spores must be ingested, germinate, proliferate and produce toxin. Gut microorganisms offer a degree of protection against *C. difficile* colonisation ('colonisation resistance'), but the mechanisms of this process, and the roles and relative importance of different gut microflora populations remain unclear. Probiotic treatment and prophylaxis regimens are based on the exploitation of colonisation resistance, although the effectiveness of these strategies has not yet been proven in large scale clinical trials. Lactobacillus spp. populations are common constituents of probiotic products.

Methods. The in vitro human gut model has been validated as a reflective model of *C. difficile* infection (CDI) in vivo. The gut model was inoculated with a 10% faecal slurry to establish stable gut microflora populations. These populations were monitored daily by culture on selective agars. Clindamycin instillation (33.9 mg/L qds, 7days) was used to induced *C. difficile* germination, proliferation and toxin production. *C. difficile* viable counts and spores were enumerated on Brazier's CCEYL, and toxin levels were measured by cell cytotoxin assay. Further co-culture experiments were carried out anaerobically in BHI broth for 48 h and toxin determination was performed by cell cytotoxin assay.

Results. Following clindamycin dosing of the human gut model, as expected germination and proliferation of *C. difficile* ribotype 027 spores occurred. However, these events were not accompanied by measureable high level toxin production as observed previously. Simultaneously, a prolific lactobacillus strain was isolated from the model, which was identified by 16S PCR as Lactobacillus casei. Subsequent experiments showed that, whilst the co-culture of *C. difficile* with this L. casei strain did not affect viable counts of either organism, the detection of cytotoxin was substantially reduced compared with toxin levels measured when *C. difficile* alone was cultured.
Conclusions. The presence of L. casei does not have a deleterious effect on *C. difficile* populations. However, toxin detection is impaired when this L. casei strain is co-cultivated in vitro either in a CDI gut model or in batch culture. The mechanisms for this remain unclear. The phenomenon has the potential for therapeutic or preventative exploitation.

P60

A NOVEL ECOSYSTEM THERAPEUTIC FOR THE TREATMENT OF *Clostridium difficile* INFECTIONS

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The human gut contains a diverse bacterial community that contributes to overall health. In some individuals, dysbiosis in this community can lead to many gut diseases. In particular, frequent use of antibiotic therapies can cause a shift in beneficial gut microbes and allow pathogenic interactions to predominate. *Clostridium difficile* associated disease (CDAD) is strongly associated with prior antibiotic exposures, and is a particular problem in the hospital setting. Treatment of CDAD, particularly recurrent CDAD, is often difficult; conventional antibiotic treatment can, ironically, exacerbate the underlying dysbiosis in the gut microbiota. In contrast, microbiota replacement strategies such as fecal transplants have shown more promising results in curing disease and restoring health. However, fecal transplants carry a significant amount of risk to the patient because the exact composition of the donor stool is largely unknown and, despite extensive screening protocols, may harbour pathogenic microorganisms. This study focuses on the preparation and use of a Defined Ecosystem Therapeutic (DET) for CDAD treatment based on known bacterial isolates from healthy donor stool. Bacteria were isolated from a fresh stool sample using various culture conditions. A chemostat was also inoculated with the same sample and allowed to reach steady state for further enrichment and isolation of bacterial diversity. Bacterial isolates were identified by 16S rRNA sequencing, and antibiotic resistance profiles were determined for each strain. The composition of the final formulation was based on acceptable antibiotic resistance profiles and representative proportions of bacterial genera from the healthy human gut. The DET was administered in one dose to two patients with severe CDAD and resulted in complete and rapid resolution of disease, with no recurrence to date. 16S rRNA gene sequence profiling suggested that the ecosystem persisted in these patients for several months following the procedure.

P61

ANALYSIS OF THE MICROBIOTA OF SYRIAN HAMSTERS FOLLOWING CLINDAMYCIN TREATMENT AND IMPACT ON *Clostridium difficile* SUSCEPTIBILITY

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Clostridium difficile infections (CDI) are caused by the outgrowth of toxigenic strains of the bacterium, usually following the use of antimicrobial therapies. A better understanding of the protective commensal gut microbiota could provide critical information about the risk of acquiring CDI and inform the development of treatments. Here, we utilized the lethal enterocolitis model in Syrian golden hamsters to analyze the microbiota changes associated with susceptibility to *C. difficile* infection following administration of a single dose of clindamycin. Fecal samples collected from Syrian hamsters following treatment with clindamycin (1 or 30 mg/kg) were profiled through Second Genome's Microbiome Signature Discovery™ platform using the PhyloChip™ assay, a microarray-based method that measures the abundance of 16S rRNA genes representing 58,443 bacterial taxa. Microbiome dissimilarity was evaluated with both weighted and unweighted UniFrac. To determine *C. difficile* infection susceptibility in vitro, spores of strain VPI 10463 were cultured with and without soluble fecal filtrates for 72 hrs at 37°C under anaerobic conditions. Cultures were analyzed by qPCR to determine *C. difficile* copy number and by EIA to determine toxin production. *C. difficile* culture growth and toxin production was inhibited by the presence of naïve hamster fecal extracts but not extracts collected 5 days post-administration of clindamycin. Inhibition was re-established by 15 days after treatment, which corresponded with the animals' susceptibility to challenge. CDI susceptibility also correlated with a substantial microbiome shift upon treatment with clindamycin, but not PBS. Time course analysis revealed a significant disturbance at day 5 through day 9, then a return to near baseline by day 20. A comparison of the microbiota before and 5 or 9 days after treatment with 30 mg/kg of clindamycin showed a profound decrease in Bacteroidetes and corresponding increase in Proteobacteria. A correlation was observed between time from disruption to restoration of the microflora and the ability for *C. difficile* to grow in vitro and in vivo. Soluble factors produced by the gut microbiota may be responsible for the suppression of *C. difficile* growth and toxin production under normal conditions.

P62

GUT MICROBIOTA ASSOCIATED WITH *Clostridium difficile* COLONIZATION IN INFANTS INVESTIGATED BY PYROSEQUENCING

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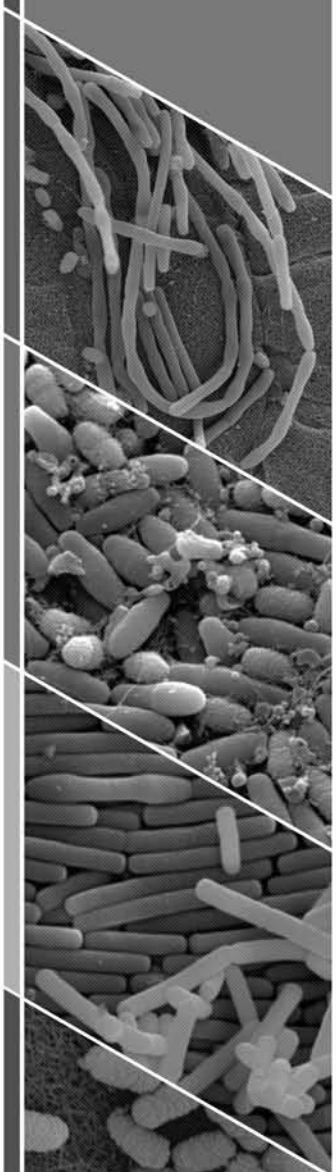
Introduction. *Clostridium difficile* infection (CDI) is likely a result of imbalanced ecosystem in the gut leading to an inability of the commensal microbiota to resist to *C. difficile* colonization. During early infancy, asymptomatic colonization by *C. difficile* is common for unknown reasons. In order to investigate the potential relationship between the commensal microbiota composition and the implantation of *C. difficile* in the gut, *C. difficile* colonization was kinetically followed in infants and faecal microbiota before, during and after *C. difficile* colonization were compared.

Methods. Faecal samples of five infants were collected monthly from birth to one year of age and presence of *C. difficile* was investigated by culture. For four infants, faecal samples were obtained before, during and after *C. difficile* colonization. In one infant, colonization started at birth and was used as control. Microbiota composition was assessed by 454 pyrosequencing in order to highlight bacterial signatures of the gut microbiota associated with *C. difficile* colonization.

Results. High inter-individual variability of microbiota composition was observed mostly related to diet (breastfed or formula fed). Breastfed infants had higher percentages of Actinobacteria (mainly Bifidobacterium) and Bacteroidetes whereas formula fed infants had high levels of Firmicutes and Proteobacteria. Bacterial species diversity (Chao1 and Shannon index) was not affected by *C. difficile* colonization. The kinetics analyse of the microbiota revealed that Ruminococcus gnavus ($p = 0.007$) was significantly associated with *C. difficile* colonization independently of the age of the infants. 16S rDNA sequences related to R. gnavus were more frequent ($p = 0.026$) and abundant during colonization (3.37% of sequences) than before colonization (1.91% of sequences) ($p = 0.007$).

Conclusions. This work highlights that gut microbiota modifications, especially the presence of R. gnavus, is associated with *C. difficile* colonization in infants. Elucidate if specific species favour *C. difficile* implantation or elimination may be useful to prevent CDI in adults.

4th International *Clostridium difficile* Symposium



Abstracts of poster presentations

(section 2 P63 to P120, Saturday)

First author	#	Title
Bahadur, T.	P63	Identification of Clostridium difficile infection in antibiotic associated diarrhea patients in Indian population
Belanger, J.	P64	Retrospective analysis of human biotherapy treatment for Clostridium difficile
Benea, Ş.	P65	Clostridium difficile infections hospitalized in Romanian Institute of Infectious Diseases during the first three months of 2012
Butt, E.	P66	Derivation and validation of a simple, accurate and robust prediction rule for risk of mortality in patients with Clostridium difficile infection
Faires, M.C.	P67	Clostridium difficile contamination in the medical and surgical wards of a community hospital
Hell, M.	P68	Oral multispecies probiotic as adjunctive therapy for Clostridium difficile Infection: case series involving 10 patients (5 of them with recurrent CDI)
Hell, M.	P69	Effectiveness of a new surface disinfecting concept for reducing the risk of nosocomial Clostrium difficile infection
Kato, H.	P70	Bacteremia and splenic abscess due to Clostridium difficile without intestinal infections: a case report
Kullin, B.	P71	Clostridium difficile in a South African hospital setting: incidence and antibiotic resistance
Tagashira, Y.	P72	Two cases of fulminant colitis due to binary toxin-positive Clostridium difficile, which was neither PCR ribotype 027 nor type 078
Taori, S.K.	P73	Prospective analysis of Clostridium difficile cases in Scotland with emphasis on sporadic and outbreak strains of ribotype 078
Trajkovska-Dokic, E.		P74Prevalence of Clostridium difficile in patients with antibiotic-associated diarrhoea and pseudomembranous colitis
Avesani, V.	P75	Trends in Clostridium difficile ribotype epidemiology in Belgium 2009-2011
Banks, A.	P76	Emergence of Clostridium difficile ribotype 078 in Scotland

First author	#	Title
Beigot Glaser, S.	P77	The variability of Clostridium difficile ribotypes isolated from a single wastewater treatment plant over a six month period
Broukhanski, G.	P78	The best typing method for investigation of C. difficile outbreaks: ribotyping, MLVA, MMLVA, eMLVA or riboMMLVA?
Janezic, S.	P79	Comparison of different primer pairs and electrophoresis platforms for Clostridium difficile PCR-ribotyping
Jovanović, M.	P80	Clostridium difficile at the Clinical Center of Serbia- spread over surgical and medical clinics
Romano, V.	P81	Occurrence and genotypic characterization of Clostridium difficile in food, animals and the environment in Italy and Switzerland
Terhes, G.	P82	Prevalence of gastrointestinal disease and clinical features of Clostridium difficile- associated infections: results of a retrospective study, 200-201 in a university hospital in Hungary
Terhes, G.	P83	Changes in the epidemiology and antibiotic susceptibility pattern of nosocomial and community-acquired Clostridium difficile in Hungary
Torpdahl, M.	P84	Tandem repeat sequence typing (TRST) as a replacement for PCR ribotyping in routine surveillance of Clostridium difficile
Carson, K.	P85	Isolation of Clostridium difficile from faecal specimens – a comparison of chromid C. difficile agar and cycloserine cefoxitin fructose agar containing taurocholoate
Dobreva, E.	P86	Implementation of molecular methods for identification, detection of toxin encoding genes and typing in Clostridium difficile
Foster, N. F.	P87	Analytical sensitivity of the BD GeneOhm™ Cdiff assay, the Meridian illumigene® C. difficile and the Cepheid Xpert® C. difficile for the detection of Clostridium difficile spores
Kim, H.	P88	Comparison of sensitivity of enzyme immunoassay for toxin A/B and distribution of PCR ribotypes in South Korea
Knetsch, W.	P89	Novel multiplex RT-PCR for the detection of lineage specific Clostridium difficile strains

First author	#	Title
Koppler, T.	P90	Development of a rapid Point-of-Care test system for the detection of toxigenic Clostridium difficile infections
Lemire, A.	P91	Characterization of three new toxinotypes of Clostridium difficile
Metcalf, D.	P92	Evaluation of commercial kits for extraction of DNA and RNA from Clostridium difficile
Murabata, M.	P93	Evaluation of culture media for Clostridium difficile isolation from environmental surfaces
Narender, K.	P94	Development, optimization and qualification of a sensitive high throughput cell-based neutralizing antibody assay for Clostridium difficile toxin A and B
Senoh, M.	P95	Rapid detection method of live Clostridium difficile
Shin, B.	P96	Effectiveness of C. difficile chrome agar for rapid diagnosis of C. difficile infection
Thanki, A.	P97	The development of a rapid-phage based biosensor for the identification of Clostridium difficile
Van Broeck, J.	P98	Evaluation of a chromogenic medium for the isolation of Clostridium difficile from stool samples using an automated inoculation system
Eckert, C.	P99	Detection of glutamate dehydrogenase in stool samples: evaluation of the IMMUNOQUICK C. DIFFICILE GDH ASSAY (Pro-Lab)
Eckert, C.	P100	Evaluation of VIDAS® Clostridium difficile Toxin A&B on colonies
Eckert, C.	P101	Isolation of Clostridium difficile in 24 hours with chrom-ID® C. difficile agar, a new chromogenic medium
Eckert, C.	P102	Contamination of ready-to-eat raw vegetables with Clostridium difficile, France
Avbersek, J.	P103	Clostridium difficile in goats and sheep in Slovenia
Avbersek, J.	P104	Detection of Clostridium difficile in animals: comparison of LightCycler and Taq-Man real-time PCR with the culture method
Blanco, J.L.	P105	Is Clostridium difficile prevalence in animals underestimated?

First author	#	Title
Blanco, J.L.	P106	Zero prevalence of Clostridium difficile in canary birds under antibiotic treatment
Blanco, J.L.	P107	Isolation of Clostridium difficile from puppies: a longitudinal study
Blanco, J.L.	P108	High recovery rate of Clostridium difficile PCR ribotype 078 in Iberian free-range pigs
Drigo, I.	P109	Clostridium difficile isolated from rabbits in Italian commercial farms
Drigo, I.	P110	Molecular characterization of Clostridium difficile isolated from pigs and humans in North-Eastern Italy
Mentula, S.	P111	Matching Clostridium difficile isolates in piglets and humans in Ostrobothnia area in Finland
Niwa, H.	P112	Isolation of PCR ribotype 078 Clostridium difficile from five cases of fulminant colitis in Thoroughbred racehorses
Pirs, T.	P113	Antimicrobial susceptibility testing of animal and human isolates of Clostridium difficile by broth microdilution
Pirs, T.	P114	Clostridium difficile in poultry production: detection and characterisation
Richa, S.	P115	Surveillance of Clostridium difficile in retail chicken: it's antimicrobial and molecular characterization
Rodriguez, C.	P116	Clostridium difficile in intestinal contents and on carcasses of pigs and cattle at slaughterhouse in Belgium
Squire, M.	P117	A novel molecular type of Clostridium difficile in neonatal pigs in Australia lacks tcdA and tcdC but causes greater morbidity than 078 strains
Zidaric, V.	P118	Clostridium difficile in meat products, eggs and vegetables in Slovenia
Zidaric, V.	P119	International Clostridium difficile animal strain collection
Škraban, J.	P120	Different composition patterns of microbiota in C. difficile colonised and non - colonised humans and animals

P63

IDENTIFICATION OF *Clostrium difficile* INFECTION IN ANTIBIOTIC ASSOCIATED DIARRHEA PATIENTS IN INDIAN POPULATION

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Background: *Clostrium difficile* is an obligate anaerobic Gram-positive bacillus. *Clostrium difficile* infection accounts for 15% - 25% of Antibiotic Associated Diarrhea (AAD). Incidence of AAD varies with the choice of antimicrobial agent and route of administration. Diarrheal symptoms are caused by production of toxins A and B.

Aim: To determine the presence of *C. difficile* in Antibiotic Associated Diarrhea patients.

Methods: A total of 876 stool samples were collected from patients with antibiotic associated diarrhea. All the samples were processed for culture of *C. difficile* and detection of enterotoxin and cytotoxin (toxin A and toxin B) on the stool samples by Enzyme immunoassay (Premier toxins A & B; Meridian Diagnostics, Inc., Cincinnati, Ohio, USA). For *C. difficile* isolation faecal samples were treated with 95% ethanol. After treatment, samples were inoculated on selective media i.e. Cycloserine Cefoxitin fructose agar (CCFA) and Brain heart infusion agar (BHIA). Plates were incubated anaerobically at 37°C in an anaerobic jar. The pure colonies were identified by biochemical tests and subjected to PCR for amplification of Tox A and B targeting tcdA and tcdB gene by using publish primer (Gumerlock et al.,1993).

Results: A total of 876 Samples were received between 2008 and 2011. Of these 51/876 (5.8%) were ELISA positive and 15/876 (1.7%) were culture positive. Out of culture positive 7/15 (46.6 %) were positive for both tcdA and tcdB gene whereas either tcdA or tcdB gene alone were positive in 4 additional isolates respectively and rest of 4 isolates were negative for both the gene.

Discussion: C.dfficile is important infectious agent of AAD. Application of molecular tool will help in detection of toxin gene to determine the virulence of isolated strains . Surveillance and investigation of *C. difficile* is required in different hospitals in order to implement appropriate preventive and therapeutic strategies.

