# 3rd iCDS

# International *Clostridium difficile* Symposium

## abstract book

September, 22nd to 24th 2010, Bled, Slovenia

ZAVOD ZA ZDRAVSTVENO VARSTVO MARIBOR

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# International *Clostridium difficile* Symposium

## abstract book

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**3rd International Clostridium difficile Symposium**
Abstract book
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55 Abstracts of poster presentations (section 1 P1 to P51, Thursday)

109 Abstracts of poster presentations (section 2 P52 to P102, Friday)

165 ICDS Participants

# WELCOME

Dear colleagues,

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

Clostridium difficile is still one of the most important human pathogens. However, over the years the clinical as well as the research focus has shifted somewhat and this is also apparent in the programme of the 3rd ICDS. While the main clinical issues are still the prevention, control and epidemiology of disease, especially of recurrent infections and of outbreaks, research has gained new perspectives. This followed the publication of several genomes and development of methods for the genetic manipulations of C. difficile. Host responses and host factors such as gut microbiota, which influence the development of C. difficile infection, are now becoming more important.

ICDS is now established as a traditional C. difficile meeting and the venue is also traditionally located in Slovenia (Kranjska Gora in 2004; Maribor 2007). This year we have chosen the Bled region. 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

# ICDS PROGRAMME

**Wednesday, 22nd September**

| SESSION 1 | | |
|---|---|---|
| 16.15 | Rupnik M. | OPENING |
| 16.30-17.00 | Miller M. | L1 - THE ROLE OF INFECTION CONTROL IN *Clostridium difficile* OUTBREAKS: THERE IS SO MUCH WE DON'T KNOW |
| 17.00-17.20 | Siu K.H.G. et al. (Yam W.C.) | O1 - MOLECULAR EPIDEMIOLOGY OF *Clostridium difficile* IN HONG KONG |
| 17.20-17.40 | Indra A. et al. | O2 - HIGH OUTPUT PCR-RIBOTYPING OF *Clostridium difficile* DURING AN OUTBREAK OF PCR-RIBOTYPE 027 IN VIENNA, AUSTRIA IN 2009 |
| 17.40-18.00 | Pillai D.R. et al. (Broukhanski G.) | O3 - DEFINING CRITERIA TO INTERPRET MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS TO AID *Clostridium difficile* OUTBREAK INVESTIGATION |
| 19.00 | | WELCOME RECEPTION (Hotel Golf) |

**Thursday, 23rd September**

| SESSION 2 | | |
|---|---|---|
| 9.00-9.30 | Kelly C.P. | L2 - HOST RESPONSE TO Clostridium difficile INFECTION |
| 9.30-9.50 | Papatheodorou P. | O4 - CLOSTRIDIAL GLUCOSYLATING TOXINS ENTER CELLS VIA CLATHRIN-MEDIATED ENDOCYTOSIS |
| 9.50-10.10 | Popoff M.R. and Geny B. | O5 - HOW DO LARGE CLOSTRIDIAL TOXINS MODIFY THE ACTIN CYTOSKELETON AND INTERCELLULAR JUNCTIONS AND ARE ALL CELLULAR EFFECTS DEPENDENT ON THEIR ENZYMATIC ACTIVITY ON SMALL GTPases? |
| 10.10-10.30 | Miura M. et al. | O6 - A NOVEL VIRULENCE FACTOR *Clostridium difficile* SRL MODULATES TOXIN B SENSITIVITY OF INTESTINAL EPITHELIAL CELLS |
| 10.30-11.00 | | COFFEE BREAK |

| SESSION 3 | | |
|---|---|---|
| 11.00-11.30 | Malozzi M.M. et al. (Vedantam G.) | L3 - *Clostridium difficile* PATHOGENESIS MEDIATED BY NON-TOXIN VIRULENCE FACTORS |
| 11.30-11.50 | Joshi L.T. et al. | O7 - CHARACTERISATION OF THE SPORE SURFACE OF *Clostridium difficile* AND ITS ROLE IN ADHERENCE |
| 11.50-12.10 | Reynolds C.B. et al. (Fairweather N.F.) | O8 - VARIATION AND CONSERVATION: THE REGULATION OF *Clostridium difficile* CELL WALL PROTEIN CWPV |
| 12.10-12.30 | Fortier L.C. et al. | O9 - THE MULTIPLE FACETS OF BACTERIOPHAGES AND THEIR ROLE IN THE DIVERSITY AND VIRULENCE OF Clostridium difficile |
| 12.30-14.00 | | LUNCH (Hotel Golf) |
| 14.00-16.00 | | POSTER SESSION I (P1-P51) |
| 16.00-16.30 | | COFFEE BREAK |

| SESSION 4 | | |
|---|---|---|
| 16.30-16.50 | Buckley A. et al. | O10 - INFECTION WITH DIFFERENT TOXINOTYPE STRAINS OF *Clostridium difficile* RESULTS IN CLEAR PATHOLOGICAL DIFFERENCES IN THE HAMSTER MODEL |
| 16.50-17.10 | Zidaric V. et al. | O11 - COLONIZATION OF CALVES WITH *Clostridium difficile* IN BELGIUM |
| 17.10-17.30 | Squire M.M. et al. | O12 - A NOVEL MOLECULAR TYPE OF *Clostridium difficile* IN NEONATAL PIGS IN AUSTRALIA |
| 17.30-17.50 | Medina-Torres C. et al. (Weese J.S.) | O13 - PREVALENCE AND CHARACTERIZATION OF *Clostridium difficile* IN HORSES |
| 19.00 | | DINNER (Hotel Golf) |

**Friday, 24rd September**

| SESSION 5 | | |
|---|---|---|
| 9.00-9.30 | Lepage P. et al. | L4 - HUMAN GUT MICROBIOTA: FUNCTIONALITY AND BACTERIA-HOST CROSSTALK |

| | | |
|---|---|---|
| 9.30-9.50 | Kuehne S.A. et al. | O14 - THE ROLE OF TOXINS IN *Clostridium difficile* INFECTION |
| 9.50-10.10 | Bobo L.D. et al. (Dubberke E.R.) | O15 - MITOGEN ACTIVATED KINASE ACTIVATED PROTEIN KINASE- 2 (P-MK2): RATIONALE FOR CONSIDERING THIS PROMISING BIOMARKER IN *Clostridium difficile* INFECTION |
| 10.10-10.30 | Lawley T.D. et al. | O16 - HYPERVIRULENT *Clostridium difficile* INDUCES INTESTINAL DYSBIOSIS TO ENHANCE TRANSMISSIBILITY |
| 10.30-11.00 | | COFFEE BREAK |
| 11.00-12.30 | | POSTER SESSION II (P52-P103) |
| 12.30-14.00 | | LUNCH (Hotel Golf) |

| SESSION 6 | | |
|---|---|---|
| 14.00-14.30 | Riley T. | L5 - CLASSICAL AND NOVEL APPROACHES IN *Clostridium difficile* DIAGNOSTICS |
| 14.30-14.50 | Tenover F.C. et al. (Baron E.J.) | O17 - IMPACT OF *Clostridium difficile* RIBOTYPE ON PERFORMANCE OF ANTIGEN-BASED DIAGNOSTIC ASSAYS AND RESULTS OF PCR FOR REPEAT SAMPLES ON SAME PATIENT |
| 14.50-15.10 | Norén T. et al. | O18 - RAPID AND SENSITIVE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR *Clostridium difficile* DIAGNOSIS CHALLANGE CYTOTOXIN B CELL TEST AND CULTURE AS GOLD STANDARD |
| 15.10-15.30 | Babakhani F. et al. | O19 - KILLING KINETICS OF FIDAXOMICIN AND ITS MAJOR METABOLITE (OP-1118) VS. *Clostridium difficile* |
| 15.30-16.30 | | COFFEE BREAK (with poster session) |

| SESSION 7 | | |
|---|---|---|
| 16.30-17.00 | Ben-Horin S. | L7- GUT IMMUNITY |
| 17.00-17.20 | Spencer J. et al. | O20 - THE POTENTIAL USE OF REPEAT REGIONS IN THE BINDING DOMAINS OF TOXIN A AND TOXIN B FROM *Clostridium difficile* AS A VACCINE CANDIDATE |
| 17.20-17.40 | Mulvey G.L. et al. (Armstrong G.D.) | O21 - THERAPEUTIC POTENTIAL OF EGG YOLK ANTIBODIES FOR TREATING *Clostridium difficile* INFECTION |

| | | |
|---|---|---|
| 17.40-18.00 | Tatarowicz W. et al. | O22 - SAFETY AND TOLERABILITY OF AN ORAL SUSPENSION OF VP 20621, SPORES OF A NON-TOXIGENIC *Clostridium difficile* STRAIN, IN HEALTHY OLDER SUBJECTS |
| 19.00 | | CONGRESS DINNER (Hotel Toplice) |

**Saturday, 25th September**

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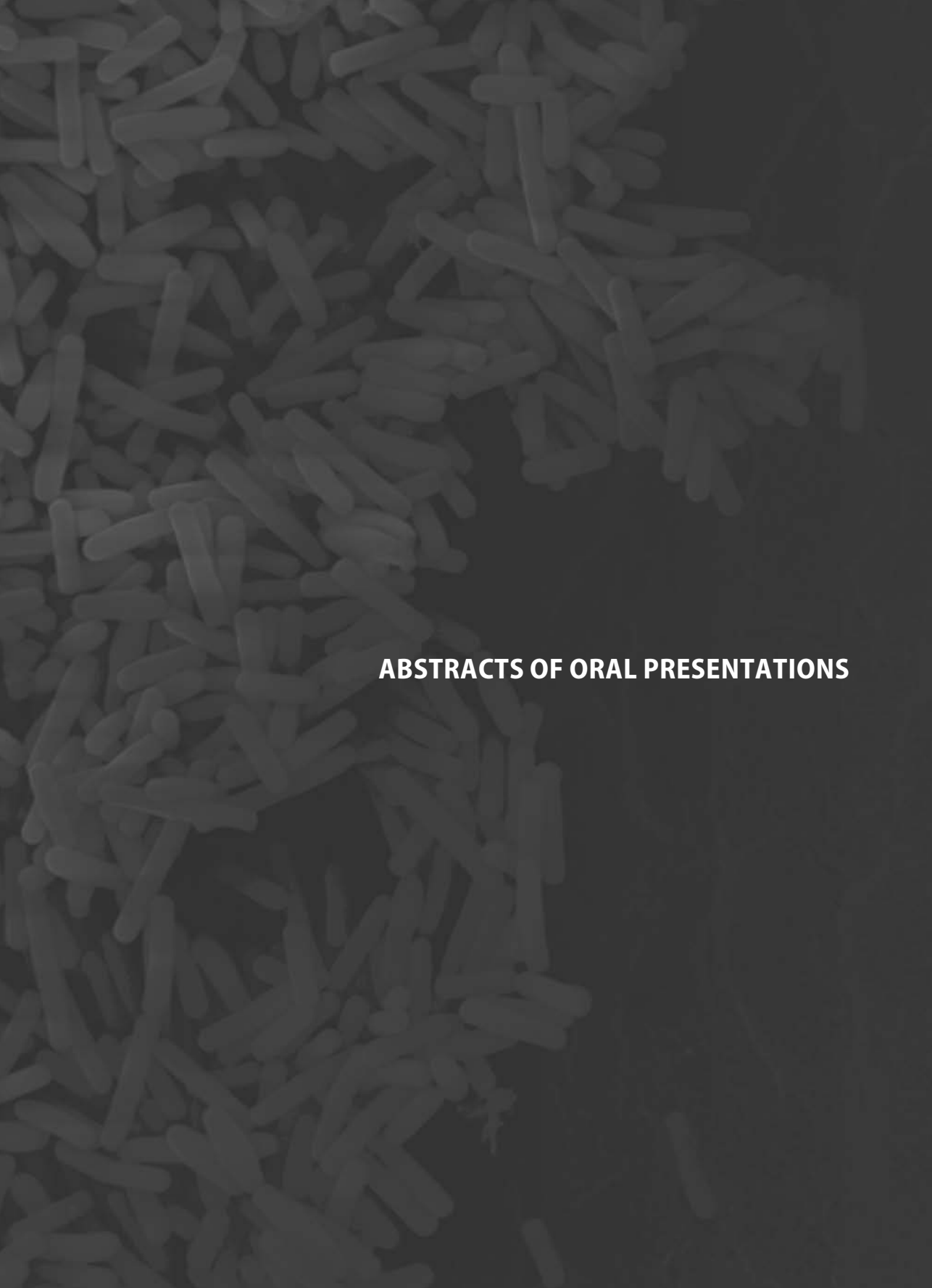
| CONGRESS EXCURSION TO LOKEV & SLOVENIAN COAST |



# ABSTRACTS OF ORAL PRESENTATIONS

**L1**

# THE ROLE OF INFECTION CONTROL IN Clostridium difficile OUTBREAKS: THERE IS SO MUCH WE DON'T KNOW

M. Miller

Jewish General Hospital and McGill University, Montreal, Canada

Outbreaks of *Clostridium difficile* infection (CDI) in healthcare facilities worldwide have occurred and been documented for decades. However, since 2002, outbreaks in some North American and European centers have caused CDI rates much higher than previously witnessed, and have been associated with morbidity rates (i.e. colectomy, need for intensive care) and mortality rates much higher than ever recognized before.

The emergence of "hyper-virulent" CDI has caused increased interest in the optimal methods of conducting "infection prevention and control" for this pathogen. Protocols consisting of multiple methods simultaneous "bundled" interventions (e.g. as implemented in Quebec, Canada in 2004 and elsewhere) have succeeded in decreasing the incidence and complication rates of CDI. However, the exact role and imporatance of each intervention remain largely unknown.

In addition, there are many unresolved and important questions related to the control of CDI in healthcare establishments, such as:

- what is the optimal duration of contact precautions?
- should we be looking for and isolating asymptomatic carriers of CD spores?
- what is the best method of disinfecting contaminated surfaces?
- should intensive housekeeping be aimed at only symptomatic patients or all patients?
- does early empiric therapy of diarrhea contribute to control of transmission?
- are any therapies more successful than others at controlling the spread of CD?

The current evidence, knowledge gaps, and future challenges to the control of CDI will be discussed.

**O1**

# MOLECULAR EPIDEMIOLOGY OF *Clostridium difficile* IN HONG KONG

K.H.G. Siu[1], V.C.C. Cheng[1], K.Y. Yuen[1], L.Y. Tsang[1] and W.C. Yam[1]

[1]Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, Hong Kong

Backgound: The incidence of *C. difficile* infection has increased markedly recently in many countries, notably associated with the epidemic spread of the hypervirulent strain with PCR-ribotype 027 (NAP1). Accurate surveillance is essential so that appropriate infection prevention measures can be implemented. Up-to-date, the epidemiology of *C. difficile* in Hong Kong has not been fully elucidated.

Aim: To investigate the molecular epidemiology of *C. difficile* in Hong Kong.

Results: In period of January to December in 2009, a total of 2,277 stool specimens were collected from non-duplicated patients suffering mild to severe diarrhea, of which 469 (21%) specimens were culture positive for *C. difficile*. The HeLa cell cytotoxin assay revealed 132 (28%) specimens were cytotoxin positive for both stool filtrate and the corresponding cultured isolates whereas 285 (61%) specimens showed cytotoxic effect only in cultured isolates. Among 285 cytotoxic isolates, 36 (13%) of them belonged to ribotype-002 which is the most predominant ribotype in Hong Kong, followed by ribotype og39 (3.5%) and ribotype-012 (3.2%). No ribotype-027 was isolated during the study period. The only reported local case was documented in late 2008. Multiple-locus variable-number tandem-repeat analysis revealed that the strain belonged to a historical type 027 instead of the epidemic one. All the ribotype 002 isolates harbored a wildtype tcdC allele and were capable of producing both toxin A and B but not cdtA and cdtB binary toxins. The enzyme immunoassay revealed that 12 (33%) of ribotype-002 isolates had toxin yields ranged from 151.5U/ml to 191.6U/ml, which were compatible to the historical and epidemic type 027 isolate (toxin yield= 189U/ml) and were at least 5-fold higher than the historical type 002 isolates (toxin yield =29U/ml). Our aero-tolerant and sporulation experiment showed 4 of these hyper-toxigenic isolates had sporulation frequencies of about 60% at 48h, which was similar to that of the epidemic 027 strain. Patients infected by these hyper-toxigenic isolates with high sporulation frequencies had developed recurrent episode while one suffered form of intestinal mucormycosis.

Conclusion: Our data showed that type 002 *C. difficile* is the predominant ribotype in Hong Kong. High capacities for both toxin and spore production may contribute to disease severity, therapy failure, relapse, and spread in our region.

**O2**

# HIGH OUTPUT PCR-RIBOTYPING OF *Clostridium difficile* DURING AN OUTBREAK OF PCR-RIBOTYPE 027 IN VIENNA, AUSTRIA IN 2009

A. Indra[1], S. Kasper[2], M. Blaschitz[1], S. Huhulescu[1], S. Kernbichler[1], D. Schmid[2], A. Fiedler[1] and F. Allerberger[1]

[1]Institute for Medical microbiology and Hygiene, Vienna, Austria, Austrian Agency for Health and Food Safety, [2]Competence Center for infectious epidemiology, Vienna, Austria, Austrian Agency for Health and Food Safety

From January to August 2009, 174 cases of *C. difficile* infections (CDI), including 38 cases of infection with *C. difficile* ribotype 027(027CDI) occurred in a Viennese hospital (HX). Previously, in 2008, 11 cases of 027CDI were identified in Austria.

PCR-ribotyping was performed using capillary-sequencer-based PCR-ribotyping in combination with an internet based Database - AGES-WEBRIBO (webribo.ages.at) - a high output method for typing. This method utilizes the advantage of PCR-ribotyping and bypasses the problematic data-interpretation and laboratory interchangeability issues by using a capillary-sequencing machine and an internal size-standard with every sample. To cope with variations in the capillary-sequencer-dependent results from different types of sequencing machines, a newly developed algorithm used in AGES-WEBRIBO (webribo.ages.at) was applied after capillary gel electrophoresis based *Clostridium difficile*-PCR ribotyping in order to analyse the typing results.

During this outbreak, a total of 1320 *Clostridium difficile* isolates, including 720 from Vienna, were PCR-ribotyped using capillary-sequencer-based PCR-ribotyping in combination with AGES-WEBRIBO (webribo.ages.at). Of these samples, the most common strain found was PCR-ribotype 053, 014 and 027.

Non-compliance to evidence based control measures, recommended by ESGCD, resulted in a rapid spread of a high virulent CD strain, which also caused a very high patient mortality. Using capillary-sequencer-based PCR-ribotyping for typing all *Clostridium difficile* isolates in Vienna during this outbreak helped to quickly identify the spread of these ribotypes not only in hospital HX but also to 5 other hospital in the area. Following the measure implementation, the hospital-based CD surveillance, supported by the PCR-ribotyped results, indicated a decreasing trend of all CDI cases in HX within the following months.

These results show the importance and practicability of this new method as a tool for real-time outbreak investigation even in elucidating links between separate hospital clusters.

**O3**

# DEFINING CRITERIA TO INTERPRET MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS TO AID *Clostridium difficile* OUTBREAK INVESTIGATION

D.R. Pillai[1,2], G. Broukhanski[1,2] and A. Simor[1]

[1]Department of Laboratory Medicine & Pathobiology, University of Toronto, Ontario, Canada, [2]Public Health Laboratory, Toronto

It is widely accepted that pulsed field gel electrophoresis (PFGE) is useful for national surveillance for *C. difficile* but lacks discriminatory power to aid outbreak investigation. Compounding this has been the high baseline rates of the epidemic North American pulsotype (NAP) 1 strain in hospitals. Multi-locus variable number tandem repeat analaysis (MLVA) appears to have superior discriminatory power but criteria to define clonality have not been set. We conducted surveillance for toxin-positive *C. difficile* infection (CDI) at a single academic health sciences centre between September, 2009 and April, 2010. Seventy four (n=74) patients' specimens resulting in 86 discrete CDI episodes were subjected to PFGE and a modified MLVA method (N. Results were analyzed using Bionumerics software to generate phylogentic trees and compared with unit location, date of admission, date of toxin positivity, new versus recurrent case, and community versus hospital onset infection. Population analysis was conducted on several colonies within a stool specimen. Demographic breakdown revealed 62% were female with an average age of 72 for all patients. Seventy five percent were health care-associated, the remainder community-acquired. Sixty four patients (86.5%) were single episodes with 10 (14.5%) patients experiencing recurrent CDI. 32 CDI were due to the NAP1 pulsotype (37%), 3 (3.5%) NAP 7, 2 (2.3%) NAP4, 1 (1.2%) NAP 2, with the remainder (56%) due to arbitrarily assigned pulostypes. Both categorical and distance-based clustering algorithms were evaluated for modified MLVA. Amongst the NAP 1 strains, 2 distinct clusters were identified by MLVA using an 85% similarity as a cut-off by Manhattan distance similarity coefficient with clustering using Ward algorithm, 5 clusters using 95%, and 7 clusters using 97% as a cut-off. Sixty percent of patient relapses were due to the NAP 1 pulsotype. Using a defined similarity cut-off based on Manhattan distance, the modified MLVA method was able to distinguish recrudescence from reinfection. Based on these observations, we recommend the use of Manhattan distance-based clustering with a cut-off of 95% to demonstrate clonality amongst CDI outbreaks in an effort to implement cost-effective infection control measures.

**L2**

# HOST RESPONSE TO *C. difficile* INFECTION

C.P. Kelly

Harvard Medical School, Boston, USA

Shortly after *Clostridium difficile* toxins were identified as major virulence factors in *C. difficile* infection (CDI) investigators noted variable adaptive immune responses in the form of anti-toxin antibodies. Furthermore, immunization against *C. difficile* toxins protected against CDI in animals. In humans, many, but not all adults, have detectable circulating and secretory anti-toxin. These findings led to the hypothesis that the adaptive immune response to *C. difficile* may influence the outcomes of CDI. Later studies support this hypothesis by showing that symptomless carriers of toxinogenic *C. difficile* and those with a single episode of CDI without recurrence show more robust anti-toxin immune responses than those with symptomatic and recurrent disease.7 8 To date, protective immunity has been linked most strongly to anti-toxin antibody responses; immune responses to non-toxin antigens are evident but their role in protection against CDI remains unclear. Accordingly, immune based approaches to CDI therapy and prevention have, thus far, focused on anti-toxin responses. The first reports of immunization in CDI used passive immunotherapy with pooled human intravenous immunoglobulin (IVIG) which contains some toxin neutralizing activity. However, the efficacy of IVIG in treating severe CDI or in preventing recurrence is unproven. In contrast, human monoclonal antibodies against *C. difficile* toxins A and B were shown, recently, to protect against recurrent CDI in a randomized controlled trial.12-14 This finding is the first clear demonstration of the efficacy of immunization against CDI in humans and highlights the important role of systemic IgG antitoxin.12 A vaccine using *C. difficile* toxoids has been reported to be immunogenic in humans but, as yet, there are very limited data on its efficacy in protecting against CDI.

It has been clear for many years that innate immune responses to *C. difficile* and its toxins are central to the pathogenesis of CDI. An acute intestinal inflammatory response with prominent neutrophil infiltration and associated tissue injury is characteristic of CDI. Furthermore, inhibiting this inflammatory response and neutrophil recruitment can protect against the intestinal effects of *C. difficile* toxins in animals. More recently a link has been reported between a polymorphism of the IL-8 gene promoter and risk for CDI. IL-8 is a potent neutrophil-directed chemokine that regulates neutrophil activation and recruitment to sites of tissue injury.

A number of studies have examined host risk factors for CDI and an initial, validated clinical prediction rule has been developed. A validated clinical prediction tool has also been developed for recurrent CDI based on prominent, independent risk factors. Risk factors for severe CDI with negative outcomes have also been identified and include marked elevation of the peripheral white blood cell count and elevated creatinine. However, further work is needed in this area to guide the clinical application of new approaches to disease prevention and treatment including new antimicrobial as well as passive and active immunization.

**O4**

# CLOSTRIDIAL GLUCOSYLATING TOXINS ENTER CELLS VIA CLATHRIN-MEDIATED ENDOCYTOSIS

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Members of the family of the clostridial glucosylating toxins (CGTs) are important pathogenicity factors, responsible for the outcome of diverse human diseases. *C. difficile* toxin A (TcdA) and B (TcdB) cause antibiotic-associated diarrhea and pseudomembraneous colitis. C. sordellii lethal toxin (TcsL) is involved in toxic shock syndrome after abortion and C. novyi alpha-toxin (TcnA) in gas gangrene development. CGTs enter cells via receptor-mediated endocytosis and require an acidified endosome for translocation of the catalytic domain into the cytosol. In the present study, we aimed to clarify the endocytic processes that mediate cell internalization of the CGTs. Intoxication of cells was monitored by analyzing cell morphology, status of Rac glucosylation in cell lysates and transepithelial resistance of cell monolayers. We found that the intoxication of cultured cells by CGTs was strongly delayed when cells were preincubated with dynasore, a cell-permeable inhibitor of dynamin, or chlorpromazine, an inhibitor of the clathrin-dependent endocytic pathway. Additional evidence about the role of clathrin in the uptake of the prototypical CGT family member toxin B was achieved by expression of a dominant-negative inhibitor of the clathrin-mediated endocytosis (Eps15 DN) or by siRNA against the clathrin heavy chain. Accordingly, cells that expressed dominant-negative caveolin-1 were not protected from toxin B-induced cell rounding. In addition, lipid rafts impairment by exogenous depletion of sphingomyelin did not decelerate intoxication of HeLa cells by CGTs. We conclude from our data that the endocytic uptake of the CGTs involves a dynamin-dependent process that is mainly governed by clathrin.

**O5**

# HOW DO LARGE CLOSTRIDIAL TOXINS MODIFY THE ACTIN CYTOSKELETON AND INTERCELLULAR JUNCTIONS AND ARE ALL CELLULAR EFFECTS DEPENDENT ON THEIR ENZYMATIC ACTIVITY ON SMALL GTPASES?

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Large glucosylating clostridial toxins (LGCT) are 250-300 kDa proteins encompassing *Clostridium difficile* toxins A and B (TcdA and TcdB), the main toxins involved in pseudomembanous colitis and post-antibiotic enteritis, Clostridium sordellii lethal toxin (TcsL), and hemorrhagic toxin (TcsH)), as well as Clostridium novyi α-toxin TcnA). LGCT enter cells by receptor-mediated endocytosis and deliver the N-terminal catalytic domain into the cytosol by an autoproteolytic process. TcdA and TcdB inactivate through glucosylation Rho, Rac and Cdc42, whereas TcsL only modifies Rac among the Rho-GTPases but inactivates Ras proteins. In polarized epithelial cells, TcsL preferentially induces a depolymerization of basolateral actin filaments, whereas TcdA and TcdB alter apical and also basolateral actin filaments. Therefore, TcsL preferentially perturbs the adherens intercellular junctions (AJ) by removing the whole E-cadherin-catenin complex from the membrane to the cytosol, and TcdA and TcdB preferentially alter the apical tight junctions. Thus, Rac has a pivotal role in the control of E-cadherin junctions and acts through several effectors such as IQGAP, which directly regulates the assembly of E-cadherin-catenin complexes. However, TcsL does not alter the Rac-IQGAP pathway but modifies E-cadherin junctions via Rac and actin filament depolymerization. We showed that TcsL induces a rapid dephosphorylation of paxillin, a protein regulating focal adhesion (FA), independently of inactivation of paxillin kinases such as Src, Fak and Pyk2 and in a Rac-dependent manner. TcsL inactivated Rac modifies interactions between proteins from AJ and FA complexes. Indeed, in Triton X-100 insoluble membrane proteins from these complexes, namely E-cadherin, β-catenin, p120-catenin, and talin, as well as phosphoinositide content are decreased upon TcsL intoxication. We propose that Rac inactivation by TcsL alters phosphoinositide metabolism leading to FA and AJ complex disorganization and actin depolymerization. In addition, TcsL as well as TcdB and TcdA activate the three MAPkinase pathways (ERK, JNK, p38). But only a permeable and specific JNK inhibitor, JNK inhibitor II, prevents TcsL-dependent actin depolymerization and cell rounding but not that of TcdB. We show that JNK activation is dependent on entry of the toxin N-terminal domain into the cytosol but independent of small GTPase glucosylation. However, TcsL activated JNK pathway facilitates GTPase glucosylation. Indeed, LGCT activates certain cell signal cascades independently of GTPases glucosylation which contribute to their effects on actin cytoskeleton.

**O6**

# A NOVEL VIRULENCE FACTOR *Clostridium difficile* SRL MODULATES TOXIN B SENSITIVITY OF INTESTINAL EPITHELIAL CELLS

M. Miura[1], H. Kato[2] and O. Matsushita[3]

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Retrograde transport through the Golgi complex to the endoplasmic reticulum (ER) has been shown to be employed by some bacterial toxins for efficient and maximal biological activity. The conserved C-terminal KDEL or KDEL-like sequence in these toxins is required for retrograde movement through the Golgi to the ER.

We report here that CD2298 of *Clostridium difficile* has a KDEL-like motif at its C-terminus and could modulate toxin sensitivity of intestinal epithelial cells. We therefore renamed CD2298 as "Srl" (Sensitivity regulation of *C. difficile* toxins) and discuss its involvement in the retrograde pathway and intracellular fate of *C. difficile* toxins.

Phalloidin staining demonstrated that *C. difficile* culture supernatant-treated MDCK cells exhibit a cytopathic structure, referred to as an "actin aggregate," at the polar juxtanuclear position. Because the actin aggregate co-localized with the Golgi complex, we hypothesized that a factor which has a KDEL or KDEL-like motif produced in the *C. difficile* culture supernatant reaches the Golgi complex, where it induces actin aggregation. Using a bioinformatics approach, we focused on Srl and purified the recombinant Srl protein. GST-tagged Srl caused actin aggregation in MDCK cells in a toxin B-dependent manner. It localized in the Golgi complex at the site of actin aggregation. GST-Srl treatment alone did not show any cytopathic effects, although toxin B treatment combined with GST-Srl decreased the viability of T84 cells to a greater extent than toxin B treatment alone.

These data indicate that Srl reaches the Golgi complex and causes formation of actin aggregate thereby increasing the toxin sensitivity of the cells and that direct or indirect interaction between Srl and toxin B is involved in increasing toxin sensitivity of the cells.

The increase in sensitivity to toxin and toxin B-dependent localization of Srl in the Golgi complex at the juxtanuclear position imply a guidance role of Srl to toxin B as a novel toxin pathway-modulating factor.

**L3**

# *Clostridium difficile* PATHOGENESIS MEDIATED BY NON-TOXIN VIRULENCE FACTORS

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*Clostridium difficile* infection (CDI) currently affects >400,000 patients annually in the USA alone, with an added burden to the healthcare system of $3 billion. Disease recurrences is a hallmark of CDI, with older/immune-compromised patients particularly affected due to a preponderance of risk factors.

*C. difficile* (CD) epidemiology has altered markedly in the past 10 years. Virulent "variant" strains have emerged, that are associated with outbreaks, severe disease, increased recurrence rate(s) and community onset of disease. Little is known about the virulence of CD epidemic-associated strains. MLST/micro-array analyses reveal that epidemic strains clade into a discrete group. In addition, most epidemic strains also harbor a point mutation as well as deletions in the negative modulator of toxin production, TcdC. CD toxin gene expression is modulated by the regulators TcdC, TcdR, CodY, as well as additional molecules. tcdC gene deletions in most epidemic strains are functionally irrelevant, since the upstream point mutation results in a frame-shift and, consequently, a truncated TcdC protein. Absence of functional TcdC does not result in early toxin production [and only very moderate increases (2-3 fold) in toxin amount] in epidemic strains, since the profoundly-important positive activator (TcdR) is intact and fully regulatory in these strains.

What then, is a distinguishing feature of epidemic-associated CD? Several groups including ours have shown that NAP1/027/BI strains have higher sporulation efficiencies than comparable non-epidemic, non-NAP1 strains. Increased spore numbers could contribute not only to infectious particle dissemination, but also serve as reservoirs for recurrent disease. Persistent CD colonization is another factor that could profoundly influence the ultimate virulence phenotype. We have recently obtained data that argue strongly in favor of this contention: multiple proteins, all predicted to be involved in CD colonization, are altered in NAP1 strains. These include surface molecules, transporters and hose innate immunity modulators. Therefore, non-toxin virulence factors play a critical role in CD pathogenesis.

**O7**

# CHARACTERISATION OF THE SPORE SURFACE OF *Clostridium difficile* AND ITS ROLE IN ADHERENCE

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*Clostridium difficile* is an anaerobic Gram positive bacterium implicated in causing antibiotic associated diarrhoea (CDAD). The ability of *C. difficile* to produce highly resistant endospores facilitates environmental survival and disease transmission. For this reason we sort to determine the contribution of the spore to surface adherence and biocide resistance. Initially the ability of a diverse collection of 21 clinical isolates representing different ribotypes to form spores in brain heart infusion and Columbia broths was determined. While spore yields varied between 104-108cfu/ml we found no obvious correlation between yield and ribotype. The surface properties of these spores were subsequently compared using a Hexadecane-based partition assay which determines relative hydrophobicity. The results revealed a relationship between hydrophobicity and ribotype, with hypervirulent isolates tending to be hydrophobic suggesting that they are more adept at binding to hydrophobic materials such as stainless steel. Indeed this hypothesis was confirmed using a novel replica plating method which found that hydrophobic spores bound more firmly to stainless steel discs than their less virulent (hydrophilic) counterparts. Subsequent examining of spore structure via electron microscopy found that hydrophobic (virulent) spores possessed a more pronounced exosporial layer than their hydrophilic counterparts. The exosporial layer has been found to play an important role in facilitating the adhesion of spores to human cell substrates, agar and glass surfaces during spore germination. The adhered exosporium also maintains the emergence of newly formed bacterial cells, ensuring surface colonization and spread. To determine if exposure to sub-lethal concentrations of biocide affect the ability of spores to adhere to surfaces such as stainless steel we exposed spores to 1000ppm of the biocide Sodium dichloroisocyanurate (NaDCC). While exposure to biocide had no effect on viability or hydrophobicity it did increase the ability of virulent spores to adhere to stainless steel. This is of particular interest as adherent bacteria show increased resistance to biocide. Given these results it is intriguing to speculate that exposure to sub-optimal concentration of biocide could potentiate the adherence of hypervirulent spores, thus aiding environmental contamination and onward patient transmission.

