2nd iCDS
International
Clostridium difficile
Symposium

Clostridium difficile:
Organism, Disease, Control & Prevention

ABSTRACT BOOK
http://www.clostridia.net/
ICDS would like to acknowledge the contributions of

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WELCOME

Dear colleagues,

It is a pleasure to welcome you to the 2nd International *Clostridium difficile* Symposium. *C. difficile* has received much attention in the last few years and this has been reflected in the interest in this meeting.

Many new themes have emerged in the *C. difficile* field and we hope to cover most of them. The main interest of the *C. difficile* community currently is still the worldwide spread of different virulent types, especially the BI/NAP1/027 strain. New methods for strain characterization have been developed and additional virulence factors are being investigated. Much novel data are available on structure/function of the toxins, and on the molecular biology of toxins and the entire *C. difficile* genome. Last, but by no means least, the association with animals and food is becoming increasingly important.

As the 2nd International *Clostridium difficile* Symposium is one of a series of Marie Curie training conferences and workshops on Clostridia, the organizers have limited the number of invited speakers and given a larger number of young researchers the opportunity to present their work. Additionally, a new Meet the Expert session has been introduced with the aim of enhancing communication between participants.

The Organizing Committee hopes that you will enjoy the meeting and that it will stimulate new ideas and new collaborations.
## ICDS PROGRAMME

### Wednesday, 6th June

**17.00 Opening**

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<th>EPIDEMIOLOGY – geographic distribution and prevalence</th>
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<td><strong>17.50 - 18.10</strong></td>
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**19.30 Welcome reception and dinner (Hotel Habakuk)**
The changing epidemiology of Clostridium difficile in Quebec, Canada

A comparative study in the hospitalised elderly population in Edinburgh: what makes a patient susceptible to Clostridium difficile?

Three cases of Clostridium difficile infection in children

Epidemiological features of patients with Clostridium difficile–associated diarrhea

C. difficile S-layer proteins: characterisation and potential in nanotechnology

Detection of the toxin B gene of Clostridium difficile in stool specimens by loop-mediated isothermal amplification (LAMP) and direct typing of the SlpA gene by sequencing

International multi-laboratory comparison of seven Clostridium difficile typing techniques

CwpV, a variable cell wall protein of Clostridium difficile
### Friday, 8th June

#### Session IV

**PATHOGENESIS AND MOLECULAR BIOLOGY**  
Chair: I. Poxton

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<td>Castagliuolo I.</td>
<td>Immune responses and neuronal effects mediated by <em>C. difficile</em>-derived proteins</td>
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<td>9.00 - 9.20</td>
<td>Deneve C., C. Janoir, C. Deloménie, A. Collignon</td>
<td>Antibiotics involved in Clostridium difficile – associated disease increase colonization factor gene expression</td>
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<td>Merrigan M. M., J. Roxas, V. K. Viswanathan, N. Fairweather, D. N. Gerding and G. Vedantam.</td>
<td>Hypervirulent <em>C. difficile</em> strains have altered surface proteins and increased adherence to human intestinal epithelial cells</td>
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<td>9.40 - 10.00</td>
<td>Dupuy B., R. Govind and S. Matamouros</td>
<td>TcdC, the negative regulator of toxin synthesis in <em>C. difficile</em></td>
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<td>10.00 - 10.20</td>
<td>Dineen S. S., A. C. Villapakkam, J. T. Nordman, and A. L. Sonenshein</td>
<td>Repression of <em>Clostridium difficile</em> toxin gene expression by CodY</td>
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#### Session V