P64

RETROSPECTIVE ANALYSIS OF HUMAN BIOTHERAPY TREATMENT FOR *Clostrium difficile*

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Clostrium difficile infection (CDI) is one of the most common causes of nosocomial infection and a retrospective review of data from 27 subjects with recurrent CDI and treated with Human Biotherapy (HBT) was conducted and published in Kassam et al, Arch Intern Med, 172(2): 191-2. The covariates reported above are CDI treatments (in total days) prior to HBT which consist of: metronidazole monotherapy; vancomycin monotherapy; metronidazole+vancomycin; vancomycin taper; and probiotics. Our interest is in the relationship between these covariates and the resolution of CDI following a single HBT. Through the use of exact logistic regression (due to the small sample size), of all the covariates, metronidazole+vancomycin, turns out to be the only statistically significant covariate. Indeed it is a negative significance leading to the suggestion that the practice of first prescribing metronidazole, followed by vancomycin, leads to a statistically significant decrease in the probability of resolution of recurrent CDI when HBT is administered for the first time.

P65

Clostrium difficile INFECTIONS HOSPITALIZED IN ROMANIAN INSTITUTE OF INFECTIOUS DISEASES DURING THE FIRST THREE MONTHS OF 2012

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Objectives In Romania the incidence of Infection with *Clostrium difficile* (CDI) is largely undervaluated due to the insufficient clinical awareness of this problem and due to the lack of microbiological diagnosis capacities; however, since 2011, the incidence raised dramatically and we aimed to determine the clinical aspects and evolution of the patients hospitalized at Romanian Institute for Infectious Diseases with CDI.

Methods: We retrospectively listed the patients discharged from our hospital during three consecutive months (January to March 2012) with one of the next diagnosis "*Clostrium difficile* infection (or diarrhea)", " antibiotic-associated diarrhea ", "pseudomembranous colitis", and cases with other diagnoses at discharge, but with positive bacteriological samples. Because the tests for *Clostrium difficile* toxin detection wasn't available for all patients, we retained for the final analysis 93 cases considered as CDI or „probable CDI" (defined as " antibiotic-associated diarrhea " and positive culture from feces for *Clostrium difficile* or, at least, leukocytes and red blood cells in the feces).

Results The mean of monthly number of cases is abouth six times higher than in the same period of 2011: 31 cases vs 4.8 cases/month), $p < .001$. In the studied group there were more women (55.9%), and the average age was 65.2 years, with extremes between 22 and respectively 93 years. In 75.3% of cases cultures were positive, suggestive changes for colitis in the stool exam were detected in 50.5% of cases. As origin, 74 cases were healthcare facility-associated, 10 cases were community-associated, and the other 9 cases were classified as indeterminate. At least 60.2% of patients received antibiotics recently (more than one antibiotic were used in 42.5% of cases), and 33.3%of patients have undergone recent surgery. We were able to calculate the ATLAS score in 63.4% of cases, which was higher than 5/10 for 21 patients (35.6%). The serum procalcitonin was tested for 29 patients (31.2%), being higher as 0.5 ng/mL in 51.7% of them. Patients received specific therapy with metronidazole (78.9%), glycopeptides (67.1%) and tigecycline (11.8%). Unfortunately, in 50.5% of cases other antibiotics were associated. There were 10 deaths recorded (10.75%) in patients with a mean age of 78.4 years and with an average value of ATLAS score of 5.9, higher than 3.39 in survivors, p=0.00012. All deceased patients patients received associate systemic antibiotic treatment during hospitalization, had hyponatremia and 8 of them had elevated creatinine. The procalcitonin was higher than 0.5 ng/ml in 6 of deceased patients. In patients with

increased level of procalcitonin, the systemic antibiotics were associated; when compared with carbapenems, the use of tigecycline improved significantly the outcome ($p = 0.04$, Fisher's exact test).

Conclusions: *Clostrium difficile* infection incidence recorded an increase from previous years. We found a widespread distribution of *Clostrium difficile* in hospitals from Bucharest area. ATLAS score proved to be useful in assessing the evolution of this infection. Tigecycline therapy increases survival in patients with elevated procalcitonin level, probably with systemic iunfections caused by enteric germs.

P66

DERIVATION AND VALIDATION OF A SIMPLE, ACCURATE AND ROBUST PREDICTION RULE FOR RISK OF MORTALITY IN PATIENTS WITH *Clostrium difficile* INFECTION

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Background and Aims *Clostrium difficile* infection poses a significant healthcare burden. However, derivation of a simple, clinically applicable prediction rule to assist patient management has not been described This study aimed to identify such a prediction rule to stratify hospital inpatients at initial diagnosis of infection according to risk of all-cause mortality.

Method Univariate, multivariate and decision tree procedures were used to deduce a prediction rule from over 186 variables; retrospectively collated from clinical data for 213 patients. Cohort inclusion criteria included those who had a frozen sample of *C. difficile* toxin A/B positive stool, and were recent inpatients on a *Clostrium difficile* cohort ward at an acute teaching hospital. The resulting prediction rule was validated on independent data from a cohort of 158 patients described by Bhangu et al. (2010)1.

Results Serum albumin levels (g/L) (P= 0.000), respiratory rate (resps /min) (P=0.002), C-reactive protein (mg/L) (P=0.034) and white cell count (mcL) (P=0.049) were predictors of all-cause mortality. Threshold levels of serum albumin ≤ 24.5 g/L, C- reactive protein >228 mg/L, respiratory rate >17 resps/min and white cell count >12 mcL were associated with an increased risk of all-cause mortality. A simple four variable prediction rule was devised on based on these threshold levels and when tested on the initial data, yield an area under the curve score of 0.754 (P<0.0001) using receiver operating characteristics. The prediction rule was then evaluated using independent data, and yield an area under the curve score of 0.653 (P=0.001).

Conclusions Four easily measurable clinical variables can be used to assess the risk of mortality of patients with *Clostrium difficile* infection and remains robust with respect to independent data. This simple clinical prediction rule could be easily used by any clinician in much the same way as CURB-65 score is widely used in assessing pneumonia.

P67

Clostrium difficile CONTAMINATION IN THE MEDICAL AND SURGICAL WARDS OF A COMMUNITY HOSPITAL

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Introduction: Longitudinal studies investigating *C. difficile* contamination in the hospital environment have not been conducted. The objectives of this study were to determine the prevalence of *C. difficile* contamination in patient rooms and the general environment and determine what environmental surfaces, their location, and type of surface material are contaminated.

Methods: Environmental surfaces in patient rooms and the general environment in the medical and surgical wards of a community hospital were sampled six times over a 15 week period. Sterile electrostatic cloths were used for sampling and information pertaining to surface material and location was recorded. Enrichment culture was performed and isolates were characterized by ribotyping, toxinotyping, and investigated for the presence of toxin genes by PCR. *C. difficile* isolates from patients hospitalized during the study period were also obtained.

Results: In patient rooms, *C. difficile* was isolated from 5.4% (14/257) of surfaces, including bulletin boards, chair backs, end of beds, overbed tables, and televisions. The highest prevalence of contamination (18.2%, 6/33) occurred on cork bulletin boards. Four different ribotypes were identified, including ribotypes 078 (n=9) and 027 (n=1). In the general environment, 6.3% (16/253) of surfaces were contaminated with *C. difficile* with a high prevalence of contamination occurring on fabric surfaces located in visiting rooms. Nine different ribotypes were identified, including 078 (n=6) and 027 (n=2). Overall, 21 *C. difficile* patient isolates were collected during the study period. Seven patients were identified with ribotype 027 and only one patient was identified with ribotype 078. Toxinotype variants accounted for 18/21 (86%) patient isolates, including toxinotypes III (n=10), IX (n=3), XXIV (n=3), V (n=1), and XII (n=1).

Conclusion: The identification of specific locations, materials, and surfaces contaminated with *C. difficile* suggests common contact sites that may be frequently touched by staff, patients, and visitors. Contamination may have been attributed to lack of hand hygiene and/or inconsistent cleaning and disinfection. The reason that ribotype 078 accounted for 50% of the isolates from patient rooms and the general environment, yet uncommon in people with CDI, is unknown. This hospital serves a rural community, which may increase the potential for community-associated ribotype 078 colonization among patients, staff, and visitors. A higher priority for cleaning and disinfecting specific types of surfaces contaminated with *C. difficile* may help to limit the transmission and dissemination of this pathogen.

P68

ORAL MULTISPECIES PROBIOTIC AS ADJUNCTIVE THERAPY FOR *Clostrium difficile* INFECTION: CASE SERIES INVOLVING 10 PATIENTS (5 OF THEM WITH RECURRENT CDI)

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Background:
Evidence supporting the benefit of probiotics in *Clostrium difficile* Infection (CDI) treatment remains sparse. We report a descriptive case series of ten patients with CDI of a single university hospital (five different departments, Nov. 2010 to July 2011) who received adjunctive probiotic multispecies therapy (at least for 2 weeks). Five out of these suffered from recurrent - and three from severe CDI.

Results:
The mean age of patients was 82 years (range 72-89); the majority were men (7 out of 10). All patients were hospitalized at the onset of CDI. Only one of the 10 patients had no history of antimicrobials; 9 of 10 received antimicrobial medication (range: 1 to 6, mean: 3.7 different antibiotics) in the last three months before onset of CDI. The most frequently administered antibiotics were ciprofloxacin and amoxicillin+clavulanic acid (each 5 of 9 patients) and piperacillin+tazobactam (4 of 9 patients). 8 patients got PPIs and/or cortison before and during the CDI-therapy. Complete resolution of clinical presentation occured in eight patients (80%). Two relapses (both in patients with recurrent CDI) were noted and only one of the observed subjects died within a 3-months follow up period. This patient suffered from pneumonia which led to the fatal course. No adverse events were reported. A repeated stool testing was performed in 9 of 10 patients and these proofed to be negative. Molecular characterization of the strains was done in 70% (7 out of 10); PCR-ribotyping revealed thereby 5 different strains (2 x 014, 2 x 053, and one of each: 023, 433, 413). No clustering or transmission was seen among the investigated patients.

Conclusion:
In this case series administration of adjunctive multispecies probiotic therapy appeared to be a safe, practical and effective therapy for (severe) CDI and recurrent CDI.

P69

EFFECTIVENESS OF A NEW SURFACE DISINFECTING CONCEPT FOR REDUCING THE RISK OF NOSOCOMIAL Clostrium difficile INFECTION

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Background: *Clostrium difficile*, an important nosocomial pathogen is the leading cause of hospital-acquired diarrhea associated with high risk of fatal outcome. A conventional, sporocidal surface disinfection applied in response to the occurrence of CDI was compared with daily application of a new sporocidal agent at two wards of a university hospital with respect to the risk reduction of CDI.

Methods: The routine CDI-surveillance data indicated a high endemic incidence of community-acquired (CA)-CDI and health-care associated (HA)-CDI (n_{total}=118) at two wards (A, B) of an internal medicine department. CDI patients were classified into CA-CDI and HA-CDI according to ECDC definitions. Two intervention studies with a before-after design were performed at the wards A and B: ward A including four to six-bed-rooms and ward B single or two-bed-rooms. At ward A the "pre-intervention phase", in which the hospital policy based usual disinfection procedure (sporocidal surface disinfection in response to occurrence of nosocomial CDI only) was applied, took place from November 2007 until April 2009; the intervention phase, in which a new liquid oxygen releaser disinfectant with high sporocidal activity was daily used regardless of CDI occurrence, took place from May 2009 until July 2010. At ward B the pre-intervention phase lasted from August 2009 until June 2010 and the intervention phase from July 2010 until May 2011.

Results: A total of 67 cases of CDI (including 26 CA- and 41 HA-CDI cases) occurred at ward A and 51 cases (including 34 CA- and 17 HA-CDI cases) at ward B. At ward A, the incidence rate difference of nosocomial CDI cases (0.4/1.000 hospital days) observed was insignificant. At ward B, the incidence rate of nosocomial CDI at the end of the pre-intervention phase was 1.84/1.000 hospital days compared with an incidence rate of 0.77/1.000 at the end of the intervention phase, resulting in a rate difference of 1.07/1.000 hospital days at borderline significance (p=0.10).

Conclusion: We concluded that the daily use of a liquid oxygen releaser disinfectant with high sporocidal activity at a ward with single or two bed-rooms was superior in preventing occurrence of nosocomial CDI to the sporocidal surface disinfection, applied as needed.

P70

BACTEREMIA AND SPLENIC ABSCESS DUE TO *Clostrium difficile* WITHOUT INTESTINAL INFECTIONS: A CASE REPORT

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Extra-intestinal infections caused by *Clostrium difficile* are uncommon. In most of these cases, mixed infections with other organisms are found. We report a fatal case of bacteremia and splenic abscess caused by *C. difficile*.

Case report: A-65-year-old female had a past medical history of chronic nephritis and had been on dialysis since 1984. In February 2011, she needed to have arteriovenous graft in her thigh as hemodialysis access. At the beginning of April 2011, she was given a diagnosis of pneumonia caused by Haemophilus influenzae. A blood culture performed before administration of antimicrobial agents yielded no organisms. She had ceftriaxone followed by levofloxacin with recovery of pneumonia. In late May, she complained of her left flank pain but had no intestinal symptoms including diarrhea. On the next day, she had septic shock and died. A blood culture performed before her death grew only *C. difficile*. Computed tomography of the abdomen revealed a splenic abscess. At autopsy, the abscess content was collected and *C. difficile* was solely recovered from it. No evidence of colitis was noted at autopsy. *C. difficile* isolates recovered from both the blood culture and the abscess were toxin A-positive, toxin B-positive, binary toxin-negative, and typed as PCR ribotype 002.

Discussion: This patient suffered from bacteremia and splenic abscess, both of which were monomicrobial-infections by *C. difficile*. She had arteriovenous graft in her thigh, which might be the portal of entry for the infections. Since she presented no intestinal symptoms, her fecal specimens were not examined for *C. difficile*. In this case, it was evident that *C. difficile* in extra-intestinal infections contributed to her death.

P71

Clostrium difficile IN A SOUTH AFRICAN HOSPITAL SETTING: INCIDENCE AND ANTIBIOTIC RESISTANCE

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Routine typing and antibiotic susceptibility testing of *Clostrium difficile* strains causing infection is not carried out in South Africa. Detection of *C. difficile* is only performed on request if patients develop diarrhoea in hospital and the methods used currently, which are based on the direct detection of toxins A and B in faecal material, suffer from inferior sensitivity and specificity. Consequently, there is little reliable data about the incidence of this infection in local hospitals. In this preliminary study, a total of 100 stool samples from patients at Groote Schuur Hospital in Cape Town, South Africa were screened for the presence of *C. difficile* using a combination of immunological (MiniVidas and Immunocard *C. difficile* assays) and molecular (Cepheid GeneXpert) methods. The samples were submitted by attending clinicians based on clinical suspicion of *C. difficile* associated diarrhoea. Viable *C. difficile* strains were also isolated from the study samples using selective culture media and assayed for resistance to metronidazole, vancomycin, ciprofloxacin and erythromycin. Isolates were screened for the presence of the tcdA, tcdB, and binary toxin genes and classified according to their ribotype. Overall, *C. difficile* strains were detected in approximately 25% of the samples analysed. Results obtained using the GeneXpert method correlated well with those obtained by selective culture, while the Minividas and Immunocard methods were less sensitive. A large proportion of the isolated *C. difficile* strains belonged to the ribotype 017 class and showed resistance to ciprofloxacin and erythromycin. No metronidazole or vancomycin resistant strains were isolated, although a trend towards decreased susceptibility to metronidazole (MIC ≥ 2 µg/ml) was observed in some of the ribotype 017 isolates. In conclusion, this is the first study to report active surveillance of *C. difficile* in the South African hospital environment and provides preliminary epidemiological data that suggest a relatively high incidence of *C. difficile* infection amongst GIT patients in Groote Schuur hospital.

P72

TWO CASES OF FULMINANT COLITIS DUE TO BINARY TOXIN-POSITIVE *Clostrium difficile*, WHICH WAS NEITHER PCR RIBOTYPE 027 NOR TYPE 078

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Introduction It has been documented that patients infected with *Clostrium difficile* producing binary toxin (CDT), such as PCR ribotype 027, are more likely to develop severe disease. In Japan, while outbreaks caused by type 027 have not been documented so far, the epidemiology of *C. difficile* infections (CDI) due to CDT-positive strains is unknown. The fatal cases of CDI caused by CDT-positive *C. difficile*, which was neither PCR ribotype 027 nor type 078 have been reported in Japan.

Case reports and bacterial identification A 60s suffered from subcortical hemorrhage and was admitted to hospital. He developed aspiration pneumonia, and ampicillin-sulbactam was administered. 7 days after antimicrobial therapy, he showed symptoms of severe watery diarrhea and hypovolemic shock, leading to admission to the ICU. The stool specimen was tested positive for toxins A and B by EIA, and vancomycin was administered. Two days after his onset of colitis, he died. The findings in an autopsy revealed pseudomembrane formation in the entire colon and marked edema in the mesentery. 11 weeks after the onset of colitis in the 1st patient, a 60s woman who was hospitalized in the same ward, developed fulminant colitis. She suffered from subarachnoid hemorrhage and showed symptoms of pyelonephritis, for which piperacillin-tazobactam was administered. She had watery diarrhea after 18-day antimicrobial therapy and was diagnosed as CDI by EIA test detecting toxins A and B. Computed tomography of her abdomen showed that the entire colon was markedly edematous. 5 days after administration of vancomycin and metronidazole, she died. *C. difficile* isolates recovered from both patients were toxin A-positive, toxin-B-positive and CDT-positive. Both isolates were identical by PCR ribotyping and the banding pattern of the isolates differed from that of PCR ribotype 027 as well as that of PCR ribotype 078. The sequences of the slpA gene of the isolates were same; other clinical isolates of the same slpA sequence type have not been found in our investigation in Japan so far.

Discussion Fulminant colitis due to *C. difficile* positive for CDT occurred in two cases in 11 weeks. *C. difficile* recovered from the patients may be a new hypervirulent strain. Further studies for virulence factors and epidemiology of this strain are required.

P73

PROSPECTIVE ANALYSIS OF *Clostrium difficile* CASES IN SCOTLAND WITH EMPHASIS ON SPORADIC AND OUTBREAK STRAINS OF RIBOTYPE 078

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Clostrium difficile ribotype 078 has been increasing in incidence in some countries over the past few years. It is widely regarded as a hypervirulent strain because a few reports demonstrated an increased severity in patients affected by this strain and it also has genomic similarities with ribotype 027. We performed a prospective study in Lothian, UK, from Aug 2010 to July 2011 to determine the prevalence of ribotypes in this region and to compare clinical severity associations of ribotype 078 with other prevalent ribotypes. During the follow up of this study, an outbreak of ribotype 078 occurred in Jan 2012.We also analysed these additional cases in comparison to the study cohort.