**O8**

# VARIATION AND CONSERVATION: THE REGULATION OF *Clostridium difficile* CELL WALL PROTEIN CWPV

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Apart from two cytotoxins, little is known about the molecular mechanisms underpinning *C. difficile* pathogenicity. *C. difficile* must employ surface proteins to interact with host tissues mediating colonisation of the gut. A family of cell surface proteins has been identified, but their roles during infection are largely uncharacterised. This study focuses on one of these proteins, CwpV. In a manner analogous to SlpA, CwpV is post-translationally cleaved and expressed on the cell surface as two proteins. These form a stable complex, with one subunit responsible for the non-covalent cell wall anchoring of the second, large repetitive subunit. CwpV is expressed in a phase-variable manner controlled by an invertible DNA switch. The novel mechanism controlling this phase variation has been charaterised using enzymatic reporter assays.

The site-specific recombinase catalyzing the inversion of the CwpV DNA switch was identified and, by knocking out this recombinase in *C. difficile*, we have constructed CwpV-locked ON and locked OFF strains. These strains exhibit different colony phenotypes, which may reflect a role for CwpV during infection. Due to the significant genetic heterogeneity of *C. difficile*, the characteristics of CwpV across a panel of strains was investigated. The CwpV switch, the recombinase responsible for phase variation and the N-terminus of CwpV are well conserved across diverse *C. difficile* strains. However, the repetitive domain of CwpV varies markedly. Five different repeat types have been identified which are antigenically distinct. Over-expression of the five CwpV types in a ⊠cwpV strain revealed that CwpV mediates a distinctive colony phenotype and bacterial aggregation which can be visualised both at the macrocscopic and microscopic levels. The biological implications of these functionalities and the phase variation and strain-strain variation will be discussed.

**O9**

# THE MULTIPLE FACETS OF BACTERIOPHAGES AND THEIR ROLE IN THE DIVERSITY AND VIRULENCE OF *C. difficile*

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The TcdA and TcdB exotoxins are the main virulence factors in *C. difficile*. Other factors probably contribute to the virulence of this pathogen, but little is known about their nature and their role. Genomic microarray studies and whole bacterial genome sequencing revealed the presence of multiple mobile genetic elements in *C. difficile*, including several prophages. Integrated bacteriophages often play a key role in genome evolution and virulence, but only a few phages have been fully characterized in *C. difficile* so far, so their contribution is unclear. Recent data from our lab suggest that prophages may be more important than previously thought, stressing the need to characterize these elements.

We have induced several prophages from clinical isolates of *C. difficile* using mitomycin C and UV irradiation. Free phage particles were also found in stools of CDI patients, and we showed that they were spontaneously released from *C. difficile* during infection. The isolated phages were characterized and we sequenced the whole genomes of phages phiCD38-2, phiMMP02 and phiMMP04. These three phages share little genetic similarity and are different from other known phages in *C. difficile*. Known virulence factors or toxin genes were not detected in these phages, but we identified putative membrane and secreted proteins in genomic regions that could have been acquired through horizontal gene transfer and that could be involved in lysogenic conversion. Interestingly, phage phiCD38-2 was able to infect 99 of 207 clinical isolates representing 11 different PCR ribotypes, including most of the hypervirulent NAP1/027 isolates (79/89). More interestingly, upon integration of phiCD38-2 into the chromosome of a NAP1/027 representative isolate, toxin production increased by more than 2-fold in culture supernatants, as revealed by ELISA assays and immunodot blotting using monoclonal antibodies against TcdA and TcdB. Real-time qRT-PCR analyses showed that the levels of toxin mRNA were up to 5-fold higher in the lysogen carrying phiCD38-2. The exact mechanism by which this prophage exerts its effect on toxin production is currently under investigation.

In summary, our results show that prophages are highly diverse and mobile and that they likely participate to horizontal gene transfer during CDI. Moreover, they can alter toxin production in vitro, suggesting that other elements besides typical tcdC deletions may affect toxin production in this important pathogen. A better characterization of the prophage content in *C. difficile* is highly warranted.

**O10**

# INFECTION WITH DIFFERENT TOXINOTYPE STRAINS OF *Clostridium difficile* RESULTS IN CLEAR PATHOLOGICAL DIFFERENCES IN THE HAMSTER MODEL

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*Clostridium difficile* is the main cause of antibiotic associated disease, a disease of high socio-economical importance that has recently been compounded by the global spread of Ribotype 027 (BI/NAP1/027) distinguished by increased morbidity and mortality rates. Whilst the majority of *C. difficile* cases have been attributed to toxin A+B+ strains, some recent outbreaks have been associated with toxin A-B+ strains, which have high recurrence rates (up to 36%) and increased disease severity. The hamster model of infection is widely accepted as an appropriate model for studying aspects of *C. difficile* host-pathogen interactions. Using this model we characterised the pathological differences in infection with a toxin A-B+ strain (M68) and a toxin A+B+ strain (R20291), comparing survival times, spore shedding, bacteriological analysis, histology and toxin production. Hamsters infected with *C. difficile* R20291 succumbed to infection on average 51h post challenge but varied between 31-66h, whilst those infected with *C. difficile* M68 did not reach clinical end point (>14 days post challenge). Hamsters infected with M68 were shedding detectable spores up to 8 days post infection, c.a. 2.3x103 CFU/ml. Furthermore onset of clinical symptoms in these animals was preceded by an increase in internal body temperature, with the magnitude of this temperature spike associated with the severity and duration of symptoms. Bacteriological analysis at post-mortem showed that hamsters infected with R20291 were highly colonised in the caecum and colon, c.a. >106 CFU/ml where bacteria associated with the lumen were predominantly spore form whilst those associated with the tissue were in vegetative form. This is in contrast to M68 where, although they were colonised to a similar extent at post mortem, bacteria associated with the lumen were predominantly vegetative cells and those with the tissues were in spore form. In vivo toxin analysis and histology from gut samples showed differences between the two strains. Using the hamster model of *C. difficile* infection we show that *C. difficile* strains R20291 and M68 have different pathological differences.

**O11**

# PREVALENCE AND TYPING OF *C. difficile* IN A GROUP OF WHITE VEAL CALVES

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The white veal calf industry is specialized in rearing young dairy or beef calves on an exclusively milk powder diet. Colonization with *C. difficile* was followed in a group of 62 veal calves on a Belgian veal farm. The calves came from different herds of birth and arrived at the veal unit at an age of 14 days. During the sampling period (February and March 2010) the calves were housed in individual calf boxes in the same room. Objectives of the study were to determine the prevalence of *C. difficile* at different sampling points and to determine the genotype of the isolates. Rectal swabs were collected from 50 randomly selected calves on five different occasions: 14 (arrival), 18, 25, 32 and 46 days of age. Rectal temperature and fecal score were recorded. The swabs were cultured in enrichment media, and after alcohol shock plated on commercial selective agar plates. One to four isolates per sample were characterized by toxinoyping and PCR ribotyping. Out of 250 samples collected, 21 (8,4 %) were *C. difficile* positive. The prevalence of *C. difficile* positive calves was 10,0 %, 16,0 %, 12,0 %, 2,0 % and 2,0 % at age 14, 18, 25, 32 and 46 days, respectively. Altogether 15 calves (30%) were *C. difficile* positive in at least one sampling. One fourth of *C. difficile* positive calves were colonized on different sampling points, usually in two consecutive samplings and with the same genotype. Seventy five percent of the toxigenic *C. difficile* positive calves were asymptomatic (no fever, no diarrhea) at the time of sampling. Both, nontoxigenic and toxigenic strains of three different toxinotypes (0, XIb and V) comprising 6 different PCR ribotypes (SLO 010, 033, 033-like (SLO 060), 012, 066, 078/126) were detected. The highest diversity of strains was observed at first sampling on the day of arrival. In further samplings, isolates of variant toxinotype V and PCR ribotype 078/126 prevailed, accounting for 83,9 % of isolates vs. 28,6% identified at the first screening. PCR ribotype 078 has already been reported as common among pigs and calves. In 2 out of 21 *C. difficile* positive samples more than one PCR ribotype was detected. This study demonstrates that age dependent colonization with *C. difficile* occurs in both healthy and diarrheic veal calves under commercial conditions. Our results also suggest that one genotype can prevail on a single calf farm over a certain time period.

**O12**

# A NOVEL MOLECULAR TYPE OF *Clostridium difficile* IN NEONATAL PIGS IN AUSTRALIA

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*Clostridium difficile* causes intestinal disease in humans and a variety of animals. It has recently emerged worldwide as a major cause of intestinal disease in pigs less than 7 days of age, and has now become the most diagnosed cause of enteritis in pigs in this age group in the USA. *C. difficile* and its toxins can also be found in approximately 75% of apparently healthy pigs, suggesting that all pigs may be colonized with this organism soon after birth. There is also evidence that *C. difficile* is part of a zoonosis. The incidence of community-acquired disease is escalating and there is increasing overlap between the PCR ribotypes and toxin profiles of *C. difficile* causing human and animal disease, particularly in the community. Transmission may be via food-borne spores as *C. difficile* is found in up to 45% of retail meat, including pork and pork smallgoods, although this is yet to be proven.

The prevalence of *C. difficile* in Australian pigs is currently unknown. It is likely that the epidemiology of *C. difficile* disease in pigs is different in Australia due to geographical isolation and strict quarantine laws on the importation of livestock and animal products. We examined the PCR ribotypes and toxin gene profiles of *C. difficile* isolated from faecal material from 220 pigs less than 7 days of age from herds across Australia with a veterinary diagnosis of intractable scours. We also analysed samples from a herd of healthy pigs with no history of neonatal scours.

We found that toxigenic strains of *C. difficile* are very common in these herds, with an overall prevalence of 64%. The carriage rate in apparently healthy neonates from scouring and non-scouring herds was 94%. Clonal PCR ribotypes dominated in each herd. One of these ribotypes (UK 237) from the herd with the worst scours had not previously been described in Australia or elsewhere. All ribotypes from scouring pigs matched human isolates retrieved from our collection, but did not correlate with the dominant Australian human ribotype (UK 014). Ribotype 078, the most common porcine ribotype identified worldwide, was not found. Nor was epidemic *C. difficile* ribotype 027. PCR for toxin A, toxin B and binary toxin (CDT) was used to establish toxin profiles. Two toxin profiles, A-B+CDT+ and A+B+CDT-, were identified in scouring herds. Isolates from the asymptomatic herd were A-B-CDT+. The variation in toxin profiles raises questions about which toxins are essential for porcine *C. difficile* disease. These results confirm that *C. difficile* is present in neonatal pigs in Australia in numbers that mirror international prevalence figures, but that the epidemiology of porcine *C. difficile* disease in Australia is unique.

**O13**

# PREVALENCE AND CHARACTERIZATION OF *Clostridium difficile* IN HORSES

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Introduction: *Clostridium difficile* in an important equine pathogen, particularly as a cause of community-associated enterocolitis. Despite this, there is limited information regarding the prevalence and types of *C. difficile* in horses on farms and in training facilities, and little information about differences in prevalence between horse types.

Methods: Feces were collected from a Standardbred and Thoroughbred race-horses in active training on 4 racetracks, broodmares and foals on 2 breeding farms, and horses admitted to an equine veterinary hospital. Only horses with normal feces were included. Enrichment culture was performed and isolates were characterized by ribotyping and detection of tcdA, tcdB and cdtA using established PCR methods.

Results: *Clostridium difficile* was isolated from 52/742 (7.0%) horses ; 41/540 (7.6%) racehorses, 7/120 (5.8%) mares and foals on breeding farms and 4/82 (4.9%) horses at the equine hospital. There were no statistically significant differences in prevalence between the different groups (all P>0.08). There was also no influence of age on prevalence. 17 ribotypes were identified. 14 ribotypes were toxigenic, accounting for 87% of isolates. Ribotype 027 (tcdA, tcdB, cdtA) was the most common strain (27%) followed by ribotype 001 (tcdA, tcdB, 25%), a non-toxigenic strain (9.6%) and ribotypes 078 (tcdA, tcdB, cdtA) and 017 (tcdB) (5.8% each). 13/14 toxigenic ribotypes, accounting for 93% of toxigenic isolates, have been previously found in humans in the province. Overall, 38.5% of isolates possessed tcdA, tcdB and cdtA.

Discussion: *Clostridium difficile* was relatively common in healthy horses. The predominance of strains associated with human disease, particularly ribotypes 027 and 001, support the notion that *C. difficile* can be exchanged between humans and horses, however the direction, frequency, risk factors and clinical implications of this (for humans or horses) are not known. The lack of a difference in prevalence between racehorses and broodmares is in contrast with the high prevalences reported in some broodmare studies. Similarly, the lack of an association between young age and colonization is in contrast to some earlier reports of higher prevalences in foals, although the low number of foals may have played a role. It is also possible that age-related risk of *C. difficile* shedding is not consistent or is confined to specific age groups, such as very young foals, given evidence of marked changes in prevalence over short periods of time in neonatal piglets.

**L4**

# HUMAN GUT MICROBIOTA: FUNCTIONALITY AND BACTERIA-HOST CROSSTALK

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The human intestinal tract counts up to 1014 bacteria. In terms of cell number, this bacterial community represents 100 times the total number of tissue cells in the entire body, and the number of species has been estimated to reach 1,000 per individual's gut microbiota (Tap et al., 2009). The complex intestinal microbiota plays a beneficial role for its host in the metabolism of non-digestible food components (dietary fibers), in producing vitamins and short-chain fatty acids and shaping host physiology. Dysbiosis or dysfunctions of the intestinal microbiota are also suspected to be involved in several pathological situations, from focal diseases (e.g.: inflammatory bowel disease, colorectal cancer) to systemic ones (e.g.: allergy, obesity). However, no conclusive demonstration of a causative link has been given to date, due to our limited knowledge of the intestinal microbiota, mainly because of the difficulty to cultivate in vitro the largest part of the bacterial diversity (around 80% to 90% of total diversity). This represents a major technological hurdle. Thus, in order to better understand the implication of the intestinal microbiota in homeostasis and sustained human health, it is necessary to use and adapt the most innovative molecular technologies.

Today, via the metagenomics approach, the bacterial genomic content of an ecosystem can directly be accessed from the environment, without any cultivation step. Coupling this approach with DNA chips, quantitative PCR and other molecular techniques now allows quantitative and qualitative analyses of a microbial community within all its complexity. Furthermore, it is now established that each human being has its own gut microbiota composition. Recently, Tap et al. demonstrated the existence of a phylogenetic core within the human gut microbiota, representing a total of 66 bacterial species that are shared by at least 50% of a healthy human population. The existence of a common bacterial core composed of 75 bacterial species has further been proposed by the MetaHit consortium by applying Metagenomics (Qin et al, 2010). Yet very few study aim at describing functions expressed by these bacteria at the interface with intestinal epithelium. Getting insight into the functionality of these uncultivable bacteria and into the interactions that take place at the mucosal interface will constitute a major step toward the understanding of microbiome homeostasis and deregulation impact.

**O14**

# THE ROLE OF TOXINS IN *Clostridium difficile* INFECTION

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The two large toxins, TcdA and TcdB, are thought to be the main virulence factors in *Clostridium difficile* infection (CDI). In recent years so-called 'hypervirulent', epidemic strains of *C. difficile* have emerged, which reportedly produce more toxin A/B than the 'non-epidemic' strains. In addition to their individual roles it is of interest to compare toxin activity in vitro and in vivo between these two types of strains. Many of the epidemic strains carry a third toxin, a two-component ADP-ribosyltransferase (CDT). Little is known about the importance of this toxin in infection. Up to date it has been difficult to study the relative roles of the toxins in disease due to lack of genetic tools in Clostridia. Historically, toxin A was believed to have a dominant role in disease and toxin B only being able to evoke symptoms if prior damage to the gut mucosa occurred. However with the emergence of A-B+ strains it was recognised that toxin B alone can also cause disease. A recent study, in which unstable, single crossover mutants were generated, indicated that toxin A alone cannot cause disease in hamsters (1). This leaves a paradox over the individual importance of toxin A and toxin B in CDI. Making use of newly available genetic tools for Clostridia, we constructed stable, isogenic single and for the first time double mutants of tcdA and tcdB. The mutants were generated using the ClosTron mutagenesis system(2). They were characterised, analysed and tested in vitro (including cytotoxicity assays) and in vivo using the hamster infection model. Each toxin alone displayed cytotoxic effects, whereas no toxicity was measured in the double mutant (A-B-). Furthermore our findings re-establish the importance of both toxin A and toxin B and show that each of those two toxins alone can cause fatal disease in the hamster infection model.

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**O15**

# MITOGEN ACTIVATED PROTEIN KINASE ACTIVATED PROTEIN KINASE-2 (P-MK2): RATIONALE FOR CONSIDERING THIS PROMISING BIOMARKER IN *Clostridium difficile* INFECTION

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Background. *C. difficile* infection (CDI) can be difficult to diagnose on both clinical grounds and with laboratory methods to identify *C. difficile* or its toxins. This is due to asymptomatic colonization, multiple potential causes of diarrhea in hospitalized patients, and wide variation of sensitivity and specificity of *C. difficile* diagnostics. The signaling molecule, p38, the pre-cursor of P-MK2, is stimulated by *C. difficile* toxin A (tcdA). In addition, siRNA screening of the human kinome indicated Shiga toxin stimulates phosphorylation of MK2, and subsequent inflammatory and apoptotic cascades that may potentiate intestinal pathology. Based on these studies, we have evaluated P-MK2's role in CDI. Methods. An in-house EIA for P-MK2 was developed to measure levels in human fecal samples and toxin-stimulated T-84 and HT-29 colonic intestinal cells. Pure tcdA, and culture filtrates from high toxin producer, TW 10463, strain#76, a nontoxigenic *C. difficile* (no tcdA/B genes), and strain #70, a non-cytotoxic *C. difficile* isolate with a tcdB mutation were used in stimulation assays. P-MK2, IL-8, and effect of p38 and P-MK2 inhibitors were also analyzed by Western Blot. Results. P-MK2 EIA was sensitive to 30 ng/ml compared with 150 ng/ml by Western blot. Stimulation of T-84 and HT-29 cells with either tcdA or culture filtrate of TW 10463 exhibited a time and dose-dependent effect by either Western blot or P-MK2 EIA. The EIA detected significant P-MK2 production starting at 5 min, with Interleukin (IL)-8 produced between 60-180 min. Both p38 and P-MK2 inhibitors significantly decreased P-MK2's downstream target, Hsp 27, and subsequent IL-8 production. TW 10463 filtrate stimulated the highest level of P-MK2 of 450 pg/ml at 120 min from HT-29 cells by EIA, and P-MK2 production was inhibited by the addition of antitoxin. Strain #76 filtrate induced no more then 55 pg/ml of P-MK2. Strain #70 was isolated from the stool of a patient with hematochezia and diarrhea, and colonoscopy did not reveal pseudomembranes. Fecal cytotoxicity was negative, but tcdB was detected from stool with two commercially available PCR-based detection tests (BD GeneOhm Cdiff, Cepheid Xpert *C. difficile*). P-MK2, IL-8 and lactoferrin were not detected in stool, and strain #70 filtrate was not cytotoxic in Vero and HT-29 cells. Additionally, #70 stimulated <60 pg/ml of P-MK2 from HT-29 cells. Western blot of this isolate showed that it produced tcdA, but that tcdB was truncated. Conclusions. P-MK2 may be a useful adjunct in assessing whether the presence of *C. difficile* is significant in clinical samples, especially when diagnostic tests are conflicting.

**O16**

# HYPERVIRULENT *Clostridium difficile* INDUCES INTESTINAL DYSBIOSIS TO ENHANCE TRANSMISSIBILITY

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Hypervirulent *Clostridium difficile* (027/BI/NAP1) has emerged globally as a significant cause of antibiotic-associated diarrhea and healthcare-acquired infection. However, it is not known how hypervirulent *C. difficile* disseminates so efficiently and outcompetes other disease causing variants. We show that mice infected with hypervirulent *C. difficile* rapidly develop a highly contagious and persistent supershedder state and, as a result, hypervirulent *C. difficile* can outcompete other *C. difficile* within murine populations. We demonstrate that supershedding hypervirulent *C. difficile* are key components of a characteristic intestinal microbiota that is distinct in bacterial composition, diversity and metabolites compared to a healthy intestinal microbiota. We propose that the persistent supershedder state is linked to homeostatic imbalances within the intestinal microbiota that permits high-level hypervirulent *C. difficile* colonization and promotes both intestinal disease and efficient spore-mediated transmission. Importantly, restoration of a healthy intestinal microbiota with bacteriotherapy, but not vancomycin therapy, rapidly resolves relapsing intestinal disease and significantly decreases host contagiousness. Thus, our results suggest that hypervirulent *C. difficile* has evolved the ability to establish a persistent supershedder state in infected hosts, facilitating enhanced transmission by outcompeting inhibitory components of the indigenous microbiota.

**L5**

# CLASSICAL AND NOVEL APPROACHES TO *C. difficile* DIAGNOSTICS

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Since the discovery of *Clostridium difficile* as a cause of antibiotic associated diarrhea (AAD) and pseudomembranous colitis (PMC) over 30 years ago much has been written about *C. difficile* diagnostics. For many years faecal cytotoxin detection was regarded as THE test to do by many, particularly in the USA, despite its many flaws. This was replaced by enzyme immunoassays (EIAs), mainly to reduce turn-around-time. Unfortunately, EIAs were often evaluated against faecal cytotoxin detection as the "gold standard" making them appear better than they were. Lately, the problem of poor diagnostics for *C. difficile* infection (CDI) has again been high-lighted with the emergence of epidemic strains of *C. difficile* and their spread world-wide. Laboratory diagnostic methods can be divided into 3 groups; traditional faecal cytotoxin detection (with or without culture), EIAs and molecular methods. Faecal cytotoxin detection is specific but lacks sensitivity, culture is sensitive but lacks specificity without further analysis of isolates. Both are slow and require expertise which appears to be lacking in the modern scientist! Current PCR methods have the potential for great sensitivity and specificity, but are only available in larger well-funded laboratories, may have DNA extraction problems and are not always as sensitive as they should or could be. In-house tests can be quite cheap but commercial assays are still too expensive for widespread adoption. There is also the issue of *C. difficile* in developing countries where access to cheap, simple, reliable tests is crucial. Therefore the recently much maligned EIAs still have a role in such places, as well as in small to medium sized laboratories, providing there is recognition of their limitations. In addition, diagnostic companies need to strive to improve commercially-available tests, and/or lower the cost. Whatever method is chosen, it is necessary for the laboratory to have as fast a turn-around-time as possible, particularly in an outbreak situation. This allows infection control initiatives to be implemented quickly to avoid further spread. In the current situation of less than ideal cheap tests being available, the idea of using a rapid sensitive screening test in a 2-step algorithm has merit. It is also necessary to understand the molecular epidemiology of an outbreak. While in the future this may be achievable directly on the specimen, at present all laboratories should have the capacity to culture *C. difficile* and access to molecular typing facilities.

**O17**

# IMPACT OF *C. difficile* RIBOTYPE ON PERFORMANCE OF ANTIGEN-BASED DIAGNOSTIC ASSAYS AND RESULTS OF PCR FOR REPEAT SAMPLES ON SAME PATIENT

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Six medical centers from the United States and one from Canada used their own standard diagnostic assays and the Cepheid GeneXpert® C difficile assay to test a total of 2,296 unformed stools for the presence of toxigenic *Clostridium difficile*. Stools were cultured onto *C. difficile* selective agar directly or after broth enrichment. Isolates from cultures were identified with GLC and conventional methods and tested for cytotoxin production in a cell culture cytotoxin neutralization assay. 338 toxigenc strains were further characterized by pulsed-field gel electrophoresis, restriction enzyme analysis, and PCR-ribotyping. Using Xpert® C difficile PCR as the comparator, differences based on ribotype were seen in the accuracy of glutamate dehydrogenase (GDH) assays for 47 strains (two different commercial assays represented) and toxin A and B enzyme immunoassays (EIA) for 151 strains (two different commercial assays represented) among the 151 strains for which PCR ribotypes were assigned. Ribotype 027, which comprised around 25% of all isolates in the U.S. and > 50% of Canadian isolates, was detected with equal sensitivity (91%) by GDH and PCR. For non-027 strains, PCR was significantly more sensitive than GDH (92% vs. 72%). Sensitivity of the EIA assays varied dramatically among ribotypes, but PCR was ≥ 20% more sensitive for all ribotypes except ribotype 078 (which yielded an 18% difference). Conclusion: PCR and toxigenic cultures are the best methods for laboratory diagnosis of *C. difficile* infection. Toxigenic culture results take too long to be clinically useful.

A second PCR study involved a test for two molecular *C. difficile* targets performed on stool samples submitted from the same patients over periods of time varying from 1 to 14 days. Positive PCR results were confirmed using toxigenic culture. Of > 400 samples tested, only 10 were positive on the second or subsequent test. Only 3 (< 1%) positive PCR results were seen among > 250 additional tests performed < 7 days after the original negative result, confirming previous recommendations not to test additional samples from PCR-negative patients for at least one week.

**O18**

# RAPID AND SENSITIVE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR *Clostridium difficile* DIAGNOSIS CHALLANGE CYTOTOXIN B CELL TEST AND CULTURE AS GOLD STANDARD

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Currently, epidemics of *C. difficile* infection (CDI) featuring higher morbidity and mortality are experienced in both North America and Europe. Rapid diagnosis, inciting therapy, most likely prevent early progression of disease and avoid delays from excluding alternative pathology. The diagnosis of CDI is usually based on clinical history in combination with several laboratory tests, that for optimal sensitivity require both costs and labour. From rapid toxin A and B enzyme immunoassays with suboptimal sensitivity to the recent years of various PCR techniques directly on faeces these requirements have been met to some extent. Still, standard *Clostridium difficile* diagnostics using cytotoxin B cellculture assay (CTBA) and positive culture of toxogenic isolates (TC) is often referred to as the most sensitive method and considered the "gold standard". We added for comparison prospectively a new diagnostic approach using loop-mediated isothermal amplification (LAMP) assay (Illumigene™, Meridian Bioscience Inc., Cincinnati, USA) targeting a highly conserved 204 bp sequence within the tcdA region of the *C. difficile* pathogenicity locus (PaLoc) to our CTBA and TC used in clinical routine. A total of 272 consecutive faecal specimens from May to June 2010 were referred for *C. difficile* testing. Faecal culture on CCFA anaerobically for 48 hours and faecal filtrate (20 µl) incubated on a 96-well microtitre plate containing cultured McCoy cells for 48 hours at 37ºC were applied to all specimens as well as LAMP analysis. As a result 53 (19%) of samples turned out positive by LAMP of which 49 were also positive by TC+CTBA and one were positive in TC but negative in CTBA and LAMP. An additional positive result by rpoA PCR was confirmed among the four positives only in LAMP and this could indicate a higher sensitivity. Only 68% (38/53) were positive only by CTBA and TC tested positive in multiple days time added almost one third of the positives.

In conclusion, our LAMP result was produced within one hour turn-around time with a sensitivity and specificity of 0.98 and positive predictive value (PPV) and negative predictive value (NPV) of 0.92 and 0.99, respectively. LAMP thus compete excellent with "gold standard" presenting result to the clinician in average 55 hours ahead of CTBA+TC, enabling early diagnosis and treatment of potential importance in current severe CDI.

**O19**

# KILLING KINETICS OF FIDAXOMICIN AND ITS MAJOR METABOLITE (OP-1118) VS. Clostridium difficile

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Fidaxomicin (FDX) (formerly OPT-80 and PAR-101) is the first in a new class of narrow spectrum macrocyclic antibiotic drugs that is being developed for the treatment of *Clostridium difficile* infections (CDI). In a recent Phase 3 clinical trial, FDX demonstrated significantly lower recurrence (P = .004) and better global cure rate (P = .006) compared to vancomycin. The clinical success may be attributed to targeted bactericidal activity against C difficile and minimal disruption of the gut flora. In this study we examined the kinetics of bacterial killing for FDX and its major metabolite (OP-1118) vs. *Clostridium difficile* strains, which included two hypervirulent restriction endonuclease analysis (REA) group BI clinical strains (ORGs 1687 and 1698), ATCC strain 43255, and two laboratory derived mutant strains with increased FDX MIC values (ORGs 919 and 1620). Vancomycin, a known bacteriostatic drug, was included as control during the studies. Broth dilution method with Brucella broth supplemented with vitamin K and hemin was used for MIC determination and for killing kinetic studies.

Both FDX and its major metabolite demonstrated time-dependent killing of *C. difficile* strains, whereas vancomycin, as expected, was bacteriostatic vs. all strains tested. FDX (at 4x MIC) reduced bacterial counts of the ATCC strain and the two laboratory-generated mutant strains by > 3 logs and bacterial counts of the BI strain ORG 1698 by 3 logs within 48 hours of exposure. The other BI strain, ORG 1687, was only tested at 2x MIC concentration of FDX with bacterial counts dropping one log in 48 hours. Exposure to OP-1118 (at 4x MIC) also resulted in >3 log drop in CFU counts for ATCC 43255 and the mutant strain ORG 919, and by 3 log for ORG 1687. Higher concentrations of the major metabolite (32x MIC) were required for a 3 log drop in CFU counts for ORG 1698.

In summary, results indicate that both FDX and its major metabolite are bactericidal vs. *C. difficile* strains, including the hypervirulent REA group BI strains, at concentrations that are many fold below the detected fecal concentrations of these compounds after oral administration of FDX.

**L6**

# GUT IMMUNITY

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A delicate balance exists in the gut for maintaining the commensal organism population yet fending off intrusive pathogens, while also avoiding an exaggerated immune reactivity that may culminate in disorders such as inflammatory bowel disease. Barrier function of the epithelial lining and adjacent mucoid layer has long been recognized to be important in maintaining immune homeostasis in the intestine. However, data accumulating in recent years transformed our understanding of gut immunity to encompass active immune functions exerted by non-immune cells of the epithelium. It became apparent that the epithelium is capable of constitutive and common bacterial motif-driven secretion of luminal mediators (mostly peptides) with anti-bacterial functions. In so doing, the epithelium has evolved to be integrated in the innate immune system which constitutes the rapid but non-selective defense response against intrusive pathogens. Discoveries in the last decade have also unraveled unique mechanisms by which the recruitment of the adaptive immune system cells (T & B cells) takes place. Trans-migration and chemokine-driven migration of immunocytes has been elucidated and used for development of novel biologic antibody drugs to block these functions. Moreover, our sheer concept of immune memory has evolved to the understanding that memory is both for the antigen as well as for the intestinal locale where it was first encountered. We shall review these recent discoveries, and will provide overview of perturbances in these mechanisms that can lead either to increased susceptibility to certain pathogens as *C. difficile*, or conversely, bring rise to chronic intestinal inflammation in the form of ulcerative colitis and Crohn's disease.

**O20**

# THE POTENTIAL USE OF REPEAT REGIONS IN THE BINDING DOMAINS OF TOXIN A AND TOXIN B FROM *Clostridium difficile* AS A VACCINE CANDIDATE

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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) have largely attributed to the production of two exotoxins, Toxin A and B. Previously, it has been shown that vaccination with toxoided version of these proteins provides protection against severe disease in the hamster model of this infection. This model is an acute model of the infection with symptom onset being observed 36-48h post challenge of clindamycin treated animals with *C. difficile* spores.

Using recombinant proteins encoding the repeat regions from the binding domain (B-domain) of each toxin, we have determined combinations of antigens that can protect animals against severe disease. Results have shown that vaccination with either antigen individually could not prevent death in the hamster model, however, combinations of these antigens could protect against 100% of animals challenged with 630 and 83% of animals challenged with B1. Whilst animals could be protected from death by this vaccine most suffered from short bouts of diarrhea. These coincided with an elevation of approximately 1oC in body temperature, which also occurred in control animals, however, unlike these animals whose temperature subsequently dropped below the endpoint of 35oC, these animals temperatures returned to normal.

All surviving animals from the vaccination studies shed *C. difficile* in their faeces, with 90% of animals still colonised 14 days post challenge. Bacteriology in the caecum and colon of animals at 14 days showed that recovered bacteria were mainly in the vegetative form with only a relatively low number of spores present. In contrast, control animals which were culled during the acute phase of the disease showed high levels of spores in both the caecum and the colon. All strains of *C. difficile* recovered from the animals were confirmed to be the inoculation strain by multilocus variable repeat analysis (MVLA.

Toxin measurements of caecal and colon contents showed that low levels of toxin were present in the colon and caecum of vaccinated animals compared to controls.

**O21**

# THERAPEUTIC POTENTIAL OF EGG YOLK ANTIBODIES FOR TREATING *Clostridium difficile* INFECTION

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Herein we present evidence for the therapeutic potential of colonization factor (CF) -specific egg yolk antibodies for treating acute and recurring *C. difficile* infection (CDI) in humans. The study involved cloning, expressing as 6xHis-tagged-proteins in E. coli, and Ni-affinity purifying three previously identified CFs from *C. difficile*. The recombinant CF antigens were then used to immunize Leghorn chickens. Eggs produced by the immunized chickens were used to prepare lyophilized egg yolk preparations containing CF-specific IgY antibodies. The presence and titers of *C. difficile* CF-specific IgY antibodies in the egg yolks were assessed by the ELISA and western immunoblotting techniques. The potential efficacy of these antibodies in preventing *C. difficile*-mediated morbidity and mortality was subsequently investigated in Syrian hamsters. In these experiments, hamsters were pre-treated by gavage with clindamycin phosphate 5 days prior to administering (by gavage) 1 x 103 *C. difficile* strain 630 spores admixed with a 1:1:1 mixture of the CF-specific egg yolk preparations or purified IgY antibodies prepared from these egg yolks. The animals then received the mixture of CF-specific egg yolk preparations or purified IgY antibodies every 24 hours thereafter. Control groups received either 0.1M carbonate buffer (pH 9.5) (used to rehydrate the lyophilized egg yolk preparations) alone, or an egg yolk preparation produced from eggs laid by unimmunized chickens. Beginning 48 hours post-infection, and for 11 days thereafter, the animals were closely monitored every 4 hours for signs of *C. difficile* infection, including the appearance of peri-anal fecal staining and/or lethargy. Animals exhibiting these signs were immediately euthanized by CO2 asphyxia. The results revealed that hamsters treated with the cocktail of *C. difficile* CF-specific egg yolk preparations or a purified CF-specific IgY preparation were significantly protected from *C. difficile* strain 630 infection relative to control animals treated with carbonate buffer alone or an egg yolk preparation produced from unimmunized chicken eggs. The results suggest that egg yolk preparations obtained from chickens immunized with recombinant *C. difficile* CFs may represent an effective, safe, and cost-effective treatment option in humans suffering from CDI.

**O22**

# SAFETY AND TOLERABILITY OF AN ORAL SUSPENSION OF VP 20621, SPORES OF A NON-TOXIGENIC *C. difficile* STRAIN, IN HEALTHY OLDER SUBJECTS

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Background: Colonization of hamsters with non-toxigenic strains of *Clostridium difficile* is protective against subsequent challenge with toxigenic strains. Recurrence of *C. difficile* infection (CDI) might be prevented in humans by colonizing patients with non-toxigenic *C. difficile* (NTCD) after successful therapy for CDI.

Methods: A Phase 1 study was conducted to assess the safety and tolerability of an oral suspension of spores of a non-toxigenic strain of *C. difficile* (VP 20621) in healthy adult subjects. VP 20621 (104, 106, or 108 spores) or placebo was administered as a single dose to subjects age 18-45 (Group 1) or >60 years of age (Group 2). In Group 3 an oral suspension of 108 spores or placebo was administered twice daily for 5 days to patients >60 years of age. In Group 4 subjects >60 years of age received 5 days of oral vancomycin followed by 14 days of once daily VP 20621 (104, 106, or 108 spores) or placebo. All subjects were followed through Day 28. *C. difficile* stool cultures were performed at various time points. *C. difficile* isolates were tested for the production of toxin by enzyme immunoassay.

Results: VP 20621 was well tolerated; no serious or severe adverse events (AEs) were reported and no subjects discontinued study drug. In Groups 1-3 there were no subjects with AEs of diarrhea or change in stool form or frequency. In Group 4 during pre-treatment with vancomycin, 7% of subjects had mild diarrhea. During subsequent dosing with study drug, gastrointestinal AEs were reported in 22% (6/27) VP 20621 subjects (all doses) and 33% (3/9) placebo subjects; 3 (11%) VP 20621 subjects reported mild loose or watery stool on a single study day that did not require treatment and resolved despite continued dosing. Groups 1 and 2: no *C. difficile* was cultured from stool samples. Group 3: Non-toxigenic *C. difficile* was detected in stool cultures on various days from all active subjects between Days 2 and 7. Group 4: Non-toxigenic *C. difficile* was detected in all active subjects during the dosing period and in some subjects on Days 14 and/or 28. Non-toxigenic *C. difficile* was also detected in stool cultures of placebo subjects in the 108 spores cohort. Conclusion: This Phase 1 study showed VP 20621 to be well tolerated at all doses tested in younger and older volunteer subjects. Pretreatment with oral vancomycin created a susceptible environment for colonization, mimicking the clinical situation in which most CDI occurs. Non-toxigenic *C. difficile* was isolated from stool cultures of all subjects who received multiple doses of VP 20621. Future studies of VP 20621 in CDI patients are warranted.

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| Shaw H.A. | P93 | Characterising cell wall proteins of *Clostridium difficile* |
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# ABSTRACTS OF POSTERS PRESENTATIONS

SECTION 1 P1 to P51, Thursday

# HIERARCHICAL APPLICATION OF PCR-RIBOTYPING AND MULTILOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS (MLVA) AS A METHOD FOR EXAMINING THE MOLECULAR EPIDEMIOLOGY OF OUTBREAKS OF *Clostridium difficile* INFECTION IN SCOTLAND

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Objectives. Since late 2007, PCR-ribotyping has been used as a tool to support the National Mandatory Surveillance Programme for *Clostridium difficile* infection (CDI) in Scotland. From 2007 to 2009, ribotypes 106 (29.4%), 001 (22.0%) and 027 (12.6%) were predominant. Other frequently observed types included 002, 015, 014, 078, 005, 023 and 020. PCR-ribotyping lacks the discriminatory power for local epidemiological investigation. The aim of this study was to investigate the ability of Multilocus Variable Number Tandem Repeat Analysis (MLVA) to provide useful data for the molecular epidemiological investigation of CDI in local outbreaks and in the context of the national population in Scotland.

Methods. 350 isolates of *C. difficile* from cases of CDI, submitted to SSSCDRL over a period of 30 months from all health board areas, from healthcare associated outbreaks, sporadic cases, and community cases as well as multiple isolates from individual patients, were analysed by the MLVA method according to van den Berg et al. Ribotypes included 001, 002, 005, 014, 015, 020, 023, 027, 078 & 106. The number of tandem repeats was determined and results for the seven MLVA loci were analysed using eBurst v3.0.

Results & Conclusions. The use of MLVA as a supplemental subtyping method to PCR-ribotyping for *C. difficile* is highly discriminatory and was able to discern outbreaks through geographical and temporal clustering. Clear regional clustering was observed for some ribotypes, possibly indicating circulation of related types within areas over extended periods. Some loci (CdF3 and CdH9) were found to be highly conserved within ribotypes. Some MLVA loci were not amplified in all ribotypes and the technique may not be universally applicable to all *C. difficile*. Variation was observed in multiple isolates from the same patient taken over time. The interpretation of MLVA data will require comparison with databases of results from a geographically and temporally diverse collection.

**P2**

# AN OUTBREAK OF *Clostridium difficile* INFECTIONS CAUSED BY TWO DIFFERENT PCR RIBOTYPES OCCURRING CONCURRENTLY AT A HOSPITAL IN JAPAN

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Backgrounds: Recently, outbreaks of *Clostridium difficile* infection (CDI) have been increasingly reported worldwide. *C. difficile* isolates causing an outbreak of CDI occurred in a 520-bed hospital in Japan were analyzed.

Methods: A total of 22 stool specimens obtained from 19 patients with diarrhea admitted to a hospital from April to June 2009 were examined. *C. difficile* isolates were analyzed by PCR ribotyping and by sequencing of the surface layer protein A gene (slpA). The toxin B gene on DNA extracted from stool specimens, which were cultured overnight in cooked meat medium (CMM), was detected by loop-mediated isothermal amplification (LAMP) assay.

Results and Discussion: *C. difficile* was isolated from 18 of the 22 stool specimens. Of the 18 isolates, 8 were toxin A-positive, toxin B-positive (A+B+), 9 were toxin A-negative, toxin B- positive (A-B+), and the remaining was toxin A-negative, toxin B-negative (A-B-). No isolates was found to be positive for the binary toxin gene. Seven of the 8 A+B+ isolates were typed into PCR ribotype y-smz and all the 9 A-B+ isolates were PCR ribotype trf. The slpA sequences of the 9 A+B+ isolates were all identical; all the 9 A-B+ isolates were typed into the same slpA sequence type. The amplification product of the toxin B gene by LAMP was obtained in all 17 specimens from which toxin B-positive *C. difficile* was cultured. In one specimen, the toxin B gene was positive by LAMP assay but a toxin B-negative *C. difficile* strain was recovered. Culture of the specimen was repeated and the resultant 10 colonies were randomly selected for the following study. Two of the 10 isolates were A+B+; 2 were toxin A-B-. One of A+B+ isolate was typed as the predominant type, PCR ribotype y-smz. These results suggest that two types of *C. difficile* were concurrently spreading in this hospital. Detection of the toxin B gene by LAMP assay from the overnight culture of stool specimens may be a useful and rapid tool for the diagnosis of CDI.

**P3**

# MOLECULAR CHARACTERIZATION AND CLINICAL OUTCOME IN PATIENTS WITH CDAD IN AN AUSTRIAN HOSPITAL

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Background: *Clostridium difficile* is responsible for a wide range of intestinal disease in hospitalized patients treated with antibiotics. Symptomes vary from mild diarrhea to pseudomembranous colitis (PMC) and death. The aim was to characterize hypervirulent strains (like ribotype 027) and to describe the clinical outcome of the patients in an Upper Austrian hospital. Methods: Suitable polymerase chain reaction (PCR) methods were established to differentiate between toxin A+B+ and toxin A-B+ variant strains (1) and to recover strains with the binary toxin (2) and variations in the pathogenicity locus (PaLoc) (3) of *Clostridium difficile*. For ribotyping the intergenic spacer region between the 16S and 23S rRNA genes was amplified (4). Patients: In our study we analysed 53 patients with an median age of 66 years. Thereof 10 patients were male and 43 female. During the last 3 years 29 of the infected persons were hospitalized for a median duration of 16,5 days. For 45 of the 53 patients a prior antibiotic treatment was documented. Results: In order to obtain molecular typing 60 isolates from 53 patients were analyzed from 11 hospital wards over a period of 2 years. Among those samples 52 were tested *Clostridium difficile* positive by determining the presence of the house keeping gene tpi. 6 samples showed no growth on selective agar plates and 2 were not further characterized. 51 of our samples tested positive for toxin A and B. One Patient sample proved to have no toxin A but toxin B (A-B+). Additionally, 3 isolates were carrying the binary toxin gene. Furthermore, 2 variant strains with deletions in the tcdC gene of the pathogenicity locus were found. The presence of certain ribotypes with dramatic outbreaks (like *C. difficile* ribotype 027) could not be located in our hospital. From 53 patients with hospital acquired *C. difficile* infections 12 showed an antibiotic-associated colitis and 2 a PMC. One patient developed a PMC with toxic megacolon and one a PMC with sepsis. 34 *C. difficile* patients had no complication and 3 were not documented. All together 8 patients died and 5 of them in consequence of *Clostridium difficile* infection.

Conclusion: From 53 patients 5 died in consequence of CDAD but we found no correlation between hypervirulent strains and mortality of the patients in our hospital. Patients which were infected with a hypervirulent strain recovered completely.

**P4**

# SUB-TYPE DIVERSITY OF CLINICAL ISOLATES OF *Clostridium difficile* RIBOTYPES 027 AND 078

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Increasing reports of *C. difficile* infection have been associated with the hyper-virulent ribotype 027/NAP-1 strain. The epidemiology of this strain in Europe and North America has been well documented. The epidemiology of *C. difficile* ribotype 078 in humans is less well understood, despite increasing in prevalence in several European countries.

In this study we used a semi-automated rep-PCR molecular sub-typing method (DiversiLab) in conjunction with ribotyping, toxinotyping and antimicrobial susceptibility testing to detect sub-types within ribotypes 027 and 078. By stratifying molecular techniques with information regarding origin of infection, healthcare facility location and date of sample acquisition, we have been able to investigate genetic diversity within each ribotype, antimicrobial-resistant sub-types, persistence of sub-types over time and the role of healthcare facility-specific clones.

Clinical isolates of ribotypes 027 (toxinotype III) (n=30) and 078 (toxinotype V) (n=23) were collected from healthcare facilities across the Republic of Ireland over 2 months in 2006 and 2009. Ribotype 027 isolates clustered into 9 rep-PCR sub-types (cut-off >90% similarity). Representatives of the largest sub-type which contained half of all ribotype 027 isolates studied, exhibited 89% similarity to a rep-PCR profile of the North American NAP-1 strain. Rep-PCR sub-typing correlated well with antimicrobial susceptibility profiles, in particular clindamycin susceptibility. Comparisons of isolates within and between healthcare facilities from 2006 and 2009, confirmed a change in epidemiology of ribotype 027 in Ireland over this time and the wide geographical clonal spread of the recent predominant sub-type.

Ribotype 027 isolates were significantly more likely to be related to each other (similarity ≥ 95%) compared to ribotype 078 isolates ($p = 0.001$). Ribotype 078 isolates clustered into 14 sub-types (cut-off >90% similarity) and almost half (45.5%) were unrelated to another isolate (<88.2% similarity). A greater diversity in antimicrobial susceptibility profiles was also seen in 078 isolates, even within a single healthcare facility.

Clonality of ribotype 027 isolates suggests effective adaptation to the human niche, whereas the considerable genetic diversity seen within ribotype 078 isolates suggests that they may have originated from a variety of healthcare and possibly non-healthcare sources.

**P5**

# PREVALENCE OF *Clostridium difficile* PCR RIBOTYPES IN BULGARIA DURING AND AFTER THE PAN EUROPEAN SURVEILLANCE STUDY 2008-2010

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Objectives: To analyze the prevalence of *Clostridium difficile* ribotypes in Bulgaria determined during the pan European Surveillance Study and the next 16 months.

Methods: For the period November 2008 - March 2010, sixty five fecal samples from patients with mild to severe enterocolitis and previous antibiotic treatment have been investigated using conventional microbiological and genetic methods. The stool samples derived from nine hospitals situated in Sofia and Plovdiv regions. Strains were typed and further characterized for the presence of toxins A (TcdA), B (TcdB)_and binary toxins (CdtA and CdtB).

Results: The incidence of *C. difficile* infections estimated recently for Bulgaria is 3.12 per 10,000 patient admissions (0.7 per 10,000 patient-days). Fifteen patients with different clinical forms of CDI were identified during the present study. Six of the isolates (40%) belonged to PCR ribotypes 017 (TcdA-; TcdB+; CdtA/B-). The second most prevalent ribotype (12%) was 078 (TcdA+; TcdB+; CdtA/B+). The remaining *C. difficile* isolates belonged to 001, 002, 012 and 046 ribotypes. All of these ribotypes have been reported to cause oubreaks worldwide.

Conclusion: During the pan European surveillance study Bulgaria initiated a laboratory-based investigation of CDI. The most prevalent *C. difficile* ribotype in our country was 017 and the second - 078 that were found to be associated with severe CDI.

**P6**

# MOLECULAR EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF *Clostridium difficile* ISOLATES IN BELGIUM

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Aim: *Clostridium difficile* associated diarrhea is a global health problem and new virulent strains are emerging. Data regarding the molecular epidemiology and antimicrobial resistance of *C. difficile* isolates in Belgium remain limited.

Materials and methods: Over a one year period from July 2008 to June 2009 our National Reference Center ribotyped 909 strains from all over the country, using PCR-ribotyping and DNA fragments analysis by capillary electrophoresis. Minimal inhibitory concentrations (MIC) of moxifloxacin, metronidazole and vancomycin were determined on 126 selected strains using E-tests susceptibility testing.

Results: Among the 909 strains a total of 119 different ribotypes were identified. Ribotype 027 (21,7%), 014 (8,8%), 002 (3,9%) and 078 (3,7%) were predominant. The ribotype 027 (N=20 strains tested for AB susceptibility) showed a clear reduced susceptibility to metronidazole compared to other ribotypes: MIC 50 was 1,0 mg/L and MIC 90 1,5 mg/L as compared to MIC 50 of 0,190 mg/L and MIC 90 of 0,380 mg/L for other ribotypes (N=106). All but one 027 strains were resistant to moxifloxacin. We detected moxifloxacin resistance in 3 other ribotypes, which all showed a deletion in the tcdC gene and the presence of the binary toxin gene. These 3 ribotypes did not show a reduced susceptibility to metronidazole. The highest MIC of vancomycin was 0,75 mg/L and there was no significant difference among strains.

Conclusion: New emerging ribotypes resistant to fluoroquinolones show up and justify molecular epidemiology and antimicrobial surveillance. No resistance was found towards metronidazole but a ribotype specific reduced susceptibility.

**P7**

# MULTILOCUS VARIABLE-NUMBER TANDEM-REPEAT ANALYSIS: AN INTERESTING TOOL TO SUBTYPE *Clostridium difficile* ISOLATES

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Molecular typing methods are frequently used to detect emergence of new clones of *C. difficile* and to investigate outbreaks. Several of these commonly used genotyping methods (i.e. PCR-ribotyping, pulsed field gel electrophoresis) lack discriminatory power, more particularly when a clone of *C. difficile* is predominant within a hospital or a country (such as PCR ribotype 027). MLVA (Multi-Locus VNTR [Variable Number Tandem Repeat] Analysis) is highly discriminatory and has been successfully used for investigating *C. difficile* outbreaks. The objective of this study was to evaluate the usefulness of MLVA for typing and subtyping strains of *C. difficile*.

Sixty strains were studied including 18 strains of toxinotype III (4 historical PCR-ribotype 027 and 14 epidemic isolates from different hospitals and various countries), 16 strains of toxinotype V (including 3 PCR-ribotype 078 and 2 PCR-ribotype 126), 19 strains of toxinotype 0 (including 3 strains from a possible transmission from a patient to a nurse and strains from a well documented outbreak in Paris) and 7 strains of various toxinotypes (VIII, IX/XXIII and IV). Stability was tested by comparing the typing results of 2 strains subcultured 11 times and by testing 4 colonies of the same fecal specimen. After DNA extraction, 7 tandem repeats loci (A6, B7, C6', E7, F3, G8, H9) from published MLVA schemes were amplified by PCR and sequenced. The distance between two strains was determined by calculating the STRD (summed tandem repeat difference). Genomic diversity was evaluated by using the minimum spanning tree (Bionumerics 5.1 software program, Applied Maths).

Among the 60 *C. difficile* isolates examined, 54 unique MLVA-types were identified suggesting a high discriminatory power. Eight clusters (defined by a STRD ≤ 10) were identified including from 2 to 14 strains. A good agreement was observed between MLVA types and toxinotypes with the exception of strains of toxinotype IV and VIII. Stability of VNTR loci was good (STRD ≤1). MLVA could separate isolates of the hypervirulent PCR ribotype 027 clone in several clusters; all 027 strains isolated within a hospital were grouped in a specific cluster. Results of MLVA also indicated that strains from toxinotype V were closely related whatever their PCR ribotypes.

This highly discriminatory method is useful to investigate outbreaks and to understand molecular evolution of *C. difficile*. MLVA remains however time consuming and expensive but is an interesting tool to subtype *C. difficile* isolates.

**P8**

# EVALUATION OF REPETITIVE-SEQUENCE-BASED PCR FINGERPRINTING COMPARED WITH PCR-RIBOTYPING OF *Clostridium difficile* STRAINS

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*Clostridium difficile* is a common pathogen of health care associated *Clostridium difficile* infection (HA-CDI). Community associated CDI (CA-CDI) seems to be increasing as well. Therefore, we need DNA typing techniques which are more discriminatory to differentiate the origin of strains.

Several molecular typing methods are used for genotyping of *C. difficile*. PCR-ribotyping and PFGE are the reference standard in typing *C. difficile* in Europe and US.

Repetive-sequence based PCR fingerprinting (rep-PCR) is a typing technique that uses primers that target noncoding repetitive sequences interspersed throughout the bacterial genome. Semi-automated rep-PCR (DiversiLab, bio-Merieux) could offer good standardization and useful discrimination power.

We compared rep-PCR with PCR-ribotyping using 103 *C. difficile* strains isolated in a tertiary care hospital in Korea. Of 103 isolates, the number of tcdA+tcdB+, tcdA-tcdB+ and tcdA-tcdB- strains were 46, 45 and 12.

Using rep-PCR method, a total 31 different rep-PCR patterns were obtained. In tcdA+tcdB+, tcdA-tcdB+ and tcdA-tcdB- strains, 12, 8 and 11 patterns were identified respectively. With PCR-ribotyping, 9, 2 and 6 patterns in tcdA+tcdB+, tcdA-tcdB+ and tcdA-tcdB- strains were observed. Most of tcdA-tcdB+ strains (97.7%) showed same pattern (PCR-ribotype 017) in PCR-ribotype but more diverse patterns in rep-PCR, which could be useful with the infection control in tcdA-tcdB+ strain prevalent areas.

This suggested rep-PCR is a reliable and valuable tool for the discrimination and delineation of *C. difficile* strains.

**P9**

# MOLECULAR CHARACTERIZATION AND RESISTANCE MECHANISMS OF MAJOR *Clostridium difficile* CLONES CAUSING SEVERE INFECTIONS IN A LARGE ITALIAN TEACHING HOSPITAL

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Objectives: *Clostridium difficile* (Cd) is recognized as the main cause of hospital acquired antibiotic-associated diarrhoea worldwide. Its virulence is attributed to the production of toxins A and B. From 2003 increasing incidence, severity and mortality of Cd infection (CDI) were reported and related to the spread of highly virulent strains, such as PCR-ribotype 027 and 078. These hypervirulent strains produce the binary toxin (CDT) and harbour mutations/deletions in the tcdC gene. We previously reported for the first time the presence of ribotypes 027 and 078 in Italy (Baldan R. et al., Clin Infect Dis 2010, 50 (1): 126-127); we now analyzed at the molecular level all the toxigenic Cd strains isolated from April 2007 to April 2010 from symptomatic patients admitted at the San Raffaele (hSR), a large Northern Italy nosocomial setting, in order to identify and characterize the main Cd clones and evaluate their spread.Methods: we characterized by PCR-ribotyping, detection of tcdA, tcdB, cdtA/cdtB genes and sequencing of the tcdC gene 213 Cd strains. All strains were investigated by PCR for the presence of ermB gene, responsible for the MLSB resistance phenotype, and of gyrA C245T mutation, associated to high-level fluoroquinolones (FQ) resistance. Selected strains were subjected to susceptibility testing to levofloxacin, moxifloxacin, metronidazole and vancomycin by E-test.Results: the main clone in hSR was the ribotype 018, accounting for 31.5% of isolates. 19% of the strains showed a band pattern closely related to ribotype 018, followed by 8% belonging to the hypervirulent ribotype 078, 6% to 020, 5% to 001 and 4.7% to 126. Only 1 case of severe CDI was caused by ribotype 027. CDT was detected in 22% of the isolates in association with tcdC deletions/mutations. 26.5% of the strains carried the ermB gene, present in half or more of strains belonging to ribotypes 078, 126 and 001. gyrA C245T mutation was detected in all isolates belonging to ribotype 018 and 027 and in more than 60% of ribotypes 078, 126 and 001. The strains susceptible to moxifloxacin but resistant to levofloxacin presented the mutation in gyrB T1246G. Interestingly, all isolates belonging to 020 ribotype, that appeared in hSR in 2009, did not harbour the C245T mutation and were fully susceptible to FQ. All strains tested were susceptible to vancomycin and metronidazole.Conclusion: the main clone detected in hSR was the ribotype 018, whose establishment was partly due to the increased use of FQ in the last years. In 2007-2008 we documented the circulation of the hypervirulent clones 027 and 078, never reported in Italy before, with 078 representing the second most prevalent ribotype in our institution. We are now investigating the adaptation mechanisms and virulence properties of our successful clone 018 contributing to its establishment.

**P10**

# CHARACTERISTICS OF *Clostridium difficile* PCR-RIBOTYPE 027 ISOLATES AND ASSOCIATION WITH DISEASE ACTIVITY IN A LARGE HOSPITAL SETTING IN LIVERPOOL

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Background: Over the past decade the morbidity and mortality associated with *Clostridium difficile* infection (CDI) has increased considerably. This has coincided with escalating prescriptions of new-generation antibiotics and the emergence of epidemic strains, particularly PCR-ribotype 027/NAP1/BI (R-027), which has caused several outbreaks in North America and Europe. R-027 has recently become the predominant strain in the United Kingdom. There is growing evidence that this strain also accounts for higher rates of severe disease and death. Furthermore, there have been reports of *C. difficile* strains becoming less susceptible to metronidazole and vancomycin, mainstay antimicrobials used for therapy of CDI.

Our objective was to investigate the profile of R-027 among 177 patients consecutively recruited to a prospective study and then assess its association with several clinical parameters used to evaluate the course of the disease. Assessment of disease severity was based on the Health Protection Agency scale. Screening of faecal leukocytes (an indicator of the degree of intestinal inflammation) was performed by observing a wet preparation of faecal sample on a slide. The MIC of metronidazole and vancomycin was determined by E-test for a collection of isolates based on their temporal distribution in our hospital.

Results: One-hundred and fifty-one *C. difficile* strains recovered from 177 clinical cases were characterised by PCR-ribotyping (85%). Of these strains R-027 was the commonest type (45%), followed by PCR-ribotypes 106 (11%) and 078 (7%). When compared to other groups, R-027 predicted increased levels of toxin in stools (OR=2.1, p=0.02), positively correlated with the presence of faecal leukocytes (coef.=+0.86, p=0.04), and was significantly associated with severity of the disease (OR=2.63, p=0.004). All isolates were inhibited by a concentration of ≤0.5 mg/L of metronidazole and vancomycin with no evidence of strains with decreased susceptibility.

Conclusion: In our derivation cohort, R-027 was correlated with CDI activity, accounting for increased severity and possibly higher levels of intestinal inflammation, with no evidence of decreased susceptibility to either metronidazole or vancomycin. Further work is warranted to validate our findings using an independent set of samples.

**P11**

# THE MOLECULAR EPIDEMIOLOGY OF *Clostridium difficile* IN CANADIAN HOSPITALS: 2005-2009

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Objective: The emergence of hypervirulent strains causing serious *Clostridium difficile* infections (CDI) is a growing concern in Canada and many other countries. This study examined the molecular epidemiology of *C. difficile* from 2005 to 2009 in Canada.

Methods: In April and May 2005, 2007, 2008, and 2009, all toxin positive stools identified from inpatients at 29 sites from the Canadian Nosocomial Infection Surveillance Program (CNISP) were submitted to the National Microbiology Laboratory. *C. difficile* was isolated using an alcohol shock procedure. PCR was used to detect the toxin tcdA and tcdB genes, to confirm the species (tpi), to detect variations in the size of the tcdC gene, and to detect the presence of binary toxin (cdtB). Isolates were typed by PFGE using SmaI and fingerprints were analysed with BioNumerics (v 5.0) software.

Results: A total of 1,225 *C. difficile* isolates were typed with 12 North American Pulsovar (NAP) types identified over the study period. The three most common strains identified were NAP1 (42.5%; n=521), NAP2 (15.5%; n=190), and NAP4 (7.9%; n=97). NAP1 increased from 28.6% (2005) to 48.8% (2007), and then slightly declined to 41% (2009); NAP2 decreased from 27.8% (2005) to 7.7% (2009); and NAP4 increased from 5.1% (2005) to 12.8% (2009). All three of these NAP types have been identified in all regions of Canada. The NAP7/8 strains have increased from 1.1% in 2005 to 2.7% in 2009.

Conclusions: In general, *C. difficile* strain types that have been reported to be more virulent due to deletions in the tcdC (NAP1 and NAP7/8) are increasing in Canada and seem to be replacing other strain types without tcdC deletions. However, the NAP4 strain with a wildtype tcdC amplicon size continues to increase as well in Canada. Further studies into the virulence and fitness of these epidemic strains should be undertaken.

**P12**

# CHANGEIN PREVALENT PCR RIBOTYPES OF *Clostridium difficile* IN A TERTIARY CARE KOREAN HOSPITAL OVER 10 YEARS

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The aim of this study was to evaluate the relevance of changes in prevalence of various PCR ribotypes of *Clostridium difficile* over a 10 year period.. A total of 1407 clinical isolates of *C. difficile* from patients with diarrhea in a tertiary care hospital in Korea from 2000 to 2009 was analyzed. Toxin production of *C. difficile* was determined by PCR. PCR ribotyping was performed by O'Neill's method. Of the total 1407 isolates, 1103 (78.5%) were toxigenic *C. difficile*. Overall recovery rates of A+B+ CDT-, A+B+ CDT+, and A-B+ CDT- strains were 58.4%, 3.1%, and 17.0%, respectively. A total of 74 different ribotype patterns were found. There were 33 patterns within A+B+ CDT- strains, 11 within A+B+ CDT+, 1 A-B+ CDT- and 29 within A-B-. Ribotype groups were designated by upper and lower-case letters combined with a number. Four ribotypes patterns showed definite change of prevalence during the study period. These four ribotype accounted for 799 of the 1407 isolates (56.8%). Ribotype AB1 (A+B+ CDT- ) was a predominant type in 2000 (65.9%, 118/179) and then rapidly decreased (11.3% in 2004, 2.1% in 2009). Ribotype aB (A-B+ CDT-) was only 4.5% in 2000, peaked in 2004 (39.6%), and then decreased to 8.5% in 2009. On the other hand, ribotype AB2 was 2.8% in 2000, increased to 17.0% in 2004, and then steadily isolated until 2009 (11.9%). Ribotype AB17 was not found until 2005, thereafter gradually increased to 15.7% in 2009 and became the most frequent ribotype in 2009 (58/239).

**P13**

# *Clostridium difficile* INFECTION –MONOCLONAL OR POLYCLONAL?

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Background/Objectives: *Clostridium difficile* is considered a leading cause of hospital-acquired diarrhea and can cause a potentially fatal illness. Recurrence of the disease and refractoriness to therapy pose major problems in treatment of *Clostridium difficile* infections. We were interested in the question whether *Clostridium difficile* infection (CDI) is of monoclonal or of polyclonal origin. Methods: In the time period April - June 2010, five different isolates were taken from each primary culture plate of routine stool cultures of 11 consecutive CDI-patients. Fifty-five isolates were then sent to the National Reference Laboratory for *C. difficile* at the Austrian Agency for Health and Food Safety (AGES) in Vienna for species-confirmation, toxin detection, in-vitro susceptibility testing against clindamycin, vancomycin, moxifloxacin and metronidazole, and PCR-ribotyping.

Results: Of 55 isolates sent, 40 could be recultured. Ten of those eleven patients were confirmed to be positive for *C. difficile*. It was possible to reculture 3 to 5 isolates from each patient. One patient had four isolates of PCR-ribotype 438 and one isolate of PCR- ribotype 232. In the other 9 patients, we found only a single PCR-ribotype. Except of the four isolates of PCR-ribotype 438 (positive for toxin A, toxin B and binary toxin), all the other 36 isolates tested positive on toxin A plus toxin B only. Antimicrobial susceptibility testing revealed that the clones did not differ among each other except in two patients. They differed in their Clindamycin MICs (intermediate to fully sensitive). The most frequent ribotypes were 053 (n=12), 018 (n=7), 538 (n=5) and 002/2 (n=5), accounting for 29 of the 40 isolates tested.

Conclusion: Monoclonal pathogenesis of CDI is more likely than polyclonal genesis. This finding underlines the value of PCR-ribotyping in epidemiological investigation of transmission in outbreaks and as a tool to differentiate CDI-recurrence from CDI-reinfection.

**P14**

# MOLECULAR EPIDEMIOLOGY OF *Clostridium difficile* AT A HOSPITAL IN JAPAN: SHIFTS IN THE PREDOMINANT PCR RIBOTYPE OVER A FIVE-YEAR PERIOD

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Objectives: In Europe and North America, large outbreaks due to hypervirulent strains such as *Clostridium difficile* PCR ribotype 027 have been reported. The aim of this study was to investigate the prevalent PCR ribotypes in a hospital in Japan and to know nosocomial transmission of specific strains of *C. difficile* including PCR ribotype 027.

Methods: Subjects were 66 patients who suffered from *Clostridium difficile* infection (CDI) from January, 2005 to December, 2009 in Gifu Red Cross hospital, a general hospital with 352 beds. CDI was defined as diarrhea and a stool sample positive for toxic-culture. *C. difficile* isolates were analyzed for the presence of the toxin genes by PCR and typed by the PCR ribotyping technique.
Results: A total of 77 isolates of *C. difficile* were recovered from 77 episodes of 66 patients. The number of isolates obtained during each yearly period was sequentially 20, 12, 8, 14, and 23. Of the 77isolates, 46 (59.7%) were toxin A-positive, toxin B-positive, and binary toxin-negative (A+B+CDT-), 5 (6.5%) were toxin A-positive, toxin B-positive, and binary toxin-positive (A+B+CDT+), and 26 (33.8%) were toxin A-negative, toxin B-positive, and binary toxin-negative (A-B+CDT-). The 77 isolates were classified into 20 types by PCR ribotyping. The most predominant type was PCR ribotype trf (26 isolates, 33.8%), the second most frequent type was PCR ribotype smz (14 isolates, 18.2%), and the third frequent type was PCR ribotype 002 (11 isolates, 14.3%). The predominant type shifted from PCR ribotype smz (A+B+CDT-) in 2005 (7/20, 35.0%) to PCR ribotype trf (A-B+CDT-) in 2006 (10/12, 83.3%), and then, to PCR ribotype 002 (A+B+CDT-) (8/23, 34.8%) in 2009. PCR ribotype 027 was isolated from one patient with pseudomembranous colitis, who had recurrent diarrhea caused by the same PCR ribotype. Of 9 patients with recurrent CDI, 8 patients relapsed with the original PCR ribotype strain and 1 patient acquired another PCR ribotype strain.

Conclusion: The molecular epidemiology of CDI in our hospital was characterized by changing twice the dominance of specific *C. difficile* PCR ribotypes over a five-year period.

**P15**

# RAPID TYPING OF *Clostridium difficile* BY MALDI-TOF MASS SPECTROMETRY

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In the past decade, incidence of *C. difficile* infections (CDI) with more severe disease has increased, which is associated with the emergence of hypervirulent PCR ribotype 027. In addition, a rise of a new hypervirulent strain has been noticed in The Netherlands due to PCR ribotype 078, affecting younger people with similar CDI severity. The aim of this study was to explore the application of matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) as a rapid technique to recognize emerging *C. difficile* strains. Since November 2009, MALDI-TOF MS is a validated and implemented technology for routine bacterial identification at the LUMC clinical microbiology lab. The MALDI-TOF Microflex system (Bruker Daltonics) is combined with the Bruker Biotyper database which contains approximately 3.500 organisms, including (n= 10) reference strains of *C. difficile* . Bacterial identification is performed using an algorithm whereby scores above 2.3 are classified as identification on species level with high certainty. First, a separate database was build for *C. difficile* using spectra from over 50 different isolates belonging to 7 different PCR ribotypes (001, 027, 078, 012, 014, 044 and 087). For this purpose only spectra of high quality were used. Whole cell protein extracts were made by performing an ethanol wash followed by a formic acid/ acetonitril extraction. 0,8 ul sample was transferred on a polished steel targetplate (Bruker Daltonics) and covered with a-Cyano-4-hydroxycinnamic acid (HCCA) matrix. Reference spectra were obtained for 7 different reference PCR ribotypes, 001, 027, 078, 012, 014, 044 and 087. Second, we typed over 50 clinical isolates obtained from the National Reference Laboratory and belonging to the above mentioned PCR ribotypes with the following results: 100% identification Type 027 (42 spectra from 7 isolates), 94.3% identification Type 078 (72 spectra from 12 isolates), 94.5% identification of Type 001 (72 spectra from 12 isolates). The other PCR ribotypes (012, 014, 087 and 044) scored lower ranging from (68.5% until 90.3%). All bacterial identification scores were high (> 2.4). Therefore, we conclude that MALDI-TOF MS is capable to recognize strains of *C. difficile* type 027 and 078, but that improvement is needed for the other types.