All patients who presented with clinical diarrhoea and were toxin positive by toxin A+B ELISA and GDH testing were included in the study. All stool samples were cultured on CCEY medium and isolates typed by PCR ribotyping using capillary gel electrophoresis. Isolates of ribotypes 078 and from patients with repeat episodes were also sub typed by MLVA to look for dominant sub types and associations with severity. All patients were followed up for 1 year post presentation to look for outcome. Epidemiological factors, hospital/community acquisition, markers of severity, co-morbidities presence of other pathogens and treatment given were noted. A total of 376 individual episodes from 345 patients were analysed. Ribotype 078 was the 6th commonest ribotype found in 7% of the episodes whereas ribotypes 001, 002, 005, 015 and 014/020 were found in 15%, 10.4%, 8.7%, 8.2% and 14% respectively. No isolates of ribotype 027 were found in this study. Cases with ribotype 078 were not found to be more likely to have severe or recurrent disease as compared to the non 078 cases (p=0.79 and 1.0 respectively), though 078 cases were less likely to be hospital acquired as opposed to community or health care associated acquisition(p=0.002). In the January outbreak four patients in related wards were affected. Initial doubts were raised on their relatedness as ribotype 078 is also known to be an animal associated strain and patients could be independent community carriers of these strains as opposed to their hospital acquisitions. Sub typing revealed that the outbreak isolates clustered closely together compared to other prevalent 078 strains.

In our study ribotype 078 cases were not associated with increased severity or recurrence compared to episodes with other ribotypes. Subtypingof ribotype 078 is technically difficult since its genome has variations in the primer annealing regions, however, using a combination of 6 published loci we were able to distinguish outbreak related isolates from endemic ones.

P74

PREVALENCE OF *Clostrium difficile* IN PATIENTS WITH ANTIBIOTIC-ASSOCIATED DIARRHOEA AND PSEUDOMEMBRANOUS COLITIS

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Clostrium difficile is the main etiological agent of antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis (PMC). Its virulence is mainly due to the production of two toxins: toxin A and/or toxin B. The aim of the present study was to assess the prevalence of *C. difficile* in stools of adult patients with AAD and PMC. Material and methods: During one year period, 68 cases of diarrhoea were reported from hospital units of infectious diseases, internal medicine and surgery. All stool samples were cultured anaerobically on a selective medium (cycloserine-cefoxitin-fructose agar, CCFA) and on a Columbia agar supplemented with sheep erythrocytes, haemin and vitamin K at 37°C for 48h. They were also investigated for *C. difficile* toxins A/B by an immunoenzyme assay (R-Biopharm). In all stool samples, the presence of aerobic normal gut flora was estimated by culturing the samples on blood agar, aerobically, for 24h. Host factors, such as age and antibiotic exposure, were evaluated. Results: 18 (26.47 %) stools were positive for *C. difficile* toxins by an EIA technique, while *C. difficile* was cultured from 31 (45.58 %) samples. *C. difficile* was isolated from 19 stools that were EIA negative. Additionally, in 19 (27.94 %) stool samples the normal gut flora was reduced or absent but Pseudomonas aeruginosa and Candida albicans isolates were detected in 11 (16.17 %) and 8 (11.76 %) cases. Ninety-five percent of the *C. difficile* - positive patients and 75 % of the *C. difficile* - negative patients were above 60 years (p value = 0.015). Conclusions: Using both toxin A/B EIA and culture, the detection of *C. difficile* in patient stools could be enhanced. Using either method would have led to false-negative results. The presence of Pseudomonas aeruginosa or Candida albicans isolates in 27.93 % of cases with AAD or PMC could have a significant importance, and it is worth to investigate their role in the pathogenesis of these conditions.

P75

TRENDS IN *Clostrium difficile* RIBOTYPE EPIDEMIOLOGY IN BELGIUM 2009-2011

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Surveillance of *Clostrium difficile* infection is compulsory in Belgian hospitals since 2007. All cases have to be reported at the Institute of Public Health. Every six months every hospital laboratory is invited to send to the *C. difficile* National Reference Center the first five strains isolated in the routine bacteriology laboratory. It is mandatory at least once a year. Since 2009, all strains are ribotyped. Here we report on the major trends in ribotype incidence during the last three years.

Methods. For ribotyping, DNA were extracted with chelex and 16S -23S rRNA intergenic spacer regions were amplified using primers as described by Barbut et al (J. Clin. Microbiol. 2000). Amplicon sizes were analysed by capillary electrophoresis using an automatic sequencer (ABI 3100 Automated Capillary DNA Sequencer) and GeneMapper analysis (Applied Biosystems, Inc.). A 35–500 bp ROX ladder (ABI) was used as internal marker. Profiles were analyzed by comparison with those of reference strains from the European collection (Brazier classification) and with our own database. Toxin genes (A, B and binary), deletion in tcdC and moxifloxacin resistance mutations were tested using GenoType CDiff (Hain Lifescience).

Results. We received 389 isolates from 117 different laboratories in 2009, 505 from 107 in 2010 and 462 from 89 in 2011. A total of 129 different ribotypes were identified, 92 of them being seen only in one case. Ribotype 027 was the most frequent in 2009 (72/389, 18.5%) but gradually decreased in 2010 (62/505, 12.3%) and 2011 (56/462, 12.1%). Ribotype 014 was the second most frequent but had an opposite trend going from 44/389 (11.3%) in 2009 to 56/462 (12%) in 2011. Ribotypes 020, 002 and 078 also gradually increased, the most marked increase being observed with ribotype 002 which went from 15/389 (3.9%) in 2009 to 21/505 (4.2%) in 2010 and 37/462 (8%) in 2011. Binary toxin and deletion in tcdC were detected in strains belonging to ribotype 027, 023 and 078. Moxifloxacin resistance mutation was detected in most of ribotype 027 strains and in some of ribotype 078.

Conclusion. Ribotype 027 is decreasing in Belgium while other ribotypes (014, 002, 078 and 020) are on the increase. Our surveillance program allows a good follow-up of the epidemiology.

P76

EMERGENCE OF *Clostrium difficile* RIBOTYPE 078 IN SCOTLAND

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Aims To report on the emergence of *Clostrium difficile* ribotype 078 in Scotland.

Methods Ribotype 078 data from samples sent to the Scottish reference service for PCR ribotyping (from severe cases and outbreaks, and isolates from a fixed proportion of all cases including mild, moderate and severe disease (Snapshot Programme)) were analysed. Samples were also susceptibility tested against a range of antibiotics and results described.

Results From November 2007 to March 2012, a total of 135 isolates of ribotype 078 were reported from severe cases and outbreaks (57% female, age range 1-98 years (median 77 years)). A total of 99 isolates were reported from the Snapshot Progamme between January 2009 and March 2012 (62% female, age range 18-97 years (median 73 years)). The proportion of ribotype 078 has increased year on year: 2.5% (2008), 3.2% (2009), 4.3% (2010) and 7.8% (2011) from severe cases/outbreaks; 3.6% (2009), 5.6% (2010), 6.4% (2011) from the Snapshot Programme. In the quarter, January-March 2012, 078 was the most commonly observed ribotype, accounting for 15% of severe cases/outbreaks and 14%, of snapshot surveillance isolates. Outbreaks due to ribotype 078 were reported for the first time in two hospitals on the East coast of Scotland in the last 6 months. Overall, cases with ribotype 078 have been observed in twelve of the fourteen NHS administrative areas in Scotland. Ribotype 078 is sensitive to vancomycin and metronidazole. A majority of the 135 cases from severe cases and outbreaks were resistant to clindamycin (98%) and cephalosporins (58%), with a lower proportion of resistance to erythromycin (18%), levofloxacin (13%) and moxifloxacin (8%). Based on the information available on the request forms ~90% were from in-patients.

Conclusions Ribotype 078 has emerged in Scotland against a background of decreasing previously 'epidemic' ribotypes 001, 106 and 027. This may suggest a strain replacement in hospitals but this requires further investigation. This type has also emerged in the UK and Europe (notably the Netherlands) and possibly represents another hypervirulent strain with links to animals and high levels of recurrence. Further characterisation of the epidemiology of CDI casued by ribotype 078 (disease severity, sources of infection and potential differences in infection prevention and control) is warranted.

P77

THE VARIABILITY OF *Clostrium difficile* RIBOTYPES ISOLATED FROM A SINGLE WASTEWATER TREATMENT PLANT OVER A SIX MONTH PERIOD

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The wastewater treatment plants (WWTP) are an environment where *C. difficile* strains present in waste waters from different environments such as hospitals, farms, households etc. WWTPs could therefore be a very suitable sampling site to assess the overall diversity of *C. difficile* in a given reservoir. The aim of this study was to detect *Clostrium difficile* in wastewater effluent and to determine variability of PCR ribotypes over a 6-month screening period.

Study was conducted between January and June 2012 on a single wastewater treatment plant. Altogether 6 samples, one per month, were collected at effluent. After heat shock at 70°C for 20 minutes water samples were filtered through a 0.2-µm pore size cellulose nitrate membrane filter (Whatman). Filters were placed on commercial selective chromID™ *C. difficile* agar (BioMérieux) and incubated anaerobically at 37°C for up to 3 days. After incubation, 20 black colonies were picked from each filter and subcultured on fresh medium. *C. difficile* was confirmed by detection of molecular marker cdd3 and characterized by toxinotyping and PCR ribotyping.

All tested samples were *C. difficile* positive and altogether 49 strains were isolated. From each sample, 2 to 13 isolates were isolated and 79.6% of all isolates were toxigenic. In samples collected in March and April most strains (13 strains each sample) and most PCR ribotypes (7 types each sample) were found. Overall, 18 different PCR ribotypes were identified. PCR ribotype 014/020 was detected in all tested samples, 010 and 046 in 66,7% of tested samples and 002 in half of all samples. All four PCR ribotypes were predominant among isolated strains, encounting for from 8,2% to up to 38,8% of all isolates per sampling. Other 14 PCR ribotypes were rare (2%-4% of all isolates) and were detected in only one to two out of all 6 tested samples. Interestingly, PCR ribotypes 014/020 and 002 are also the most prevalent PCR ribotypes in hospitalized patients in Slovenia and also frequently found in animals in Slovenia.

P78

THE BEST TYPING METHOD FOR INVESTIGATION OF C. difficile OUTBREAKS: RIBOTYPING, MLVA, MMLVA, eMLVA or riboMMLVA?

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Investigation of *C. difficile* (CD) outbreaks in hospitals successfully done before using ribotyping or PFGE currently requires more discriminatory methods due a wide spread of ribotype 027/NAP1 strain. To trace these strains more discriminatory methods are required. MLVA and its improved versions, MMLVA and eMLVA have ability not only to discriminate outbreak 027 strains from sporadic ones but also to classify other strains. Previously we expanded typing capabilities of MLVA by incorporating detection toxin genes and tcdC gene deletion, a marker for ribotypes 027 and 078, (modified MLVA, MMLVA) and developed criteria for interpretation of typing results. Other authors suggested increasing the number of loci for MLVA analysis in attempt to match typing capability of ribotyping for improved analysis of genetic diversity of CD populations. These approaches have benefits (better ability to identify strains) and disadvantages (complicated reactions setup and results interpretation). We assessed another approach, combining two typing methods, MMLVA and ribotyping, together to utilize benefits of both of these methods in a single test. To achieve this, a universal PCR protocol was developed to amplify MLVA fragments, toxin genes and ribotyping fragments (ribo-MMLVA). Using two typing methods together was successfully applied for typing of some other bacteria but never attempted before, despite obvious benefits, for typing of CD. Amplicons are analyzed with the same protocol on a capillary sequencer (ABI 3130xl) and strains are compared using BioNumerics composite data sets feature. This combination allows avoiding false classification of strains belonging to different ribotypes as closely related which happens sometimes in MLVA when number of repeats is similar and, when ribotyping data are processed using an on-line data-base (webribo.ages.at or similar), to match distribution of strains at a specific hospital to data from other hospitals which use ribotyping as a main tool. We automated PCR and fragment analysis setup of the ribo-MMLV using liquid handler and thermocycler with a 384-well adapter which allows us testing 4 colonies per specimen of up to 96 specimens at a time which meets requirements of the most extensive outbreak investigation. Details of reaction protocols and analysis will be presented. We will discuss expanded set of criteria for identification of clonality of strains and ways to scale results of analysis to meet different levels of discrimination, from a single ward affected by a CD outbreak to larger geographic areas.

P79

COMPARISON OF DIFFERENT PRIMER PAIRS AND ELECTROPHORESIS PLATFORMS FOR *Clostrium difficile* PCR-RIBOTYPING

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PCR-ribotyping, method based on size variation of intergenic spacer region (ISR) of ribosomal operons, has in recent years become a method of choice for typing of *C. difficile*. Several different primer pairs have been described for *C. difficile* PCR-ribotyping. Most commonly used are primers described by Bidet et al. (1999) and Stubbs et al. (1999). Recently, we reported modified primers which allow direct ribotyping of *C. difficile* in total DNA from stool samples (Janezic et al., 2011). In this study we have compared different primer pairs and electrophoresis platforms available for *C. difficile* PCR-ribotyping.

The ISRs of a well characterized *C. difficile* strains were amplified with two different primer pairs (Bidet et al., 1999; Janezic et al., 2011). Amplified fragments were resolved by classical agarose gel electrophoresis (GE), by QIAxcel (Qiagen) capillary electrophoresis system (Q-CE) and by capillary electrophoresis on automated sequencer (S-CE) using two different apparatus; CEQTM 8000 Genetic Analysis System (Beckman Coulter) and ABI 3130 DNA Analyser (Applied Biosystem). PCR-ribotypes were determined with WEBRIBO software (for S-CE-based ribotyping) and by comparison of banding patterns with BioNumerics software (for GE- and Q-CE-based ribotyping).

Comparability of results with both primer pairs was very good. The only difference observed between the banding patterns was the presence or absence of double peaks (± 2 bp) generated with one of the primer sets compared to the other. However, in all these cases WEBRIBO identification of the PCR-ribotype was correct regardless of the primers used. A subset of 48 strains was also ribotyped using S-CE with primers described by Stubbs et al. (1999) and for all the strains tested correct PCR-ribotypes were determined with WEBRIBO analysis. Comparison of different electrophoresis platforms showed that, as described before, capillary sequencer based PCR-ribotyping is more discriminatory and enabled us to subtype some of the PCR-ribotypes. Discriminatory power of Qiaxcel system is comparable to classical agarose gels, however, Q-CE sometimes failed to detect the highest peak leading to incorrect identification of closely related PCR-ribotypes. In conclusion, PCR-ribotyping based on S-CE is more discriminatory and accurate than GE and Q-CE. Furthermore, WEBRIBO software enables fast and reliable identification of PCR-ribotypes using any of the three primer sets described for *C. difficile* PCR-ribotyping.

P80

Clostrium difficile AT THE CLINICAL CENTER OF SERBIA- SPREAD OVER SURGICAL AND MEDICAL CLINICS

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BACKGROUND: Infection with toxin-producing *Clostrium difficile* strains is a common cause of diarrhea in the hospital setting. Nowadays it is becoming a growing problem at the Clinical Center of Serbia.

GOALS: To evaluate the yield of stool immunochromatographic testing in patients with suspected *Clostrium difficile* infection (CDI).

METHODS: Stool samples from all patients of Clinical Center of Serbia (CCS) with symptoms of CDI are processed at the Bacteriology laboratory of Clinic for Infectious and Tropical Diseases, which is a part of CCS. Our microbiology laboratory database was queried from January 1, 2009 to April 30, 2012 for all patients who underwent immunochromatographic toxin A and/or toxin B stool testing for suspected infection caused by *C. difficile*. Data collected included location of patient (various clinics).

RESULTS. Testing was performed on 2053 stool samples and 494 yielded *C. difficile*. The number of stool samples was increasing through 2009, 2012, 2011 and four months of 2012: 218, 480, 742, 613, as well as the number or positive samples: 20, 43,201, and 230 respectively. The majority of positive results are obtained from Clinic for Infectious and Tropical Diseases, where patients suffering from diarrhea caused by *C. difficile* are usually hospitalized (n=74), but also from surgical (n=78) and medical clinics (n=59) of Clinical Center of Serbia, where they reside for other diseases. In the period September 2010-June 2011 test indicating presence of toxin A and toxin B separately has been in use. Presence of toxin A only was not notified in any stool sample, toxin B only in 88 and both toxins in 44 stool samples.

CONCLUSION. The number of patients with symptoms consistent with *C. difficile* infection is increasing in Clinical Center of Serbia, as well as the number of stool samples where presence of *C. difficile* toxins is confirmed. Toxin B was much more prevalent than both toxins simultaneously in the samples processed in our laboratory.

P81

OCCURRENCE AND GENOTYPIC CHARACTERIZATION OF *Clostrium difficile* IN FOOD, ANIMALS AND THE ENVIRONMENT IN ITALY AND SWITZERLAND

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Hospitalization and antibiotic therapy has been mostly considered the main risk factor for *Clostrium difficile* infection (CDI). Recently, the emergence of community acquired cases impels to explore new reservoirs and vehicles for *C. difficile*. Environment, animals and food are all candidates for community routes and sources of infection.