**P16**

# PREVALENCE AND DIVERSITY OF *Clostridium difficile* STRAINS IN INFANTS

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Introduction. *Clostridium difficile* is the leading cause of antibiotic-associated diarrhoea and pseudomembranous colitis. Recently, the epidemiology of *C. difficile* infections have changed with the worldwide emergence of a hypervirulent and epidemic 027 PCR-ribotype strain. During early infancy, *C. difficile* is frequently recovered in the intestinal tract and mainly associated with asymptomatic colonization. The aim of this study was to update infant colonization prevalence and to characterize infant colonizing strains, in terms of their virulence factors and their phylogenetic biodiversity.

Methods. Faecal samples from 0 to 2 year old infants were submitted to toxigenic culture to detect *C. difficile* colonization. Virulence genes encoding toxins A and B (tcdA and tcdB) and binary toxin (cdtA and cdtB) were detected by PCR on isolates. Strain diversity was analysed by two molecular typing methods PCR-ribotyping and Multi-Locus Sequence Typing (MLST: aroE, dutA, gmk, groEL, recA, sodA and tpi). Infant genotypes were compared to those of a collection of strains isolated in adults from various European countries and USA.

Results. A prospective screening of 294 infants from Jean Verdier hospital identified 99 *C. difficile* carriers. Twenty-one of the 99 isolates were toxigenic. *C. difficile* colonization prevalence averaged 34% (maximum of 59% between 6 and 9 months), and 7% for toxigenic strains. Molecular characterization was performed on 91 isolates. In addition, 11 isolates from two other hospitals were studied. Among these 102 isolates, 27 were toxigenic including 25 tcdA+/tcdB+ and 2 tcdA-/tcdB+. All the isolates were negative for the binary toxin genes. Phylogenetic analysis identified 19 PCR-ribotypes (PR) ; MLST is currently performing and only preliminary results are yet available. In a same PR/ST genotype, the toxigenic status was conserved. The different PR/ST genotypes were equally distributed regardless of infant age or location. No major or emergent epidemic strain was identified among infant strains. In non toxigenic strains, the PR JV11/ST38 predominated (32/75, 43%). Most of the toxigenic strains (16/27, 59%) clustered in PR JV04/ST33 (PR014/020/077, according to Brazier's nomenclature). These results indicate that infant colonizing strains are widely distributed in adults ; thus infants may be a reservoir for adult infecting strains.

**P17**

# A NATIONAL SURVEILLANCE PROGRAMME FOR *Clostridium difficile* INFECTION (CDI) IN SCOTLAND

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Aims: To understand the epidemiology and facilitate the reduction of CDI in healthcare settings in Scotland through the implementation of a national mandatory surveillance system for CDI in conjunction with strain typing provided by a reference service.

Methods: Following a national protocol for testing and reporting, cases of CDI in patients aged 65 and above from all acute and non-acute hospitals, as well as general practices, are submitted. Samples representing severe cases and suspected outbreaks are submitted to the reference service for PCR ribotyping. Rates are calculated by NHS board using occupied bed days (OCBDs).

Results: All NHS boards across Scotland have complied with the protocol. For patients aged 65 years and above, 6430 cases were reported in 2007 (annual rate 1.34 per 1000 total OCBDs); in 2008, there were 6322 cases (annual rate, 1.23); in 2009, there were 3625 cases (annual rate, 0.71) . Overall rates for Scotland have decreased consecutively over seven quarters since the beginning of 2008, with the last quarter in 2009 having the lowest reported rate since surveillance began. Between 2008 and 2009, overall rates have decreased 42% (95% CI 38%, 46%). 64% of ribotypes reported were of three main types: 106, 001 and 027, with 106 predominating.

Conclusions: The national mandatory surveillance programme has led to harmonisation of testing, diagnosis and reporting of CDI, with the same case definition used by all clinicians in all NHS boards across Scotland. Surveillance, along with the implementation of guidance, has raised awareness of CDI and supported the significant reduction in reported rates. Typing of isolates from severe cases and outbreaks suggest that 106, 001 and 027 have become predominant in Scotland. Type 106 is usually found in the UK compared to the wider distribution of 027 and 001.

**P18**

# RESULTS OF A NEW DUTCH NATIONAL SENTINEL SURVEILLANCE; ANOTHER NEW EMERGING *Clostridium difficile* PCR RIBOTYPE?

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In the period between May 1st 2009 and May 1st 2010, a national sentinel surveillance for *Clostridium difficile* infection (CDI) was started in the Netherlands. This surveillance was initiated to obtain continuous incidence rates of CDI in Dutch hospitals, to identify new circulating PCR ribotypes and correlate them to clinical and epidemiological characteristics. Nineteen hospitals (20% of all hospitals) participated in the surveillance. In the survey, all patients were included above the age of two years, with diarrhoea and a positive toxin test for *C. difficile*. Stool samples were cultured and sent to the LUMC where PCR ribotyping was performed. Web-based questionnaires were used to collect clinical and epidemiological data.

In total, 533 patients were included in the study and 396 *C. difficile* isolates were obtained. The mean incidence was 15 per 10,000 hospital admissions. Type 001 was the most frequently found type (18%), followed by type 014 (14%), type 078 (11%) and 002 and type 044 (both 6%). Type 027 was found in 2%. Eleven outbreaks were observed. Of the 533 patients included in the surveillance, 268 were male (50.3%) and 265 were female (49.7%). The mean age was 65 years. Of 533 patients, 333 patients (62.5%) were defined as health-care associated CDI. Most patients (64.9%) used antibiotics prior to the start of diarrhoea. A total of 133 patients (27.9%) had severe CDI. After 30 days, 69 patients with CDI (14.8%) died; two deaths were attributable to CDI and 16 deaths were contributable to CDI. *C. difficile* PCR ribotype 044 was, until 2009, not a frequently found type but is now responsible for one outbreak and several sporadic cases. The clinical characteristics of this type did not differ from CDI due to other types. Extrapolating the data of sentinel surveillance to all hospitals in The Netherlands, it is estimated that more than 2700 hospitalized patients annually will develop CDI of which 100 will succumb attributable or contributable to CDI. In these estimations, the impact of CDI in other healthcare facilities than hospitals was not included.

We conclude that the epidemiology of CDI is continuously changing with types 001 and 014 as the predominant types. In The Netherlands, the incidence rate of *C. difficile* PCR ribotype 027 is decreasing in comparison with previous years. Types 078 and 044 are new emerging types, of which type 078 is of importance due to its microbiological similarity with 027 and its relatedness with animal *C. difficile* strains.

**P19**

# INTESTINAL COLONIZATION OF *Clostridium difficile* IN PEDIATRIC CANCER PATIENTS AND THE CONTAMINATION OF THE WARD ENVIRONMENT WITH THIS ORGAMISM

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Background: Pediatric cancer patients receiving antineoplastic chemotherapy often have diarrhea during their hospitalization. Excretory care in such children is done not by healthcare personnel but by their families. In this study, we investigated intestinal colonization by *C. difficile* in children undergoing antineoplastic chemotherapy. In addition, the environmental surfaces of the pediatric ward where they were hospitalized were sampled and examined for the presence of *C. difficile*.
Methods: From November 2005 to May 2009, stool specimens were obtained from 15 children (5-15 years of age) who were admitted to a pediatric ward for antineoplastic chemotherapy and were subjected for *C. difficile*-culture.Sampling from environmental sites considered to be commonly exposed to patients and health care staff (e.g. sinks, toilet seats, door knobs, bed frames, call buttons, bedside tables, commodes, and floors) was performed with moistened cotton swabs to culture in cycloserine - cefoxitin - mannitol broth. *C. difficile* isolates from patients and environments were analyzed by PCR ribotyping.

Results and Discussion: *C. difficile* was isolated from 13 of the 15 children examined, and 2 of them were given the diagnosis of *C. difficile* infection (CDI) and treated by vancomycin. In 9 of the 13 children, *C. difficile* was not recovered on admission, suggesting that the acquisition and/or growth of *C. difficile* during chemotherapy. Among 72 isolates recovered from the 13 children, 19 different PCR ribotypes were found. Typing results showed that nosocomial spread among 10 patients.A total of 502 environmental surfaces were examined and 39 were positive for *C. difficile*. The 39 *C. difficile* isolates were classified into 21 different PCR ribotypes. In 2 patients, PCR ribotypes isolated from stool specimens were identical with those from environmental surfaces of their rooms. These findings indicate that environmental contamination may contribute to *C. difficile* transmission among pediatric cancer patients. In conclusion, a CDI prevention program including hand hygiene and healthcare environment cleaning must be provided to healthcare personnel as well as to patients and their families.

**P20**

# TWO OUTBREAKS OF *Clostridium difficile* INFECTIONS SIMULTANEOUSLY IN ONE HOSPITAL

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Background: Outbreaks of *Clostridium difficile* infection (CDI) due to PCR ribotype 027 (type 027) are emerging worldwide, while some countries report an increase of toxin A negative PCR ribotype 017 (type 017). We encountered a unique outbreak due to types 027 and type 017 occurring simultaneously in a teaching hospital in The Netherlands.

Methods: We performed a case control study from May 2005 through January 2007 to investigate general and type specific risk factors as well as outcome parameters associated with CDI. Clonal dissemination was investigated by multi locus variable number of tandem repeat analysis (MLVA). Results: Of 168 patients with CDI, 57 (34%) was associated with *C. difficile* type 017, 46 (27%) with type 027, and 65 (39%) with other types. Controls consisted of 77 non-CDI diarrheal patients and 162 patients without diarrhea. Risk factors for CDI were nasogastric intubation, recent hospital admission and use of second generation cephalosporins and clindamycin. Age above 65 years was a specific risk factor for types 017 and 027; use of macrolides, clindamycin and immunosuppressive agents were associated with type 017 and use of fluoroquinolones with type 027. The overall mortality at one year follow-up among patients with types 017, 027, other types, non-CDI diarrheal patients and non diarrheal patients was 67%, 63% and 23%, 20% and 21%, respectively. MLVA showed persistent clonal persistence of types 017 and 027 throughout the hospital, despite thorough disinfection.

Conclusions: Distinct risk factors and higher mortality were found for types 017 and 027, compared to patients with other types and control patients, with clonal persistence throughout the hospital.

**P21**

# SPOROCIDAL DISINFECTION OF THE ENVIRONMENT OF CDI - PATIENTS - AN ESSENTIAL STRATEGY FOR PREVENTION OF NOSOCOMIAL TRANSMISSION OF *Clostridium difficile* SPORES?

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Background/Objectives: *Clostridium difficile* infection (CDI) has been changing in its clinical severity and epidemicity. Hypervirulent strains of *Clostridium difficile* are causing hospital outbreaks in the US and Europe. This phenomenon is associated with particular PCR ribotypes including ribotype 027. This spore-forming bacterium, is highly resistant in the environment. Therefore sporocidal disinfecting concepts have to be in place for surfaces adjacent to patients.

Whether routine cleaning and and non-sporocidal disinfection of the patient environment could be sufficient to prevent transmission for C diff. Spores was investigated by two different sampling methods. Methods: The Two different methods used for recovery of *Clostridium difficile* spores were bloodagar contact plates (Biotest) and single cotton-tipped plastic swabs (COPAN). In March and April 2010 from a total of 12 patients , from different departments of a university hospital, 118 samples in total were taken, containing one sample each method, on 5 defined sites adjacent to the patient before sporocidal surface disinfection was started. The samples were cultivated on *Clostridium difficile* selective agar (bioMérieux), and, together with the contact plates, incubated for 48 hours under anaerobic conditions. Any colonies were sent to AGES in Vienna for PCR-ribotyping.

Results: None of the 118 environmental samples showed a positive result, although of clinical manifestation of CDI and proof of toxin A and toxin B in all 12 patients. Out of these 12 only 7 patients showed a positive toxigenic culture result. Among the 5 culture-negative patients, contamination of the environment was very unlikely. 5 out of the 7 isolates of the culture-positive patients were available for PCR-ribotyping. 3 isolates were identified as PCR-ribotype 053, 1 as PCR-ribotype 050 and 1 as AI-8/0. Conclusion: There was seen no difference among the two methods. During the investigation-period no clusters of CDI were observed. Therefore it can be assumed, standard cleaning and non-sporocidal disinfection of surfaces adjacent to the patients seem to be sufficient to prevent transmission of *C. difficile*-spores.

For clarification of insufficient sensitivity of the used methodology, the investigation of the surfaces should be extended in frequency and area.

**P22**

# TOXIN A-NEGATIVE, TOXIN B-POSITIVE *Clostridium difficile* IN AUSTRALIA

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Toxigenic strains of *C. difficile* typically produce both toxin A and toxin B (A+B+), however, toxin A-negative, B-positive (A-B+) strains have been well described. One particular A-B+ strain, PCR ribotype 017, has dominated international typing studies of A-B+ isolates, and been responsible for epidemics in Canada, the Netherlands and Ireland. The aims of our study were to determine the frequency and molecular diversity of A-B+ strains among Australian

*C. difficile* isolates. PCR was used to detect *C. difficile* toxin genes and isolates were typed by PCR ribotyping. A total of 907 recent Australian *C. difficile* isolates from both humans and animals were examined. Of these, 593 (65.3%) were determined to be toxin A+B+, while 152 (16.8%) were A-B+ and 162 (17.9%) were A-B-. Binary toxin genes were detected significantly more often ($P < 0.0001$) among the A-B+ isolates (151/152, 99.3%), compared with 43/593 (7.3%) A+B+ isolates and 12/162 (7.4%) A-B- isolates. PCR ribotyping could distinguish 15 distinct types among 152 A-B+ isolates. Only one isolate was PCR ribotype 017. Representatives from all but one of the 15 A-B+ ribotypes were further characterised by toxinotyping. Three ribotypes belonged to toxinotype XVII, while one ribotype each belonged to toxinotypes XVI and VIII. Four ribotypes belonged to two toxinotypes not described previously. Three ribotypes could not be toxinotyped due to the absence of the B1 fragment, suggesting those strains produced neither toxin A or B. A-B+ strains occurred frequently among Australian *C. difficile* isolates, they were unique with respect to their genetic diversity and contained a high proportion of binary-toxin positive types.

**P23**

# EFFICACY OF DISINFECTANTS AND DECONTAMINANTS AGAINST *Clostridium difficile*

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*Clostridium difficile* is a Gram positive, anaerobic, spore forming bacillus that causes a spectrum of disease from asymptomatic carriage and self-limiting diarrhoea to pseudomembranous colitis, toxic megacolon and even death, collectively referred to as *Clostridium difficile* infection. *C. difficile* enters the body of the host as spores acquired usually exogenously or possibly endogenously. Patients suffering from CDI excrete large amounts of vegetative cells as well as spores into the environment. These spores can remain viable for months on contaminated surfaces and are resistant to several common disinfectants. Thus, effective decontamination of the environment is essential in preventing the transmission of *C. difficile*, not only in hospitals, but also in the laboratory. The efficacy of four disinfectants, Actichlor, MicroSol 3+, TriGene Advance and Virkon, and one decontaminant, Decon 90, against both vegetative cells and spores of *C. difficile* was tested. Five strains were selected for the study: strains 630 and VPI 10463, and the three epidemic strains in Scotland, PCR ribotypes 106, 001 and 027. Minimum inhibitory concentrations were determined by agar dilution as well as broth microdilution. All the agents tested inhibited the growth of vegetative cells of the selected strains at concentrations below those recommended by their respective manufacturers. Additionally, their effect on spores was determined by exposing the spores of these strains to different concentrations of the agents for different periods of time. For some of the agents, an exposure of ten minutes was required for sporicidal activity. Further investigations into the efficacy of these agents in a real-life setting are being performed using different types of contaminated surfaces commonly found in the hospitals and laboratories in order to determine the most suitable method to eliminate *C. difficile* from the environment.

**P24**

# PREVALENT *Clostridium difficile* GENOTYPES AMONG HUMAN AND ANIMAL ISOLATES OTHER THAN RIBOTYPE 078

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*Clostridium difficile* PCR ribotype 078 is currently the most frequently isolated strain from pigs and calves and is also increasingly reported in human population. In Slovenia PCR ribotype 078 is very rare and strains other than ribotype 078 are prevalent in humans and animals. The aim of this study was to compare prevalent ribotypes isolated from humans and different animals with pulsed-field gel electrophoresis and antimicrobial susceptibility testing.

Altogether 50 representative *C. difficile* isolates from 7 most frequently isolated PCR ribotypes (014/020, SLO 010, SLO 055, 023, 029, 002 and SLO 011) were analyzed. These included 32 human strains isolated as a part of routine *C. difficile* testing from three different Slovenian hospitals and 18 animal strains isolated from pigs (n=3), poultry (n= 8), cat (n=1), calves (n=2) and dogs (n=4) isolated from different Slovenian farms and households. Selected strains had been isolated between 2006 and 2009 and had been previously characterized by toxinotyping and PCR ribotyping. All strains were further characterized with pulsed-field gel electrophoresis (PFGE) with two different restriction enzymes (SmaI and SacII).Minimum inhibitory concentrations (MICs) for 6 antimicrobial agents (rifampicin, moxifloxacin, erythromycin, tetracycline piperacillin/tazobactam and clindamycin) were determined by E-test method.

With PFGE five animal-human isolate groups had indistinguishable banding patterns (100 % similar) when digestion was performed with SmaI. The first and second group contained poultry and human isolates of ribotype 002 and 023, respectively. The third group consisted of three animal (cat, dog and poultry) and four human isolates, all of ribotype 014/020. The fourth group contained two dog isolates and one human isolate, all of them were PaLoc negative (ribotype SLO 010 and SLO 055). However, when restriction was performed with SacII, only one pig isolate had banding pattern indistinguishable from the human one. Both human and pig isolate were of ribotype 029 (toxinotype 0) and had similar MIC values for all antibiotics tested. The other animal isolates from other four groups were similar but not identical to any of human isolates when restriction was performed with SacII. In general, human isolates had higher MICs90 and MICs50 values than animal isolates for all antibiotics tested except tetracycline, for which MIC50 was higher for animal isolates and MICs90s were the same. For rifampicin all animal and human isolates had MICs <0,002 mg/l. Our results show that human and animal isolates from the same PCR ribotype are genetically similar and that the use of two different restriction enzymes can improve PFGE typing of *C. difficile*.

**P25**

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

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BACKGROUND: *Clostridium difficile* infection (CDI) is the most common cause of infectious diarrhea occurring in hospitalized patients in developed countries. An increase in the incidence and severity of CDI in some hospital centers has been linked to epidemic virulent CD strains, an aging population, inadequate infection control, and increased use of certain antibiotics. There are little data on the natural history of CD. A study was designed to describe CD acquisition, colonization and infection in the hospital setting.

METHODS: A multi-site, hospital-based prospective study is being conducted in the U.S. and Canada. Patients admitted to general medical and surgical units, on antibiotics, and over 60 years of age are enrolled in the study. Patients negative for CD colonization at enrollment are followed prospectively to determine the timing of possible CD acquisition, and the rates of CD colonization and symptomatic CDI. Stool specimens or rectal swabs are collected every 3 days from enrollment to hospital discharge. Stool specimens are tested for CD toxin if patients develop diarrhea. CDI cases are defined as patients without CDI symptoms and culture-negative for CD at the time of enrollment that later develop a diagnosis of CDI. Asymptomatic carriers are those patients without CDI symptoms and culture-negative for CD at the time of enrollment that later develop a positive stool culture for CD while hospitalized, but do not develop CDI during follow-up. HindIII restriction endonuclease analysis (REA) typing is performed on all cultured CD isolates.

RESULTS: As of April 2010, 570 patients were enrolled in the study; 57 (10%) of those patients were colonized with CD at enrollment. In the 513 patients who were negative for CD at enrollment, 11 patients became asymptomatic carriers (2.1%) and 6 became CDI cases (1.2%). The average age of CDI cases was 79.2 years versus 74.8 years for asymptomatic carriers (p=0.28). Four of the 6 (67%) CD isolates from CDI cases were REA group BI, compared to 1 of the 11 isolates (9%) from the asymptomatic carriers (p=0.09). The mean time from hospital admission to colonization was 9.8 days (range 3-31; CDI cases: 10.3 days and asymptomatic carriers: 9.5 days).

CONCLUSION: Data from this large epidemiologic study on the natural history of CDI will help further our understanding of the timing of hospital-associated CD acquisition and symptomatic infection. They will also assist in the planning of future clinical trials designed to evaluate candidate preventive and therapeutic CDI agents.

**P26**

# ROLE OF FLAGELLA IN ASPECTS OF *Clostridium difficile* VIRULENCE

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*Clostridium difficile* is a major cause of nosocomial antibiotic associated diarrhoea and colitis. It is a serious and costly public-health concern worldwide. Two large toxins, A and B, have been described as its main disease causing components, however other accessory virulence factors such as flagella are undoubtedly involved in establishing infection. Flagella play important roles in numerous gastrointestinal pathogens as surface adhesins, in motility or as secretion apparatus for virulence factors. Previous studies have shown that *C. difficile*'s flagellar proteins (FliD and FliC) contribute to attachment to the protective mucus barrier, the first step during the colonization process. In the present study flagellar mutants have been made in the cap (fliD), the filament (fliC) and the hook (flgE) to investigate the role of flagella in *C. difficile* virulence. They were characterised in vitro by TEM, showing no flagella, and motility assays, proving them non-motile. Surprisingly when comparing those mutants to *C. difficile* 630Δerm, a non-epidemic toxigenic strain, in a cell-based adherence assay, they adhered more than the wild-type. This could be due to an increased binding of cell surface components by the non-flagellated bacteria as a result of closer contact. Furthermore, it was shown, in vivo, that the fliC-mutant colonized better compared to the wild-type using the mouse model. In the hamster infection model the fliC-mutant was more virulent than the wild-type. This could be due to increased toxin secretion as a cell toxicity assay showed higher toxin levels for this mutant. Further investigation of this interesting hypothesis is underway. In addition the role of flagella in a 'hypervirulent' *C. difficile* strain, ribotype 027 is being investigated.

**P27**

# NEW ASPECTS IN THE STRUCTURE-FUNCTION RELATIONSHIP OF *Clostridium difficile* TOXIN B

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Toxin B (TcdB) is one of the major virulence factors of *Clostridium difficile*, causing antibiotic-associated diarrhea and pseudomembranous colitis in severe cases.

Toxin B glucosylates and thereby inactivates small GTPases of the Rho family. The inactivation of such Rho GTPases leads to cell rounding and cell death, since important cellular signaling pathways like the re-organisation of the actin cytoskeleton are interrupted.

Upon binding to a so far unknown receptor on the cell surface, toxin B is taken up into endosomes via clathrin-mediated endocytosis. The low pH within the endosomal lumen induces conformational changes within the toxin, resulting in the insertion into the endosomal membrane and the translocation of the enzymatic component into the cytosol.

Clostridial glucosylating toxins are described to consist of at least four subdomains, resulting in the so-called ABCD model. The N-terminus of the protein harbors the biologically active portion (A), the glucosyltransferase domain, whereas the C-terminal part is determined as the receptor binding domain (B). In between these two domains, a subdomain (D) required for membrane insertion and delivery of the enzymatic part into the cytosol has been postulated. An intrinsic cysteine protease domain (C) releases the glucosyltransferase moiety into the cytosol upon activation through binding of a small molecule, inositol hexakisphosphate.

Here we discuss new findings about structural elements present in subdomain D of toxin B that are involved in the delivery of the glucosyltransferase domain into the cytosol.

**P28**

# THE HOLIN LIKE PROTEIN TCDE MEDIATES *Clostridium difficile* TOXIN SECRETION

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Most clostridial toxins are released into the extracellular environment via the general secretion pathways. *Clostridium difficile* toxins A and B have no export signature and their secretion is not explained by cell lysis, suggesting that they might be secreted by an unusual mechanism. The TcdE protein encoded within the *C. difficile* pathogenicity locus (PaLoc) has structural features similar to those of bacteriophage holin proteins. During many types of phage infection, host lysis is driven by an endolysin that crosses the cytoplasmic membrane through a pore formed by holin oligomerization. We demonstrated that TcdE has a holin-like activity by functionally complementing a λ phage deprived of its holin. Similar to λ holin, TcdE expressed in E. coli and *C. difficile* formed oligomers in the cytoplamic membrane. A *C. difficile* tcdE mutant strain grew at the same rate as the wild-type strain, but secreted a dramatically reduced amount of toxin proteins into the medium. There was no difference in the levels of tcdA and tcdB gene expression and several cytoplasmic proteins were equally abundant in the mutant and the wild-type strains. In addition, TcdE did not affect the membrane integrity of *C. difficile*. Thus, TcdE acts as a holin-like protein to facilitate the release of *C. difficile* toxins to the extracellular environment but, unlike the phage holins, does not cause the non-specific release of cytosolic contents. TcdE appears to be the first example of a bacterial protein that releases toxins into the environment by a phage-like system.

**P29**

# CYTOTOXIC PROPERTIES OF *C. difficile* TcdA AND TcdA LACKING THE PROPOSED RECEPTOR BINDING DOMAIN

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The *Clostridium difficile* associated disease is primarily linked to the two exotoxins TcdA and TcdB. These intracellular acting toxins modify and thereby inactivate Rho GTPases by monoglucosylation. They enter host cells via receptor- mediated endocytosis and release the N-terminal catalytic moiety from an acidic endosomal compartment into the cytosol. Although the molecular mode of action of the toxins A and B is well understood, far less is known about binding structure and the internalization pathway of the target cell. Indeed, the C- terminal located clostridial repetitive oligopeptides (CROPs) are proposed to be involved in receptor binding to a so far unknown receptor. However, a novel identified cytotoxin from Clostridium perfringens, homologous to TcdA and B from *C. difficile*, has cytotoxic activity towards target cells although lacking the supposed receptor binding domain. Based on this finding, we dispute the necessity of the CROP domain concerning functional properties of TcdA and TcdB. Therefore, we generated a truncated TcdA mutant lacking CROPs (TcdA1 1874) and studied its cytotoxic impact towards different cell types in comparison to the full length toxin. Interestingly, TcdA1 1874 induced time and concentration dependent cell rounding and Rac1-glucosylation; however, depending on the cell line, with up to 10-fold reduced potency. FACS and Western blot analysis revealed a correlation between CROP-binding to the cell surface and toxin potency. This coherence is supported by the observation that addition of zinc cations enforces both, CROP-binding as well as toxin- catalyzed Rac1-glucosylation. These findings refute the accepted opinion of a solely CROP- mediated toxin uptake. We propose an additional alternative uptake process at least for TcdA which might be based either on the recognition of different receptor structures or on the use of other associated endocytotic pathways. To substantiate this hypothesis, we analyzed toxin potency following apical or basolateral uptake as model for different receptor structures. In fact, cells were hardly susceptible towards apically applied TcdA1-1874, whereas basolateral treatment causes a pronounced cytopathic effect. In contrast, potency of full length TcdA was almost identical and independent of the side of application. Thus, different routes for cellular uptake might enable the toxins to enter a broader repertoire of cell types leading to the observed multifarious pathogenesis of *C. difficile*. The characterization of alternative endocytotic pathways used by the *C. difficile* toxins might therefore be the basis to investigate the opportunity of toxin uptake inhibition as therapeutic option.

**P30**

# *Clostridium difficile* IN A FARROWING PEN

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*Clostridium difficile* is an important cause of enteric disease in humans. In pigs *Clostridium difficile* can cause neonatal enteritis and can be isolated from faeces from diseased and healthy animals. According to recent research, isolates from humans and animals show genetic and phenotypic overlap. In The Netherlands, strains isolated from diseased piglets were indistinguishable from strains isolated from Dutch patients. These strains belonged to ribotype 078. Because pigs can either be clinical hosts and/or may be a possible reservoir more understanding of the epidemiology of *Clostridium difficile* among pigs is needed. The objectives of this study were to specify whether, how and when newborn piglets get infected by *Clostridium difficile* for the first time. With this intention, six sows, their farrowing crates and litters (71 piglets) at one farm were sampled around the day of birth of the piglets. Furthermore, 38 piglets born by caesarean section, in a sterile surgical theatre, were sampled immediately after birth. Within 48 hours after birth, all sampled 71 piglets at the farm became positive for *Clostridium difficile* ribotype 078. Moreover, all sows became positive within 113 hours after birth of the piglets and the farrowing crates were intermittently positive during the sampling period. The piglets born by caesarean section were negative at birth. This research shows that the sow, the farrowing crate, the air and the teats of the sow are possible transmission routes of *Clostridium difficile* ribotype 078 and that vertical transmission of *Clostridium difficile* seems unlikely. This information might help to advise farmers on taking measures against *Clostridium difficile* infections in neonatal piglets.

**P31**

# *Clostridium difficile* IN WILD SMALL MAMMALS

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Introduction: *Clostridium difficile* can be found in an impressive range of animal species, including humans, domestic pets and food animals. Limited study has been performed on wildlife, yet the organism has been isolated from diverse species such as elk, penguins and bears. While direct contact of humans and domestic animals with wildlife is limited, wildlife are able to serve as reservoirs or vectors of various infectious agents, and can be involved in transmission of pathogens between farms and between food animal and human environments. The objectives of this study were to determine the prevalence of *C. difficile* in selected wildlife populations and characterize recovered strains.

Materials and Methods: Small wild mammals were trapped on and around swine, beef and dairy farms in Ontario, Canada. Fecal swabs were obtained for enrichment culture, along with characterization of isolates by ribotyping and PCR detection of tcdA, tcdB and cdtA. Fecal specimens were also obtained from raccoons living freely (i.e. not part of the zoo animal collection) at a large zoological park.

Results: *C. difficile* has been isolated from 3/58 (5.2%) animals from 12 farms to date; 3/34 (8.8%) raccoons, 0/9 deer mice, 0/8 house mice, 0/4 rats and 0/1 groundhog, shrew and vole. *C. difficile* was not isolated from any of 216 samples from raccoons from the zoo. Two of the positive raccoons were from one beef farm while the other was from a swine farm. None had evidence of gastrointestinal disease. All three isolates were indistinguishable and were a toxinotype 0 ribotype that has been found in humans and calves in Ontario. The isolates possessed tcdA and tcdB but not cdtA. Comparison with *C. difficile* from food animals on those farms is pending.

Discussion: *Clostridium difficile* can be isolated from a small percentage of wild animals living on and around farms, potentially because of exposure from the farm. The strain that was found in raccoons has been found in both humans and food animals, so inferring the source is difficult, but the presence of such strains in raccoons suggests that some wild animals could act as a source of *C. difficile* transmission between farms or as a bridge between farms and other environments or individuals. Further study of the potential role of wildlife in the epidemiology of *C. difficile* is required.

**P32**

# *Clostridium difficile* IN HORSES IN AUSTRALIA

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*Clostridium difficile* causes infectious diarrhoea in humans and animals. It has been found in both diarrhoeal and non-diarrhoeal pigs, horses, and cattle, suggesting a potential reservoir for human infection, and in 20-40% of meat products in Canada and the USA, suggesting the possibility of food-borne transmission. PCR ribotype 078 is the most common ribotype of *C. difficile* found in pigs (83% in one study in the USA) and cattle (up to 100%). Equine and human isolates appear to be more diverse. Horses overseas have died of fulminant *C. difficile* colitis. During a 24-month period from 2007 to 2009, 169 faecal specimens from horses (predominantly from WA) were tested for *C. difficile*. *C. difficile* was isolated from 16 (30%) of 54 diarrhoeal animals and none of 116 healthy adult horses. These isolates were toxin profiled by PCR for toxin A, toxin B and binary toxin (CDT), and ribotyped. Ten of the equine isolates were A+B+CDT-, as well as A-B-CDT+ (1 isolate), A+B+CDT+ (1), A-B+CDT+ (1), and A-B-CDT- (3). There were 8 different equine ribotypes, ribotype 012 being the most common with seven isolates. Two horses had two different strains of *C. difficile*. None of the equine isolates was ribotype 078 which is A+B+CDT+. Ribotype 078 has only been detected twice in WA, both times from a human. All isolates were susceptible to metronidazole and vancomycin. These results suggest that the epidemiology of *C. difficile* in horses is currently similar to other parts of the world, but requires further surveillance to monitor changes.

**P33**

# *Clostridium difficile* IN VEGETABLES AND SEAFOOD

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Introduction: *Clostridium difficile* has been isolated from various food products, particularly meat, and concern has been expressed regarding food as a possible source of human *C. difficile* infection (CDI). Determination of the presence of *C. difficile* in a wide variety of food sources is needed as part of the investigation into the potential role of food in CDI. Here, we sought to isolate *C. difficile* from a variety of vegetables and seafood obtained from local grocery stores and to characterize these isolates.

Methods: Vegetables (carrots, potatoes, beets, garlic, eddoes, ginger, mushrooms, radishes, lettuce, green onions, broccoli, asparagus, turnips, and beets) were purchased from 11 different grocery stores in Guelph, Ontario, Canada between May and August 2009. Seafood and fish (raw and cooked shrimp, scallops, oysters, mussels, calamari, and others) were also purchased from grocery stores in Guelph between May and July 2010. Seafood samples were also obtained from across Canada as part of the active retail sampling component of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). Enrichment culture was performed and isolates were characterized by ribotyping, PFGE, toxinotyping and PCR detection of tcdA, tcdB and cdtB.
Results: *C. difficile* was isolated from 4.5% (5/111) of vegetables (2 carrots, 2 ginger, 1 eddoes). Three isolates were ribotype 078/NAP 7/toxinotype V, possessing all 3 toxin genes. The other 2 isolates were a toxinotype 0/NAP4 ribotype possessing tcdA and tcdB but not cdtB that has been previously found in humans with CDI in Ontario. *C. difficile* was isolated from 4.8% (5/104) of seafood (1 scallop, 1 perch, 1 salmon, 2 shrimp. 4 isolates were ribotype 078/toxinotype V and possessed tcdA, tcdB and cdtB. The other isolate was non-toxigenic.

Conclusions: The prevalence of contamination of vegetables and seafood in this study is similar to some reports of retail meat, and suggests that there are many possible sources of foodborne exposure. The predominance of ribotype 078, a strain increasingly associated with community-associated CDI in humans is of concern. This study, combined with studies of other food sources, suggests that widespread contamination of food is common and that food may be a regular source of human exposure. There are various potential sources of contamination with the food-animal-associated ribotype 078, including contamination at source from agricultural run-off and cross-contamination during processing and sale. Further study of the role of food in human *C. difficile* infection is required.

**P34**

# THE PREVALENCE AND IMPACT OF TETRACYCLINE RESISTANCE IN *Clostridium difficile* IN CALVES

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Introduction: Resistance of *Clostridium difficile* to tetracycline has been reported, however the prevalence, mechanism and implications of resistance in food animals have not been adequately investigated. The objective of this study was to longitudinally investigate the *C. difficile* colonization in veal calves, characterize recovered isolates, to determine the impact of tetracycline treatment on tetracycline resistance and determine the impact of tetracycline resistance on prevalence.

Methods: Calves arriving on 1 veal farm were sampling at the time of arrival, as well as 1, 17 and 21 weeks later. Calves were treated with tetracycline in milk replacer for 5 days after arrival as part of normal farm protocols. Selective culture for *C. difficile* was performed. Isolates were characterized by ribotyping, PCR for tcdA, tcdB, cdtA and tet(M) and Etest for tetracycline susceptibility. Multivariable logistic regression models were used to determine the relationship between tetracycline resistance and the presence of tet(M) and the independent variables: time of sampling, rooms where animals were housed and ribotype classification.

Results: Overall, *C. difficile* was isolated from 98/200 (49%) calves, with prevalences of 32%, 51%, 2% and 2% calves during the 1st through 4th samplings, respectively. 12 different ribotypes were present at the first timepoint, with ribotype 078 being most common (66%). By the 2nd sampling time, only 3 ribotypes were present ; ribotype 078 (66%), a toxinotype 0 ribotype (33%) and a toxinotype XII ribotype (1%), all of which have been found in humans in Ontario. 21% of isolates from the 1st sample were tetracycline susceptible, compared to 6.8% from the 2nd (post-treatment) sample. tet(M) was detected in 13% of isolates from sample 1 and 45% from sample 2. Isolates from the 2nd sample were less likely to be tetracycline susceptible (OR:3.0, CI:1.2-7.2, p=0.016) and more likely to possess tet(M) (OR:5, CI:1.65-14.91, p=0.004). tet(M) was also more common in isolates from calves housed in two specific rooms (OR:18, CI: 5.11-107, p<0.001 and OR 10.9, CI: 2.59-45.5, p=0.001) compared to a referent room. Correlation between resistance and presence of tet(M) was poor (r=0.16). Many resistant isolates did not have tet(M), so investigation of other resistance mechanisms is pending.

Discussion: The increase of resistance after routine tetracycline use may have an impact on the epidemiology of *C. difficile* on farms, with impacts on strain distribution and resistance patterns, in addition to potentially affecting the prevalence of colonization. Further study of the impact of antimicrobial use and different resistance mechanisms is required.

**P35**

# PREVALENCE AND MOLECULAR CHARACTERIZATION OF *Clostridium difficile* ISOLATED FROM BEEF CATTLE UPON ARRIVAL AND EXIT FROM FEEDLOTS

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Introduction: The presence of indistinguishable strains of *Clostridium difficile* in humans, food animals and food, as well as the apparent emergence of the food-animal-associated ribotype 078/toxintype V as a cause of community-associated *C. difficile* infection have created concerns about the potential for foodborne infection. While studies have reported *C. difficile* in calves, studies of cattle closer to the age of slaughter are required.

Methods: Four commercial feedlots in Alberta, Canada were enrolled. Fecal samples were collected at the time of arrival and exit. Selective culture for *C. difficile* was performed, and isolates were characterized by ribotyping, pulsed field gel electrophoresis, and PCR for tcdA, tcdB and cdtA. Fisher's exact test was used for categorical comparisons.

Results: A total of 543 cattle were enrolled; ranging from 121-179 cattle per feedlot (mean 137). *C. difficile* was isolated from 18/543 (3.3%) of cattle at the time of feedlot arrival. Among the four feedlots, the prevalence on arrival ranged from 1.7-7.4%. *Clostridium difficile* was isolated from 18/330 (5.5%) cattle at the time of exit. At one feedlot, cattle were lost to follup for exit sampling. Overall, there was no difference in the prevalence of *C. difficile* shedding on arrival versus exit ($P = 0.16$), nor was there a difference at the individual feedlot level at three farms ($P = 0.62$; $P = 1.00$; and $P = 0.38$). At one feedlot, the prevalence was higher at exit ($P = 0.01$). When only the matched pairs were evaluated, there was a significant difference in prevalence between arrival and exit in one of the four feedlots, as well as overall ($P=0.039$), with *C. difficile* isolated from 7/330 (2.1%) cattle at arrival and 18/330 (5.5%) at exit. However, 14/18 (78%) positive exit samples were from the same feedlot, which only accounted for 3/7 (43%) positive arrival samples. No animals were positive on both samples. All the isolates recovered were ribotype 078, a toxinotype V strain possessing tcdA, tcdB and cdtA. In addition, all strains were classified as NAP7 by PFGE.

Discussion: While the prevalence was low, the significant increase at one feedlot indicates that continued study of factors potentially influencing *C. difficile* shedding is required. The dominance of ribotype 078/NAP7 was not surprising, but is somewhat different from studies of retail beef that have also found other strains, including 027/NAP1. It is apparent that C difficile is carried in the intestinal tracts of a small percentage of cattle going to slaughter. Further study of influences on *C. difficile* colonization and sources of food contamination are required.

**P36**

# PREVALENCE AND CHARACTERIZATION OF *Clostridium difficile* FROM SLAUGHTER-AGE PIGS

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Introduction: *Clostridium difficile* is an important cause of neonatal piglet disease in some regions and its presence in food animals and food has raised questions about whether it may be a zoonotic and foodborne pathogen. There is, however, a paucity of data regarding the prevalence of *C. difficile* shedding by pigs close to the time of slaughter, presumably the most important period for foodborne contamination. The objectives of this study were to determine the prevalence of *C. difficile* shedding by slaughter-age pigs on farms across Canada and to characterize recovered isolates.

Methods: 44 commercial swine farms from 5 Canadian provinces were recruited in conjunction with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). On each participating farm, freshly passed fecal samples were collected from pens containing grower-finisher pigs close to the time of slaughter were sampled. A single sample was collected per pen, to represent an individual pig sample. Enrichment culture was performed and isolates were characterized by ribotyping and PCR detection of tcdA, tcdB and cdtB.

Results: *Clostridium difficile* was isolated from 30/435 (6.9%) samples from 15/44 (34%) farms (only 5 samples were obtained from 1 farm). There was a significant difference between provinces ($P<0.001$), with the prevalence ranging from 0-26%. On positive farms, between 1 and 5 samples (median 1) were positive. 20/27 (74%) isolates were ribotype 078, possessing tcdA, tcdB and cdtB. This strain was found on 12 farms and 4 provinces. Four isolates were of two different ribotypes possessing tcdA, tcdB and cdtB that has been found in Canadian pigs previously. Three (11%) isolates, consisting of two ribotypes, were non-toxigenic. No disease attributed to *C. difficile* was reported on any of the participating farms.

Discussion: The relatively low prevalence reported here is in contrast with studies of younger pigs, and is consistent with the hypothesis that there is a significant age-related influence on the prevalence of colonization, either because of biological factors relating to age or because of management changes that occur during the pig's life. Because of this, studies investigating the potential for food contamination should focus on pigs approaching the age that they would enter the food chain. The predominance of ribotype 078 is consistent with other studies of food animals, however 078 is not the only strain that can be found in food animals. This study cannot answer any questions pertaining to animal or public health risks, but provides more information about the wide dissemination of this strain in food animals.

**P37**

# *Clostridium difficile* IN LOCALLY PRODUCED SALAMI IN NORTH-EASTERN ITALY

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Background: A recent cross-sectional study conducted in 2009 in our laboratory demonstrated that ground beef and pork sold at retail in North-Eastern Italy do not represent a risk factor for human infection with *Clostridium difficile* (CD).

Objective: The aim of this work was to evaluate the presence of CD spores in locally produced pork sausages and salami in Treviso and Venice provinces.

Methods: In total 416 meat samples were analyzed: 152 fresh spiced minced pork 81 fermented salami after a mean seasoning of 20 days and 183 after a mean seasoning of 60 days. In order to increase the sensitivity, the presence of CD was assessed using three different methods: microbiological examination in CD selective medium (Cefoxitine Cycloserine Fructose broth added with Taurocolate), real time PCR for specific identification of CD DNA (Penders et al., 2005) and toxins detection using a commercial membrane enzyme immuno assay kit (C. DIFF QUIK CHECK COMPLETE KIT, Techlab). For all the fresh meat samples the presence of CD was evaluated at the same time in faeces obtained from the slaughtered pigs. To the purpose of estimate the faecal contamination during meat processing enumeration of Enterococci and sulphite-reducing Clostridia was also performed.

Results:
- None of the 416 analyzed meat products were positive for CD or toxin A and B.
- Clostridial spores and Enterococci counts differed greatly in magnitude between samples
- Five over 123 faeces collected from slaughtered animals resulted positive for CD both to microbiological analysis and toxins detection.

Conclusions:
The low percentage of faeces positive for CD (4%) obtained from slaughtered swine is consistent with the low prevalence of this microorganism in grower-finisher pigs reported in previously published studies. The low carriage rate within finishing pigs and a proper meat handling procedures seem to make pork products at low risk for CD transmission into the food chain.

**P38**

# COLONIZATION RATES OF *Clostridium difficile* IN BROILER PRODUCTION CYCLE

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*Clostridium difficile* appears to be an important cause of enteric disease in different animal species. Recently published studies suggest that poultry can be an important reservoir for *C. difficile* with high colonization rates (up to 62%) and high diversity of genotypes. Chicken meat may also be the vehicle for transmission to humans and consequently potential route of infection in case of community-acquired *C. difficile* infections. The aim of this study was to screen for *C. difficile* during a broiler production cycle on one large farm in Slovenia. Fecal samples were collected from breeding flock and three age groups of broiler chickens originating from the same breeding flock. Broiler neck skins were taken at slaughter line.

Fecal samples from breeding flock (n=30), one day old (n=30), 16 days old (n=30), and 32 days old (n=30) broiler chickens were analyzed for the presence of *C. difficile*. Neck skin samples (n=10) were taken at the slaughter line. The samples were inoculated in cycloserine-cefoxitin fructose enrichment broth supplemented with 0.1% sodium taurocholate and processed as described before (Arroyo et al., 2005). The isolates were identified based on morphological criteria and the identification was confirmed by multiplex PCR targeting tpi, tcdA, and tcdB (Lemee et al., 2004). Binary toxin gene (cdtB) was amplified by in-house LightCycler real-time PCR.

All samples from breeding flock and one day old broiler chickens (n=60) were negative for *C. difficile*. Among 16 days old broiler chickens 12/30 (40%) samples were positive. Six strains were nontoxigenic and seven strains were A+B+CDT-. In one sample we isolated nontoxigenic and toxigenic strain of *C. difficile*. In the oldest group of broiler chickens (chickens before slaughter) *C. difficile* was isolated from 2/30 (6.7%) samples. One strain was nontoxigenic and one was toxigenic (A+B+CDT-). All the neck skin samples were negative for *C. difficile*.

The results of the present study indicate that broiler chickens are colonized in different proportions regarding age. The colonization rate decreased from 40% to 6.7% in two weeks. These could explain previously reported low levels of *C. difficile* in retail chicken meat. We can assume that breeding flock is not the source of *C. difficile* for broiler chickens. Possible source of *C. difficile* could be environment, feed and water. Further studies are needed to get better insight into epidemiology and transmission of *C. difficile* in poultry.

**P39**

# COMPARISON OF A TAQMAN-BASED REAL-TIME PCR ASSAY TO A COMMERCIAL PCRFAST *Clostridium difficile* A/B TEST FOR DETECTION OF TOXIGENIC *Clostridium difficile* IN ANIMALS

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*Clostridium 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 new TaqMan- based real-time PCR (TMrtPCR) targeting genes for toxins A, B, and binary toxin to a commercial PCRFast *Clostridium difficile* A/B test (ifp Institut für Produktqualitet, Germany) (PCRFast).

DNA was extracted from rectal swabs from piglets and stool samples from calves (n=76) by using QIAamp DNA Stool Mini Kit (Qiagen, Germany). We developed and validated a TMrtPCR targeting tcdA, tcdB and cdtB genes by using TaqMan probes with the ABI Prism 7000 instrument (Applied Biosystems, USA). The PCRFast test was performed on an ABI Prism 7000 instrument, using TaqMan Universal PCR Master Mix (Applied Biosystems, USA). The amplification program was modified according to the manufacturer's instructions.

All nine tested toxinotypes (0, I, IIIb, IV, V, VI, VIII, X, XII) of *C. difficile* were detected by both assays. Toxinotype XIa (A-B-CDT+) was negative with PCRFast. In case of toxinotypes VIII (A-B+CDT-) and XIa (A-B-CDT+) the TMrtPCR assay targeting tcdA was positive, while these toxinotypes have gene for toxin A, but it is not expressed. Among 76 samples eight positive and 38 negative samples were in complete concordance, while 30 samples were positive only with TMrtPCR assay. In four negative samples inhibition was observed. Samples were defined as positive with TMrtPCR, if test was positive for all genes previously detected by other methods (data not shown).

With TMrtPCR we perform triple test for one sample, while in PCRFast test the primers and probes for both genes are mixed in one reaction and it is not possible to distinguish between A-B+ and A+B+ strains. Furthermore, PCRFast test does not detect gene for binary toxin, which is often present in animals. These preliminary results indicate that TMrtPCR is more suitable that PCRFast, while according to manufacturer's instructions it is not specify to be validated on animal samples. In spite of these results further testing of PCRFast with other PCR-reagents is required.

**P40**

# THE PREVALENCE OF *Clostridium difficile* IN PORK PROCESSING FACILITIES

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Introduction: Community associated *Clostridium difficile* infection has become an increasingly relevant issue in the past several years. The prevalence of *C. difficile* in food animals such as pigs and cattle is well documented, and recent studies have found retail meats contaminated with the pathogen. However, there is a lack of research investigating the slaughterhouse environment as a potential source of *C. difficile* to the community. The objectives of this study were to investigate *C. difficile* contamination of the pig slaughterhouse environment, as well as pig carcasses during processing.

Methods: Environmental samples were collected from a high capacity (5500 animals/day) pig slaughterhouse in Southern Ontario, Canada. Samples were obtained on two separate occasions from the plant in March and April 2010. Samples were collected from floors and walls in the animal holding area and various locations throughout the production line (e.g. scald water, polishers). Sponge samples were taken from carcasses along the line in the bleeding area, pre-evisceration and post evisceration, and in the cold storage room. Pooled manure samples from daily accumulated feces at the plant were also collected from pigs prior to slaughter. Enrichment culture was performed and isolates were ribotyped.

Results: *C. difficile* was isolated from 25% (33/130) of samples taken overall. 80% (16/20) of manure samples were positive, while 45% (9/20) of holding area floor and wall samples, 15% (3/20) of post bleeding carcasses, 15% (3/20) of post evisceration carcasses and 10% (2/20) of bleeding area carcasses sampled were positive. *C. difficile* was not isolated from scald water samples, polisher units or on carcasses in the cold room at the end of the line. 14/14 tested isolates were ribotype 078.

Discussion: These results indicate that *C. difficile* can be commonly found in the pork slaughterhouse environment at various locations in the processing line, with the expected predominance of the food-animal-associated ribotype 078. Despite a high prevalence in pooled manure samples and the clear entry of *C. difficile* into the slaughterhouse, processed carcass contamination was not identified. Further longitudinal study in slaughterhouses, plus investigation of other possible sources of contamination are required.

**P41**

# PERFORMANCE OF SEVEN DIFFERENT DIAGNOSTIC TESTS FOR *C. difficile* INFECTION IN PIGLETS

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*C. difficile* is increasingly recognised as a cause of neonatal enteritis in swine, but the prevalence of CDI in general pig populations is unknown. A prerequisite to study the prevalence and transmission of *C. difficile* in pig populations is the application of reliable detection methods for this bacterium. The detection methods for *C. difficile* are extensively evaluated for use in humans, but their performance in animal samples is largely unknown.

We comparatively studied seven diagnostic tests for the detection of *C. difficile* or its toxins in feces of pigs. In total, 176 samples were taken from piglets between 0 to 7 days old, with and without diarrhea. The piglets were selected from 25 herds suspected to be positive for the presence of *C. difficile*, based on recurrent problems with neonatal diarrhea that was irresponsive to antibiotic treatment or where vaccination was unsuccessful.

Samples were either stored at 2-4˚C and processed within 48 hours after sample taking, or frozen directly upon arrival at -20˚C and thawed only once before processing. The presence of *C. difficile* or its toxins was determined with cytotoxicity assays (CTA) and toxigenic culture as reference methods and with the following tests: Premier tox A/B® (Meridian), Immunocard tox A/B® (Meridian), VIDAS® (bioMérieux), GeneOhmTM® Cdiff RT-PCR (BD) and the in house RT-PCR method from the LUMC. Preliminary results reveal that of 168 samples, 76 (45 %) were positive with CTA. Compared with CTA, sensitivity of the tests ranged from 53% to 95%, specificity from 38% to 86%, the positive predictive value ranged from 54% to 74% and the negative predictive value ranged from 71% to 92%. We concluded that the tests used in human diagnosis of CDI are applicable for detection of *C. difficile* in pigs with a lower sensitivity and specificity than reported for human stool samples.

**P42**

# GLOBAL REGULATION IN RESPONSE TO CYSTEINE AVAILABILITY IN *Clostridium difficile*

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*Clostridium difficile* pathogenicity is linked to its ability to produce two toxins: TcdA and TcdB. The level of toxin synthesis is influenced by several environmental signals such as PTS sugars, biotin and amino acids like cysteine. We are interested in understanding the molecular mechanisms of cysteine-dependent repression of toxin production. We first performed a reconstruction of sulfur metabolism in *C. difficile* and tested the growth of the bacterium in the presence of various sulfur sources to confirm the metabolic pathways identified. We also noticed that providing large amount of cysteine to *C. difficile* correlates with hydrogen sulfide production indicating that cysteine is actively metabolized by cysteine desulfhydrases of the bacterium.

Then we looked at the regulatory networks involved in cysteine-dependent response by a global approach. The transcriptomic analysis of the C630E strain indicated that 242 genes were differentially expressed after addition of high concentration of cysteine in a rich medium: 105 genes are repressed whereas 137 are induced. The most highly regulated genes by cysteine are those involved in cysteine metabolism, fermentation and energy metabolism, amino acids biosynthesis and stress response. Interestingly, most genes belonging to the Fur regulon are also induced as if hydrogen sulfide production chelated iron mimicking an iron depletion of the media. However, the cysteine-dependent repression of toxin synthesis seems to be independent of the Ferric uptake regulator (Fur) since a fur mutant still exhibits an efficient cysteine toxin repression. In addition, cysteine-dependent repression of toxins production is not mediated by the TcdC antisigma factor. Actually, we showed that toxin expression is efficiently repressed in the epidemic strain 027, which lack TcdC production, in comparison to the isogenic strain complemented for TcdC expression.

Among the genes induced or repressed by cysteine, we found four regulators of unknown function. We also identified a regulator Cd1278 that shares 40% identity with CymR, the global regulator of cysteine metabolism in Bacillus subtilis. Thus, we are currently constructing mutants in the genes encoding these five regulators to determine their possible involvement in cysteine-dependent regulation of toxin synthesis.

**P43**

# GENETIC RELATEDNESS AMONG *Clostridium difficile* ISOLATES FROM VARIOUS ORIGINS BY TRIPLE-LOCUS SEQUENCE ANALYSIS BASED ON TOXIN REGULATORY GENES tcdC, tcdR AND cdtR

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A triple-locus nucleotide sequence analysis based on toxin regulatory genes tcdC, tcdR and cdtR was initiated in order to assess the sequence variability of these genes among *Clostridium difficile* isolates and to study the genetic relatedness between isolates. A preliminary investigation on the variability of tcdC gene was done on fifty-seven clinical and veterinary isolates. Twenty-three isolates representative of 9 main clusters were selected for tcdC, tcdR and cdtR analysis. The number of alleles found for tcdC, tcdR and cdtR were 9, 6 and 5, respectively. All strains possessed a cdtR gene except toxin A-negative toxin B-positive variants. All but one binary toxin CDT-positive isolates harboured a deletion (> 1-bp) in tcdC gene. The combined analysis of the 3 genes allowed to distinguish 5 lineages correlated with the different types of deletion in tcdC, i.e.: 18-bp (associated or not with a deletion at position 117), 36-bp, 39-bp, 54-bp and the wild type tcdC (no deletion). tcdR and tcdC genes though located within the same pathogenicity locus (PaLoc) were found to have evolved separately. Co-evolution of the three genes was only noted with strains harbouring a 39-bp or a 54-bp deletion in tcdC that formed two homogeneous separate divergent clusters. Our study supported the existence of the known clones (PCR-ribotype 027 isolates and toxin A-negative toxin B-positive *C. difficile* variants) and evidenced clonality of isolates with a 39-bp deletion (toxinotype V, PCR-ribotype 078) that are frequently worldwide isolated from human infections and from food animals.

**P44**

# ANALYSIS OF THE *Clostridium difficile* BINARY TOXIN LOCUS

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Introduction: The function and regulation of the *C. difficile* binary toxin (CDT) is currently poorly understood. The independent role of this toxin in pathogenesis or the possible symbiotic relationship with the major *C. difficile* toxins has yet to be defined. The objective of this study was to compare full binary toxin loci sequences from different *C. difficile* ribotypes in an effort to further the understanding of the regulation of the binary toxin and its putative regulator. We also sought to perform sequence and phylogenetic analysis to determine concordance between ribotype and binary toxin sequence relatedness.

Methods: Approximately 6500 bp of sequence, covering the binary toxin locus (from the promoter of the binary toxin regulator gene, cdtR, to the 3' end of the cdtB gene), was sequenced in 10 *C. difficile* strains from various different ribotypes and toxinotypes. The sequences were analyzed for polymorphisms, non-sense mutations, promoter features, signal sequences, and used for phylogenetic analysis. Quantitative polymerase chain reaction (qPCR) was also used to detect expression of the binary toxin enzymatic component gene, cdtA.

Results: Several consensus promoter features were identified. Various polymorphisms were identified ranging from single nucleotide polymorphisms to deletions of 9 nucleotides. A non-sense mutation in cdtR was identified in one strain, ribotype 078, that would result in a predicted severely truncated protein being produced. Despite this mutation, cdtA expression was still detected by qPCR. Dendograms based on total sequence indicated that isolates belonging to the same ribotype, shared the greatest similarity within the binary toxin locus.

Conclusion: The variability of the *C. difficile* binary toxin locus sequence supports the notion that this organism is very heterogeneous. Although cdtR has previously been demonstrated to be involved in regulation of cdtA expression, a nonsense mutation in the regulator did not inhibit expression of cdtA, suggesting either the truncated protein is functional, or another regulator of the binary toxin exists. Overall, sequence similarity was usually highest amongst isolates belonging to the same ribotype.

**P45**

# REFERENCE GENE EVALUATION AND BINARY TOXIN GENE EXPRESSION IN *Clostridium difficile*

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Introduction: Quantitative real-time PCR is a sensitive and efficient technique for studying gene expression. Identification of stably expressed reference genes is necessary to avoid bias yet potential reference genes do not necessarily behave the same between (or even within) bacterial species. Specific validation of candidate genes is required for *C. difficile*. The objectives of this study were to identify stable reference genes to be used to normalize *C. difficile* expression data, and compare expression of cdtA, and its putative regulator (cdtR), and tcdB during exponential (Expo) and stationary (Stat)phases, and in response to levofloxacin (Levo) exposure.

Methods: Eight genes (adk, gluD, gyrA, rho, rpoA, rpsJ, rrs, and tpi) were assessed in ribotypes 027, 078, and 001 during Expo and Stat growth phases and their stability ranked using geNorm. Expression of cdtA, cdtR, and tcdB was also evaluated in ribotypes 027 and 078, comparing Expo and Stat phase expression and assessing the effects of sub-MIC Levo exposure.

Results: rrs, adk, and rpsJ ranked among the most stable, but there were differences in the most stable genes between strains. There was an effect of growth phase on expression of cdtA, with 12.3X higher expression in Stat vs Expo phase for 027 but only a 1.6X increase for 078. Despite this, there was no difference in cdtR expression between phases for 027, and a decreased expression in Expo in 078. Expression of tcdB was 2.5X higher in Stat in 027 and 10X higher in 078 ($P<0.001$). In 027, exposure to Levo increased expression of tcdB (3.8X). but repressed expression of cdtA (0.5X) and cdtR (0.96X) in Stat phase. Increased tcdB expression (2.23X) was noted during Expo phase, along with reduced cdtR expression (0.04X) and no change in cdtA. An increase in cdtA (5.0X), cdtR (4.2X), and tcdB (4.2X) expression was detected in Stat vs. Expo in the presence of Levo. In 078, Levo repressed expression of all 3 genes in both phases (for cdtA 0.42X in Expo, 0.55X in Stat; for cdtR 0.37X in Expo, 0.79X in Stat ; for tcdB 0.27X in Expo, 0.1X in Stat). Despite overall lower expression of cdtR, and cdtA in response to Levo, a significant increase in expression in response to growth phase in the presence of Levo was still detected in the 027 strain with cdtR (20 fold, $P=0.013$) and tcdB (4 fold, $P=0.016$), and in the 078 strain with tcdB (2.5 fold, $P=0.016$).

Conclusion: Identification of strain dependent gene stability suggests selection of reference genes in this heterogeneous species requires assessment of the stability of multiple genes in the strains being studied. Variation in gene expression between growth phases and strains is consistent with studies of toxin production. The effect of Levo on gene expression, particularly increased tcdB expression in 027, may have clinical relevance.

**P46**

# tcdR TRANSCRIPTION CORRELATES WITH EXPRESSION OF tcdA AND tcdB

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Introduction: The rate and severity of *Clostridium difficile* infection (CDI) has increased in recent years and is at least in some cases associated with hyperproduction of toxins A and B, encoded by tcdA and tcdB, respectively. These genes are positively regulated by TcdR, and negatively by TcdC protein (Dupuy). Previous work suggested a negative correlation between tcdC expression and toxin expression in *C. difficile* VPI10643 (Hündberger 1997).

Materials and Methods: Single colonies of *C. difficile* strain 630 were used to inoculate pre-reduced Brain Heart Infusion broth supplemented with Yeast extract and L-cysteine. The inoculated bacteria were incubated at 37°C and anaerobic conditions. Samples for RNA preparation and toxin detection were taken at 2, 4, 6, 9 and 24 hours post inoculation. In addition, the optical density of each sample was determined to measure the growth of the bacteria. Total RNA was extracted using Trizol according to the manufactures' protocol, followed by a DNase step. cDNA was generated from total RNA and transcription levels of tcdA, tcdB, tcdC, tcdE and tcdR were determined by real-time PCR. The levels of toxin expression were determined with a commercial ELISA (Techlab) for TcdA and TcdB according to the manufactures' protocol.

Results: Transcription levels of tcdA, tcdB, tcdE and tcdR increases in time, with a concomitant increase in the production of Toxin A and B. tcdC demonstrates a distinct expression profile, with an initial increase followed by a moderate decrease in transcription levels. Overall, the ratio of TcdC/TcdR decreases in time.

Conclusion: We demonstrate a clear correlation between tcdR, but not tcdC, transcription levels and expression of the toxin genes tcdA and tcdB. Our data suggest that regulation of tcdR transcript is the dominant determinant. We propose that positive auto-regulation by TcdR is crucial for toxin expression and that this is affected by TcdC protein levels rather than transcription levels.

**P47**

# DEVELOPMENT OF A NOVEL MOLECULAR TECHNIQUE FOR THE IDENTIFICATION OF ESSENIAL CLOSTRIDIAL GENES

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For colonisation of *C. difficile* to occur in the gut of an individual, the resident gut flora must be quantitatively or qualitatively altered, normally through antibiotic treatment for an unrelated infection. Due to this antibiotic associated alteration, most broad-spectrum antibiotic treatments predispose a patient to becoming colonised. At present, oral vancomycin and metrondiazole are the antibiotics of choice, but these broad-spectrum antibiotics disturb normal gut flora, and suffer from poor recurrence rates and the emergence of resistance. Narrow-spectrum antimicrobials would circumvent these problems, but depend on the identification of novel drug targets. The products of essential genes may be good drug target candidates, and essential genes unique to an organism may equate to narrow-spectrum drug targets. Molecular techniques available to identify and study essential genes in other organisms have not yet been applied to *C. difficile*. One such strategy is to show that an essential gene cannot be knocked out unless a second copy of the gene is present in the genome. Here we use this strategy, which required the development of a novel method, and demonstrated a candidate narrow-spectrum drug target is indeed encoded by an essential gene.

**P48**

# RE-ANNOTATION OF THE GENOME SEQUENCE OF *Clostridium difficile* STRAIN 630

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The first complete genome sequence of *Clostridium difficile* (Sebahia, M., et al., Nature Genetics 38: 779-786), led to the development of high throughput projects such as comparative genomics, transcriptomics, proteomics and metabolomics. However the relevance of these experiments will depends on the information carried by the genes and their functions. Therefore a regularly update of the annotation is a key to maintain it accurate and relevant, reinforced recently with the outbreak of multiple genomic project (Stabler, R.).

Four years after the first publication, we manually re-annotated each of the coding sequence (CDS), using a high-level annotation platform MicroScope (Vallenet, D., et al., Database (Oxford) 2009 - PMID 20157493 -). This method is based on software pre-computed results allowing the re-annotation of CDS systematically proceeding by inference. Thus, we combined synteny groups (i.e. conservation of chromosomal co-localization between pairs of orthologous genes), and results from localization program (TMhmm, SignalP, PsortB), sequence alignment (Blast) and protein signatures (InterProScan). Furthermore several metabolic pathways were reconstructed using a metabolic pathway database (MicroCyc).

The function of more than 300 genes previously putative has been re-annotated based on new sequence similarities with proteins whose functions are identified by experimental data from the literature (2100 new PubMed references are added during the re-annotation process). We also modified several CDS starts (156), detected new CDS (16) and added 227 new enzyme commission (EC) numbers. Finally an intensive work for standardization of product name and gene name were carried out (PTS system, ABC-type transport ...).

All these data were stored in a relational database available soon on the MicroScope platform, (http://www.genoscope.cns.fr/agc/mage) as project ClostridioScope. We hope that the present effort will be benefit to the community.

**P49**

# MICROEVOLUTION OF EPIDEMIC *Clostridium difficile* 027 STRAINS REVEALS CHANGES IN ANTIBIOTIC RESISTANCE, FLAGELLA-MEDIATED MOTILITY AND PCR RIBOTYPE

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*Clostridium difficile* is the most common cause of antibiotic-associated diarrhea worldwide. Recently, the incidence and severity of disease has increased in the United States and United Kingdom due to the emergence of a highly virulent clone designated PCR ribotype 027. We compared 027 isolates from the US and UK isolated between 1988-2008 and show that antibiotic resistance has increased over time and that isolates from the UK are more motile than their US counterparts. Additionally, we demonstrate that some of the strains typed as BI by restriction endonuclease analysis, and presumed to be PCR ribotype 027 are in fact other PCR ribotypes. These observations show that 027 strains are continuing to evolve and that 050, 176 and new PCR ribotypes are potentially highly virulent.

**P50**

# AN lgt MUTANT OF *Clostridium difficile* EXHIBITS DIFFERENT LIPOPROTEIN PROTEIN PROFILES

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Intestinal diseases caused by the Gram-positive bacterium *Clostridium difficile* range from mild diarrhoea to life-threatening and sometimes fatal pseudomembranous colitis (PMC), and are almost always associated with the administration of antibiotics. *C. difficile* is one the most important causative agents of health care-acquired infections and are most commonly seen in the hospitalised elderly. Thus, identifing more virulence factors of *C. difficile* is an emerging issue. This study was aimed to investigate whether lipoproteins of *C. difficile* are involved in pathogenesis.
Gram positive bacterial lipoproteins are cell surface associated molecules that have been shown to contribute towards many cellular processes and several are recognised by immune cells suggesting that they may play a role in the virulence of certain pathogens. Similar to many other Gram positive bacteria, *C. difficile* possesses the enzymes necessary for the biosynthesis and processing of bacterial lipoproteins. Lipoprotein diacylglycerol transferse (Lgt) is responsible for attaching the protein to the extracellular side of the cytoplasmic membrane using membrane lipid components as an anchor.

We used the ClosTron system to knock-out the lgt gene of *Clostridium difficile* 630 and performed experiments to compare the protein profiles of the mutant and the wild-type strain. Our findings show that the loss of lgt has an effect on the expression profile of a subset of *C. difficile* lipoproteins that are recognised by the immune system. Further characterisation of the mechanisms of *C. difficile* protein acylation are being undertaken.

**P51**

# CHARACTERIZATION OF FIBRONECTIN BINDING PROTEIN A (FBPA) MUTANT FROM *Clostridium difficile*

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*Clostridium difficile* is a frequent cause of severe, recurrent post-antibiotic diarrhoea and pseudomembranous colitis. Its pathogenicity is mediated mainly by two toxins, TcdA and TcdB. However, different adhesins were also described as important colonization factors. We focused our interest on one of these adhesins, the Fibronectin Binding Protein A (FbpA). An FbpA mutant (ΔfbpA) was constructed in *Clostridium difficile* 630 erm strain by the Clostron technology.

We previously showed that the FbpA mutant did not adhere any more onto immobilized human fibronectin (hFn) when compared with isogenic wild type (WT) strain. In order to determine the site of interaction between FbpA and fibronectin we tested the binding of *C. difficile* 630Δerm strain to the N-terminal fragment of fibronectin (Heparin-Gelatine Binding Domain, HGBD 70 kDa fragment) as well as to the Heparin Binding Domain (HBD) and the Gelatine Binding Domain (GBD) of fibronectin. Our results showed that *C. difficile* 630Δerm strain binds to all of these fragments, thus suggesting that the whole N-terminal fragment of fibronectin (HGBD) plays a role in FbpA binding to hFn. As expected, in contrast to WT strain, ΔfbpA adhered neither to N-terminal fragment HGBD nor to HBD and GBD of fibronectin.