The aim of this work was to investigate the occurrence of *C. difficile* ribotypes in sea food, animals and the environment in Southern Italy and in Ticino Canton (Switzerland). A comparison of ribotypes with those associated to human CDI in Ticino Canton was also carried out. Samples of seawater (25), zooplankton (5), sea sediments (5), shellfish (105), river water (5), lake water (8), lake sediments (8) and raw (1) and treated wastewater (1) were collected in different areas of Campania and Basilicata regions, in Southern Italy. In Ticino, rectal swabs from ruminants (150) and piglets (2) and samples of influents (9) and effluents (9) from the 9 largest wastewater purification plants of the region were also collected. Molecular identification, toxin genes detection, toxinotyping and ribotyping were carried out on the isolates. In Italy, *C. difficile* was isolated from seawater, zooplankton, edible shellfish, river water, lake sediments and in both samples of raw and treated wastewater analyzed. In Ticino Canton *C. difficile* was recovered from ruminants, piglets and wastewater. Taking all the environmental strains into account, toxinotype V/ribotype 078 was the most common isolate, followed by toxinotype 0/ribotypes 014 and 070, and nontoxigenic strains/ribotype 010. Toxinotype 0 strains were the most frequently isolated in all the meat producing animals investigated, while the most common ribotypes were 001 and 066. Shellfish, a popular food in Southern Italy usually eaten raw or lightly cooked, were found to be contaminated with toxigenic *C. difficile*; ribotypes 014 and 078 were the most commonly found among these isolates. Some of the ribotypes of *C. difficile* isolated in Ticino Canton overlapped with those responsible of human CDI in 2010 in the same geographic area. The lack of epidemiological data in Southern Italy on the incidence of CDI prevented us from drawing a similar correlation. The results of the 2008 European survey about the incidence of CDI in European hospitals highlighted that the most prevalent clinical ribotypes recovered in Italy were 078, 001, 014, 106, 126, 018, the same ribotypes we found in shellfish, lake sediments and wastewater in this area. This work points out a possible health risk associated to the widespread diffusion of virulent *C. difficile* strains, including the hypervirulent *C. difficile* ribotype 078, in environmental, animal and sea food sources in both Ticino Canton and Southern Italy.

P82

PREVALENCE OF GASTROINTESTINAL DISEASE AND CLINICAL FEATURES OF *Clostrium difficile*- ASSOCIATED INFECTIONS: RESULTS OF A RETROSPECTIVE STUDY, 200-201 IN A UNIVERSITY HOSPITAL IN HUNGARY

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Objectives: Recent spread of severe cases of *C. difficile*-associated diarrhoea (CDAD) reported in different parts of of Hungary have emphasized of an ongoing epidemiological surveillance of CDAD. Recent reports have documented that *C. difficile* infections (CDIs) are occuring among patients without traditional risk factors. Our objective was to determine the epidemiology of CDAD over years 2000-2010 in a 1200-bed university hospital in Hungary by estimating the incidence of CA-CDIs and HA-CDIs, and identifying patient-related risk factors.

Methods: All CDAD cases diagnosed from Jan. 01 2000 to Dec. 31. 2010, were reviewed. A CDAD case was definied as diarrhea in a hospitalized patient who had a stool specimen that tested positive for *C. difficile* cytotoxin or had a positive toxigenic culture reult. CDAD was considered community acquired if the diarrhea occured in the patient within 72 h after admission and if the patient had no history of hospitalization in the previous month; otherwise CDAD was considered healthcare-associated.

Results and conclusion: In the year of 2000, 3081 faecal samples were screened for bacterial enteric pathogens in our laboratory, and only 945 of them for the presence of *C. difficile* toxins, of these stool samples, 178 (18.9%) were toxin positive. Our first survey sholud draw attention of local clinicians to the role of toxin-producing *C. difficile* as a major hospital-acquired pathogen in patients who have subjected to a prolonged hospital stay. During the examined 10-year period, the number of patients with *C. difficile*-associated diarrhea has dramatically increased. Since 2008, increase in the number of toxin tests for the detection of CDI in our lab colud be observed, simultaneously our finding showed that the prevalence of CDI is also increasing. In 2010, already 3839 faeces samples were investigated for *C. difficile* toxins, 975 samples of 689 patients (25.9%) proved to be positive. The age, gender, co-morbidity, antibiotic usage and surgical intervention of patients were evaluated in this study period. The incidence of community-onset cases of CDAD may increase outside known risk groups, that are currently characterized by prior hospitalization, antibiotic usage, older age and significant co.morbidity. This single-center surveillance project, which established CDAD rates at fequencies currently reported from international surveys, is useful as benchmark and will help in understanding patterns and impact of CDAD at regional, or local level. Our study documented, that the epidemiology of CDI is changing, with CA-CDI occuring in populations not traditionally considered "high-risk" for the disease.

P83

CHANGES IN THE EPIDEMIOLOGY AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF NOSOCOMIAL AND COMMUNITY-ACQUIRED *Clostrium difficile* IN HUNGARY

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Objectives: The rising incidence of C. diffcile infection is getting more and more important, this has been partly attributed to the increased consumption of broad-spectrum antibiotics among hospitalized patients and in the community. The increased antibiotic consumption may have impact on the emergence of multiple resistant *C. difficile* strains too; therefore our aims were to continue our previous investigations, in which changes in the antibiotic susceptibility patterns of toxigenic *C. difficile* isolated from human diarrhoeal faeces were determined. In Hungary, the first recognized infection due to *C. difficile* PCR ribotype 027 was detected in 2007, after this case, isolate belonged to this PCR ribotype could not be found among those strains, which were sent to the Anaerobe Reference Laboratory for further analysis, but since May, 2010 increased number of binary toxin positive strains has been detected, because of this, more than 1000 strains were investigated for the presence of toxin genes to characterize the strains isolated from outbreak situation.

Methods: Up to this point, 1085 *C. difficile* strains isolated after May, 2010 in three Hungarian laboratories represented West, East and South Hungary from diarrhoeal faecal samples of both inpatients and outpatients were analysed for the presence of toxin genes and PCR ribotype 027. Antibiotic susceptibility of 200 toxin-positive strains isolated between 2008 and 2011 was determined by MIC Test Strip (Liofilchem, Italy) for metronidazole, moxifloxacin, clindamycin, erythromycin and rifampicin. The results were compared with our earlier findings given in 2007.
Results and conclusion: Until this time, 46.2% of the isolated strains carried binary toxin genes and 97.8% of these strains belonged to PCR ribotype 027. Among the examined isolates, the emergence of toxin A-negative and toxin B-positive strains could be also observed. All of the tested isolates were susceptible to metronidazole during the recent study period. 22.8% of the isolated strains showed moxifloxacin resistant phenotype between 2008 and 2011. The prevalence of erythromycin resistant isolates was 28.7%, while 19.9% of tested strains proved to be resistant to clindamycin. 12.3% of the isolates were resistant to rifampicin. In comparison of the given results with our data from a previous study, in Hungary, all of the tested isolates were sensitive to metronidazole, the prevalence of isolates resistant to moxifloxacin, or erythromycin, or clindamycin has not been increased, while the prevalence of rifampicin resistant isolates was higher between 2008 and 2011.
This work was supported by ESCMID/bioMérieux grant 2010.

P84

TANDEM REPEAT SEQUENCE TYPING (TRST) AS A REPLACEMENT FOR PCR RIBOTYPING IN ROUTINE SURVEILLANCE OF *Clostrium difficile*

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In Denmark, since 2009, the surveillance of *Clostrium difficile* PCR ribotype 027 has been intensified. This includes reporting infections with 027 to the national public health institute for infectious diseases, Statens Serum Institut (SSI). Also, when 027 or other virulent PCR ribotypes are suspected but not verified by the local clinical department, isolates are sent to SSI for further characterization.

PCR ribotyping was performed according to the method published by O'Neill (Anaerobe, 1996). PCR ribotypes were named after comparison with the profiles obtained from the international reference collection and new profiles were assigned an arbitrary local number. From January 2012 we included TRST (Zaiβ et al., BMC Microbiology, 2009) in the routine surveillance of all incoming isolates as well as on selected PCR ribotypes from previous years. TRST types were assigned using BioNumerics with the Polymorphic VNTR Typing plugin. In order to have a central source for nomenclature we have created the PubTRST web site (http://pubtrst.org/) which has been set up in accordance with nomenclature from Zaiβ et al. The website is continuously updated with new TRST types and everyone is encouraged to contribute.

In total, 450 surveillance isolates as well as 100 project and reference strains have been characterized using both PCR ribotyping and TRST. In general there was high concordance between TRST and PCR ribotyping. A few PCR ribotypes were split into several TRST types and some TRST types included several PCR ribotypes. Because of the selection criteria used when choosing isolates for surveillance, 43% of all the isolates analyzed with TRST have the PCR ribotype 027. Of these, 97% were also found to have the same TRST type (tr027), while the remaining 3% were given new TRST types. When creating a phylogenetic tree based on the TRST sequences, these remaining types were closely related to each other as well as to 027 and more distantly related to all other types assigned.

Based on these findings we therefore conclude that TRST is a good alternative to PCR ribotyping for routine surveillance of suspected virulent *Clostrium difficile*. TRST is furthermore easy to perform in the laboratory and types are automatically assigned using the TRST type definition from PubTRST.

P85

ISOLATION OF *Clostrium difficile* FROM FAECAL SPECIMENS – A COMPARISON OF CHROMID C. DIFFICILE AGAR AND CYCLOSERINE CEFOXITIN FRUCTOSE AGAR CONTAINING TAUROCHOLOATE

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The rapidly changing epidemiology of *Clostrium difficile* infection (CDI) over the past 10 years highlights the need for improved and continuing surveillance, and the on-going requirement for stool culturing to facilitate molecular tracking. Here we compare bioMérieux ChromID Cdiff (CDIF) to pre-reduced CCFA with 1% sodium taurocholate (TCCFA) for the isolation of *C. difficile* from faecal samples that were glutamate dehydrogenase positive, either PCR-positive or -negative, and processed by direct culture or alcohol shock. For direct culture, all liquid stools were plated undiluted and solid stools were diluted 1:2 in sterile saline before plating. Growth of *C. difficile* and other faecal flora was recorded semi-quantitatively as 1, 2 or 3. All plates were incubated in an anaerobic chamber and read at both 24 and 48 h with no plate >15 min outside the chamber during examination and/or manipulation. Presumptive *C. difficile* colonies were sub-cultured onto blood agar and PCR used to detect toxin A, toxin B and binary toxin genes. Isolates were also PCR ribotyped.

Direct culture on CDIF had a sensitivity of 100% and recovery of 94% while for TCCFA these were 87% and 82%, respectively. There was a significant difference between growth on CDIF at 24 h and TCCFA at 48 h (p 0.007) and between the two media at 48 h ($p < 0.001$). The difference between growth on CDIF at 24 and 48 h was also significant (p 0.0068). Contingency tables were constructed and analysed by the Chi squared test for independence. CDIF grew more *C. difficile* than did pre-reduced TCCFA (24 h p 0.0162 and 48 h p 0.0457) and less faecal flora (24 h $p < 0.0001$ and 48 h p 0.0002). Of the 100 PCR-positive alcohol-shocked faecal samples, *C. difficile* grew on 95 CDIF plates at 24 h and 92 TCCFA plates at 48 h which gave similar recovery and sensitivities to CDIF with direct plating. Of the 84 PCR-negative faecal samples, *C. difficile* grew on 41 CDIF at 24 h and 36 TCCFA at 48 h. When these results were analysed by Fisher's Exact Test no significance difference was found. Of the 96 isolates from PCR-positive samples, 81 were $A^+B^+CDT^-$ and belonged to several different ribotypes with 24 being 014. Five isolates were $A^+B^+CDT^+$ and could not be assigned a UK ribotype. Of the 46 isolates from PCR-negative samples, 11 were $A^+B^+CDT^-$ and one was $A^-B^+CDT^-$. Seven, including the $A^-B^+CDT^-$ strain could not be assigned a UK ribotype. CDIF with direct plating had similar sensitivity and recovery to both CDIF and TCCFA with alcohol shock. CDIF was the more selective agar with greater growth of *C. difficile* and less contaminating faecal flora with the added advantage of growth at 24 h.

P86

IMPLEMENTATION OF MOLECULAR METHODS FOR IDENTIFICATION, DETECTION OF TOXIN ENCODING GENES AND TYPING IN *Clostrium difficile*

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Clostrium difficile (CD) is associated with asymptomatic colonization, mild to severe diarrhea and life-threatening gastrointestinal diseases. It is considered a causative agent in 25% of the total cases of antibiotic-associated diarrhea (CDAD). Asymptomatic carriers are an important hidden reservoir of CD. The main predisposing factor for *C. difficile* infection (CDI) is the use of antimicrobial agents. The enteropathogenicity of CD is mediated by two toxins: enterotoxin A (TcdA) and cytotoxin B (TcdB). Some strains of clinical significance also produce a third chromosomally encoded binary toxin (CDT). We have analyzed 110 stool samples from patients with mild to severe enterocolitis and history of previous antibiotic therapy. Toxin A/B production was analyzed by EIA. Detection of toxin encoding genes tcdA, tcdB, cdtA/cdtB and gluD was performed by EvaGreen Real-time PCR methods. PCR-ribotyping and MLVA7 were applied for genotyping of the strains. Ribotype patterns were compared to reference the 25 ECDC CD collection.

Toxin production of toxin A/B by EIA was registered in 27,3% (30/110) of the stool samples whereas culture isolation rate was higher 33,6% (37/110). GluD gene however was confirmed in only 24,5% (27/110) of the isolates indicating a significant false positive results in EIA and culture. TcdA was determined in 88,9% (24/27) while 25% (6/24) of the strains harboured the tcdA deletion (714bp). TcdB gene was observed in 92,6% (25/27). We have developed multiplex PCR method for detection of tcdB and gluD genes. The sensitivity of the method was 24 CFU for gluD and 2,4 CFU for tcdB respectively. All three toxigenic variants were distinguished among our isolates: 66,7% (18/27) toxin A+B+; 29,6% (8/27) A–B+ and 3,7% (1/27) A-B- . The binary-toxin genes cdtA/cdtB were detected in two of the A+B+ strains. Eight ribotypes were confirmed and the most prevalent one was 017-29,6% (8/27) followed by 014/020– 18,5% (5/27), 001, 002; 012 by 7,4% and 046, 070, 078 represented by 3,7% each. Eighteen percent of CD (5/27) corresponded to non-reference PCR- ribotypes.

A total of 18 MLVA7 genotypes were detected in our strains, distributed as follows: four for ribotype 017; two for 001, 002, 014/020 respectively; one for ribotype 046, 070 and 078 respectively. Four patients from the same hospital in Sofia were infected with CD ribotype 017 and two of them had lethal outcomes. The significant number of diagnosed CD cases with outbreak ribotypes might represent a problem in our country in the future. The results of the present study should be strongly supporting and improving the diagnostic and therapeutic preparedness of the Bulgarian hospitals when dealing with CDI.

P87

ANALYTICAL SENSITIVITY OF THE BD GENEOHM™ CDIFF ASSAY, THE MERIDIAN ILLUMIGENE® C. DIFFICILE AND THE CEPHEID XPERT® C. DIFFICILE FOR THE DETECTION OF *Clostrium difficile* SPORES

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Rapid diagnosis of *C. difficile* infection (CDI) is important for early implementation of infection control measures to limit the transmission of infection. Historically, *C. difficile* detection involved toxigenic culture, a process that took several days and required skilled personnel. Therefore, the much more rapid and simple enzyme immune assays designed to detect toxin A, and now also toxin B, the two major virulence factors of *C. difficile*, grew in popularity despite having lower sensitivity. Real-time PCR (RT-PCR) and isothermal loop-based (LAMP) technologies are now being utilised to detect *C. difficile* toxin genes in patient specimens as diagnostic tools. Three assay systems that use these technologies are the BD GeneOhm™ Cdiff assay (RT-PCR), the Meridian illumigene® *C. difficile* (LAMP) and the Cepheid Xpert® *C. difficile* (RT-PCR). While the manufacturers provide details of the analytical sensitivity of these systems, there is a lack of independent studies of this nature. In this study, the analytical sensitivities of these assays were determined and compared to culture on CCFA+T (cycloserine, cefoxitin, fructose agar containing 0.1% taurocholate) before and after broth enrichment. This was achieved by independently testing four human pooled faecal samples seeded with known 10-fold serially diluted concentrations of spores of *C. difficile* PCR-ribotypes 014, 027 and 078. All molecular assays were performed according to the manufacturers' instructions. Direct culture used 10 µl of specimen spread for isolated colonies on pre-reduced CCFA+T and incubated anaerobically for 48 h. Broth enrichment used 100 µl of specimen in Robertson's cooked meat broth containing gentamicin, cycloserine and cefoxitin, with ethanol shock performed at 7 d then subcultured onto CCFA+T.

Direct culture and broth enrichment enabled detection of 1.47×10^3 and 2.61×10^2 CFU/g, respectively. All molecular assays performed similarly to the manufacturers' specifications. The expected and observed sensitivities were (in order of decreasing sensitivity): GeneXpert, 6.32×10^3 and 1.08×10^3 CFU/g; GeneOhm, 6.67×10^4 and 6.81×10^3 CFU/g; illumigene, 5.1×10^5 and 2.61×10^4 CFU/g. The difference in sensitivity between studies was possibly due to difficulties commonly experienced in recovering all spores by culture when determining the inoculum concentration. The likely limiting factor affecting sensitivity for the molecular assays is the volume of specimen utilised, with direct and enrichment culture using the largest or least dilute amount of specimen. Considering that patients with CDI excrete an estimated $104\text{-}10^7$ *C. difficile* per gram of faeces, these molecular assays appear adequately sensitive. Ultimately it will be their accuracy in the diagnosis of CDI that will set them apart.

P88

COMPARISON OF SENSITIVITY OF ENZYME IMMUNOASSAY FOR TOXIN A/B AND DISTRIBUTION OF PCR RIBOTYPES IN SOUTH KOREA

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Enzyme immunoassays (EIAs) for toxin A/B are most commonly used for the laboratory diagnosis of *Clostrium difficile* infection due to their rapidity and ease of use, however, their sensitivity varies greatly (38-81.6%). The predominant PCR ribotypes of *C. difficile* vary according to the region or country studied and, recently, it was reported that the sensitivity of EIA was affected by strain type. The objective of this study was to compare the sensitivity of EIAs in relation to PCR ribotype in South Korea.

A total of 910 toxigenic *C. difficile* recovered from patients with diarrhea in tertiary teaching hospitals from 2006 to 2009, and 2011, were analyzed. The detection of toxin genes of *C. difficile* was determined by PCR and PCR ribotyping was performed by O'Neill's method. Toxin detection was performed by TOX A/B Quick check (TechLab) from 2006 to 2009 and VIDAS *Clostrium difficile* A & B assays (bioMérieux) in 2011.

The sensitivity of TOX A/B Quick check and VIDAS *Clostrium difficile* A & B assays for the frequent 5 ribotypes was as follow: 66.7% and 0% for ribotype AB1 (UK 001); 25.3% and 36.3% for ribotype AB2 (UK 014); 13.0% and 24.6% for ribotype AB3; 56.6% and 71.7% for ribotype AB17(UK 018) ; 48.6% and 54.3% for ribotype aB (UK 017), respectively.

The prevalence of the predominant 3 ribotypes (AB1, AB17, and aB) which have higher detection rate than other ribotypes by EIA changed in this study period: 41.2% in 2006, 44.8% in 2007, 33.75% in 2008, 25.4% in 2009, and 39.4% in 2011. These correlated strongly with the positive rate of EIA for toxigenic *C. difficile*: 47.7% in 2006, 40.0% in 2007, 38.7% in 2008, 29.4% in 2009, and 46.3% in 2011.

Although the overall sensitivity of the VIDAS *Clostrium difficile* A & B assays was higher than that of TOX A/B Quick check, the sensitivity of VIDAS *Clostrium difficile* A & B assays was significantly lower than that of the TOX A/B Quick check for detection of ribotype 001.

We concluded that the sensitivity of EIA for each country would vary depending on the distribution of common ribotype.

P89

NOVEL MULTIPLEX RT-PCR FOR THE DETECTION OF LINEAGE SPECIFIC *Clostrium difficile* STRAINS

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Clostrium difficile, an anaerobic gut pathogen, has rapidly emerged as a leading cause of antibiotic associated diarrheal disease in humans. Since 2002, the rate and severity of hospital acquired *C. difficile* infections (CDI) increased coincident with the emergence of two problematic PCR ribotypes (RT) 027 (NAP01) and 078 (NAP07/08). Rapid identification of problematic strains is essential for preventing the spread of these strains. Recently, our laboratory described two genetic markers that are present in the genomes of PCR RT027 and 078 strains and several other highly related PCR RT strains. Comparative genome analysis (Multi locus sequence typing on 7 housekeeping genes) confirmed that PCR ribotype strains sharing the same genetic marker belong to phylogenetically coherent lineages (i.e. lineage 2 and 5).

In the present study primers and probes were developed to target the genetic markers as well as the toxin B (tcdB) gene and the glutamate dehydrogenase (GDH) gene. All targets were amplified in one multiplex real time (RT) PCR reaction. The developed multiplex assay was validated on a large collection of clinical samples. In total, 526 diarrhea samples were prospectively collected and included in the study, of which 101 samples (19.2%) were positive in cytotoxigenic culture. The results were compared to the appropriate gold standard (toxigenic culture, culture and PCR ribotyping).

Compared to cytotoxigenic culture, sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) for the tcdB probe was 91%, 97%, 87%, and 98%. Compared to Culture, sensitivity, specificity, PPV, and NPV for the GDH probe was 83%, 97%, 87%, and 96%. Compared to PCR ribotyping (culture positive fecal samples only), sensitivity, specificity, PPV, and NPV for the 027 probe was 90%, 96%, 87% and 98% and for the 078 probe was 75%, 100%, 100% and 99%.

Our multiplex RT PCR assay is rapid and displays sufficient performance (sens, spec, PPV and NPV) to use it as a first screening test in an algorithm for diagnosing CDI. Simultaneously to diagnosing CDI, this multiplex assay is able to detect strains belonging to lineage 2 (PCR RT027) & lineage 5 (PCR RT078).

P90

DEVELOPMENT OF A RAPID POINT-OF-CARE TEST SYSTEM FOR THE DETECTION OF TOXIGENIC *Clostrium difficile* INFECTIONS

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Background: Most of the currently available diagnostic tools for the detection of *Clostrium difficile* infections (CDI) are either time consuming and labor intense or alarmingly insensitive. The sequential use of at least two different test systems, e.g. a GDH antigen assay followed by a toxin assay was proposed to overcome some of the individual disadvantages. Here we present a new point-of-care (POC) test system that is both, fast and sensitive.

Material and Methods: We have previously developed a generic platform for rapid multi-parameter quantitative molecular diagnostics at the POC. The platform combines a disposable microfluidic test chip with a compact device for chemiluminescence-based optical detection called GENSPEED® Reader. *Clostrium difficile*-specific probes were printed onto the test chip together with three controls. Prior to the analysis on the test chip stool samples were prepared using a newly developed pre-analytics protocol followed by rapid PCR amplification. PCR products were mixed with a hybridization buffer, denatured at 95 °C and applied onto the designated GENSPEED® Cdiff test chip. The system uses an enzyme complex and a chemiluminescence substrate, to detect specific DNA-binding. Signals were read in the GENSPEED® Reader and quantified by the GENSPEED® software.

Results: To obtain PCR-ready DNA from native stool samples we developed a simple, yet effective pre-analytics protocol that requires only 5 minutes hands-on time. PCR was optimized using a proprietary *C. difficile* master mix (Amplex Diagnostics). Test chips were designed to include three controls (PCR-control, hybridization control and negative control), to detect the toxin B gene and distinguish *C. difficile* ribotype 027. However, the test system may be extended to identify up to 8 analytes (including controls). Eighty native stool samples were analyzed and compared to the results obtained by bacterial culture and toxin assay. Our fast and simple pre-analytics method allowed efficient PCR amplification with less than 4 % PCR-inhibition. Forty-two samples were culture positive of which 38 appeared positive in a commercial toxin B assay. Twenty-seven samples (out of 38) were tested positive and 42 (out of 42) samples tested negative using our point-of-care test system, thus reaching a sensitivity of 71 % and a specificity of 100 %. With a total hands-on time of less than 15 minutes, results are obtained within 90 minutes.

P91

CHARACTERIZATION OF THREE NEW TOXINOTYPES OF *Clostrium difficile*

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Background: Toxinotyping is a PCR-based method for differentiating *Clostrium difficile* strains according to the changes in their toxin genes when compared to strain VPI 10463. As of July 2012, 31 toxinotypes are identified. We describe here 3 additional toxinotypes isolated from patients with *C. difficile* antibiotic associated diarrhea.

Methods: Five strains (CD10-055, CD10-165, RA09-070, SA10-35 and SA10-050) were sent to the national reference laboratory for *C. difficile* for characterization. They were isolated from 5 patients with *C. difficile* diarrhea. One patient (CD10-165) died from a severe form (toxic megacolon, septic shock, leukocytosis>50,000/mm3 and colectomy). Two strains (SA10-35 and SA10-050) were isolated from two patients hospitalized at the same period in the same ward. Identification was confirmed using MALDI-TOF mass spectrometer. The entire PaLoc was explored using PCR A1, A2, A3, B1, B2, B3, and tcdC. Toxinotyping was performed according to the method described by Rupnik et al. (J. Clin. Microbiol., 1998). Binary toxin genes were amplified by PCR. Toxin production was assessed using the cytotoxicity assay and the Vidas Toxin A/B assay (BioMérieux). Strains were characterized by PCR ribotyping and MLST (http://www.pasteur.fr/recherche/genopole/PF8/mlst/references_Cdifficile.html)

Results: All the strains were negative for binary toxin genes and for tcdC gene. CD10-055, CD10-165; SA10-35 and SA10-050 produced toxin B but not toxin A. Conversely RA09-070 produces only toxin A but not toxin B. The PCR ribotypes were different (except for SA10-35 and SA10-050 which gave a similar profile) and did not belong to the most frequent PCR ribotypes (001,002,003,005,014/20/77, 015, 017, 019, 023, 012/048, 027, 046, 053, 070, 075, 78, 0126, 87, 106). CD10-055 was negative for A1, A2, A3 and B3 and positive for B1 and B2. Digestion of B1 by AccI and HincII showed a new type. CD10-165, SA10-35 and SA10-050 were negative for A1, A2, A3 and B3 and positive for B1 (type 5) and B2. RA09-070 was negative for B1, B2, B3, A3 and positive for A1 and A2. Each strain exhibited a previously unknown Sequence Type (ST) by MLST: RA09-70 ST was very close to ST65. CD10-165 and SA10-35/50 were only different on AroE gene sequence (they were both negative for DutA).

Conclusion: These new toxinotypes have large deletions in the Paloc. For the first time, we describe a variant strain producing the toxin A but not the toxin B.

P92

EVALUATION OF COMMERCIAL KITS FOR EXTRACTION OF DNA AND RNA FROM *Clostrium difficile*

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Introduction: Commercial nucleic acid extraction kits are commonly used in the extraction of DNA and RNA, yet there has been no assessment of these kits for production of the required high purity, quality and DNA or RNA yield from *C. difficile*. The objectives of this research were to evaluate commercial RNA and DNA extraction kits in terms of cost, processing time, labour intensity, quantity, purity and quality of nucleic acid preparation, and the potential for downstream applications.

Methods: Four DNA (Maxwell® 16 SEV Tissue DNA kit using the Maxwell® 16 automated system, High Pure PCR Template Preparation Kit, MagNA Pure LC DNA Isolation Kit III (RMD) with the MagNA Pure LC 2.0 System, and the UltraClean® Microbial DNA Isolation Kit) and 3 RNA (RNeasy mini kit (QR), Maxwell® 16 LEV Cell RNA preparation kit , and the MagNA Pure LC RNA Isolation Kit (RMR)) were evaluated using *C. difficile* ribotype 027 and 078 spores and vegetative cells.

Results and Discussion: DNA yield ranged from 20.9 - 546 ng/ml, with A260/280 ratios of 1.92 - 2.11. There was no difference between spores and vegetative cells. The RMD yielded the highest concentration of DNA and samples among the highest purity. Electrophoretic visualization of the RMD DNA indicated substantial shearing in all samples, but a gluD gene fragment was amplified by qPCR with similar efficiency and specificity as the other kits, and a 3.1kb tcdA fragment was also generated, indicating some DNA degradation may not significantly impair all downstream processes. The other kits were all suitable for DNA extraction from both spores and vegetative cells. For RNA extraction, the QR kit yielded samples of the highest concentration (189 ng/ml) and highest RNA integrity number (RIN) (9.1). Differences in yield were also apparent through assessment of gluD expression, where the Ct was 3 cycles lower for QR vs RMR. Evaluation of the quality of the RMR samples using the Agilent Bioanalyzer was not possible due to the failure of the kit to remove small RNAs which were interpreted as degradation and scored a low RIN despite no visual evidence of degradation, indicating that this assessment method is inappropriate for RNA prepared by this system. RNA preparations from all kits required multiple DNAse treatments due to DNA contamination.

Conclusion: Good DNA and RNA yield are critical but methods are often overlooked. This study highlights the potential for critical variation between established commercial systems and the need for assessment of any extraction methods that are used.

P93

EVALUATION OF CULTURE MEDIA FOR *Clostrium difficile* ISOLATION FROM ENVIRONMENTAL SURFACES

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Background: *Clostrium difficile* is one of the major nosocomial pathogens. In some situations, it is valuable to investigate *C. difficile* strains in environmental sites of health-associated facilities to compare those recovered from patients with *C. difficile* infections. Especially, hospital environment in pediatric wards can be more frequently contaminated with *C. difficile*, because toilet support such as diapering is performed not by nurses but by the parents of infants. We evaluated two media, cycloserine-cefoxitin-mannitol media (CCMM) and reinforced clostridia media(RCM), for the recovery of *C. difficile* spores.

Materials and Methods: Both CCMM and RCM were prepared in semisolid media. After 48h-incubation at 35°C, *C. difficile* culture was heated for 30 minutes at 75°C to select spores. The each of a 100µl spore suspensions containing 10, 10^3, or 10^5 CFU/mL were used for the following experiments. The spore suspensions were put on sterile plastic plates and then dried out. After incubation for 1 minutes, 6 hours, 24 hours, 3 days, 7 days, 1 month, and 6 months at room temperature, the surfaces of the plastic plates were rubbed with moistened cotton swabs. Those swabs were then incubated anaerobically in CCMM and RCM for 5 days at 35°C. Colony counts were performed on cycloserine-cefoxitin-mannitol EX agar plates. The experiments were repeated five times.

Results and Discussion: *C. difficile* spores were able to be recovered from plastic surfaces by using CCMM even after 6 month-incubation. However, RCM yielded no growth of *C. difficile* spores in any tests. These results indicate that the use of CCMM may result in the excellent recovery of *C. difficile* from environmental sites such as the surface of plastic devices. In contrast, RCM had low ability to recover *C. difficile* spores in this study, although this medium is widely used for the recovery of clostridia. In conclusion, CCMM may be useful for the epidemiological study of *C. difficile* infection in children as well as elderlies.

P94

DEVELOPMENT, OPTIMIZATION AND QUALIFICATION OF A SENSITIVE HIGH THROUGHPUT CELL-BASED NEUTRALIZING ANTIBODY ASSAY FOR *Clostrium difficile* TOXIN A AND B

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Background: Toxin A (TcdA) and toxin B (TcdB) are the major virulence factors of *C. difficile* and are attractive targets for vaccine development. A robust, high throughput (HTP) neutralization (neut) assay for toxin A and B was required to measure the functional antibodies in both preclinical and clinical samples.

Methods: Anti-toxin neutralizing activity was measured by antibodies inhibition of toxin-mediated cytotoxicity. Briefly, a 96-well culture plate was seeded with human fibroblast cells (IMR-90). The following day, equal volumes of antiserum and toxin were mixed together. After incubation for 90 min, the mixture was transferred to the cell culture plate and incubated at 37°C for 72h. The monolayer was then treated with CellTiter-Glo® (Promega) reagent generating luminescent signals proportional to the amount of cellular ATP. The neutralization antibody concentrations in neut units/mL were determined from anti-toxin A and B Reference Standards.

Results: Traditional methods measure the level of cytotoxicity by observing the extent of cytopathic effect on the cell monolayer by microscopic evaluation and assigning an arbitrary score. Such methods are subjective, tedious and not amenable to automation. We used luciferase-based CellTiter -Glo® to determine the number of viable cells based on the measurement of cellular ATP. A multivariate Design of Experiment (DOE) was used to optimize assay conditions during development. Although TcdA and TcdB share partial sequence identity, the assay was shown to be specific to the type of toxin used. The assay demonstrated excellent accuracy and precision within the quantitative range of 21 – 176 and 40 – 1280 neut units/mL for TcdA and TcdB respectively.

Conclusions: A specific and reliable HTP neut assay was developed and qualified to measure anti-toxin A and B antibody responses. The assays were shown to be robust and to reproducibly measure functional anti-toxin antibody levels in serum samples from preclinical, epidemiological and clinical studies.

P95

RAPID DETECTION METHOD OF LIVE *Clostrium difficile*

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Clostrium difficile is one of the pathogens causing antibiotic-associated diarrhea and colitis. There are several methods to detect *C. difficile* or toxins in stool specimen in the laboratory, such as culture, enzyme immunoassay (EIA), and PCR. However, EIA and PCR can not distinguish between live and dead cells. Although the culture method detects only live cells, the turnaround time of testing is longer than that of EIA. We focused that RNA is transcribed in only live cells. To establish rapid detection method of live *C. difficile*, Reverse Transcription PCR (RT-PCR) was performed.

C. difficile strains KO20 (A+B+CDT-), TR15 (A-B+CDT-), and JND06-50 (A+B+CDT+) were used as the source for RNA preparation. RNA was extracted and purified by ISOGEN according to the manufacturer's instruction. A part of toxin A non-repeating sequence region was amplified by RT-PCR. RT-PCR which used RNA extracted from pure culture of *C. difficile* succeeded in these 3 strains. The detection limit was final concentration of RNA of 10 pg/ml in the reaction mixture. The spike test was also performed. The pure culture of *C. difficile* strain KO20 was added to the stool specimen which is *C. difficile* negative, then RNA was extracted and purified by ZR Soil/Fecal RNA MicroPrep from the mixture of stool and *C. difficile* culture. By RT-PCR followed by RNA extraction from the stool specimen, the correct size of amplicon was represented.

It was demonstrated that mRNA of toxin A of *C. difficile* could be detected by this method, even RNA was extracted from the stool specimen, directly. Whereas the culture method takes at least 16 hours to get the result, this method completed within several hours.

P96

EFFECTIVENESS OF C. DIFFICILE CHROME AGAR FOR RAPID DIAGNOSIS OF C. difficile INFECTION

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C. difficile infection is one of the major pathogens causing hospital associated infection. Recently, community associated *C. difficile* infection is also increasing in prevalence. Therefore, rapid diagnosis of *C. difficile* is important for infection control and treatment of C. difficle infection.

Traditionally, *C. difficile* culture has been used for diagnosis of *C. difficile* infection and epidemiologic study. Culture media using cycloserin cefoxitine fructose agar or *C. difficile* Selective Agar has been used for isolation of *C. difficile*. However, culture is time-consuming process and needs more than 2-5 days. *C. difficile* Chrome Agar (Bio-Merieux sa, France) is a agar medium for specific isolation of *C. difficile*.

We evaluated *C. difficile* Chrome Agar for rapid and specific diagnosis of *C. difficile*. A total of 326 diarrheic stools were simultaneously inoculated on *C. difficile* Chrome Agar (CA) and *C. difficile* Selective Agar (CDSA; Becton-Dickinson, US). We observed the growth of *C. difficile* colonies for 3 days and identified the colonies using Vitek Automated Identification Systems.

The final detection rates of CA and CDSA for *C. difficile* were 91.3% and 83.5%, respectively. The positive rates of growth on Chrome Agar were 56.5% in 1st day and 84.3% in 2nd day compared with those of CDSA, which were 15.7% in 1st day and 76.5% in 2nd day. Therefore, *C. difficile* Chrome Agar could be used for rapid diagnosis of *C. difficile* infection.

P97

THE DEVELOPMENT OF A RAPID-PHAGE BASED BIOSENSOR FOR THE IDENTIFICATION OF *Clostrium difficile*

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Background: *Clostrium difficile* is the most common cause of infectious diarrhoea in hospitals, responsible for 8,462 cases during 2011-2012 in the UK. The standard method of genotyping *C. difficile* is based on the size and number of the variable intergenic 16S-23S rRNA spacer regions of the bacteria. Some ribotypes, such as 027 and 078 are thought to be more 'virulent' in causing infection in patients. There is currently no specific diagnostic method available to identify *C. difficile* in healthcare settings and thus validate appropriate cleaning has occurred, which inevitability poses a risk of infection to new patients. Bacteriophages (phages), viruses, which bind very specifically to their target organism, could be exploited to produce a bacteriophage-based assay to detect the presence of *C. difficile* in the environment. They adsorb to the bacteria, use the bacterium's resources to propagate and before the subsequent release of new phages, lyse their host cells. These specific interactions have the potential to be exploited to produce a test.

Aims: This study aims to identify appropriate phages which could be used to develop a detection test and to determine the most effective measurable output. Specifically the ability of a test based on the initial adsorption of phages to *C. difficile* was investigated.