The FbpA mutant was also analysed in monoxenic and dixenic mice model by following the kinetic of intestinal implantation (faecal shedding) and caecal colonization (at day 7). In monoxenic mice, ΔfbpA shed in faeces at the same rate as isogenic WT strain but it colonized less the caecal wall. In dixenic mice, shedding rate was slightly slower for FbpA mutant compared to isogenic WT strain, but no difference in caecal colonization was observed. Similar rates of intestinal implantation and caecal colonization were observed for the two strains in assays performed in human microbiota-associated mice.

Caecal colonization (at day 7) let to evaluate the capacity of one strain to interact with the intestinal epithelium while kinetics of faecal shedding measures the intestinal implantation of a strain. Our data showed that both caecal colonization (monoxenic mice) and intestinal implantation (dixenic mice) of ΔfbpA are slower than those of WT strain, thus suggesting a role for FbpA in intestinal colonization by *C. difficile*.

# ABSTRACTS OF POSTERS PRESENTATIONS

SECTION 2 P52 to P102, Friday

**P52**

# IMMUNOMODULATORY ACTIVITIES OF SURFACE LAYER PROTEINS OBTAINED FROM EPIDEMIC AND HYPERVIRULENT *Clostridium difficile* STRAINS

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Purified monocytes and monocyte-derived dendritic cells (MDDC) represent a powerful tool to analyze in vitro human inflammatory immune responses. Moreover, they are an invaluable experimental tool to predict the type of immune response induced in vivo in humans from bacterial preparations such as surface layer proteins (SLPs) and to evaluate adjuvant properties useful to improve specific and protective immune responses. In these models we tested six SLPs purified preparations from *C. difficile* obtained from the hypervirulent strain R20291 (PCR- ribotype 027-UK), AI13 (PCR-rybotype 027-EFGCD) and the epidemic strains ATCC 43598 (PCR- ribotype 017), C253 (PCR- ribotype 012-IT), 630 (PCR- ribotype 012) and 001-3 (PCR-ribotype 001- ECDC). The last two *C. difficile* isolates are international reference strains. Monocyte activation was measured by protein secretion in ELISA or/and mRNA transcription. The ability of SLPs preparations to induce MDDC maturation was assessed by phenotypic analysis, measuring the up-regulation of the maturation markers. The cytokine profile was measured by protein secretion in supernatants of MDDC cultures elicited by SLPs preparations.

The results so far obtained indicate that SLPs preparations were able to induce pro-inflammatory cytokines (IL-1beta and IL-6) in human purified monocytes. The SLPs preparations induced maturation of MDDC, which acquire crucial functions of the mature stage, such as the production of regulatory cytokines. All SLPs were able to induce production of high levels of IL-10 and in general low or undetectable levels of IL-12p70.

Overall, no significant differences were found in the activation induced in monocytes and MDDC upon SLPs preparations from epidemic or hypervirulent *C. difficile* strains for the parameters so far tested. The differences in the structures, found by Spigaglia et al, in another poster on show in the meeting, are probably not sufficient to trigger different inflammatory processes associated with different levels of severeness of infection caused by epidemic or hypervirulent strains.

In conclusion, *C. difficile* SLPs may contribute to the pathogenicity of the bacterium by perturbing the fine balance of inflammatory and regulatory cytokines. These data are of interest also in the light of the possible use of SLPs in a multi-component vaccine against *C. difficile* infections for high-risk patients.

This work was supported by grant from EC project HYPERDIFF-HEALTH-F3-2008-223585.

**P53**

# *Clostridium difficile* TOXIN A AND B DOWNREGULATE INTERLEUKIN-16 IN HUMAN MAST CELLS HMC-1

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Mast cells play a crucial role in *Clostridium difficile* induced inflammatory processes of the colonic mucosa. The pathogenic factors TcdA and TcdB are the causative agents for clinical symptoms such as secretory diarrhoea and pseudomembranous colitis. We previously reported the activation of mast cells by TcdA and TcdB regarding exocytosis and release of prostaglandins. To elucidate direct effects of TcdA and TcdB on the expression of further inflammatory genes we performed cDNA microarrays of 110 selected inflammatory genes of the human mast cell line HMC-1. Interestingly, microarrays revealed only a limited number of genes whose expression was affected by TcdA or TcdB within the early phase of treatment. Interleukin-8 (IL-8), transcription factor c Jun, and the heme oxygenase-1 were more than two-fold upregulated. In contrast, IL-16, known as a CD4+ T-cell chemoattractant factor, was down-regulated whereas the typical mast cell mediator IL-6 was not affected. A corresponding decrease of IL-16 on protein level was verified by ELISA. RT-PCRs were performed to check the regulation in more detail. Time-dependent analysis showed an inverse correlation of the expression of IL-8 and IL-16. Treatment of HMC-1 cells with IL-8, however did not induce reduction of IL-16 mRNA, indicating that downregulation of IL-16 was not a sequel of IL-8 release. Furthermore, specific inhibition of the p38 MAP kinase abolished toxin-induced expression of IL-8 but had no effect on the downregulation of IL-16. Therefore, IL-8 and IL-16 expression was regulated independently in response to treatment with TcdA/TcdB. To further investigate the mechanism leading to altered gene expression we generated glucosyltransferase-deficient mutants of TcdA and TcdB. We found that downregulation of IL 16 as well as upregulation of IL-8 strictly depended on glucosylation of Rho GTPases since glucosyltransferase-deficient toxins did not alter mRNA levels.

In addition to the well known increase in IL-8 synthesis in response to treatment with Rho-glucosylating toxins our findings show relevant alteration in the level of chemoattractant IL-16 which might contribute to pathogenesis by affecting T-cell mediated host-pathogen response.

**P54**

# DESIGN OF A TOXIN-BASED VACCINE AGAINST *Clostridium difficile*: CHARACTERIZATION OF PROTECTIVE BINDING DOMAINS OF TOXINA AND B BY IN VITRO NEUTRALIZATION ASSAY

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*Clostridium difficile* is the major cause of pseudomembranous colitis and antibiotic-associated nosocomial enterodiseases.

Toxins A and B are principally responsible for the disease manifestations associated with the infection by causing damage to human colonic mucosa and inflammation of the colon.

To prevent *C. difficile* pathogenicity, we propose the development of a toxin-based recombinant vaccine in which the administration of binding domains induces neutralizing antibodies. Total IgG response anti-toxin A and B was evaluated by ELISA in mice immunized i.p. with three doses of recombinant binding domains in presence of two adjuvants. To prove the efficacy of the anti-toxin response, we analyzed the sera raised against binding domains of both toxins by in vitro cellular toxicity assay. We show that combination of binding domains of toxin A and B induce strong neutralizing antibodies. Toxin-neutralizing titers are comparable to titers reached by immunization with toxoid A and B, demonstrating that recombinant binding domains induce antibodies able to completely inhibit the cytopathic effect of the two toxins.

The current studies are supporting the choice of the optimal domain combination that will ultimately confer protection against toxin pathology in an in vivo model of infection.

**P55**

# MICROBIOME PROFILES OF RECIPIENTS AND DONORS POST FECAL TRANSPLANTATION FOR MULTIPLY RECURRENT *Clostridium difficile* INFECTION

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Recurrent *Clostridium difficile* infection (CDI) remains one of the most vexing clinical problems since no treatments are guaranteed to stop the cycle of disease control by antibiotic suppression of the pathogen followed by recrudescence of disease within days or several weeks post treatment. Probiotics are of unproven benefit. Yet fecal flora replacement, often as a single infusion, cures the vast majority of patients with multiply recurrent disease. Which members or combinations of members of the microbiome that contribute to control of *C. difficile* colonization and infection is unknown at present. To examine which microbial genera might be associated with the multiply recurrent disease state, the composition of the microbiome was determined in recipients of fecal flora replacement before and after transplantation and compared to donor profiles. Patients undergoing fecal flora replacement had >6 months of recurrent disease, generally with 4 or more recurrences and had failed tapering of vancomycin. Donors were genetically related family members (not spouses) who had no antibiotic exposure in the previous 6 months are were not on medications. Fecal samples were obtained after stopping vancomycin and before the procedure, at 1, 3 and 6 months post procedure. The microbiome was characterized by quantitative real time qPCR following extraction of bacterial DNA from 250 mg fecal samples using previously developed primers for Bacteroides / Prevotella, C. coccoides/Erectale, C. leptum, Desulfovibrio, Atopobium, Enterobacteriaceae, *C. difficile*, Lacto/Enterococcus, Lactobacillus, Bifedobacteria and Veillonella spp. The LLOD is 2 log10. On the basis of 5 recipient / donor sets of samples comparing pre, one month post and donor feces, multiply-recurrent CDI patients have marked reductions (> 4 log10) of Bacteroides/Prevotella, C. coccoides and C. leptum groups with corresponding increases primarily of Enterobacteriaceae and Veillonella spp. Enterococcal counts appeared not to be increased before or after transplantation, perhaps due to suppression by oral vancomycin (non-VRE). No alterations in Bifedobacteria or Lactobacilli in these data sets was observed, remaining non-dominant members of the microbiome. The microbiome profiles of recipients and donors were highly similar post transplantation. Comparison of these profiles suggest that Bacteroides, Prevotella, C. coccoides and C. leptum which have the highest shifts upwards post transplantation and associated diminution of coliforms play a major role in defense against *C. difficile* disease.

**P56**

# ANTIBODY-BASED THERAPEUTICS FOR *Clostridium difficile* INFECTION

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*Clostridium difficile* infection (CDI) continues to be a major problem as a healthcare-associated infection. The bacterium produces two large protein toxins (Toxins A and B) which are the principal virulence factors and the primary cause of the symptoms of CDI which can range from mild, self-limiting diarrhoea to life-threatening colitis. Some strains of *C. difficile* also produce a binary toxin whose contribution to pathogenesis is unclear. A substantial body of evidence suggests that an immune response to Toxins A and B provides protection against CDI in animal models. In the present study, we describe the requirements of potential antibody-based therapeutics for CDI. A toolbox of assays and techniques has been established as well as the capacity to produce polyclonal antibodies to the various toxins of *C. difficile* on a large scale. In the current study, we describe data demonstrating the efficacy of these antibodies as prophylactics against CDI in passive immunisation experiments using an animal model. The neutralising antibody composition required to provide protection against CDI induced by a variety of disease isolates is discussed as well as the clinical potential of such antibody-based therapeutics.

**P57**

# EXPOSURE TO QUINOLONE ANTIBIOTICS SELECTS FOR STRAINS OF *Clostridium difficile* WITH HIGHER MICS

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*Clostridium difficile* is a major cause of antibiotic-associated diarrhoea, particularly in the hospital setting. Several studies have reported particular links between the use of fluoroquinolones and the development of *Clostridium difficile* infection (CDI) and it has been suggested that fluoroquinolones may have contributed to the spread of ribotype 027. As part of a one-month national *C. difficile* typing and enhanced surveillance project, we determined the antimicrobial susceptibility of 139 *C. difficile* isolates to 13 antibiotics by the E-test method (Biomerieux). Details of antibiotic exposure in the eight weeks preceding diagnosis of CDI were collected.

Sixty-three percent of patients had received a penicillin, 12% a cephalosporin, 7% a macrolide, 19% a fluoroquinolone, 1% a glycopeptide and 5% a carbapenem antibiotic. All isolates typed were susceptible to metronidazole, vancomycin, co-amoxiclav and piperacillin-tazobactam,. The highest MIC recorded for metronidazole was 1.0mg/L (in ribotypes 001 and 106) and for vancomycin was 1.5mg/L (in ribotype 027). All isolates typed were resistant to ciprofloxacin, levofloxacin and cefuroxime. Resistance varied from 3-57% for the other antibiotics tested, but resistance to erythromycin and all quinolones tested was universal among ribotype 027 isolates. There was no correlation between exposure to related antibiotic classes and MIC levels with metronidazole, or betalactam, macrolide, glycopeptide or carbapenem antibiotics; however there was a statistically significant association between prior exposure to any quinolone and (moxifloxacin ($p<0.0001$), levofloxacin ($p<0.0001$) and ciprofloxacin ($p=0.05$)) MIC levels, with 93% of patients exposed showing MICs of > 32mg/L to all three quinolones tested.

In addition, quinolone exposure was associated with the presence of ribotypes 001, but not ribotype 027 or 078.

These findings suggest that quinolone antibiotics may exert positive selection pressure for strains with higher MICs, and should be restricted in clinical settings where C difficile is prevalent.

**P58**

# *Clostridium difficile* ISOLATED FROM ANIMALS: SUSCEPTIBILITY TO 30 ANTIMICROBIAL AGENTS USING BROTH MICRODILUTION METHOD

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The minimum inhibitory concentration (MIC) of 30 antimicrobial agents was determined by broth microdilution method for 31 isolates of *Clostridium difficile* from pigs (21), calves (5), dogs (4) and a horse (1).

The method was performed on commercially available broth microdilution plates for monitoring resistance of anaerobic and Gram-positive bacteria (Trek Sensititre Anaerobe MIC Plate and Gram-Positive MIC Plate). The antimicrobial agents tested were ampicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, cefotetan, cefoxitin, chloramphenicol, ciprofloxacin clindamycin, daptomycin, erythromycin, gentamicin, imipenem, levofloxacin, linezolid, meropenem, metronidazole, mezlocillin, moxifloxacin, nitrofurantoin, oxacillin, penicillin, piperacillin, piperacillin/tazobactam, quinupristin/dalfopristin, rifampin, streptomycin, tetracycline, tigecycline, trimethoprim sulfamethoxazole and vancomycin.

All isolates had low MICs for metronidazole (≤0.5 µg/mL-1 µg/mL) and vancomycin (≤0.25-0.5 µg/mL). For the fluorquinolones, the MICs90 were determined for levofloxacin (4 µg/mL), moxifloxacin (2 µg/mL) and ciprofloxacin (>2 µg/mL). For levofloxacin and moxifloxacin, MICs of three strains were >4 µg/mL. For the cephalosporins, the MIC90 for cefoxitin was >32µg/mL and for cefotetan 16 µg/mL. MICs for clindamycin were distributed within whole tested range (≤0.25->8 µg/mL, MIC90 8 µg/mL). MICs for erythromycin also varied (≤0.25->4 µg/mL, MIC90 1 µg/mL). MICs for tetracycline showed bimodal distribution: 58% of isolates had MIC ≤0.25 µg/mL and 42% of isolates had MIC 4-8 µg/mL. For the penicillins, the MICs90 were determined for penicillin (2 µg/mL), piperacillin (8 µg/mL), piperacillin/tazobactam (8/4 µg/mL), oxacillin (4 µg/mL), ampicillin (1 µg/mL) and amoxicillin/clavulanic acid (≤0.5/0.25% µg/mL). For the carbapenems, MIC90 for imipenem and meropenem was 8 and 1 µg/ml, respectively. For rifampin, all isolates had low MICs (MIC50 and MIC90 ≤0.5 µg/mL), except one strain isolated from a dog (>4 µg/ml).

We found the broth microdilution method convenient to perform, reproducible and MIC endpoints were generally clearly determined. However, in the case of some antimicrobial agents, the dilution range was too narrow to identify resistant strains.

**P59**

# DISTRIBUTION OF EPIDEMIC PCR-RIBOTYPES AND RESISTANCE TO ANTIMICROBIAL AGENTS IN *Clostridium difficile* ISOLATED FROM DIARRHOEIC ADULT PATIENTS HOSPITALIZED BETWEEN 2004-2008 IN THREE HOSPITALS IN POLAND

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We investigated distribution of type of toxigenicity (TcdA, TcdB and CDT-binary toxin) and resistance to nine antimicrobial agents among 373 *C. difficile* strains isolated from diarrhoeal patients hospitalized in different hospitals in Poland (2004-2009). MICs for clindamycin, erythromycin, tetracyclin, metronidazole, vancomycin, imipenem, ciprofloxacin, moxifloxacin, gatifloxacin and rifampicin were determined. 62 *C. difficile* strains from hospital A were A+B+CDT-, 8 were A+B+CDT+, 74 were A-B+CDT- and 9 were non-toxigenic. In hospital B 87 were A+B+CDT-, 11 were A+B+CDT+, 70 were A-B+CDT- and 9 were non-toxigenic. In hospitals C 23 were A+B+CDT-, 6 were A+B+CDT+, 6 were A-B+CDT- and 6 were non-toxigenic. Among strains originating from hospital A, B and C resistance to erythromycin was found in 53,6%, 48,6% and 48,3%, respectively. Resistance to clindamycin was 53,6%, 47,5% and 53,5%; to ciprofloxacin resistance frequencies were 98%, 98,3% and 86%%. The resistance levels against moxifloxacin 38,5% (A), 38,4% (B), and 32,5% (C), to gatifloxacin 38,5% (A), 40,1% (B) and 30% (C) to imipenem resistance were 72,4% (A) and 74,4% (B) and to tetracycline resistance were 22,6% (A) and 22,6% (B) and all strains were susceptibility to rifampicyn. Among 25 binary toxin positive *C. difficile* strains isolated from Polish patients, 2 strains showed the characteristics of PCR ribotype 027 ( hospital B) and 4 strains were PCR-ribotype 176. The rest of the strains belonged to PCR-ribotypes 023, and 045. *C. difficile* strains of toxigenicity A-B+CDT- and classified as PCR-ribotype 017 were common in hospital A and B. Strains of PCR-ribotype 017 were co-resistant to clindamycin, erythromycin, and the newer fluoroquinolones. One strain belonging to PCR-ribotype 027 was resistant to erythromycin and one to clindamycin and erythromycin. All strains belonging to PCR-ribotype 176 were resistant to clindamycin and erythromycin. Among *C. difficile* strains isolated between 2005-2008 the frequency of resistance to clindamycin and erythromycin was very high. Erythromycin resistance was frequently associated with clindamycin resistance. Resistance to ciprofloxacin was very frequent. Epidemic PCR-ribotypes of *C. difficile* were detected in all hospitals. Three ongoing epidemics of *C. difficile* PCR-ribotypes 017, 027 and 176 were detected in Poland.

**P60**

# COMPARATIVE EFFICACIES OF DAPTOMYCIN AND VANCOMYCIN FOR TREATMENT OF *Clostridium difficile*-ASSOCIATED DIARRHEA IN HAMSTERS

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*Clostridium difficile*-associated colitis is an increasing cause of morbidity and mortality in hospitalized patients, with high recurrence rates following conventional therapy. Daptomycin, a non-absorbed lipopeptide antibiotic which inhibits the biosynthesis of cell wall peptidoglycan, has proved a rapid bactericidal activity in vitro against *C. difficile*. We carried out a study combining epidemiological and experimental approaches in order to evaluate the efficacy of daptomycin against *C. difficile* in vitro and in vivo in a hamster model of *C. difficile*-associated diarrhea (CDAD). The minimal inhibitory concentrations (MICs) of daptomycin were determined against a set of reference and clinical *C. difficile* strains collected from two French hospitals.

For in vivo experiments, hamsters received clindamycin by oral route five and one days before being infected with the reference *C. difficile* strain (VPI 10463). Daptomycin (50 mg/kg of body weight), vancomycin (50 mg/kg) or isotonic saline (for untreated controls) were then given orally according several therapeutic schemes: a single dose or multiple dose for five consecutive days beginning either 24 hours after the challenge with *C. difficile* or since the onset of CDAD. The respective therapeutic efficacies of both antibiotics was evaluated by Kaplan Meier survival estimates including weight gain, delay to the onset of CDAD, delay to death or the observation of recurrence within 30 days post infection in case of survival.

In epidemiological approach, the MIC50 and MIC90 for 120 strains of *C. difficile* were 0.38 and 0.75 mg/L respectively [range: 0.047-1.5]. The MIC for *C. difficile* VPI-10463 was 0.5 mg/L. Oral treatment with a single and identical dose of both antibiotics produced a similar prolonged survival of hamsters. However, although the onset of CDAD and the death were significantly delayed, death invariably occurs within 7 to 20 days after those of untreated animals. Oral treatment with a multiple dose of both antibiotics for five consecutive days showed that daptomycin was as effective as vancomycin, as reference treatment, in delaying death in hamsters. However, it has been observed fewer recurrences at 30 days among animals treated with daptomycin than in those treated with vancomycin. No resistant strains or increasing MICs were observed during all in vivo experiments. Altogether, our results indicate that daptomycin is an effective therapeutic agent for clindamycin-associated colitis in hamsters and suggest that it may be a valuable alternative for the treatment of CDAD.

**P61**

# *Clostridium difficile* STRAINS WITH INTERMEDIATE MIC LEVELS TO TETRACYCLINE: INDUCTION OF RESISTANCE AND CHARACTERIZATION OF RESISTANCE DETERMINANTS

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Aim. To characterize 31 *Clostridium difficile* strains, isolated during the European prospective study conducted in 2005, showing intermediate MIC levels for tetracycline (TC).

Materials and Methods. The E-test method was used to determine the MIC of strains. The breakpoint was 16 mg/L as suggested by CLSI. Induction of resistance to TC was evaluated by pre-growth for 18 h Brucella broth containing sub-inhibitory concentrations of the antibiotic (from 3 to 6 mg/L). Primers TETMd/TETMr, tndx1/tndx2 and INT1/INT2 were used to detect tetM, tndX and int genes, respectively (JAC. 2006, 57: 1205–1209). tndX was used as a marker for element Tn5397, specific of *C. difficile*, whereas int was used as a marker for elements belonging to the Tn916 family, widespread in a large number of bacteria. Elements belonging to the Tn916 family were characterized by PCR mapping (AAC. 2005, 49: 2550–2553).Other tet classes, tetA, A(P), O, Q, and W, were detected using the primer couples already described (JAC. 2006, 57: 1205–1209). Sequencing analysis was performed by the DNAStar's Lasergene v.8.0 software, the National Center for Biotechnology Information BLAST server and the European Bioinformatics Institute Clustal W server. Results. The MICs to TC of the 31 strains examined ranged between 6 and 12 mg/L. After induction the strains showed resistance with MICs ranging between 24 and 64 mg/L. Twenty three isolates were positive for tetM and int, 8 for tetM and tndX. Four different genetic organizations of Tn916 elements were identified in a selection of 11 representative *C. difficile* strains and two of these organizations have never been observed before. Interestingly, one isolate was positive for tetO. This is the first report of this class in *C. difficile*. The TetO encoded is a new variant showing 97% of identity with that found in Dorea longicatena DSM 13814, a member of the Firmicutes division isolated from colonic microbiota of humans. Conclusions. The present study suggests that *C. difficile* strains intermediate to tetracycline harbour a tetM gene and have an inducible resistance to this antibiotic. In the majority of the strains analyzed tetM was carried by a Tn916-like element, a family of transposons known to be able to transfer antibiotic resistance genes and virulence determinants. The co-presence of a tetM and a tetO gene, a tet class already identified in bacteria from human and animals, has been found in one isolate. Genetic exchanges of antibiotic resistance determinants among *C. difficile* and other intestinal bacteria of different origin may represent a chance for this pathogen to acquire new virulence traits with relevant clinical consequences.

**P62**

# HIGH RESOLUTION MELTING ANALYSIS FOR THE DETECTION OF SNPS IN THE RPOB GENE OF *C. difficile*

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*Clostridium difficile*, a Gram-positive, spore-forming anaerobic bacterium, is the main causative of hospital-acquired diarrhea worldwide. In addition to Metronidazole and Vancomycin, a promising antibiotic for the treatment of recurring *C. difficile* infections (CDI) is rifaximin, a rifamycin derivative. The exposure of *C. difficile* to this antibiotic has led to the development of rifaximin-resistance due to point mutations in the rpoB gene. This gene encodes the -subunit of the RNA polymerase, the target of rifaximin.

350 *C. difficile* strains of the AGES strain collection, previously phenotypically tested for rifaximin resistance, were investigated by sequence analysis for respective single nucleotide polymorphisms due to point mutations within the rpoB gene. Subsequently genotyping of the SNP hot spot region of the rpoB gene was performed by high resolution melting analysis (HRM). This method allows the detection of SNPs by detecting the alteration of the melt behaviour of mutated double-stranded DNA in contrast to the wild type.

The creation of artificial heterozygotes led to characteristic curve shapes used as comparison standards in HRM and thereby allowing differentiation between wild type strains and strains containing SNPs in the respective rpoB gene section.

In this study, we were able to identify 10 different SNPs in the rpoB hot spot region in 350 *C. difficile* strains investigated. Seven SNPs were associated with rifaximin resistance (leading to amino acid substitutions), three of which have not been previously described. Three of ten SNPs did not result in amino acid substitutions, and therefore had no impact on the rifaximin resistance in the respective isolates. HRM results correlated with sequencing analysis results and antibiotic resistance testing of all investigated *C. difficile* strains, except for two of 70 rifaximin resistant strains that could not be detected by applying the HRM method.

This investigation demonstrates that this HRM analysis is a fast and cost effective detection tool for SNPs in the rpoB gene of *C. difficile* to distinguish potentially resistant from sensitive strains. Furthermore, this method can even be used to identify specific SNPs.

**P63**

# PREVALENCE OF THE VANG-LIKE CLUSTER IN *Clostridium difficile* CLINICAL ISOLATES

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Treatment of *Clostridium difficile*–associated diarrhea requires in general cessation of their causative antibiotic and subsequent administration of metronidazole or vancomycin. Intriguingly the genome of *C. difficile* strain 630 contains a gene cluster homologous to the vanG-type operon of Enterococcus faecalis BM4518 (Sebaihia et al, 2006). However, the vanG-like cluster does not confer vancomycin resistance to *C. difficile* strain 630.

Distribution of this cluster in *C. difficile* was investigated by PCR studies on 41 clinical isolates. The vanG-like cluster was found in 35 out of the 41 isolates confirming its high prevalence in this bacterium. Amongst these 35 isolates, the cluster was found to be located at the same locus between two genes encoding a putative flavoprotein and a putative membrane protein. Interestingly, the intergenic region delimited by these genes contains a perfectly conserved 19 base-pair inverted repeat in which the vanG-like cluster can become inserted. However, analysis of the vanG-like environment does not highlight any recombinase or transposase gene, and mobility of the vanG cluster remains until now unestablished.

**P64**

# IS ANTIBIOTIC RESISTANCE OF *Clostridium difficile* PCR RIBOTYPE 078 INDICATIVE FOR ZOONOTICAL TRANSMISSION?

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*Clostridium difficile* PCR ribotype 078 is a common cause of *Clostridium difficile* infections (CDI) in both humans and animals. Currently, this type is the third most common type detected in humans the Netherlands and the most commonly detected strain in Dutch pigs. To study similarity between human and pig CDI, and indirectly the possibility of a common source or interspecies transmission, we determined the antibiotic susceptibility patterns and genetic relatedness of type 078 in the Netherlands.

We randomly selected fifty human *C. difficile* type 078 isolates from stool samples submitted to the Dutch national reference laboratory between June 2006 and May 2009. Furthermore, we selected 49 type 078 isolates from Dutch piglets with neonatal diarrhoea, with a maximum of two strains per farm (total of 25 farms).

The antibiotic susceptibility patterns of these isolates were determined using the E-test method: a suspension of *C. difficile* was cultured on non selective blood agar plates in an anaerobic environment at 37˚C. Thereafter, the strains were inoculated in PBS to a 1 McFarland suspension and plated on Brucella blood agar plates. Minimal inhibitory concentrations (MICs) for tetracycline, amoxicillin, co-trimoxazole, erythromycin, clindamycin, moxifloxacin, cefuroxim, imipemen and ciprofloxacin were determined after an incubation of 48 hours. MIC breakpoints were obtained from EUCAST. Genetic relatedness was analyzed using MLVA. Molecular characterization of antibiotic resistance is underway.

Similar antibiotic susceptibility patterns of human and porcine isolates for all tested antibiotics were observed. Almost all human and porcine isolates were susceptible to amoxicillin (100%) and clindamycin (91%), whereas resistance was found for cefuroxim and ciprofloxacin (94%). A variable resistance pattern was found for erythromycin (68% resistant), imipenem (39% resistant), moxifloxacin (16% resistant), tetracycline (14% resistant) and co-trimoxazole.

Previous studies found similar antibiotic susceptibilities in type 078 from pigs and humans, but only limited numbers of strains were investigated. This study provides an systematic, broad, view on antibiotic susceptibility patterns in CDI due to type 078 and indicates a high degree of similarity between human and pig 078 *C. difficile* type 078 strains.

**P65**

# EVALUATION OF THREE COMMERCIALLY KITS FOR TOXIGENIC *Clostridium difficile* MOLECULAR DETECTION IN STOOLS

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*Clostridium difficile* is the most common bacterial cause of nosocomial diarrhoea. A few years ago, the incidence of *C. difficile* infections markedly increased leading laboratories to perform an optimal testing strategy. The aim of this study was to evaluate performances of three commercially kits for toxigenic *C. difficile* molecular detection.

Ninety-four stool specimens were studied in a retrospective study. These stools were obtained from patients hospitalized respectively in Jean Verdier (46 patients) and in André Mignot Hospital (40 patients). Three commercially kits : BD GeneOhm Cdiff (BD Diagnostics, San Diego,CA), XPert *C. difficile* PCR (Cepheid, Sunnyvale, CA) and illumigene *C. difficile* not FDA Approved in the US at time of submission (Meridian Bioscience, Cincinnati, Ohio) were evaluated. The results were compared to the cytotoxicity assay and to the toxigenic culture (performed with ImmunoCard Toxins A and B, Meridian Bioscience) which are considered as “gold standard”.

Among the 94 stool specimens, 47 results (50%) were true negative (all the methods negative) and 39 (41,5%) were true positive (all the methods positive). For six stools (8,5%), discrepancies were observed : one false positive result with BD GeneOhm and one false positive with XPert and four false negative with illumigene. As compared to the gold standard, BD GeneOhm, XPert C difficile and illumigene demonstrated respectively a sensitivity of 100%, 100% and 91% and a specificity of 98%, 98% and 100%. The positive predictive values were respectively 97,5%, 97,5%, 100% and the negative predictive values 100%, 100% and 92%. In this retrospective study, stool specimens were frozen and thawed. This could explain three false negative with illumigene. The fourth false negative result was in a patient after 10-days treatment with metronidazole for a *C. difficile* infection.

In conclusion, the three commercially kits show good performances. For XPert, the test is easy to perform, rapid, simple but is still expensive. BD GeneOhm is rapid but needs experimented operator because of a crucial extraction step and gives many unresolved results leading to repeat testing. illumigene is rapid, easy to perform and cost effective. These results have to be confirmed in a prospective study to define a diagnostic algorithm to obtain an accurate and cost effective *C. difficile* infection diagnosis.

**P66**

# EVALUATION OF RAPID DETECTION OF TOXIGENIC STRAINS OF *C. difficile* IN DIARRHEAL STOOLS BY ISOTHERMAL AMPLIFICATION

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Background: *Clostridium difficile* is responsible for antibiotic-associated diarrhea and for virtually all cases of pseudomembranous colitis. This spore forming anaerobic bacterium is not only a major cause of hospital-acquired diarrhea but is also responsible for infections in the community and in animals. A rapid and accurate diagnosis of *C. difficile* infections (CDI) is essential not only for patient's management by also for timely implementation of infection control measures. illumigene™ is a molecular assay based on loop-mediated isothermal amplification technology (LAMP). The illumigene™ *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the 5' region of the tcdA gene. This region is found in all toxin producing strains including the A-B+ strains. Magnesium-pyrophosphate is produced as a result of the amplification which causes the reaction solution to become turbid. This turbidity is measured after 40 minutes using the Meridian illumipro-10™ Incubator/Reader.

Method: The performances of illumigene™ *C. difficile* (Meridian Bioscience Inc., France) were compared with cytotoxicity assay and toxigenic culture for the detection of *C. difficile*. Diarrheal stool samples were collected from patients suspected of having *C. difficile* infection. Cytotoxicity assay and culture were performed according to standard protocols. Discrepant results were assessed using an enrichment culture method (broth heart infusion added with 0.1% of taurocholate, 250 mg/l cycloserine and 10 mg/l cefoxitine).

Results: A total of 470 specimens were included in the study: 23 (4,9%) were positive by the cytotoxicity assay and 48 (10,2%) by the toxigenic culture. Fifty one (10.8%) were positive both by the toxigenic culture and illumigene™. Three were negative by direct culture but positive by illumigene™ but all these samples were found positive by an enrichment culture. Four specimens were positive by culture, but negative by illumigene™: 1 of these samples were found to be positive by illumigene™ after repeat testing. Overall, the sensitivity, specificity, negative and positive predictive values of illumigene™ *C. difficile* were 97%, 96,6%, 99,8% and 68% respectively as compared to the cytotoxicity assay and 94.1%, 100%, 99,2% and 100% respectively, as compared to the toxigenic culture.

Conclusions: illumigene™ *C. difficile* is a rapid and sensitive method for the detection of *C. difficile* directly from stool specimens.

**P67**

# THE USE OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD FOR IDENTIFICATION OF *Clostridium difficile* PCR RIBOTYPE 027

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Objectives: A hyper virulent *Clostridium difficile* strain, PCR ribotype 027 has been reported in North America and Europe to cause more severe disease. The earlier recognition of PCR ribotype 027 may be beneficial in countries where that PCR ribotype is epidemic as well as in countries where that type is not currently predominant. We established and evaluated a loop-mediated isothermal amplification (LAMP) method for the identification of PCR ribotype 027.

Methods: Toxin B-positive *C. difficile* isolates recovered from symptomatic patients admitted to hospitals in Japan were used. A collection of isolates for the previous collaborative typing study (Killgore G et al. 2008 J Clin Microbiol) was included. Isolates were typed by sequencing the surface layer protein A gene (slpA). For the LAMP assay, 4 primers used were derived from the sequence of the slpA gene of a US42 strain, which was typed as PCR ribotype 027 / slpA sequence type gc8-01.

Results: The isolates representing 23 slpA sequence major types and 55 subtypes were tested for the detection of slpA-gc8 gene by LAMP. The slpA-gc8 gene was positive by LAMP in isolates typed as slpA sequence major type gc8; no amplification products were obtained in isolates of other slpA sequence types. All of 26 PCR ribotype 027 isolates tested in this study belonged to slpA sequence type gc8, and were LAMP-positive. Five subtypes were found among isolates belonging to slpA sequence type gc8. Four of the 5 subtypes were toxin A-positive, toxin B-positive, and binary toxin-positive, and were typed as PCR ribotype 027. An isolate of the subtype gc8-05 was toxin A-negative, toxin B-positive, and binary toxin-positive; its PCR ribotype pattern differed from that of 027. The slpA-gc8 gene was detected in 6 isolates recovered from sporadic cases in different areas in Japan. Of these, 5 isolates were typed as PCR ribotype 027 and susceptible to both gatifloxacin and moxifloxacin. The remaining one (subtype gc8-05) was obtained from a patient, who suffered from pseudomembranous colitis. This isolate had deletions in the tcdA and tcdC genes similar to those in 8864 strain (GenBank accession number AJ011301) and resistant to gatifloxacin and moxifloxacin.