Methods: 23 *C. difficile*-specific phages were screened against 167 *C. difficile* isolates, comprising of 30 different ribotypes. The indication of a successful infection was the presence of clear or turbid plaques on *C. difficile* agar lawns. Adsorption kinetics of the phages was assessed by the number of free phage at specific time-points to 12 different *C. difficile* ribotypes.

Results: All 23 phages were able to infect at least one clinically relevant ribotype, including 027, 078 and 014/020. 4 phages were highly specific only infecting a few ribotypes, whilst 5 phages infected numerous ribotypes. The adsorption kinetics of 5 broad host range phages was studied; 3 phages bound by at least 10% to all ribotypes, even if they were unable to infect the particular ribotype. 2 phages were more specific and only bound if they infected the ribotype. The specificity of phage binding was assessed by repeating the adsorption experiment with E. coli, MRSA, and Pseudomonas aeruginosa; the phages bound by less than 5%. Ongoing work will establish if the differential binding is suitable for further development as a diagnostic.

Conclusions: Phages infecting numerous virulent ribotypes have been identified and will be used for the development of an assay. If phage adsorption is exploited to produce a test, the use of phage cocktails would help to increase specificity and will help identify virulent ribotypes. Developing an environmental test will aid in limiting and controlling the spread of *C. difficile* in healthcare settings.

P98

EVALUATION OF A CHROMOGENIC MEDIUM FOR THE ISOLATION OF *Clostrium difficile* FROM STOOL SAMPLES USING AN AUTOMATED INOCULATION SYSTEM

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Objective. Toxigenic culture remains one of the most sensitive diagnostic method for *Clostrium difficile* infection and is usually considered as the gold standard in diagnostic method evaluations. However, it is very slow as compared with other rapid tests like immunoassays or molecular biology. Here we evaluated the BD™Innova which is a new automated specimen processor and compared four different media including a chromogenic one allowing *C. difficile* isolation after only 24h incubation.

Methods. Three commercial media (Chrom ID (bioMérieux, Lyon,France), CCEY (Oxoid, Wesel,Ger.) and CDSA (BD, Sparks MD USA)) were evaluated against a home-made CCFA. Diarrheal stools were inoculated manually with 10µl loops on CCFA or were processed with a 30 µl loop on BD™Innova after dilution of about 50 µl in 2ml of Carry Blair medium (Copan,Brescia,It.). All media were incubated in anaerobic conditions at 35°C. Chrom ID plates were read after 24h incubations whereas all other plates were read after 2 night incubation. Identification was confirmed by Maldi-tof.

Results. Between Nov 2011 and Jan 2012, 290 routine stool samples were included in the study. By all means, *C. difficile* was isolated from 83 specimens (29%). CCFA manually inoculated had a much better sensitivity (80/83) than the same medium processed on BD™Innova after Carry Blair dilution (63/83). All positive specimens were recovered after 24h incubation on Chrom ID inoculated with BD™Innova whereas only 55/83 and 68/83 were recovered on CDSA and CCEY respectively. Black colonies on Chrom ID were not confirmed as *C. difficile* in 5 cases : three of them could easily be discarded by examination of the plate with a binocular and the other two were identified as C. hathewayi. On the other hand, in three cases, colonies of *C. difficile* were not coloured in black but nevertheless easily recognised by their typical appearance with a binocular. Conclusion. The new Chrom ID is an excellent medium for the detection of *C. difficile* in stool samples. Even after automated inoculation it demonstrated to be the most sensitive and allows a major reduction of the incubation period. However, our observations lead to the recommendation to examine plates with a binocular to recognise non coloured *C. difficile* colonies and black colonies that are not *C. difficile*. Another point of attention is that, after 24h incubation, the toxigenic status of the strain must be determined by a molecular biology method instead of an immunoassay.

P99

DETECTION OF GLUTAMATE DEHYDROGENASE IN STOOL SAMPLES: EVALUATION OF THE IMMUNOQUICK C. DIFFICILE GDH ASSAY (PRO-LAB)

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Background: The laboratory diagnosis of *Clostrium difficile* infection (CDI) is still difficult. Two- or three-step algorithms based on glutamate dehydrogenase (GDH) detection are now recommended by both American and European Guidelines for the diagnosis of CDI. The GDH is a constitutive enzyme produced by *C. difficile* strains and its detection in stools provides information about the presence of the bacterium. The objective of this study was to evaluate the performances of the IMMUNOQUICK *C. difficile* GDH (Pro-Lab) (also available in certain countries as Proflow GDH (Biosynex)).

Methods: This study was performed on all diarrheic stools from patients > 2 years suspected of having *C. difficile* infection and hospitalized in four different university-affiliated hospitals in Paris. The test was performed as described by the manufacturer's recommendations with a lecture at 10, 15 and 20 minutes. Culture was performed on TCCA medium (taurocholate, cycloserine, cefoxitin agar). Culture-positive but IMMUNOQUICK *C. difficile* GDH-negative stools were retested using stool aliquots stored at 4°C or -80°C. IMMUNOQUICK *C. difficile* GDH-positive but culture-negative stools were processed to enriched culture in a selective broth (containing taurocholate, cycloserine and cefoxitin) that was incubated in anaerobic atmosphere for 48 hours and 96 hours then plated on TCCA.

Results: Three hundred and four consecutive diarrheal stool samples were included between September 5th and December 5th 2011. The prevalence of positive cultures for *C. difficile* was 10.5% (71.9% toxigenic strains and 28.1% non-toxigenic strains). Compared to culture and after resolving discrepant results, sensitivity, specificity, positive and negative predictive values of the IMMUNOQUICK *C. difficile* GDH were 90.9% [CI95% 74.5-97.6], 97.4% [CI95% 94.5-98.9], 81.1% [CI95% 64.3-91.4] and 98.9% [CI95% 96.5-99.7] with a lecture at 15 min.

Conclusion: Negative predictive value of the IMMUNOQUICK *C. difficile* GDH is excellent and therefore this test represents a good screening method. However GDH is produced by both toxigenic and non-toxigenic strain and a positive result has to be confirmed by a second test for the diagnosis of CDI.

P100

EVALUATION OF VIDAS® *Clostrium difficile* TOXIN A&B ON COLONIES

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Background: Toxigenic culture (TC) is considered as a reference method for the diagnosis of *Clostrium difficile* infections (CDI). This method consists of isolating *C. difficile* on selective media and then assessing the in vitro production of toxins from colonies. However determination of the toxigenic status of a strain using immunoenzymatic assays (IEA) on colonies is not always validated. The objective of this study was to assess the performance of VIDAS *Clostrium difficile* Toxin A&B (bioMérieux) (VIDAS) directly on colonies from chromID® *C. difficile* medium (bioMérieux) (chromID).

Methods: During a study where selective media for *C. difficile* were compared, 36 strains were isolated on the 2 media tested: chromID *C. difficile* agar incubated anaerobically for 24 hours (chromID) and TCCA incubated anaerobically for 48 hours. The in vitro determination of the cytotoxicity of isolates was performed by inoculating 2 to 5 colonies isolated on TCCA into BHI that was incubated for 5 days under anaerobic conditions. The supernatant from this culture was filtered and inoculated on MRC-5 cells (TC). In parallel, the VIDAS *Clostrium difficile* Toxin A&B test was performed on colonies from chromID (from primary culture or subculture to obtain sufficient colonies). Colonies were homogenized with water to reach a 3 McFarland density. VIDAS tests were then performed according to the manufacturer's instructions. The VIDAS tests were repeated whenever results were equivocal or discordant (positive-TC and negative-VIDAS result or negative-TC and positive-VIDAS result).

Results: Among the 36 strains studied, 27 (75%) were positive and 9 (25%) negative by TC. The first VIDAS assay was performed on colonies from chromID primary culture in 29 cases (80.6%) and after subculture in 7 cases (19.4%). The sensitivity and specificity for VIDAS on colonies from chromID (1 equivocal result) compared to TC were 70.4% [49.7-85.5] and 87.5% [46.7-99.3], respectively. After resolving discordant results, the sensitivity and specificity for VIDAS on colonies from chromID (4 equivocal results) compared to TC were 83.3% [61.8-94.5] and 100% [59.8-100], respectively.

Conclusion: VIDAS *Clostrium difficile* Toxin A&B performed on colonies from chromID *C. difficile* agar (24h) is a rapid test when sufficient colonies are present on the medium. However, negative results have to be confirmed by a reference test.

P101

ISOLATION OF *Clostrium difficile* IN 24 HOURS WITH CHROMID® C. DIFFICILE AGAR, A NEW CHROMOGENIC MEDIUM

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Objective: Toxigenic culture is considered as a reference method for the diagnosis of *Clostrium difficile* infections (CDI). This method is sensitive but time-consuming and results are not available before 48 hours. Culture is also essential to obtain the strains for further testing, molecular typing and antibiotic susceptibility testing. chromID *C. difficile* agar (bioMérieux) is a new chromogenic medium for the isolation of *C. difficile* in 24 hours. The objective of the study was to evaluate the performance (in terms of sensitivity and selectivity) of the chromID *C. difficile* medium compared to two other selective media: TCCA (taurocholate, cycloserine, cefoxitine agar, home-made) and the CLO medium (cycloserine cefoxitine agar, bioMérieux)

Methods: 406 untreated diarrheic stools from patients suspected of CDI hospitalized in four different university-affiliated hospitals in Paris were collected. Suspensions of stools (50µl) were inoculated on the 3 media (TCCA, CLO, chromID *C. difficile*) according to a standardized procedure in order to perform a semi-quantification of the endogenous flora and *C. difficile* colonies. Plates were incubated anaerobically at 37°C. Cultures were read after 24 hours and 48 hours incubation for chromID *C. difficile* agar medium and after 48 hours for CLO and TCCA media. Identification of *C. difficile* was based on morphological criteria (black and irregular colonies on chromID *C. difficile* agar), Gram stain and RapID 32A strips (bioMérieux).

Results: From the 406 diarrheic stools, *C. difficile* was isolated from 54 samples (13.3%) combining the results of all the media. *C. difficile* was recovered from all the media in 29 cases (53.7%). Forty (74.1%), 47 (87%), 46 (85.2%) and 38 (70.4%) stools were positive on chromID *C. difficile* agar (24 h and 48 h), CLO and TCCA, respectively. Concerning the selectivity of these media, endogenous flora was absent in 67.7%, 30.8%, 16.5% and 3.9% on chromID *C. difficile* agar (24h and 48h), CLO and TCCA, respectively. Conversely, 3.9% of chromID *C. difficile* agar (24h), 15% of chromID *C. difficile* agar (48h), 15.3% of CLO and 59.1% of TCCA showed abundant endogenous flora.

Conclusion: chromID *C. difficile* agar allows isolation of *C. difficile* in 24 hours with good sensitivity and easy identification due to the colour of the colonies. Moreover, this new chromogenic culture medium appears to be highly selective.

P102

CONTAMINATION OF READY-TO-EAT RAW VEGETABLES WITH *Clostrium difficile*, FRANCE

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Background: Presence of *C. difficile* in food like shellfish, vegetables or meat have been reported in several publications during the past few years. Little data are available in France; a first study was conducted in France in 2007-2008 in ground meat indicated that 1.9% of ground beef was contaminated by *C. difficile* (Boutier et al, EID, 2010). Prevalence was low compared to that found in other countries. The objective of this study was to assess prevalence of contaminated ready-to-eat raw vegetables with *C. difficile*.

Methods: We evaluated 104 ready-to-eat salads and vegetables purchased from 5 urban and suburban Paris retail stores and supermarkets between September 2010 and March 2011. Twenty grams of each sample were crushed and cultured in 75 mL of prereduced BHI broth supplemented with cefoxitin, cycloserine and taurocholate. After 72h incubation in anaerobic condition, serial dilutions of the BHI broth were plated on TCCA (brain heart infusion [BHI] supplemented with 5% defibrinated horse blood, 0.1% taurocholate, cycloserine at 250 µg/ml, cefoxitin at 10 µg/ml). BHI broth was also plated on TCCA after alcohol shock. Suspected colonies were identified by conventional methods (Gram staining, odor, rapid ID32A galleries, bioMérieux) and *C. difficile* strains were characterized by PCR-ribotyping, PCR for tcdA, tcdB, tcdC, cdtA and cdtB. Susceptibilities to antibiotics were determined by the agar disk-diffusion method. The detection threshold of the enrichment method was established by spiking negative samples with known vegetative or spores of *C. difficile*.

Results: Toxigenic *C. difficile* strains were isolated in 3 samples (2.9%): 2 ready-to-eat salads (1 heart of lettuce and 1 lamb's lettuce salad) and 1 pea sprouts. Two strains belonged to PCR-ribotype 001 and 014/020/077. The PCR-ribotype of the last one was undetermined. The 3 strains were negative for binary toxin. They were all fully susceptible to metronidazole and vancomycin.The detection thresholds for vegetative cells and spores cells was 2 UFC/20 grams and 10 UFC/20 grams of salads, respectively.

Conclusion: *C. difficile* was found in ready-to-eat raw vegetables in France even if prevalence observed is lower than reported in a previous study by Bakri et al. (EID, 2009) (7.5%). Interestingly, 2 of the PCR-ribotypes isolated in this study (001 and 014/020/077) are the major PCR-ribotypes responsible for *C. difficile* infection in humans. More studies are necessary to investigate the potential role of food in *C. difficile* infection or colonization in humans.

P103

Clostrium difficile IN GOATS AND SHEEP IN SLOVENIA

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Clostrium difficile appears to be an important cause of enteric disease in different animal species, although high percentage of asymptomatic carriage is reported. To the best of our knowledge, the presence of *C. difficile* in sheep was mentioned only in a single study. The aim of this work was to screen sheep and goats (young and adult animals) for *C. difficile* on eleven different farms in Slovenia.

Faecal samples or rectal swabs from adult goats (n=10), 3 to 14 days old (n=32), 15 days to one month old (n=21), and one to four months old (n=66) goats were analyzed for the presence of *C. difficile*. Additionally, 85 sheep samples were tested (27 from adult sheep, 40 from 1-16 days old lams and 18 from 1-3 months old lambs). No goat and 24.7 % sheep had diarrhea. The samples were inoculated in cycloserine-cefoxitin fructose enrichment broth supplemented with 0.1% sodium taurocholate. The isolates were identified based on morphological criteria and the identification was confirmed by multiplex PCR targeting tpi, tcdA, and tcdB. The isolates were characterized by toxinotyping and PCR ribotyping.

All samples from adult animals were negative for *C. difficile*. *C. difficile* was isolated from two lambs (1-16 days old; 5 %), six goats (3-14 days old; 18.8 %) and two different strains from one goat (four months old; 1.5 %). All positive animals were asymptomatic. Strains belonged to four different toxinotypes, XII and XIa (isolates from sheep) and 0 and V (isolates from goat). One isolate from goat was non-toxigenic. Six different PCR ribotypes were identified of which four corresponded to the reference type strains of ribotypes 056, 045, 010 and 014/020. Two types were designated with internal nomenclature SLO 061 and SLO 151.

The results of the present study indicate that domestic small ruminants may also be the reservoir of *C. difficile* and are colonized with similar ribotypes as other animals or humans. PCR ribotype 014/020 was also isolated from poultry, calves, dog and the environment in Slovenia, while PCR ribotype 045 is prevalent on pig farms in central Slovenia. Furthermore, PCR ribotypes 045, 010, 014/020 and 056 were also isolated from humans in Slovenia. We isolated *C. difficile* from very young goats and sheep, which is in accordance with previously published studies of other animal species.

P104

DETECTION OF *Clostrium difficile* IN ANIMALS: COMPARISON OF LIGHTCYCLER AND TAQ-MAN REAL-TIME PCR WITH THE CULTURE METHOD

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Clostrium difficile-associated disease or asymptomatic carriage has been described for numerous animal species. Variant *C. difficile* strains with binary toxin CDT are often isolated from animals and it seems to be associated with community-acquired *C. difficile* infections in humans. A rapid, simple, sensitive method, capable of detection variant strains, is required for laboratory detection of *C. difficile* in animals. In this study, we compared a TaqMan- based real-time PCR (TM) (Avbersek et al., 2011) to a LightCycler real-time PCR (LC), both targeting genes for toxins A, B, and binary toxin. Additionally, both rtPCR assays were compared with the enrichment culture method (Avbersek et al., 2009). Used rtPCR assays are based on different chemistries; hydrolysis vs. hybridization detection probes.

DNA was extracted from rectal swabs from piglets and faecal samples from calves and foals (n=340) by using QIAamp DNA Stool Mini Kit (Qiagen, Germany). We developed LC targeting tcdA, tcdB, and cdtB genes by using LightCycler instrument. The TM test was performed on an ABI Prism 7000 instrument.

Culture identified 114/340 (33.5 %) samples as positive for *C. difficile*. With TM 256 (75.3 %) samples and with LC 255 (75 %) samples were in complete concordance with culture. Furthermore, TM/LC was negative in 40/39 culture positive samples and 40/38 samples were culture negative and TM/LC positive. PCR inhibition was observed in 2.2 % culture negative - LC negative samples, while with TM inhibition was not observed. Results of TM/LC for 4 and 8 culture-positive samples were not in complete concordance with A+B+CDT+ type defined for *C. difficile* isolates. Concordance between the TM and LC rtPCR was 97.6 %.

Culture-positive/rtPCR negative results could be connected with the low number of *C. difficile* cells in faecal samples or with DNA extraction failure from spores. Furthermore, culture-negative/rtPCR positive results were not regarded as false positive as every sample was tested three times (detection of three genes) with both rtPCR, expect two samples with different results with LC and TM assay. In these cases, contamination could be the reason of such results. Theoretically, LC should be more specific assay, while using two specific fluorescent probes instead of one probe used in TM assay but our results showed that both assays gave almost the same results. We can conclude that both assays could be used for screening method but further testing of DNA extraction methods is required to reduce number of samples with false-negative rtPCR result.

P105

IS *Clostrium difficile* PREVALENCE IN ANIMALS UNDERESTIMATED?

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Clostrium difficile (CD) has been isolated from a wide variety of domestic animals, showing a high prevalence in some farm species such as swine. Nevertheless, reported prevalence rates considerably differ depending on the study, the animal population surveyed and other considerations. Furthermore, it has been suggested that methods for CD recovery from fecal samples might account for some variation among the reported prevalence rates, but this issue remains to be studied in detail. In this work we assessed the performance of two different methodologies for CD recovery from swine fecal samples.

Method #1: Fecal samples were collected with the help of cotton-tipped swabs at two different swine populations characterised by a high and a low prevalence of CD: HP (1-7-day-piglets) and LP (2-3-month-piglets) populations, respectively. 18 swab samples were taken at each population. Swabs were submerged in 1 ml of 70% (v/v) ethanol for 30 s, and then the tubes were closed and left for 20 min at room temperature. Ten 75 µl-aliquots were consecutively taken from each tube and independently plated on 10 plates of solid medium containing cycloserine and cefoxitin as selective agents (CLO agar, bioMérieux). CD was recovered from a total of 16 samples (11 from the HP and 5 from the LP populations). However, if just the first of the ten cultured plates for each sample is taken into account (which is the usual procedure in most microbiology laboratories), the number positive cultures decreased to 8 (7 and 1, respectively). Therefore, the use of 10 plates per sample instead of just one, allowed us to detect a 22% of false negative samples.

Method #2: 20 swab samples from each of the HP and LP populations were cultured in parallel in solid medium (1 plate/sample) after ethanol shock and in liquid selective broth for enrichment of CD (also containing cycloserine and cefoxitin). After 8 days of incubation (37ºC, anaerobiosis), 2 ml of the liquid culture were mixed with 2 ml of absolute ethanol and left 1 h at room temperature for spore selection. Finally, tubes were centrifuged, the supernatants were discarded and cell precipitates were taken with a swab and spread on the same solid medium as above. In this case, only 4 samples, all of them from the HP population, yielded CD on the selective solid medium, while 19 samples resulted positive after enrichment in selective broth (13 from the HP and 6 from the LP population). Therefore, enrichment in selective broth considerably increased CD recovery in samples from the two surveyed populations, and allowed us to detect a 37.5% of false negative samples.

In conclusion, it seems that the procedure used for CD recovery from fecal samples can have important consequences on CD recovery. Our suggestion is that prevalence studies should rely on enrichment in selective broth before plating on solid medium, so as to increase the number of viable CD cells/spores and minimise the effect of possible false negative results.

P106

ZERO PREVALENCE OF *Clostrium difficile* IN CANARY BIRDS UNDER ANTIBIOTIC TREATMENT

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Clostrium difficile has been isolated from a wide variety of companion and farm animals. In birds, CD has been detected in poultry and wild passerine birds. For instance, in a previous prevalence study carried out in Slovenia, a 100% of fecal samples from 2-week-old chickens were culture positive for CD, and the colonization rate decreased with the age of the animals [1]. On the contrary, all 465 fecal samples from 6 different species of wild passerine birds were negative for the presence of CD [2], which represents the first report of a zero prevalence of this bacterium in an animal population. Due to the relatively high prevalence of CD in human populations, domestic animals and the environments surrounding both of them, CD is expected to be recovered also from pet birds. The aim of this work was to test this latter hypothesis using as a model a population of domestic canary birds.

The studied population was reared in a single cage composed by several enclosures harbouring a total of 28 pairs. Early in their life (from hatching to day 10) animals were fed semi-solid food formula containing ketoconazole (estimated ingested dose = 20 mg/kg/sid) and cefaclor (50 mg/kg/sid) for antifungal and antibacterial prophylaxis, respectively. A total of 47 samples were taken from day 1 to day 14 posthatching from 4 individual enclosures. Feces were collected from nests with the help of a sterile cotton-tipped swab. To increase the probability of CD recovery, fecal samples were inoculated in liquid medium and incubated in anaerobic conditions at 37 ºC for 8 days prior to ethanol shock and subsequent plating on solid selective medium (CLO agar, bioMérieux).

In spite of the culturing procedure used for the enrichment of anaerobic bacteria, CD was not recovered from any fecal sample. Therefore, here we report another avian population showing a zero prevalence of CD. Remarkably, and in clear contrast to the wild avian population studied by Bandelj et al. [2], the canary population surveyed in this study was under intense antibiotic treatment, thus potentially rendering it more susceptible to colonisation and/or infection by opportunistic microorganisms such as CD.

References:
[1] Zidaric et al. 2008. Anaerobe, 14: 325-327.
[2] Bandelj et al. 2011. FEMS Microbiology Letters, 321: 183-185.

This study was funded by grant AGL2010-18493, from the Spanish Ministry of Science and Innovation.

P107

ISOLATION OF *Clostrium difficile* FROM PUPPIES : A LONGITUDINAL STUDY

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Although a relationship between *Clostrium difficile* (CD) and development of canine diarrhea has been suggested in several case reports, most authors agree that, on the basis of current data, a causative link between CD and symptomatic diarrhea has not been established. This could be due, at least in part, to disparate results obtained in studies on the rate of the asymptomatic carriership status of dogs for CD, which, depending on the studied population, have reported prevalence rates even ≥50%. Additionally, longitudinal studies of CD presence in dogs are still lacking. The aim of this study was to assess the incidence of CD in puppies from the same litter at different ages and the length of CD shedding in feces.

Nine Black Labrador Retriever puppies (5 males and 4 females) from the same litter were sampled weekly from parturition to day 50 postpartum, resulting in a total of 63 fecal samples. All samples were plated on solid selective medium (CLO agar, bioMérieux) after ethanol shock (70%, v/v). Recovered isolates were ribotyped and further characterised by standard procedures. Of 63 samples, 15 (24%) yielded CD growth. In general, CD was recovered from samples up to day 29 postpartum, but differences on bacterial recovery were detected between age groups. CD incidence was 11.1% (1/9) in 7-day-animals, but 88.9% (8/9) at day 14. Fecal samples from one single animal never yielded CD colonies, as neither did those from ≥30-day-old puppies. All CD isolates belonged to PCR ribotype 056 and harboured the genes encoding for toxins A and B, but no binary toxin loci were detected. In addition, all isolates showed in vitro resistance to daptomycin (MIC_{50} = 2 µg/ml, MIC_{90} = 2.6 µg/ml), but were sensitive to linezolid (MIC_{90} = 1.5 µg/ml) , metronidazole (MIC_{90} = 0.38 µg/ml), moxifloxacin (MIC_{90} = 1.5 µg/ml) , rifampicin (MIC_{90} ≤ 0.002 µg/ml), teicoplanin (MIC_{90} = 0.226 µg/ml), tigecycline (MIC_{90} = 0.064 µg/ml) and vancomycin (MIC_{90} = 1 µg/ml).

We conclude that puppies carry asymptomatically toxinogenic *C. difficile* PCR ribotype 056 up to 30 days postpartum. Interestingly, CD ribotype 056, which has also been isolated from other animal species and environmental sources, has been implicated in severe cases of human CD-associated disease [1].

References:
[1] Bauer et al. 2011. Lancet, 377: 63-73.
This study was funded by grant AGL2010-18493, from the Spanish Ministry of Science and Innovation.

P108

HIGH RECOVERY RATE OF *Clostrium difficile* PCR RIBOTYPE 078 IN IBERIAN FREE-RANGE PIGS

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Iberian pigs are typically bred in free-range systems. Due to their outstanding culinary characteristics, meat products from Iberian pigs reared under 'Montanera' (a free-range system typical of SW Spain in which the animals' diet is based on acorns and grass) are highly appreciated by consumers, and usually reach higher market prices than those obtained from pigs fed with mixed diets. During the peripartum period, Iberian sows are kept in closed facilities under intensive system. Piglets are weaned at day 28 postpartum, and then kept outdoors.

In a previous study in intensively raised White pigs in Spain, we detected a 25.9% of prevalence of *Clostrium difficile* (CD) in fecal samples from newborn piglets (1-7 days old), but a total absence of this bacterium in samples from 1-2-month-old animals [1]. In that study, fecal samples were directly plated on solid selective medium (CLO agar, bioMérieux) after ethanol shock (70%, v/v).
The aim of this study was to determine the prevalence of CD in Iberian free-range pigs from various ages in Spain. A total of 175 fecal samples were analysed for CD presence using a more sensitive methodology than in the aforementioned study, which includes a pre-incubation step of fecal samples in liquid medium for 8 days for CD enrichment prior to ethanol shock and subsequent plating on solid selective medium. Sampling was as follows: i) 4 piglets from each of 5 different litters were sampled weekly during 57 days, giving a total of 160 samples; ii) additionally, 15 fecal samples were taken from the corresponding sows (3 from each of 5) at different times throughout the experiment.

CD was recovered from a 25% of piglet samples (40/160), being isolated up to day 50. However, the majority of these positive samples (75%, 30/40) came from ≤15-day animals. No CD colonies were recovered from sow samples. Six CD isolates were ribotyped and further characterised. The presence of toxin-encoding genes was assessed by PCR and antimicrobial susceptibilities were determined by the Etest (bioMérieux). All these 6 isolates belonged to the emerging PCR ribotype 078, harboured the genes encoding for A, B and binary toxins, and showed in vitro susceptibility to the following antibiotics: linezolid, metronidazole, moxifloxacin, rifampicin, teicoplanin, tigecycline and vancomycin. Only one of these isolates (16.7%) was resistant to daptomycin (MIC = 2 µg/ml), which contrasts with the relatively high prevalence of daptomycin resistance found among CD isolates recovered from Spanish White pigs (>30%, Peláez et al., unpubl. results). In conclusion, our results demonstrate that Iberian free-range piglets can be a reservoir of emerging

CD PCR ribotype 078 trains.

References:
[1] Álvarez-Pérez et al. 2009. Veterinary Microbiology, 137: 302-305.
This study was funded by grant AGL2010-18493, from the Spanish Ministry of Science and Innovation.

P109

Clostrium difficile ISOLATED FROM RABBITS IN ITALIAN COMMERCIAL FARMS

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Enteropathy due to *Clostrium difficile* has been documented in rabbits but limited data were available on the role of rabbit as carrier of pathogenic strains.

Objectives. This study aimed to investigate the presence and diversity of *Clostrium difficile* in commercial farmed rabbits in Italy.

Methods. 1179 rabbit's caecal contents were collected in 301 different farms in Italy between 2008-2011. Each sample was pre-enriched in Taurocolate Cefoxetine Cycloserine Fructose (TCCF) broth and the isolation was subsequently performed using both Columbia Agar Base added with aesculin and horse red blood cells and TCCF agar. Identification was based on morphological criteria and confirmed by PCR amplification of *C. difficile* species-specific fragment of tpi gene. All isolates were characterized by PCR amplification of tcdA, tcdB, cdtA and cdtB genes and typed by PCR ribotyping (RT) and toxinotyping (TT). tcdC gene variations were also investigated.

Results. *C. difficile* was recovered from 39/1179 animals (3.3%). Twenty-two strains (56.4%) were tcdA+/tcdB+/cdtA-/cdtB-, 4 (10.2 %) tcdA+/tcdB+/cdtA+/cdtB+ and 13 (33.4%) were non-toxinogenic. Fifteen different ribotypes were identified with RT-014/020,TT 0 (10/39, 25.6%), RT-002,TT 0 (3/39, 7.7%), RT-078, TT V (2/39, 5.1%), RT-012, TT 0 and RT-126, TT V (2/39, 5.1%) being the most frequently isolated toxinogenic strains. Among non-toxinogenic strains RT-010 has been identified but the most prevalent ribotype was the arbitrary named TV21 that didn't match with any of the profiles obtained from the strains used as reference.

Conclusions. This study represents the first report on *Clostrium difficile* ribotypes in rabbits. Our results also show that:

- *Clostrium difficile* can be isolated in a small percentage of rabbits
- All toxinogenic strains isolated in rabbits belong to ribotypes that are frequently detected also in human both in Europe and in Italy.

To date, direct transmission from animal to human has not been proved, however, the presence in rabbits of some of the most frequent types isolated in human cases of CDI (eg RT-014/020) suggests that rabbits could serve as potential carrier of pathogenic strains.

P110

MOLECULAR CHARACTERIZATION OF *Clostrium difficile* ISOLATED FROM PIGS AND HUMANS IN NORTH-EASTERN ITALY

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Objectives. This study aimed to investigate the spread of *Clostrium difficile* (CD) among pigs in Italy and to perform molecular characterization of CD of swine and human origin isolated in the same geographical area (North-East of Italy) in order to investigate the circulation of genetically correlated strains.

Methods. 366 swine faeces collected in 70 different piggeries and 119 human CD positive stool samples collected in three hospitals were analyzed for the presence of CD. Each sample was cultured in a selective medium for CD and the isolates were identified both by a commercial biochemical kit and by a species-specific PCR. Strains were characterized by tcdA, tcdB and binary toxin genes detection and by PCR-ribotyping and toxinotyping. tcdC gene deletions, ermB and tetM genes presence and mutations of the gyr genes were also investigated.

Results. CD was recovered in the 22.1% (81/366) of swine samples. The highest prevalence was detected in suckling piglets (43%) whereas only the 4.1% of finishing pigs was CD positive. All strains resulted toxinogenic with 42.5% positive also for binary toxin. Ten different PCR-ribotypes (RT) were identified, as well as different toxinotypes (TT), of these RT-150,TT 0 (46.6%, 9/70 piggeries), RT-078,T V (26%, 12/70 piggeries) and RT-014/020, TT 0 (4.1%, 3/70 piggeries) were the most frequently isolated. RT-127, TT VI was also isolated in 15.5% of the samples but it was found only in one pig farm in Treviso province. ermB and tetM genes were detected in the 5.5% and 41.1% of the strains respectively and the 23.3% of all isolates carried also the amino acid substitution Thr82 ⟶ Ile in GyrA. Among the 119 human isolates 15 different ribotypes were identified and RT-018, TT 0 (53.2%), RT-014/020, TT 0 (20%), RT-001, TT 0 (7.3%) and RT-126, TT V (6.4%) resulted the most prevalent. ermB and tetM genes were detected in 11.9% and 8.5% of the isolates. Of all strains, 73.4% carried the amino acid substitution Thr82 ⟶ Ile in GyrA and 0.9% the amino acid substitution Asp426 ⟶ Val in GyrB

Conclusions. Our results demonstrate that:

- CD is widespread in Italian piggeries and as, previously reported in other studies, the percentage of positivity decreases in a age-depending manner
- As expected, RT-078 was isolated in high percentage also in Italian pigs but in contrast with other studies the most prevalent ribotype in Italy seems to be the RT-150.

- tetM gene is widespread in swine strains and most of positive strains belong to RT-078.
- 5/10 CD ribotypes isolated from swine has been isolated also from humans
- RT-018, TT 0 is confirmed as the most prevalent CD type in human in Italy but it was not detected in pigs.
- A high percentage of human isolates shows an amino acid substitution in GyrA or GyrB and most of these strains belong to RT-18.

P111

MATCHING *Clostrium difficile* ISOLATES IN PIGLETS AND HUMANS IN OSTROBOTHNIA AREA IN FINLAND

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Objective

Clostrium difficile (CD) is an important nosocomial and emerging animal pathogen. The aim of this study was to get the first view of CD ribotype diversity of piglet isolates in Finland and to compare it in piglets with neonatal enteritis and diarrheic patients in the same geographical region during year 2010.

Materials & methods

The studied 22 pig isolates originate from 8 different farms (3 isolates/farm, range 1-6) in Ostrobothnia area, (from small intestine/ large intestine/ appendix/ feces). The 30 patient isolates originate from patients in Southern Ostrobothnia Hospital District. All strains were ribotyped.

Results & conclusions

Among the 30 patient isolates we found 12 different ribotypes (001, 002, 005, 011, 014, 018, 020, 023, 056, 070, 078, and Finnish type Un22) with types 002, 014 and 001 being most common.
Among the 22 pig isolates we found 4 different ribotypes (002, 011, 012, 135). Types 002 and 011 predominated and were found from 4 and 3 farms out of 8, and covered 55 % and 18 % of pig isolates, respectively. Within each farm, isolates were of the same ribotype except for one farm with two ribotypes (011, 135). We found two shared ribotypes among human and pig strains; ribotype 002 (12 pig and 7 patient isolates) and 011 (4 pig, 3 patient isolates). These isolates will be further typed with PFGE.

Pig isolates were less diverse than human isolates and tended to be farm specific. Certain ribotypes prevailed in the area as ribotype 002 was most common among both human and pig isolates. Ribotype 078, often reported in piglets elsewhere, was not found in pigs in this study, instead ribotypes 002 and 011 prevailed.

P112

ISOLATION OF PCR RIBOTYPE 078 *Clostrium difficile* FROM FIVE CASES OF FULMINANT COLITIS IN THOROUGHBRED RACEHORSES

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Five Thoroughbred horses presented with diarrhea or colic accompanied by leucopenia (≤ 4000 cells/μl) and a marked increase in packed cell volume (≥60%) within 2 to 4 days after surgery in a horse clinic. Four of the five horses died or were euthanized because of the severity of the illness. In these four horses, hemorrhagic and necrotizing enterotyphlocolitis was revealed by postmortem examination and *C. difficile* was recovered from the contents of the small or large intestine, or both. The remaining horse was euthanized because of marked deterioration in general condition and the development of a lung abscess, from which *C. difficile* was isolated. The horse had severe postoperative diarrhea before onset of the respiratory disorder; fecal laboratory tests for *C. difficile* were not performed. All *C. difficile* isolates were characterized as PCR ribotype 078 and were identical by both pulsed-field gel electrophoresis analysis and slpA sequence typing. This battery of cases may have been healthcare associated, although the intervals between the onset of each case were about 4 months.

P113

ANTIMICROBIAL SUSCEPTIBILITY TESTING OF ANIMAL AND HUMAN ISOLATES OF *Clostrium difficile* BY BROTH MICRODILUTION

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The purpose was to determine the MICs of 30 antimicrobials for human and animal isolates of *Clostrium difficile*. We chose 74 *C. difficile* isolates, which included 26 human isolates and 48 animal isolates from poultry (n=27), pigs (n=17), small ruminants (n=3), and a calf (n=1). The method used was broth microdilution on commercially available plates for monitoring resistance of anaerobic and Gram-positive bacteria (Trek Diagnostic Systems, Ltd., UK).