Conclusion: The LAMP assay detecting the slpA sequence of PCR ribotype 027 is likely to be a valuable tool for the discovery of *C. difficile* cases and outbreaks due to the epidemic strain that may be overlooked.

**P68**

# SURVEY OF ENGLISH NHS LABORATORY TESTING METHODS FOR *Clostridium difficile* REVEALS WIDESPREAD SUBOPTIMAL PRACTICE

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There are several options avalable for the laboratory testing of *Clostridium difficile*, however there is little standardisation of practice in the UK. Recent studies reveal significant shortcomings in the performance of toxin Enzyme Immunoassays (EIAs) which are a commonly used testing method. The UK Health Protection Agency advised against using toxin EIAs as a standalone method in March 2009.

All Acute NHS trusts in England (n=170) were surveyed to determine exact testing methods and laboratory prevalence for 2008/2009. Results were received from 168 Trusts (99% response rate) which revealed the most common testing method was a toxin EIA alone (70%). This resulted in a Positive Predictive Value (PPV) as low as 20% in some cases. Only six laboratories (3.6%) were able to offer the current gold standard of cell cytoptoxin neutralisation assay. Thirty five laboratories (19%) were using a two step algorithm which usually combined toxin A/B EIA, GDH EIA and/or PCR. However eight (5%) laboratories were using two toxin EIA's together which may not be a significant improvement on a single toxin EIA alone. The mean number of samples processed by each laboratory was 4241 in 2009 (4208 in 2008) however the mean prevalence decreased from 6.45% in 2008 to 4.47% in 2009. This can be expected to impact negatively on PPVs of diagnostic tests.

The UK government mandates reporting of cases of infection in all patients over the age of 2 years and encourages the public to compare hospital rates as a measure of quality of care and good infection control practice. This data is publicly available via a government website and is now updated on a weekly basis. Comparisons in this way may not be fair or effective given the wide disparity between testing methods and data must be interpreted with great caution (which is often not the case when viewed by the general public or media). Trusts may be averse to improving diagnostic tests for fear of increasing apparent rates of infection which could result in bad publicity and significant financial penalties amounting to several millions of pounds. There must be a clear guarantee from central government that this will not be the case in order to encourage laboratories to use the most accurate and cost effective testing methods.

The present study is the most complete data on the laboratory testing methods of English NHS trusts and clearly demonstrates widespread suboptimal testing practices. Trusts should urgently reconsider their testing strategies.

**P69**

# DEVELOPMENT OF A POINT-OF-CARE PLATFORM FOR THE RAPID DIAGNOSIS OF *Clostridium difficile*

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The rapid detection of nosocomial infections is recognised as one of the primary methods of prevention of their spread, in addition to leading to improved antimicrobial stewardship and individual patient care. *Clostridium difficile* is the leading cause of hospital-associated diarrhoea in North America and Europe with studies showing that *C. difficile* infections (CDI) cost American Hospitals in excess of 1 billion dollars annually through heightened disease severity, while the incidences of these infections is increasing. At present the timely isolation of infected patients can be hampered by the poor sensitivity and specificity of the currently available diagnostic immunoassays as well as the relatively slow turnaround time. Herein we describe the development of the hardware (Enigma ML, Enigma Diagnostics Ltd, Salibury, UK) and assays capable of detecting toxigenic *C. difficile* unambiguously in under an hour and without the need for specialist training or interpretation. Magnetic extraction methods, Enigma Diagnostics' unique Electrically Conducting Polymer (ECP) and a simple touch screen user interface with a reporting system providing a qualitative result of either "Positive", "Negative" or "Error" each contribute to an instrument that can return clear, concise results rapidly and without the need for laboratory consultation. At present the best available alternative PCR-based methods of *C. difficile* detection are the BD GeneOhm Cdiff assay (BD Diagnostics, San Diego, CA), designed to detect the tcdB gene, and the Cepheid Xpert *C. difficile* assay (Sunnyvale, CA), designed to detect the tcdB genes, as well as genes and gene sequences associated with the hypervirulent phenotype. The combination of assay and platform described in the current study is capable of detecting a larger number of target genes than the BD GeneOhm Cdiff assay and is designed to detect more clinically relevant targets than the Cepheid Xpert *C. difficile* assay. Thus the Enigma ML, in combination with the real-time PCR assays to detect the tcdA and tcdB genes, will provide rapid, concise and definitive results in less than sixty minutes. The contained nature of the Enigma ML, as well as its ease of use and portability, makes it ideal for use on hospital wards, doctors' surgeries and other point-of-care settings. With increased surveillance and reporting of these nosocomial bacteria, healthcare professionals will be better able to implement control and isolation strategies to prevent their spread.

**P70**

# RAPID IMMUNOCHROMATOGRAPHIC TEST TO DETECT *Clostridium difficile* RIBOTYPE 027 AND 017 INFECTIONS

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*Clostridium difficile* Infection (CDI) with hypervirulent strains emerged in recent years that cause endemic outbreaks of CDAD and PMC and seriously compromise the life of patients. Identification of endemic hypervirulent strains requires the immediate isolation of patients and implementation of specific hygiene measures. To simplify the detection and decrease the time to diagnosis of ribotype 027 and 017 CDI OPERON has used antibodies against TcdB developed by tgcBIOMICS to establish a novel rapid test (designated HYPERdiff) which enables identification of ribotype 027 and 017 *C. difficile* strains not only in microbial laboratories but even at the point of care. To run the test an aliquot of the stool sample is suspended in a sample buffer and following sedimentation the supernatant is used for assaying. Time from collecting the sample to receiving the results is approximately 25 minutes. No expensive equipment is needed. Results are easy to read, a blue control band indicates proper performance of the test, when positive a single second red band appears. Of these *C. difficile* ribotype 017 infections are identified with the Operon 2 A B diff test (TcdA-; TcdB+). Putative ribotype 027 infections are recognized by a positive HYPERdiff assay accompanied with two red bands (TcdA+; TcdB+) in 2 A B diff. The only exception are infections with other but more rare toxinotype III strains (like ribotype034; 075; 080) that give an 027-like assay pattern.

To evaluate the HYPERdiff assay, meaning to define the reliability of ribotype 027 and 017 detection, we made use well characterized panels of *C. difficile* strains like (i) the panel of most common *C. difficile* REA Types, (ii) the panel of most common *C. difficile* PFGE types and (iii) a third collection of the University of Mainz containing other common ribotypes isolated from fecal samples. Independent of the source of strains HYPERdiff detected all the ribotype 017 and 027 strains (and the rare other toxinotype III isolates), without exception. Our result indicate that HYPERdiff has a high predictive value for the absence of ribotype 027 and 017 strains. Negative HYPERdiff results (no red band to appear) exclude ribotype 027 and 017 infection. Only samples positive in HYPERdiff and with two bands in 2 A B diff (027-like assay pattern) need further attention, means isolation of the bacterium for typing to verify the putative 027 case. Thus HYPERdiff may be used to set the course for further diagnostic procedures meanwhile saving time, personnel and money since negative probes can be excluded from intensive and costly additional investigations. In conclusion we present promising results that show that the HYPERdiff test is a highly reliable rapid test to pinpoint to endemic, hypervirulent 017 and 027 strains as early as possible during CDI. The test is an ideal means to reduce the workload and cost of CDI and to focus on the patients that need the most attention concerning treatment and hygiene measures.

**P71**

# RAPID IMMUNOCHROMATOGRAPHIC POINT OF CARE TESTS THAT DIFFERENTIATE *Clostridium difficile* TOXINS TCDA, TCDB AND SUSPECTED HYPERVIRULENT STRAINS (AS 017 & 027)

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*Clostridium difficile* infection (CDI) is responsible for approximately 25 % of the cases of antibiotic related diarrhea. Generally, CDI is a nosocomial infection in severe cases leading to peudomembranous colitis (PMC) that may affect the patient´s life. Mortality rates of PMC are between 6% and 30%, with an increased rate when infection is due to the new hypervirulent strains of ribotype 027 that even infects people at younger age. In the hospital CDI prolongs hospital stay of the patients and produces an economic burden of $ 3.2 billion in the USA. Rapid identification of CDI is an essential step in a series of measures to block the infecting organism to spread in the local environment. Main consequences are severe hygiene measures and eventually treatment of the patient with specific antibiotics.

We here summarize data obtained with a novel one step lateral flow assay (designated 2 A-Bdiff assay) that was developed at Operon in Spain. The test is the first to differentiate between TcdA and TcdB detection in one reaction. 2 A-Bdiff has a detection limit of 3 ng/ml TcdA and 12 ng/ml TcdB which approximates the best values of ELISA systems on the market. Results are obtained within ~25 minutes from sample collection till the end of the lateral flow run. The test can be done without expensive equipment, so that the 2 A-Bdiff assay turns point of care testing to a real option. 2 A-Bdiff detects TcdA and TcdB in two separate bands.

This differentiation is needed not only due to the recent publications (1, 2) that demonstrated that lethal infections with single TcdA+ or TcdB+ mutants to be fatal and detection, but also since it allows simple and fast detection of putative ribotype 017 CDI endemic in USA (TcdA-, TcdB+). In combination with the HYPERdiff-assay of Operon 2 A-Bdiff renders the detection of putative hypervirulent ribotype 027 CDI possible. Since both tests are run with identical sample buffers they can easily be combined.

We here present data of the evaluation of 2 A-Bdiff at Saragossa Hospital. Testing was done on 215 stool samples of patients hospitalized with gastrointestinal disorders during January to November 2009. Fresh samples were tested in a cytotoxicity assay at the hospital, 2 A-Bdiff testing was performed at Operon laboratories using the same samples that had been frozen in between. As a further control a commercial ELISA was run on the frozen samples. Results with the 2 A-Bdiff

assay were identical. Of the 215 probes 70 were positive in the cytotoxicity and the 2 A-Bdiff assays, 126 negative for both techniques. Four samples were positive only with 2 A-Bdiff, 15 were positive only with cytotoxicity testing. Provided freezing did not interfere with the testing the sensitivity an specificity of 2 A-Bdiff is 82,4% and 96,9% respectively. These results will be discussed.

In conclusion, the 2 A-Bdiff test is highly reliable and the fastest approach available to diagnose CDI. Due to the simplicity of testing it can be used not only to diagnose CDI in microbial laboratories but also at the point of care. When combined with the HYPERdiff-assay of Operon the 2 A-Bdiff test is the quickest way to identify CDI due to endemic hypervirulent ribotype 027 and 017 strains.

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**P72**

# DIAGNOSIS OF *Clostridium difficile* INFECTION IN CLINICAL LABORATORIES : A DIFFICULT COMPROMISE BETWEEN PERFORMANCE, PRACTICABILITY AND COST

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The current guidelines for the diagnosis of *Clostridium difficile* infection (CDI) combine rapid detection of toxins A and B and toxigenic culture. However, there is no definitive consensus on the best tests and their combination. Thus, the diagnosis of CDI still remains tricky for many clinical microbiology laboratories. The results of an audit conducted during 2008 in our institution highlighted the limits for the rapid detection of both toxins and a low rate of isolation of the bacteria and suggested several propositions for improving our algorithm.

The aim of our study was to evaluate prospectively technical characteristics, feasability, and performance of several commercially available tests performed systematically for rapid detection of toxin A and B (ImmunoCard® toxins A & B (ICTAB, Meridian), Vidas® *C. difficile* A & B, (CDAB, bioMérieux)), toxigenic culture, and real-time PCR GeneOhm CDiff (BD). All consecutive diarrheic stools from adult patients, sent to the laboratory for suspicion of CDI during a five-month period, were included in the study. The results of each tests were compared to the cytotoxcity neutralization assay (CCNA), as reference test, and the clinical data for each episode of CDI (≥ 3 months between two episodes for the same patient). A total of 28 cases of CDI (11.8%) were diagnosed among the 237 fresh stools tested (215 patients). Systematic and rapid culture of sample, use of UV (312 nm) enrichment step in broth to isolate and identify suspect colonies have significantly improved the rate of isolation of *C. difficile* (Se: 93%, Spe: 100%). Toxigenic culture, using the ICTAB test, showed a total concordance with the CCNA. The performance of both rapid tests Vidas CDAB and ICTAB were similar (Se: 77.5%, Spe: 98% ; Se: 78.5%, Spe: 96.5% respectively). We observed an important number of equivocal results (2.5%) with VIDAS CDAB distributed either within positive (n=2) or negative (n=4) cases. Real-time PCR Cdiff GeneOhm provides rapid and relevant results (Se: 100%, NPV: 100%). One false positive result has been reported and may be explained by a succession of freeze-thawing of the sample. The choice of tests for CDI diagnosis must not be only based on their performance but should also integrate cost, feasability, and practicability for a routine use in clinical laboratory. The analysis of the results of this study led to the optimization of our organization and to define the respective position for each test in a new algorithm. It associates rapid detection of toxins and toxigenic culture, whereas CCNA is still used as a confirmatory technique for difficult or discordant cases. Despite excellent sensitivity, PCR was ruled out because of excessive costs for yet.

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# A TWO STEP ALGORITHM FOR THE DIAGNOSIS OF *Clostridium difficile* INFECTION: SCREENING WITH A RAPID IMMUNOASSAY FOR THE DETECTION OF GLUTAMATE-DEHYDROGENASE AND TOXINS A AND B FOLLOWED BY A REAL-TIME PCR FOR *Clostridium difficile*

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Aim: The Techlab ® C. diff Quik Chek Complete TM (Techlab Blacksburg,VA,USA) is a rapid immunoassay for the detection of Glutamatedehydrogenase and toxins A and B in fecal specimens, using specific antibodies. The GeneXpert ® C difficile ( Cepheid Sunnyvale, CA 94089, USA) is a real-time PCR assay targeting the toxin B gene (TcdB) of *Clostridium difficile*, binary toxin and a deletion in the tcdC gene with a fluorogenic target-specific hybridization probe for the detection of the amplified DNA. With toxigenic culture (TC) as gold standard, we evaluated the performances of the combination of these tests for the diagnosis of *C. difficile* infections (CDI) on stool specimens.
Materials and methods: Stools were from inpatients of the St Luc-UCL University hospital (890 beds), older than 2 years and suffering from diarrhoea. Cell cytotoxicity (CTA) was performed on MRC5 cells. Cultures were performed on CCFA. In case of positive culture and negative CTA, colonies were tested for 'in vitro' toxin production (TC). Specimens giving discordant results were repeated on CCFA with added taurocholate (TCCFA). Real-time PCR and the rapid immunoassay were performed according to the manufacturer's instructions.

Results: A total of 236 stool specimens were freshly tested during march and april 2010 , by GeneXpert. Twentythree samples were shown to contain toxigenic *C. difficile* by CTA and/or toxigenic culture (prevalence : 9,75%). Five specimens gave invalid results by GeneXpert. The sensitivity, specificity, PPV and NPV of GeneXpert were respectively : 87%, 96,7%, 74% and 98,6%. Those of C. diff Quik Chek Complete TM were for GDH: 95,7%, 93,9%, 62,7% and 99,5%, for toxin A&B: 69,6%, 100%, 100% and 96,8%. CTA values were 69,6%, 99%, 88,9% and 96,8% . Screening with C. diff Quik Chek Complete TM followed by GeneXpert on GDH positive, toxins A and B negative samples gave sensitivity, specificity, PPV and NPV of respectively : 82,6%, 98%, 82,6% and 98,7%.

Conclusion: The combination of theTechlab ® C. diff Quik Chek Complete TM and GeneXpert ® Cdifficile demonstrated a very good sensitivity much superior to that of CTA and a good specificity. It is a rapid method allowing result in less than two hours.

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# EVALUATION OF VIDAS® *C. difficile* TOXIN A IGG AND VIDAS® *C. difficile* TOXIN B IGG PROTOTYPES ON BLOOD BANK SERA CHARACTERIZED WITH CYTOTOXICITY NEUTRALISATION ASSAY

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The aim of this study was to evaluate the results obtained on blood bank samples characterized with cytotoxicity neutralisation assay on the VIDAS® *C. difficile* Toxin B IgG (CDB IgG) prototype and on the VIDAS® *C. difficile* Toxin A IgG (CDA IgG) prototype.

Methods: The prototypes VIDAS® CDB IgG and VIDAS® CDA IgG use specific native Toxin B and Toxin A antigens respectively coated on the solid phase with the help of specific monoclonal antibodies against these toxins respectively. During the first step of the test, the specific IgG were captured by this solid phase. The complexes are revealed during the second step using an anti-human IgG conjugated to Alkaline Phosphatase (PAL).

Forty two blood bank sera were tested on VIDAS with each prototype. The duration of the protocol is 35 minutes and the serum volume is 100 µl for each test. These sera were characterized with a cytotoxicity neutralisation assay.

Results are given as a relative fluorescence value for each sample and compared to results obtained with cytotoxicity neutralisation results.

Results: Of 18 samples characterized as positive in the cytotoxicity neutralisation assay, 18 samples showed positive results with the VIDAS® CDB IgG prototype and 9 with the VIDAS® CDA IgG prototype. Of 24 samples characterized as negative in the cytotoxicity neutralisation assay, 18 samples showed negative results with the VIDAS® CDB IgG prototype and 17 with the VIDAS® CDA IgG prototype. The agreement with the cytotoxicity neutralisation assay is 86% for Vidas CDB IgG prototype and 62% for Vidas CDA IgG ($p<0.05$).

Conclusions: With the VIDAS® *C. difficile* Toxin A IgG prototype and the VIDAS® *C. difficile* Toxin B IgG prototype, it is possible to detect the *C. difficile* toxins A and B IgG in blood sample. The results obtained with the VIDAS® *C. difficile* Toxin B IgG prototype are better correlated with the cytotoxicity neutralisation assay than the results with the VIDAS® *C. difficile* Toxin A IgG prototype ($p<0.05$). These preliminary results have to be confirmed on samples from hospitals in the context of *C. difficile* infection.

**P75**

# COMPARISON OF *Clostridium difficile* DIAGNOSTIC ASSAYS WITH PROSPECTIVE PATIENT EVALUATIONS

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Background: *Clostridium difficile* infection (CDI) is a clinical diagnosis with laboratory confirmation. Prospective evaluation of patient symptoms is not typically conducted when comparing diagnostic assays for CDI. The purpose of this study was to compare CDI diagnostic assay performance including a prospective patient assessment. Methods: 100 consecutive unformed stool specimens that met inclusion and exclusion criteria with sufficient volume were collected and stored at -70˚C until tested. Only first specimen sent for CDI testing from inpatients at Barnes-Jewish Hospital (BJH) were included. Specimens from patients unable to provide a history were excluded. All specimens were cultured on cycloserine-cefoxitin fructose agar with taurocholate and all *C. difficile* isolates were tested for neutralizable cytotoxin using tissue-cultured cells (toxigenic culture). The following assays were compared: BD GeneOhm Cdiff (BD), Cephied Xpert *C. difficile* (XC), tissue culture cytotoxicity on stool (TC), TechLab Tox A/B II (TOX), TechLab Quik Chek (QC), and Remel ProSpecT (PT). Patients were examined and asked about number of bowel movements (BM) per day. Patients selected their BM consistency from the Bristol Stool Chart (BSC). Clinically significant diarrhea was defined as ≥3 diarrheal BM (≥6 BSC) per day or diarrhea plus abdominal pain. The gold standard definition of CDI was isolation of toxigenic *C. difficile* in a patient with clinically significant diarrhea. Fisher's exact test or Mann-Whitney U was used for comparisons, as appropriate.

Results: Eleven (11%) patients met the gold standard criteria for CDI. There was no difference between patients with or without CDI who were female (46% v. 51%), white (82% v. 70%), or median age (57 v. 61 years old). Patients with CDI had more BM per day (median 5.5 v. 3.0; p=.025) but had similar stool consistency (median BSC type 7 v. 7) compared to patients without CDI. The sensitivity (%)/specificity (%) of the tests were as follows: toxigenic culture 100/99, BD 100/91, XC 100/93, TC 91/99, TOX 82/97, QC 100/88, PT 91/85. Screening with QC would increase the specificity of XC and PT to 94 and 97, respectively, with no change of other tests. For every 1,000 tests the number of true positives v. false positives identified with 11% CDI prevalence would be: toxigenic culture 110 v. 10, BD 110 v. 84, XC 110 v. 62, TC 100 v. 10, TOX 90 v. 30, QC 110 v. 110, PT 100 v. 134, QC then XC 110 v. 50, QC then PT 100 v. 30.

Conclusions: Specificity of molecular diagnostics was lower in this study compared to other studies, possibly due to including clinical presentation in the gold standard. Small changes in specificity result in large changes in the number of false positive tests.

**P76**

# *Clostridium difficile* INFECTION (CDI): PREDICTORS OF DISEASE SEVERITY AND DURATION OF SYMPTOMS IN A PROSPECTIVE COHORT

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*Objective*: There is evidence for increased severity of *Clostridium difficile* infection (CDI) with a higher frequency of associated complications and extended bed utilisation. Identifying at-risk patients is essential to improve triage, management and care of the patients. The present work used a prospectively evaluated cohort to identify clinical and demographic predictors of severe CDI illness and duration of symptoms.

*Methods*: Admission, clinical and microbiological information was collected at recruitment for 155 consecutive patients at The Royal Liverpool Hospital, UK, who were prospectively followed for 4 weeks. Information included demographics (age, gender), admission data (medication history, comorbidities), clinical (serological markers) and microbiological factors (toxin results, strain types). Logistic and negative binomial regression models were used to identify which factors predicted severe cases (based on the Health Protection Agency scale) and the duration of the CDI symptoms, respectively. These predictors were included in the relevant multiple regression models to assess how much variability was explained for these two outcomes and to identify independent predictors.

*Results*: Independent predictors of severity were C-reactive protein levels, previous use of fluoroquinolones and respiratory comorbidities. The independent predictors of duration were variation in the creatinine levels and the number of stools passed per day during the acute period. The multivariable logistic model explained 51.7% of the variability in severity and was good in discriminating between patients who were and were not severely affected (area under the ROC curve - AUC=0.87). The multivariable negative binomial model explained 33.7% of variability in the duration of symptoms.

*Conclusions*: The present study has identified a range of clinical factors that help predicting disease severity and prolonged duration of symptoms. Although our findings do not suggest the existence of a single modifier with major effect in relation to both outcomes, our multivariable analyses have

showed that it is possible to explain a large proportion of this variability by incorporating a limited number of parameters, which can easily be extracted or generated from hospital settings. This work serves as basis for the development of robust predictive models to improve management of at-risk patients and provide professionals with means to intervene at an earlier stage.

**P77**

# PREVALENCE OF BINARY TOXIN GENE AND tcdC DELETIONS AND ASSOCIATION WITH DISEASE SEVERITY IN *Clostridium difficile* CLINICAL ISOLATES 2008 - 2010

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Previous studies have demonstrated (at least in vitro) increased toxin A/B production in some so called 'hyper-virulent' strains of *C. difficile*. tcdC is a negative regulator of toxin production and several mutations in this gene have been described in these strains including a frameshift mutation due to a single base-pair deletion at position 117. Additionally, some strains produce an actin specific ADP-ribosyltansferase (binary toxin) encoded by cdtA and cdtB. Binary toxin induces formation of microtubules which may increase adherence to gut epithelium, enhancing virulence. It is unclear whether presence of binary toxin gene, or tcdC deletions result in increased severity. We investigated 194 cases of CDI over a two year period (June 2008 - June 2010). All isolates were from patients with a first episode of CDI and repeat specimens were excluded. Isolates were extracted and screened for presence of binary toxin genes (cdtA and cdtB), and tcdC in a multiplex PCR, using previously published primers. Isolates with tcdC deletions were amplified to yield a 718bp product which represents the whole of the tcdC gene before being sequenced. All isolates were ribotyped according to standard techniques.

Data was collected retrospectively on all 194 cases and (where available) included; patient age, total peripheral white cell count (WCC), C-reactive protein (CRP), serum albumin, acute rise in creatinine (>50% of baseline), admission to Intensive Therapy Unit (ITU), 30 day all cause mortality and presence of severe or pseudomembranous colitis (PMC). Presence of severe / PMC was dependent on direct visualisation at sigmoidoscopy, histological evidence, radiological evidence of severe colitis or disease requiring colectomy.

45 isolates (23%) had a tcdC deletion. There was no significant difference in age, CRP, albumin, creatinine, ITU admission, presence of PMC, and 30 day mortality, however patients with a tcdC deletion carrying strain were more likely to have a higher WCC than those with intact tcdC gene. 41 isolates (21%) were positive for binary toxin. There was no statistically significant difference in albumin, creatinine, ITU admission, presence of PMC, and 30 day mortality, however patients with a binary toxin gene carrying strain were more likley to be older, have a higher WCC, and have a higher CRP than those without.

**P78**

# MODELLING RECURRENT *Clostridium difficile* INFECTION THROUGH WHOLE GENOME SEQUENCING

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*Clostridium difficile* is a Gram positive, anaerobic, spore forming, rod shaped bacterium, associated with nosocomial infection. Symptoms of infection range from asymptomatic carriage, mild diarrhoea and from moderate to fulminant pseudomembranous colitis (PMC). One of the most difficult challenges faced when dealing with *C. difficile* infection (CDI) is prediction and treatment of patients with recurrent infection, which occurs in approximately 20% of patients following withdrawal of antibiotics.

Approximately 200 patients with positive C. diff stool samples have been collected from the Royal Devon and Exeter hospital during 2007-2009. From these samples 55 patients had recurrent CDI with two (n=48) or three (n=7) episodes. PCR ribotyping of four strains revealed that three patients showed recurrent CDI with the same strain. Whole genome sequencing of two of these isolates has revealed two non-synonymous SNPs that differ between the strains in the primary (1o) and secondary (2o) infection. These SNPs occur within putative genes involved with antibiotic resistance and thus could infer some kind of selective advantage in the host environment that has occurred within the period between the 1o and 2o infection for this clinical strain. Both the host, and bacterial genetic factors play a strong role in the manifestation of CDI and subsequent recurrences, and it is envisaged that by investigating significant clinical risk factors, along with the genomes of clinical isolates, that a robust model for predicting recurrent CDI could be developed and possible genes involved identified.

**P79**

# IS PSEUDOMEMBRANE FORMATION A RISK FACTOR FOR ADVERSE OUTCOME IN *Clostridium difficile*-ASSOCIATED DIARRHEA?

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*Background*: *Clostridium difficile*-associated diarrhea (CDAD) is a common nosocomial infection with a clinical spectrum ranging from uncomplicated diarrhea to toxic megacolon and death. Several factors have been associated with worse prognosis. Limited data suggest that one of these factors is the presence of pseudomembranous colitis on endoscopy.

We investigated whether the formation of pseudomembranes in CDAD patients is associated with a severe outcome of disease.

*Method*: We retrospectively screened all hospitalized CDAD patients from 01/2003-12/2009 and identified those patients who underwent lower endoscopy (colonoscopy or sigmoidoscopy). The medical records of all these patients were reviewed in detail. The primary outcome was mortality within 30 and 60 days of diagnosis. The secondary endpoints were a composite outcome of other adverse events (including colectomy within 60 days, toxic megacolon, peritonitis, respiratory insufficiency or hemodynamic shock) as well as laboratory parameters known to be associated with increased mortality in CDAD including hypoalbuminemia of less than 2.5 g/dl and white blood cell count above 20X103/mm3. The data were compared between CDAD patients with and without pseudomembranes on lower GI endoscopy.

*Results*: Fifty seven CDAD patients were included, 16 with pseudomembranes and 41 without. The mean age of the patients was 64 years. 3/16 patients with pseudomembranes died within 60 days compared to 7/41 in the non-pseudomembrane group (P=0.29, Fisher exact test). There was also no difference in the rate of other adverse outcomes between the two groups.

*Conclusion*: Our study suggests that pseudomembrane formation is not associated with adverse outcome in CDAD patients.

**P80**

# CLINICAL OUTCOMES AND IMMUNE RESPONSE ASSOCIATED WITH *Clostridium difficile* RIBOTYPE 027 INFECTION

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*Introduction*: *Clostridium difficile* infection (CDI) outbreaks due to PCR ribotype 027 have been reported with significantly increased morbidity and mortality. Other reports suggest that this strain is not associated with poorer outcomes in a non-epidemic setting.

*Methods*: We conducted a prospective study of all consecutive cases of hospital-onset CDI in two Dublin hospitals. We collected data on comorbidity, CDI risks, disease severity, recurrence and mortality. Initial episode severe was defined using recent SHEA guidelines as leukocytes >15,000 cells/µL and/or creatinine rise of 50% above baseline. Outcomes included recurrence rates, duration of diarrhoea, ICU admission, colectomy and death. Stools were cultured and *C. difficile* isolates were ribotyped using PCR. Serum IgG antibody levels against toxins A and B were measured using ELISA. *C. difficile* colony counts on selective agar (CCFA) after alcohol shock were recorded. Participants were followed for 60 days.

*Results*: We recruited 150 out of 181 eligible CDI cases. Median age was 77.5 (IQR 64-84) years. Of the successfully ribotyped isolates, 46% were ribotype 106, 16% ribotype 174, 11% ribotype 027, 8% ribotype 001 and 7% ribotype 014. IgG antibody levels against toxin A were higher on days 1 ($p=0.06$), 3 ($p=0.01$), 30 ($p=0.02$) and 60 ($p=0.02$) after CDI onset in 027 cases compared to cases associated with all other ribotypes. IgG anti-toxin B levels were also significantly higher in ribotype 027 cases on days 1, 3, 30 and 60 ($p<0.05$). Median peak leukocyte counts within 3 days of CDI onset were significantly higher ($p<0.05$) in 027 cases. On selective agar plates, ribotype 027 strains were significantly more likely to have higher colony densities (>5000 colonies/plate) compared to other ribotypes ($P=0.02$). Recurrence rates, duration of diarrhoea and mortality were similar for all ribotypes.

*Conclusions*: We found that *C. difficile* ribotype 027 was associated with higher serum IgG responses to toxins A and B and higher peak leukocyte counts. Despite this, outcomes were not significantly different compared to other ribotypes. The higher colony density counts suggest that this strain may have greater propensity to colonise the intestine or enhanced sporulation activity.

We speculate that enhanced faecal shedding of ribotype 027 may be instrumental in causing CDI outbreaks and epidemics. The observed higher anti-toxin antibody and leukocyte counts may reflect a more robust activation of host immune responses by this virulent strain.

**P81**

# *Clostridium difficile* INFECTION IN HAEMATO-ONCOLOGIC PATIENTS - A TWO YEAR SINGLE CENTRE SURVEY

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*Objectives*: *Clostridium difficile* infection (CDI) has been changing in its clinical severity and epidemicity. Hypervirulent strains of *Clostridium difficile* (CD) are causing hospital outbreaks in the US and Europe. This phenomenon is associated with particular PCR ribotypes including ribotype 027.

Patients with hematologic malignancies are of specific concern in this context, especially when they are receiving immunosuppressive chemotherapy.

The aims of this survey were to ascertain the incidence and 30-day mortality of CDI in oncology-patients at a university hospital and to analyse the case fatality ratio by PCR- ribotypes of the causative CD strains.

*Methods*: From January 2008 to December 2009, all patients with diarrhea admitted at the haemato-onocology department were tested by stool culture for toxigenic CD.

The identification of a case of CDI and severe CDI occurred according to the case definition proposed by the German hospital associated infection surveillance system protocol for CDI, 2009 (CDAD KISS-protocol).

*Results*: In the study period of 24 months, 122 patients fulfilled the definition of a case of CDI. Out of these, 22 patients (18 %) fulfilled the critieria of severe CDI. The CDI fatality was 16% (19/ 122 cases). Among these patients with lethal outcome, 16 had solid tumors and 3 hematologic malignancies as underlying diseases. Isolates obtained from 66 CDI-cases were available for PCR-ribotyping. PCR-Ribotype-053 was with 15% (10/ 66) the predominant strain; ribotype 027 was not found. 053 PCR ribotype was also the dominant strain among the fatal cases.

*Conclusion*: We found a high fatality among oncology patients with CDI, PCR ribotype 053 was the dominating strain among the CDI oncology patients. CDI must be taken as a serious complication in the oncology patient group and infection control measures and antibiotic stewardship are challenged to prevent these nosocomial infections.

**P82**

# DOMINANT INTESTINAL MICROBIOTA DIFFERENCES BETWEEN *Clostridium difficile* COLONIZED AND NON-COLONIZED INFANTS

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*Introduction*: *Clostridium difficile* is a major enteric pathogen, principal responsible for post-antibiotic diarrhoea and pseudomembranous colitis. Host susceptibility to the development of *C. difficile* infections and recurrences could be influenced by the commensal intestinal microbiota ability to resist to *C. difficile* colonization. During early infancy, asymptomatic intestinal colonization by *C. difficile* is common and faecal microbiota shows a lower complexity than in adulthood. Thus, in order to investigate the potential relationship between the dominant commensal microbiota composition and the establishment of *C. difficile*, faecal microbiota of infants colonized and not colonized by *C. difficile* were compared.

*Methods*: Faecal samples from 53 infants, 27 with negative *C. difficile* culture and 26 with positive *C. difficile* culture, ranging from 1day old to 1 year old, were collected. Dominant microbiota profiles were assessed by 16S rDNA PCR followed by Temporal Temperature Gradient gel Electrophoresis (TTGE). Bacterial signatures of the intestinal microbiota associated with colonization by *C. difficile* were deciphered using this molecular profiling technique coupled with Principal Component Analysis (PCA). Resulting bands of interest in TTGE profiles were excised, sequenced, and corresponding bacterial species determined with nucleotide blast (NCBI).

*Results*: A high dispersion of banding pattern was observed. The presence of *C. difficile* in the intestinal microbiota of infants could not be associated with any clustering of TTGE profiles. However, PCA on instrumental variables highlighted a significant difference on dominant bacterial species between *C. difficile* colonized and non-colonized infants ($p = 0.017$). Seven bands were identified as specifically linked to the presence or absence of *C. difficile*. 16S rDNA sequences related to Ruminococcus gnavus, Clostridium nexile and Klebsiella pneumoniae for bands associated with *C. difficile* colonized infants. For non-colonized infants, homology was found with Staphylococcus epidermidis, Bifidobacterium longum and Escherichia coli.