Low MIC_{90} values were found for metronidazole (1 mg/L), vancomycin (0.5 mg/L), tigecycline (0.06 mg/L), linezolid (2 mg/L), quinupristin/dalfopristin (1mg/L) and rifampicin (≤0.5 mg/L). However, one human isolate (PCR ribotype 078) and two isolates from piglets (045) had metronidazole MIC values of 2 mg/L, which is at the recently published EUCAST breakpoint. For rifampicin, two human isolates, both of ribotype 017, displayed MIC >4 mg/L. One of them also showed decreased susceptibility to chloramphenicol (16 mg/L), clindamycin (>8 µg/ml), tetracycline (16 µg/ml) and moxifloxacin (>4 µg/ml). MICs of daptomycin were above EUCAST breakpoint (>4 µg/mL) for two human isolates. These belong to ribotype 078 and 126, respectively. There were also animal isolates with decreased susceptibility to multiple antibiotics. These belong to ribotype 046 (poultry isolate) and ribotype 150 (toxinotype 0, piglet isolates). One goat isolate of ribotype 010 (non-toxigenic) displayed increased MIC values for erythromycin (>4 mg/L) and clindamycin (>8 mg/L).

MICs of clindamycin were distributed within whole tested range (≤0.25- >8 mg/L), but 66.7 % of animal isolates and 34.6 % of human isolates had MIC ≥8 µg/mL. Both human and animals strains had a wide range of susceptibility to erythromycin (≤0.25->4 mg/L, MIC_{50} 0 mg/L, MIC_{90} >4 mg/L) and to tetracycline, which showed bimodal distribution. For tetracycline, 27 % of human strains (among them all of three tested ribotypes 078) and 20.8 % of animal strains had MIC ≥8 mg/L. For penicillins and combinations, MIC90s were calculated for penicillin (4 mg/L), piperacillin (8 mg/L), piperacillin/tazobactam (8/4 mg/L), mezlocillin (8 mg/L), ampicillin (2 mg/L), ampicillin/sulbactam (1/0.5 mg/L), oxacillin (>4 mg/L) and amoxicillin/clavulanic acid (1/0.5 mg/L). MIC_{90}s for imipenem and meropenem were >8 and 2 µg/ml, respectively. MICs of cefoxitin were >32 mg/L for all isolates and MIC_{90} of cefotetan was 16 mg/L.

P114

Clostrium difficile IN POULTRY PRODUCTION: DETECTION AND CHARACTERISATION

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This study aimed to evaluate the presence and characteristics of *Clostrium difficile* in poultry production. A total of 678 faecal samples from broiler (n=501) and laying hen poultry farms (n=177) were collected from 7 different locations.

Given the expectation of age-related variation, the flocks were screened through the production cycle. Samples were examined by enrichment culture. Overall, we found low colonization rate of *C. difficile* in breeder flocks: 4.7 % in broiler breeder flocks (4/85) and 0 % in laying breeder flocks (n=60). Their progeny was sampled at different age and in one-day and three-day chickens colonization was from 0-13.3 % (n=90). On day 6-8 colonization rates increased on two farms, where broilers were positive in 85 % (17/20) and young laying hens in 70 % (20/31), respectively. At the age around 14 days the colonization rates on these farms were still as high as 70 % (14/20) in broilers and 70.4 % (19/27) in laying hens. Our results show significant difference between farms at these age groups, as the colonization rate ranged from 0–85 %. *Clostrium difficile* prevalence declined with the age and shortly before the time of slaughter or starting laying eggs, the colonization was low, ranging from 0-6.7 %. Additionally, 20 litter and water samples were examined from the environment (2 positive) and 46 samples from the slaughter house (1 positive). Isolates were typed by PCR ribotyping and toxinotypes were determined. The diversity of ribotypes was high, which is consistent with the previous study from our geographic region. 22 different PCR ribotypes were identified that were non-toxigenic or belonged to toxinotypes 0, V, XII and XXIV. Ribotype 014/020 was found the most prevalent strain and the majority of types are overlapping with isolates from human and from other animal species in our region.

P115

SURVEILLANCE OF *Clostrium difficile* IN RETAIL CHICKEN: IT'S ANTIMICROBIAL AND MOLECULAR CHARACTERIZATION

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Clostrium difficile is a Gram positive, spore forming, strictly anaerobic bacillus and is considered to be a potent nosocomial agent in both the developed and developing countries. *C. difficile* infection (CDI) has been associated with antibiotic exposure which leads to severity of disease ranging from mild diarrhea to pseudomembraneous colitis.In past few years, a flow of literature reporting prevalence of *C. difficile* in food animals has alarmed a situation where CDI is considered more than an unpleasent complication following antibiotic exposure. These reports have also hypothesized foodborne transmission as a threat following community associated infection. Although reports claiming overlapping of animal and human isolates are less but the seriousness of animal contamination cannot be overlooked.

The present work was aimed to study the prevalence of *C. difficile* in retail chicken collected from butcheries of Central India. The collected isolates were futhur characterized according to their antimicrobial resistance and molecular arrangement. Total 254 samples were collected and processed on CDMN agar plates in anaerobic workstation following selective enrichment of spores. Initially isolates were picked and cultured on their morphological similarity to *C. difficile*. These isolates were subjected to a battery of biochemical tests. Suspected isolates were then subjected to 16s rRNA sequencing and sets of multiplex PCR. These PCR were designed for the identification of the organism and detection of toxin genes. *C. difficile* positive isolates were tested against antibiotics by disk diffusion method and detection of antimicrobial resistant gene cassette, thus isolates were characterised for their antibiotic resistance both phenotypically and genotypetypically. The presence of multiple genotypes was studied by subjecting isolates to PGFE. The evolutionary distances between the isolates were computed to generate a dendogram showing the variation among the isolates.

The study results suggested out of 254 collected samples 9 were culture positive for *C. difficile* marking Indian prevalence of 3.45%. Biochemical test showed similarity of the isolates with standard *C. difficile*. The toxinotyping revealed that 86.5% of isolates were toxin AB positive while 13.4% were found to be non toxigenic. AMR studies of these isolates showed very interesting pattern of resistance. The Especially in the case of metronidazole, 83% of the isolates were resistant to metronidazole. Apart from that most of the isolates were resistant to fluroquinolones, quinolones and sulfonamides. 40-70% of the isolates were resistant to macrolides and lincosamides. PFGE finger print analysis of isolates showed variations and when these fingerprints were compared with clinical isolates showed similarity in band pattern.

P116

Clostrium difficile IN INTESTINAL CONTENTS AND ON CARCASSES OF PIGS AND CATTLE AT SLAUGHTERHOUSE IN BELGIUM.

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Clostrium difficile is a major cause of nosocomial acquired diarrhoea and colitis after use of antibiotics. With the recent isolation of this bacterium in healthy carrier food animals and retail meats, the possibility for foodborne transmission is a current concern. The objective of this study was to determine the presence of *Clostrium difficile* in intestinal samples and on carcasses of pigs and cattle at slaughterhouse in Belgium and identify the main PCR-ribotypes. Intestinal contents from pigs (100) and cattle (100) were collected from the slaughter line, directly from the large intestine in the viscera processing area. Carcass swabs (100 from pigs and 100 from cattle) were collected post slaughter, after entry into the chilling room. Surface swab samples of 1600cm2 on cattle and 600cm2 on pig carcasses were collected. Culture was carried out using an enrichment step. Briefly, one gram of feces was inoculated into 9 ml of cycloserine cefoxitin fructose taurocholate enrichment broth (CCFBT) freshly prepared in the laboratory and incubated anaerobically for 72h at 37°C. As for the carcass samples, the four cottons used for swabbing each individual carcass were homogenized with 40 ml of CCFBT and incubated anaerobically for 72h at 37°C. Subsequently, 10µl of the enrichment broth of each type of sample was spread onto cycloserine cefoxitin fructose taurocholate agar (CCFAT) and incubated at 37°C for two days. An identification of the isolated colonies was done by PCR detection of tpi, tcdA, tcdB and cdtA genes. Toxic activity was also confirmed by a fecal cytotoxin inmmunoessay. Further characterization was performed by PCR ribotyping. *Clostrium difficile* was found in 10% and 1% of the cattle and pigs intestinal samples respectively. Concerning carcass samples, *Clostrium difficile* was recovered from 7% of the swabs from cattle and 6% for pigs. A total of 15 different PCR ribotypes were identified including PCR ribotype 078, 014, 015, 023 and 081.The results of this study confirm that *Clostrium difficile* is carried in the intestinal tracts of food animals arriving at slaughterhouse and is present on carcasses at Belgium slaughterhouse. Some of these isolates are commonly associated with human *Clostrium difficile* infection. This fact shows that there is a clear potential for contamination of meat in the slaughterhouse. Further studies will be conducted in order to identify the presence and the prevalence of the bacterium in retail meat in Belgium.

P117

A NOVEL MOLECULAR TYPE OF *Clostrium difficile* IN NEONATAL PIGS IN AUSTRALIA LACKS tcdA AND tcdC BUT CAUSES GREATER MORBIDITY THAN 078 STRAINS

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Clostrium difficile has recently emerged as the causative agent of high morbidity enteritis ('scouring') in neonatal piglets 1-7 days of age throughout Canada, the USA and Europe, usually with PCR ribotype 078 strains. The organism and its toxins are also found in approximately 75% of apparently healthy piglets, suggesting that all pigs may be colonized with this organism soon after birth. The role of *C. difficile* in disease in Australian piglets has not previously been investigated systematically. We hypothesised that *C. difficile* was associated with an idiopathic enteritis in neonatal piglets reported by pig farmers and veterinarians Australia-wide. Because of Australia's geographical isolation, strict quarantine laws pertaining to the importation of livestock, and low human population and pig density, we also hypothesised that *C. difficile* strains in Australian piglets would be different to the rest of the world. To test these hypotheses, we conducted a pilot study of *C. difficile* prevalence in herds of scouring neonatal piglets and isolated *C. difficile* from 114/185 piglets (62%) overall. Isolates were clonal, consisting of the same novel PCR ribotype 237, not previously described in animals in Australia or elsewhere. All isolates were tcdA-tcdB+cdtA+cdtB+. In this presentation, we report on the evaluation of isolates from these piglets using toxin gene PCR, PCR ribotyping, toxin cytotoxicity testing, animal infection analysis and whole genome sequencing in relation to one strain (AI35) of PCR ribotype 237.

Whole genome sequencing revealed a PaLoc unique to this strain with tcdA and tcdC removed by a large deletion and a novel tcdE variant. The binary toxin locus was complete with an intact cdtR unlike ribotype 078 strains isolates that encode a cdtR with a premature stop codon. Despite these variations, multi-locus sequence typing showed that AI35 belonged to the same clade (clade 5) and sequence type (ST11) as ribotype 078 strains. In toxin expression analyses AI35 produced 25-fold less toxin B than ribotype 027 and 078 strains, and CPE on Vero cells was similar to that reported for *C. difficile* strain 8864, a toxin A-B+ human strain with mutations affecting its glucosylation substrate specificity. AI35 was not fatal in a mouse model of infection but caused significantly greater weight loss than a ribotype 078 strain. This study shows that a unique molecular type of toxigenic *C. difficile* is present in some Australian neonatal piglets with idiopathic enteritis. Despite lacking toxin A and producing variant toxin B at low levels, mice infected with AI35 demonstrated increased morbidity when compared to those infected with an 078 strain. This may result from enhanced binary toxin production under optimal regulation by cdtR. Investigation into the role of binary toxin in the pathogenesis of porcine infection is planned.

P118

Clostrium difficile IN MEAT PRODUCTS, EGGS AND VEGETABLES IN SLOVENIA

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Clostrium difficile is an important nosocomial enteropathogen. Recently, an increase in community acquired *Clostrium difficile* infection (CDI) has been observed and was followed by raised interest on community sources of CDI that could include animals and food. The proportion of *C. difficile* positive samples in different European countries (Austria, Sweden, the Netherlands, Switzerland, Scotland, France, Italy) is low (0, 0% - 7, 5%) and PCR ribotypes of the detected food isolates partially overlap with PCR ribotypes found in animals or in humans in a given country.

In order to determine *C. difficile* food contamination in Slovenia, 59 meat samples and meat products, 8 samples of ready-to-eat salads and sprouts and 49 chicken eggs were tested. Enrichment cultures were prepared either in selective CDALT broth (*C. difficile* agar base prepared without agar (Oxoid) supplemented with *C. difficile* selective supplement (Oxoid), sodium choleate and lysozyme) or in BHI (Biolife) supplemented with sodium choleate, yeast extract and cysteine. For BHI enrichment samples were subjected to heat shock. After 5 days of incubation under anaerobic conditions, spores were selected by alcohol shock and plated onto selective CDALT agar (*C. difficile* agar base (Oxoid) supplemented with *C. difficile* selective supplement (Oxoid), sodium choleate and lysozyme) or on commercial selective chromID *C. difficile* (BioMerieux) agar plates. Recovered *C. difficile* colonies were identified by morphology, confirmed by detection of molecular marker cdd3 and characterized by toxinotyping and PCR ribotyping.

C. difficile was not detected in any of the tested meat and vegetable samples. A single chicken egg out of 49 (2, 0%) was positive. Four obtained colonies were characterized and all 4 isolates belonged to toxinotype 0 but to two different PCR ribotypes: 014/020 and 002. Both PCR ribotypes are currently most prevalent in hospitalized patients in Slovenia and are also among most prevalent European human isolates. They are also frequently isolated from poultry in Slovenia. In conclusion, low prevalence of *C. difficile* in meat and meat products in our study correlates to other European reports. Contamination of chicken eggs together with reports on relatively high prevalence in poultry highlights the importance of poultry as *C. difficile* reservoir and adds to its potential as a source of infection for humans.

P119

INTERNATIONAL *Clostrium difficile* ANIMAL STRAIN COLLECTION

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Animals have been recognized as an important potential reservoir of *Clostrium difficile* and according to recent studies the overlap between PCR ribotypes of human and animal isolates seems to be increasing.

Here we report on an International *Clostrium difficile* animal strain collection that was established to enhance the comparative studies on animal-associated strains. The goal of the collection is to include one PCR ribotype per species per country/laboratory.

At the time 12 different countries are participating (Austria, Belgium, Canada, Czech Republic, Denmark, Germany, Italy, Scotland, Slovenia, Spain, Switzerland and USA). The collection includes 103 strains with 1 to 18 strains and up to 15 different PCR ribotypes per country. Strains originate from 11 different animal species, including pets, horses and food animals such as cattle, pigs and poultry. Sixty strains (58.3 %) are from cattle and pigs. All 103 included strains were distributed into 38 different PCR ribotypes and up to 15 different PCR ribotypes can be found within a single animal species. Six strains are nontoxigenic while toxigenic strains account for 94.2% and belong to 11 different toxinotypes: 0, II, III, IV, V, VI, VIII, XI, XII, XIII and XIX. PCR ribotypes 078, 126, 014/020, 012 and 002 that are frequently associated with animals represent 29.1 % of all strains.

In conclusion, this *C. difficile* animal strain collection is unique as it contains genetically diverse *C. difficile* strains from different geographical origin and different animal species. It can provide useful data on animal-associated strains and contribute to interlaboratory exchange of strains.

P120

DIFFERENT COMPOSITION PATTERNS OF MICROBIOTA IN C. difficile COLONISED AND NON - COLONISED HUMANS AND ANIMALS

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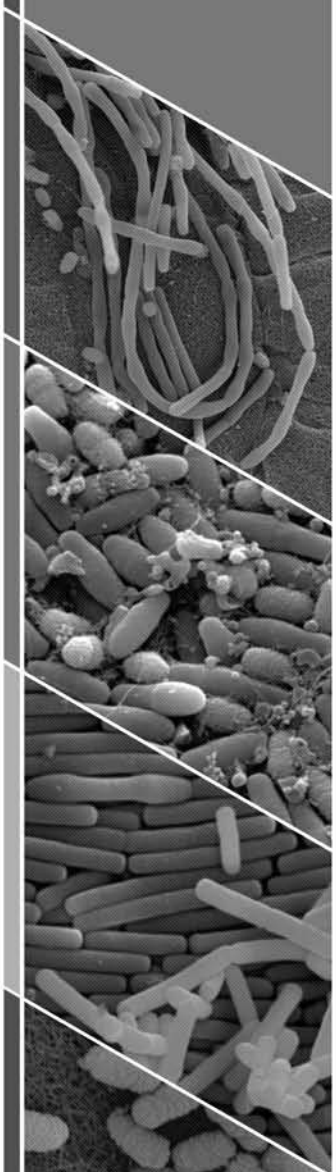
An unperturbed intestinal microbiota is important in conferring the colonisation resistance against *C. difficile* infection (CDI). Studies on the association of gut microbiota and *C. difficile* are still rare and all of them deal only with the bacterial microbiota, not including archaea and fungi. In this study, we have used a simple molecular method (DHPLC - denaturing high pressure liquid chromatography), combined with machine learning analysis methods, to identify different patterns in the composition of microbiota in *C. difficile* colonised and non - colonised humans and chickens.

We have analysed 208 human faecal samples, of which 171 were routine samples and 37 were from healthy volunteers. Out of the 171 routine samples, 105 were *C. difficile* positive and 66 negative. In chickens, 143 faecal samples from a single poultry farm were collected in seven consecutive samplings: 86 were *C. difficile* positive and 57 were *C. difficile* negative. The total DNA was isolated from the faecal samples by a standard procedure and bacterial, archaeal and fungal genes (16S rRNA or ITS2) were amplified. After DHPLC (which separates DNA amplicons based on fragment size and sequence), 23 bacterial groups, 9 fungal groups and 2 archaeal species were differentiated in human samples. In the chicken samples, 15 bacterial, 10 fungal groups and a single archaeal species were identified. A machine learning method for building decision trees, i.e., the WEKA J48 implementation of the C4.5 algorithm, was used to analyse the different patterns in faecal microbiota. We were able to show that certain patterns of microbiota composition are associated with the type of *C. difficile* colonization, e.g., the pattern Bifidobacterium longum positive / Enterococcaceae negative / Streptococcus sp. - Enterococcus sp. positive was highly associated with the presence of *C. difficile* in humans. The microbes associated with *C. difficile* colonization were found to be different in humans and chickens. In humans, the key predictor associated with *C. difficile* negative samples was Bifidobacterium longum. The presence of Streptococcus sp. - Enterococcus sp. was linked to the faecal samples colonised with the PCR ribotype 027. In chickens, the absence of Acidaminococcus intestini was recognised as the main predictor of good *C. difficile* growth.

In conclusion, this is the first study to show that, in addition to bacteria, fungal microbiota is also important in *C. difficile* colonisation and that the patterns in the composition of the gut microbiota (rather than a single microorganism) are predictive of *C. difficile* colonization in humans and poultry.

4th International *Clostridium difficile* Symposium



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ICDS Participants	Country	Institution E-mail	Presentation
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