*Conclusion*: We demonstrated that the presence of *C. difficile* in the intestinal microbiota of infants was associated with changes in the composition of this ecosystem. These results suggest that the composition of the gut microbiota might be crucial in the colonization process.

**P83**

# IRON UPTAKE SYSTEMS IN *Clostridium difficile*

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*Clostridium difficile*, a Gram-positive, anaerobic, spore-former is the major cause of antibiotic associated diarrhoea and is also associated with more severe, sometimes life threatening disease. To date little research has been carried out on iron uptake mechanisms and their regulation in *C. difficile*. In common with other pathogens, iron is likely to be an essential growth factor necessary for the survival of the organism and analysis of available genome sequences reveals the presence of several potential iron uptake systems and regulators. Initially we have focussed studies on homologues of the E. coli ferrous iron uptake system (FeoB) and the ferric uptake regulator Fur. The ClosTron mutagenesis system developed in Nottingham has been used to generate knockout mutants in two of the 3 annotated feoB homologues and in the single fur homologue in *C. difficile*630Δerm. Comparisons of the growth characteristics suggest FeoB1 but not FeoB3 is involved in ferrous iron uptake. Co-culture experiments showed that wild type out-competed the FeoB1 mutant in the presence of 2,2,-dipyridyl. As has previously been reported with another anaerobe - Shewanella oneidensis, fur mutant showed a growth defect under iron replete conditions and enhanced growth under iron limitation compared to wild type and was hypersensitive to hydrogen peroxide.

**P84**

# INVESTIGATING THE INITIATION OF SPORULATION IN *Clostridium difficile*

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*Clostridium difficile* has emerged in recent years as a major nosocomial pathogen, in which the highly resistant spores are the infective agent of disease. Despite the spores being of central importance to disease transmission little is known about the mechanisms by which they are formed. We aim to elucidate some of the molecular mechanisms that control the initiation of sporulation, which may identify novel targets in the prevention or identification of this disease.
Sporulation is energetically expensive and commits the cell to a major change in lifestyle; as a result it is a tightly regulated process. In the Bacillus subtilis paradigm sporulation is controlled by the master regulator Spo0A. The activation of Spo0A by phosphorylation affects the expression of many regulatory genes, which leads to a complex cascade of gene activation and repression and eventually a spore is formed. Spo0A is also essential to integrating information from several different intracellular and extracellular factors that affect the decision to sporulate. If the balance of these factors is in favour of vegetative growth sporulation will not be initiated, however if it shifts and a threshold of phosphorylated Spo0A is reached then the cascade will be triggered. The genes involved in initiating sporulation via Spo0A have been well characterised in B. subtilis.

Spo0A is also the master regulator of sporulation in *C. difficile* however bioinformatic analysis yields few homologues of the B. subtilis genes involved in its activation and repression. In this study we took a genomic, transcriptomic and genetics approach to identify proteins that potentially affect the initiation of sporulation in *C. difficile*. Using clostron technology mutations were made in any genes found to be of interest and their sporulation phenotypes characterised. Here we present the results of this study.

**P85**

# UNDERSTANDING THE MECHANISMS OF *Clostridium difficile* SPORE GERMINATION

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*Clostridium difficile* is the leading cause of hospital-acquired diarrhoea and a major burden to healthcare services. Endospore production plays a pivotal role in infection and disease transmission, but in order to cause disease these spores must germinate and return to vegetative cell growth. Therefore, knowledge of spore germination is important and may have direct applications in future disease prevention. Germination has been well studied in Bacillus and in some Clostridia, but the mechanisms of *C. difficile* spore germination remain unclear. Apparent homologues of genes important for germination in other spore formers have been identified in the *C. difficile* genome and we used ClosTron technology to inactivate homologues of sleC, cspA, cspB and cspC (Clostridium perfringens) and cwlJ, sleB and cwlD (Bacillus subtilis) in both *C. difficile* 630Δerm and a B1/NAP1/027 isolate (a 'hypervirulent' type associated with outbreaks of increased disease severity). Using a combination of several different assays to study these mutants in detail, we have identified a number of genes that are essential for germination and outgrowth of *C. difficile* spores. This is the first work using reverse genetics to study the germination of *C. difficile* spores and the first gene characterisation by mutagenesis in a B1/NAP1/027 isolate of *C. difficile*. Furthermore, this work has uncovered evidence that differences exist in the sporulation and germination characteristics of different *C. difficile* types.

**P86**

# AUTOLYSINS OF *Clostridium difficile* INVOLVED IN VEGETATIVE GROWTH

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Autolysins are potentially lethal enzymes capable of hydrolyzing peptidoglycan, the major structural component of bacterial cell walls. However, these enzymes are implicated in many physiological processes such as cell separation, sporulation or germination and would be also involved in antibiotic-induced lysis. As autolysins are critical to the maintenance of cell wall integrity, the transcription of autolysin genes and their activity is under tight control.

We identified a putative autolysin in *Clostridium difficile* by sequence similarity analysis with other autolysins of Gram-positive bacteria. The corresponding protein Acd, expressed in Escherichia coli, displayed a lytic activity on the walls of Micrococcus lysodeikticus, and revealed N-acetylglucosaminidase hydrolytic specificity on Bacillus subtilis peptidoglycan (RP-HPLC and MALDI-TOF MS analysis of digestion generated muropeptides). The study of acd expression at the transcriptional (qRT-PCR) and translational levels (Western-blot) during various growth phases of *C. difficile* showed that acd is expressed during the vegetative growth and that the Acd protein is accumulated during the late phase of growth. These results suggest that 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 on the walls of M. lysodeikticus in the acd mutant. However, to date no particular phenotype could be detected in an acd mutant either in cell separation (vegetative growth) or in sporulation. At least 28 putative autolysins encoding genes were identified on the genome of *C. difficile*. A functional compensation by another autolysin is therefore likely to exist. Several strategies have been used in order to identify new autolysins which are important for vegetative growth and which might be able to compensate the Acd autolysin. The first approach was to search in the *C. difficile* genome for genes homologous to major autolysin encoding genes in Bacillus subtilis. The corresponding genes were mutated using the ClosTron method in order to study their physiological functions. The second approach was to investigate the sigma factor σD, a putative regulator of autolysins. We inactivated sigD and performed phenotypic tests to establish the possible link between this sigma factor and the control of autolysins expression.

**P87**

# VARIATION IN SURFACE LAYER PROTEINS OF 4 DIFFERENT *Clostridium difficile* STRAINS

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In the past decade, the incidence of *Clostridium difficile* infections (CDI) with more severe disease has increased, which is associated with the emergence of hypervirulent PCR ribotype 027. In addition, the rise of a new hypervirulent strain PCR ribotype (078) has been noticed in The Netherlands. Type 078 affects younger people with similar CDI severity as Type 027. Assays for the rapid detection and discrimination of these hypervirulent strains are essential to prevent the spread of CDI. Currently, there are no such rapid tests available for the new hypervirulent *C. difficile* strains. Our research is focused on identifying novel markers for hypervirulent *C. difficile*. With these novel markers we would like to develop a diagnostic assay which is able to rapidly detect CDI and discriminate between hypervirulent strains and strains associated with less severe disease.

*C. difficile* expresses a surface layer coat consisting of a high molecular weight surface layer protein (HMW-SLP) and a low molecular weight surface layer protein (LMW-SLP). The SlpA gene encodes for a precursor protein which is cleaved into the two mature surface layer proteins by CWP84, a protease located on the cell wall. In this study, the SLP's of 4 different *C. difficile* PCR ribotypes (012, 078, 001 and 027) were compared. Surface layer extracts were made by using different methods (Glycine and Urea treatment) and analyzed by SDS-PAGE followed by conventional staining methods. In addition, we sequenced the Type 078 SlpA gene strain and compared it with SlpA sequences of other PCR ribotypes.

After glycine extraction, a difference in gel mobility of the SLP's was observed between the different ribotypes, especially PCR ribotype 078. We sequenced the SLPA gene of the PCR ribotype 078 and compared it with published SlpA sequences of other PCR ribotypes. Alignment of the SlpA sequences showed high homology of the HMW-SLP amongst the four different PCR ribotypes, whereas the LMW-SLP was less conserved. Furthermore, the predicted molecular weight of the different SLPs did not differ much. In addition, we analyzed if glycosylation of the SLP was responsible for the observed difference in gel behavior. The surface layer proteins were deglycosylated using a de-glycosylation kit (Sigma), after which the gel behavior was analyzed. Gel-mobility of the SLPs was unaltered after the de-glycosylation treatment.

Neither the predicted molecular weight nor glycosylation provides an explanation for the observed difference in gel mobility. Currently, we are exploring other possible explanations for the observed difference in gel mobility.

**P88**

# ANALYSIS OF SURFACE-LAYER (S-LAYER) PROTEINS OF THE HYPERVIRULENT *Clostridium difficile* PCR-RIBOTYPE 027 IN COMPARISON WITH THOSE OF OTHER PCR-RIBOTYPES

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*Aim*: To analyze the surface-layer (S-layer) proteins, high and low-molecular weight (HMW and LMW), of the hypervirulent *Clostridium difficile* 027 in comparison with those of other PCR-ribotypes.

Materials and Methods: Eight different *C. difficile* 027 and one PCR-ribotype 018 were sequenced for the slpA gene using different sets of primers, the DNAStar's Lasergene v.8.0 software, the National Center for Biotechnology Information BLAST server and the European Bioinformatics Institute Clustal W server. Amino acidic sequences were analyzed using the SignalP 3.0 Server and the ExPASy Proteomics Server. Amino acid comparisons were accomplished using the European Bioinformatics Institute ClustalW server and the output was used for the construction of the phylogenetic tree by TreeView 1.6.6. Visualization of the LMW structure for the identification of surface exposed regions was performed using Swiss-Pdb Viewer. The S-layer proteins of eight different PCR-ribotypes (001, 014, 017, 018, 020, 027, 078, 126) were obtained by a low pH glycine extraction and separated by SDS-PAGE. Immunoblotting assays using acute and convalescent sera from patients with severe *C. difficile* 027 infection was also performed.

*Results*: All strains 027 showed the same slpA nucleotide sequence. The molecular mass of the LMW and HMW proteins was calculated as 33.871 kDa and 44.174 kDa, respectively. These protein sizes were confirmed by SDS-PAGE analysis. Interestingly, the phylogenetic tree, obtained by comparison with the deposited amino acid sequences of other PCR-ribotypes, showed that *C. difficile* 027 SlpA was strongly related (identity 89%) to that of the epidemic PCR-ribotype 001. PCR-ribotype 018 showed a new SlpA variant that has 88% identity with that of PCR-ribotype 078. Alignment of the LMW amino acid sequences from PCR-ribotypes 001, 012, 014, 017, 027 and 078 has shown that there are ten regions with sequence identity between the 001 and 027 ribotype and, at the same time, low conservation with the other PCR-ribotypes. In particular, two of these regions correspond to areas of the LMW peptide that are predicted to be surface exposed. Immunoblotting results indicate cross-reaction of the sera with S-layer proteins from PCR-ribotype 027 and 001.

*Conclusions*: The results obtained provide convincing evidence that the S-layer is extremely conserved in *C. difficile* 027 strains. In presence of a high variability of S-layer proteins among the different PCR-ribotypes, the high identity found between the SlpA of *C. difficile* 027 and that of the

epidemic PCR-ribotype 001, the other type of *C. difficile* frequently responsible for severe CDI worldwide, intriguingly suggests that the S-layer of these virulent strains presents peculiar and common characteristics that could be an advantage during the infection.

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**P89**

# BIOGENESIS OF THE *Clostridium difficile* S-LAYER

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S-layers are regular two-dimensional paracrystalline arrays found on the surface of many Bacterial species. S-layers can perform many important functions for the cell: acting as scaffolds for other surface proteins, providing a molecular sieve to limit the access of large molecules to the cell surface and contributing to direct interactions with the host. The *Clostridium difficile* S-layer is composed of two proteins, termed the high molecular weight (HMW) and low molecular weight (LMW) S-layer proteins (SLPs), which are encoded by a single gene slpA. The biogenesis of the S-layer is a complex multistep process starting with the initial high-level expression of slpA gene and culminating with the final assembly of the completed S-layer and represents one of the most energy demanding processes carried out by the cell. The S-layer precursor, SlpA, has a 24 amino acid signal sequence which directs translocation across the cytoplasmic membrane. Upon translocation the signal sequence is removed by a signal peptidase and the mature SlpA then undergoes a second cleavage event to produce the final LMW and HMW SLPs. Our group and others have recently demonstrated that this second cleavage event is mediated by a cell surface cysteine protease Cwp84. The LMW and HMW SLPs form a high-affinity heterodimer, termed the H/L complex, which represents the basic subunit of the assembled S-layer. We have previously identified the domains in both the LMW and HMW SLP that mediate complex formation and elucidated the low-resolution solution structure of the H/L complex as well as the high-resolution structure of the surface-exposed domains of the LMW SLP. Little is known of the processes involved in translocation of SlpA or of the requirements for attachment of the H/L complex to the cell wall. Here we report our further characterisation of the molecular mechanism of H/L complex formation as well as our current understanding of the other steps in this complex biogenesis process.

**P90**

# A PROTEOMIC APPROACH TO IDENTIFY SURFACE EXPOSED AND SECRETED PROTEINS OF *Clostridium difficile*

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*Clostridium difficile*, a Gram-positive, spore forming, anaerobic bacterium is the leading cause of infectious diarrhoea in hospitals worldwide. Enteric infections caused by *C. difficile* have been associated with antibiotic treatment and disruption of the normal gastrointestinal flora. Due to the emergence of new hypervirulent strains (BI/NAP1/027), the importance of *C. difficile* in human infections has increased in the last few years, highlighting the acute medical need for a vaccine.
The cell surface of *C. difficile* is poorly characterized. The most widely studied components are the proteins that constitute the S-layer, a paracrystalline array surrounding the cell. Few other putative cell wall proteins (CWPs) have been reported, however their functions remain unclear. The availability of different genomic sequences of *C. difficile* has recently provided an excellent opportunity to identify novel CWPs. Characterization of the bacterial surface can be done using a 'surfome' analysis, which involves surface proteolysis of intact cells and identification of released peptides by mass spectrometry. The secreted proteins (secretome) can also be identified, by performing similar analysis on bacterial culture supernatants.

While surface proteolytic methods have been successfully applied to other bacteria, they have never been used for sporulating or anaerobic bacteria. We aim to study the surfome and secretome of natural *C. difficile* isolates and in conditions that mimic disease physiology. Preliminary work has been performed to set up reproducible conditions for preparation of intact vegetative cells, cell surface digestion, and mass spectrometric analysis. Comparison of surface profiles of clinically important *C. difficile* strains would provide valuable information for the selection of effective vaccine candidates.
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**P91**

# MOLECULAR ANALYSIS OF SURFACE PROTEINS OF *Clostridium difficile*

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*C. difficile* is an important nosocomial pathogen linked to the use of broad-spectrum antibiotics. The production of two protein toxins, TcdA and TcdB can lead to diarrhoea, which can progress to pseudomembranous colitis. Attachment of bacteria to the intestinal epithelium and other aspects of disease are likely to be mediated proteins at the bacterial surface. Recent studies and the availability of genome sequence data has helped to identify real and putative candidate surface proteins such as Acd, Cwp66, GroEL, CspA, SlpA, FliC, FliD, Cwp84, FbpA and putative sortase enzymes. For many of these proteins, their contribution to virulence is uncertain, and for some, their exposure at the pathogen surface is unknown. The aim of this study was to understand better the role of these candidate proteins and to establish their location using recombinant antibodies isolated from phage display libraries. The candidate genes were amplified by PCR using a high fidelity polymerase, and ligated into the pET-32 EK/LIC expression vector. After confirming the sequences of the cloned DNA, plasmids were transformed into E. coli BL21 (DE3), its pLysS derivative and the C43 (DE3) strain. The mass of the recombinant proteins was checked by Western blotting using an attached tag sequence and their identity was confirmed by mass spectrometry. Proteins were purified by Ni-chelating chromatography using stepwise elution with different imidazole concentrations optimised for each protein. The purified proteins were then used as targets in phage display to extract specific scFv antibodies from the Tomlinson phage display libraries. Specificity was checked by ELISA. The scFvs will be used in immunoprecipitation, immunofluorescence and immunogold-labelled electron microscopy to establish if they recognise the native forms of the target proteins and the exposure of these potential virulence factors at the surface of *C. difficile*.

**P92**

# IDENTIFICATION OF NOVEL SURFACE EXPOSED *Clostridium difficile* PROTEINS POTENTIALLY INVOLVED IN THE COLONIZATION OF INTESTINAL MUCOSA

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Pathogenicity of *Clostridium difficile* is mediated by toxin A and B and extensive studies have defined the precise modality of action of the two toxins and their interferences in cellular pathways of the host.

Delivery of toxins follows colonization of the gut epithelial cells, which require bacterial adherence to the mucosa. However, 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 recent availability of the whole *C. difficile* genome allows the identification of putative surface proteins of *C. difficile* that could mediate the adherence to epithelial cells and the colonization of the intestine. Searching for sequence homologies to known virulence factors and protein motifs involved in interaction with the host, we have selected 14 proteins of *C. difficile* exposed on the bacterial surface with a potential ability to adhere to human tissues.

Preliminary studies aimed to characterized the selected proteins have been carried-out by two approaches: i) ability of the recombinant proteins to bind human cells and tissues; ii) expression on the surface of Lactococcus lactis to evaluate their capacity to confer adhesiveness to intestinal cells.

**P93**

# CHARACTERISING CELL WALL PROTEINS OF *Clostridium difficile*

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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. Up to 30 minor cell wall proteins (CWPs) are also present within the S-layer. We have created deletion mutants in several of these genes and have characterised the cell wall protein composition of these mutants. Using a recently described mouse model of colonisation and transmission, we have investigated the behaviour of several of these mutants and we will report the results.

Sortases are bacterial enzymes that covalently attach specific proteins to the Gram-positive cell wall. Sortase and sortase substrates have been well described in Gram-positive bacteria such as Staphylococcus aureus, but little is known about the action of this enzyme or the role of its substrates in *Clostridium difficile*. *C. difficile* 630 and R20291 each have one functional copy of the sortase gene. Using bioinformatics analysis, we have identified potential sortase substrates in *C. difficile* 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. 8 potential "LPxTG"-like proteins have been identified in the 630 genome and 5 of these were also found in R20291. Expression of these LPxTG proteins in strains 630 and R20291 has been investigated using techniques such as RT-PCR.

**P94**

# THE INTEGRITY OF THE CELL WALL OF *Clostridium difficile* REQUIRES THE CYSTEINE PROTEASE Cwp84

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The external surface of *Clostridium difficile* is coated by a regularly arranged lattice of proteins termed the S-layer. The S-layer is mainly composed of two proteins, the LMW and HMW SLPs, which were shown to act as adhesins. Interestingly, these proteins are derived from a single precursor, SlpA, which is cleaved by the cysteine protease Cwp84 after translocation of SlpA to the cell surface. The products of this cleavage, the LMW SLP and the HMW SLP, form a stable complex which is responsible for the integrity of the S-layer. We have co-expressed in E. coli active Cwp84 and a fusion protein consisting of the entire LMW SLP, the domain of the HMW SLP that interacts with the LMW in the complex and a C-terminal GST tag. By generating alanine substitutions, we have confirmed that Cysteine-116 is an active site residue in Cwp84 and that Lysine-320 in the LMW SLP is essential for the cleavage of SlpA by Cwp84. We knocked-out Cwp84 in *C. difficile* 630, resulting in both the presence of full length SlpA on the cell surface and shedding of this protein into the culture supernatant. Compared to the parental strain, the colonies of 630 ⌒cwp84 display a completely different morphology, suggesting an effect on bacterial-bacterial interactions. Both the cleavage of SlpA and the wild-type colony morphology were restored by complementation with the wild-type cwp84 expressed from the cwp2 promoter. The cwp84-C116A mutant could not complement either phenotype. Analysis of the synthesis and localization of several other cell wall proteins in 630 ⌒cwp84 revealed major differences, with some proteins being largely absent from the cell wall due to shedding into the culture supernatant. These results suggest that cleavage of SlpA and formation of the LMW-HMW complex is essential for the integrity of the S-layer.

**P95**

# MODIFICATION OF COLONIZATION FACTORS EXPRESSION OF *Clostridium difficile* BY THE GLUCOSE

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*Clostridium difficile* pathogenicity is mainly due to toxins A and B expression but also requires attachment of bacteria to the gut that involves various surface proteins including the high molecular weight S-layer protein (HMW-SLP), the two adhesins Cwp66 and Fbp68, the flagellar proteins FliC and FliD and the protease Cwp84, needed to cleave the precursor of S-layer proteins, SlpA. It has been demonstrated, that expression of genes encoding virulence factors could be modified in vitro by some environmental conditions such as pH, CO2, glucose and antibiotics. As an example, transcription of tcdA and tcdB genes encoding toxins was repressed by the presence of the glucose in the medium. We know that in Gram-positive bacteria, the regulatory protein CcpA plays a major role in the carbon catabolite repression (CCR). It has been recently shown that CcpA mediates toxin expression in response to carbon sources. In this study, we wanted to analyse the effect of glucose on surface proteins expression of *C. difficile* strain 630.

When the 630 strain grew with or without glucose in the medium,, we observed no striking difference in the proteome profiles of surface proteins. However, by immunoblotting analyses, we can see that the amount of HMW-SLP, Cwp66, FliC and Cwp84 was increased in presence of glucose. This up regulation was observed at the level of the transcription of the slpA and cwp84 genes. To explore further the effect of glucose on *C. difficile* surface proteins expression, we used the knock-out mutant 630ΔccpA to assess the role of the CCR in this response. When we compared the cell wall fractions of the 630ΔccpA and the wild-type strains (630Δerm) by two-dimensional electrophoresis, we observed that the profile of the HMW-SLP, the LMW-SLP and GroEL were not changed. However, the precursor SlpA was present as three different isoforms in the mutant 630ΔccpA and not in the WT strain. On the other hand, by immonoblotting there was no significant difference in the amount of HMW-SLP between the WT and mutant 630ΔccpA, while Cwp84 was present to a lesser degree in the WT strain. Our results suggest that CcpA could regulate expression and/or post-translational modification of some surface proteins, and therefore may have an indirect role in the colonization process of *C. difficile*.

**P96**

# ROLE OF THE HEME OXYGENASE/CARBON MONOXIDE PATHWAY IN *Clostridium difficile* TOXIN A-INDUCED ENTERITIS IN MICE

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Clostridium difficile is the major cause of antibiotic-associated colitis, a disease with significant morbidity and mortality. This study investigated the role of the hemeoxygenase (HO)/carbon monoxide (CO) pathway in *Clostridium difficile* toxin A-induced enteritis in mice. Hemin (4.5 µmol/kg), HO substrate, zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor (4.5 µmol/kg), dimanganese decacarbonyl (DMDC), a CO donor (25 µmol/kg) or equivalent volume of their respective vehicles were injected systemically (subcutaneously) 30 min prior to local challenge with toxin A (25 or 50 µg/ileal loop) or phosphate buffered saline (PBS). Intestinal ileal loop weight/length ratios were calculated 3 h later. Ileal tissues were collected for histopathology and measurement of myeloperoxidase activity, and tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL- 1β) production by ELISA. The HO-1 protein expression of the ileal tissue was determined by immunohistochemistry method. The treatment of mice with hemin (HO substrate) or DMDC (a CO donor) prevented edema, mucosal disruption, and neutrophil infiltration observed in histopatology and measured by myeloperoxidase activity and also decreased the toxin A-induced TNF-α or IL-1β production. In contrast the specific HO-1 inhibitor (ZnPPIX) enhanced all these evaluated parameters. In addition, Toxin A 50 µg enhanced HO-1 protein expression in ileal tissue. In conclusion, our data suggest that hemeoxygenase and carbon monoxide contribute to the pathogenesis of the *Clostridium difficile* toxin A-induced enteritis.

**P97**

# CHARACTERISATION OF VARIANT *C. difficile* MORPHOTYPES - MICROSCOPIC EXAMINATION AND PROTEOME ANALYSIS

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The occurrence of variant colony morphologies has been reported in a number of microorganisms and has been associated with culture conditions, metabolite production, antibiotic resistance and virulence.

Clinical isolates of *C. difficile* representing diverse ribotypes were found to form two distinct colony morphotypes when grown on a range of culture media; the predominant morphotype (M1) was 4-6 mm in diameter colony, ground glass in appearance, grayish-white and irregular in shape while the minor morphotype (M2) was smaller <2 mm diameter and produced round colonies with a glossy appearance.

Microscopic examination revealed that the predominant colonial type (M1) was comprised of encapsulated Gram positive rods arranged in a chain. In contrast colonies of the minor morphotype contained Gram positive, filamentous rods with no visual evidence of capsule formation. Transmission Electron Microscopy confirmed the presence of structurally distinct capsules and what appeared to be the presence of pilus-like structures projecting from the surface of M2 variants.

Subsequent analysis of the proteome of the two distinct variants using 2 D-gel electrophoresis revealed marked differences in protein composition suggesting that the phenotypic divergence may extend beyond capsule formation. All of the isolates of *C. difficile* examined in this study produced both colonial variants and each variant upon subculture yielded both morphotypes suggesting that the different cell types are the consequence of differential gene expression.

The significance of this phenomenon is yet to be determined but given the role of capsule and surface structures in cell adhesion it is intriguing to speculate that these different morphotypes may vary in their ability to adhere to the gut mucosa. Indeed studies in our laboratory are currently investigating the ability of the two cell types to adhere to CaCo-2 cells.

**P98**

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

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Aim: To analyze the distribution and conservation of cwp genes among clinical isolates of *Clostridium difficile*.

Materials and Methods: Forty different *C. difficile* clinical isolates representing 6 of the prevailing PCR-ribotypes (001, 014, 018, 012, 078, 126) from different regions of Italy were screened by multiplex PCR for the presence of the 28 cwp genes so far identified in *C. difficile* using different sets of primers. Several of the most variable cwp genes were sequenced in at least one isolate per PCR-ribotype using DNAStar's Lasergene v.8.0 software, the National Center for Biotechnology Information BLAST server and the European Bioinformatics Institute Clustal W server. Amino acidic sequences were analyzed using the SignalP 3.0 Server and the ExPASy Proteomics Server. Amino acid comparisons were accomplished using the European Bioinformatics Institute ClustalW server and the output was used for the construction of the phylogenetic tree by TreeView 1.6.6.

Results: An analysis to determine the presence/absence of cwp genes showed that all PCR-ribotype 078 and 126 strains display genetic relatedness with the PCR-ribotype 027 for several of the variable cwp genes indicating a common origin for these PCR-ribotypes. Furthermore, sequence analysis of several cwp genes enabled the construction of a phylogenetic tree for the strains investigated.
Conclusions: The results obtained form the basis for a reliable and simple method for typing newly emerging strains of *C. difficile*.

**P99**

# DIMERIZATION OF THE *Clostridium difficile* ANTI-SIGMA FACTOR TcdC

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Background: *Clostridium difficile* is the main cause of nosocomial diarrhoea.

Well known virulence factors for *C. difficile* are the presence of the two large clostridial toxins, TcdA and TcdB and the binary toxin. In addition, high virulence is associated with deleterious mutations in the gene coding for the negative regulator of toxin expression, TcdC.

Toxin expression is positively regulated by the sigma factor TcdR, which is essential for recognition of tcdA and tcdB promoters by the RNA polymerase. TcdC has been described to act as an anti-sigma factor, which means that it prevents toxin promoter recognition by the RNA polymerase/TcdR complex, and thus is responsible for inhibition of transcription of the toxin genes. Methods: To investigate the biochemical properties of TcdC, TcdC and mutants thereof were expressed in E. coli and purified. Mutants had deletions of the hydrophobic N-terminus and the proposed dimerization domain. In addition, some point mutations were introduced in the putative dimerization domain. The dimerization of the mutants was compared to wild type TcdC using crosslinking followed by SDS-PAGE and gel filtration.

Results: TcdC formed dimers and multimers as shown by cross linking and gel filtration experiments. Deletion of the putative dimerization domain of TcdC resulted in a strong inhibition (shown by cross-linking) or a complete lack (shown by gel filtration) of the dimerization of TcdC.
Conclusions: TcdC dimerizes through a coiled-coil domain, as was previously suggested. We propose that the dimerization of TcdC is essential for its function.

**P100**

# ENDOSPORULATION: ORIGIN AND EVOLUTION OF A PROKARYOTIC CELL DIFFERENTIATION PROGRAM

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Bacterial endospores are the most resistance cell type known to man, able to withstand extremes of physical and chemical injury, and time. They are the infective particle in a variety of human diseases that cause the death of hundreds of thousands every year, and are emerging bio-terror agents. They are also part of the normal intestinal flora, and are gaining relevance as agents of probiosis, and in several other biomedical and biotechnological applications. Endosporulation is characterised by the morphogenesis of a specialised dormant and highly resistant endospore within a mother cell. The process involves a modified cell division, and the engulfment of the prespore compartment (the future spore) by the mother cell. The spore will later be released upon lysis of the mother cell, and will germinate once appropriate environmental cues are sensed. Little is known about the origins and evolution of the differentiation program that controls endosporulation. Here we take the program as defined in the model organism Bacillus subtilis and characterise its conservation pattern in a variety of sporulating and non-sporulating bacteria. We define a conserved group of ~100 genes that are present in the majority of endosporulating bacteria, and that we propose represents a minimal core for endosporulation. The core includes about 110 genes that were previously known to be essential for endosporulation. On the basis of their phylogenetic profile, several previously uncharacterized genes were predicted to be important for endosporulation, and we experimentally tested five of these genes. While one was shown to be an essential gene, four other were indeed shown to be specifically required for sporulation in B. subtilis and their expression to be developmentally regulated. Other than offering new insights into the genetic control of spore morphogenesis, our analysis also opens novel possibilities for spore identification and inactivation, specifically in the case of the pathogenic sporeformers. Moreover, our results indicate that endosporulation was likely invented once, at the base of the Firmicutes phylum (c.a., 3 Billion years ago), that it is unrelated to other bacterial spore differentiation programs, and that it involved the invention of new genes and functions, as well as the co-option of ancestral, house keeping functions.

**P101**

# INTESTINAL MICROBIOTA IN *Clostridium difficile* POSITIVE AND NEGATIVE ADULT HUMANS STUDIED BY DHPLC

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*C. difficile* can colonize the gut successfully only if the normal intestinal microbiota is disrupted, which is usually the result of treatment with antibiotics. It is also a bacterium commonly present in infants, where the normal intestinal microbiota is not yet fully functional. Changes in intestinal microbiota during colonization of *C. difficile* have thus become an important part of research on the ecology and pathogenesis of CDI (*C. difficile* infection). Molecular approaches like clone libraries, quantitative real time PCR, TTGE, DGGE and recently DHPLC have been used for analysis of fecal microbiota. The characteristics of fecal microbiota after recurrent *C. difficile* infection is significantly reduced species diversity. In this study we used a new high-throughput method for DNA fragment analysis called denaturing high performance liquid chromatography (DHPLC) to analyse and compare *C. difficile* positive and negative fecal samples from adult human patients.

Variable region V6 - V8 of eubacterial 16S rRNA gene was amplified by PCR using DNA isolated from 27 *C. difficile* positive and 18 negative fecal samples. The 470 bp amplicons were separated on the WAVE system (Transgenomics) and distinct peak profile for each sample was obtained. Peaks represent different species/groups of bacteria.

The number and distribution of the peaks show a high diversity and differences in the composition of fecal microbiota among individuals, as expected from previous studies. *C. difficile* positive samples show significantly lower number of peaks when compared to *C. difficile* negative samples, which would also be in agreement with previous studies. Certain areas of the peak profile tend to be more prominent in *C. difficile* positive samples and some are better represented in negative samples. However no specific profile nor peak could be specifically linked to the presence or absence of *C. difficile*.

**P102**

# LC-MS BASED SECRETOME ANALYSIS OF VARIOUS *Clostridium difficile* STRAINS

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*C. difficile* is an anaerobic gram-positive spore forming pathogen causing antibiotic associated gastrointestinal infections from mild *C. difficile* associated diarrhea (CDAD) to severe pseudomembranous colitis (PMC). Since 2003 new hypervirulent *C. difficile* strains (PCR ribotype 027) spread across North America and Europe accompanied by dramatically increasing morbidity and mortality rates. The secretomes of three *C. difficile* strains were analyzed by MS techniques and compared: R20291 (ribotype 027, isolated 2006), CD 196 (ribotype 027, isolated 1985), and CD 630 (ribotype 012, isolated 1982) as a reference strain.

*C. difficile* strains were cultivated in modified defined growth medium and brain heart infusion broth under anaerobic conditions at 37°C. Samples were withdrawn during stationary growth phase. Proteins of the culture supernatants were precipitated by chloroform-methanol and were separated by one-dimensional SDS-PAGE and lanes were cut into 10 pieces followed by tryptic in-gel digestion. Peptide analysis was performed by RP-HPLC (1100 series, Agilent) coupled online to an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics). MS data were searched against strain specific protein databases using Mascot. All identified proteins were processed with Secretome P and Signal P.

*C. difficile* was grown in brain heart infusion broth and in defined medium with nearly similar growth rates but different final optical densities. As evident from SDS-PAGE, protein pattern varied only slightly in different growth phases and after growth in complex or defined medium. LC-MS analysis revealed about 90 different proteins in the supernatant of *C. difficile*. Most of the identified proteins originate from the cytoplasm and might be present due to cell lysis. However, only a subset of cytoplasmic proteins was identified, indicating that protein release was somehow specific. 21 proteins in R20291, 25 in CD 196 and 26 in CD 630 contained a signal sequence and should be secreted by *C. difficile* strains. Proteins known to be secreted were mainly S-layer proteins, substrate binding proteins of ABC - transporters, cell-wall hydrolases, pilin and unknown hypothetical proteins. The major pathogenenic factors toxinA and toxin B were only identified after growth in brain heart infusion medium. Only in the hypervirulent ribotype 027 strains the ADP-ribosyltransferase binding component protein was identified which is a part of the binary toxin CDT.

The analysis of the secretome of *C. difficile* strains revealed several different proteins that might contribute to the different pathogenicity.

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