**C. difficile TOXINS**  
Chair: N. Minton

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#### Session VI

**ANTIBIOTIC RESISTANCE**  
Chair: G. Ackermann

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<td>16.30 - 16.50</td>
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<td>Emergence of a clindamycin resistant clone of <em>Clostridium difficile</em> PCR-027 in Dublin, Ireland</td>
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<td>16.50 - 17.10</td>
<td>Goh S., K. P. Song, T. V. Riley and B. J. Chang</td>
<td>Phage-mediated transfer of antibiotic resistance genes in <em>Clostridium difficile</em></td>
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<td>17.10 - 17.30</td>
<td>Norén T., I. Alriksson, M. Unemo</td>
<td>Longitudinal monitoring of <em>Clostridium difficile</em> in Sweden in the context of antibiotic resistance and emergence of epidemic strains</td>
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### Saturday, 9th June

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<td><strong>10.30 - 12.00</strong></td>
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<td><strong>12.00</strong></td>
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<td><strong>14.00 - 17.00</strong></td>
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ISOLATION AND CHARACTERIZATION OF \textit{CLOSTRIDIUM DIFFICILE} RESPONSIBLE FOR COMMUNITY-ASSOCIATED DISEASE

B. M. Limbago\textsuperscript{1}, C. M. Long\textsuperscript{1}, A. D. Thompson\textsuperscript{1}, G. E. Killgore\textsuperscript{1}, G. Hannett\textsuperscript{2}, N. Havill\textsuperscript{3}, S. Mickelson\textsuperscript{4}, S. Lathrop\textsuperscript{5}, T. F. Jones\textsuperscript{6}, M. Park\textsuperscript{7}, A. Cronquist\textsuperscript{8}, K. H. Harriman\textsuperscript{9}, L. C. McDonald\textsuperscript{1}, F. J. Angulo\textsuperscript{1}

\textsuperscript{1}Centers for Disease Control & Prevention, Atlanta, GA, \textsuperscript{2}Wadsworth Center, New York State Health Dept, Albany, NY, \textsuperscript{3}Hospital of Saint Raphael, New Haven, CT, \textsuperscript{4}Maryland Dept of Health & Mental Hygiene, Baltimore, MD, \textsuperscript{5}University of New Mexico Health Sciences Center, Albuquerque, NM, \textsuperscript{6}Tennessee Dept of Health, Nashville, TN, \textsuperscript{7}Georgia Pubic Health Laboratory, Atlanta, GA, \textsuperscript{8}Colorado Dept of Public Health & Environment, Denver, CO, \textsuperscript{9}Minnesota Dept of Health, St. Paul, MN.

\textit{C. difficile} is the leading cause of antibiotic-associated diarrhea and colitis among inpatients of healthcare facilities (HCF) and is emerging in the community, where \textit{C. difficile} associated disease (CDAD) is occurring in patients without recent HCF exposure. Emergence of an epidemic \textit{C. difficile} strain, NAP1/BI, has led to increased incidence and severity of CDAD in HCF, but little is known about what strains are responsible for community-associated CDAD (CA-CDAD). We collected and characterized CA-CDAD isolates from diverse geographical regions to better understand epidemiology of this disease. Ten FoodNet sites conducted CA-CDAD case finding and performed stool cultures for \textit{C. difficile} over 3 months. Presumed CA-CDAD was defined in an ambulatory patient with a \textit{C. difficile} toxin-positive stool who, based upon available medical record review, had no overnight stay in a HCF in the preceding 3 months. Isolates were sent to CDC for confirmatory identification and characterization, including toxinotyping, pulsed-field gel electrophoresis (PFGE), evaluation of \textit{tcdC} and \textit{cdtB}, and antimicrobial susceptibility testing. A total of 174 presumed CA-CDAD cases were identified; \textit{C. difficile} was isolated from the stool specimens of 89 (57\%) of 155 patient specimens cultured (range 29-100\% recovery at each site). CA-CDAD isolates (n=89) comprised 9 toxinotypes (TT) and 31 PFGE patterns. TT 0 isolates were most common (n=46; 51.7\%), followed by TT III (n=18; 20.2\%) and V (n=9; 10.1\%). \textit{C. difficile} isolates from presumed CA-CDAD were genetically diverse, indicating that CA-CDAD is not caused by a single strain. Strains that commonly cause HCF-associated CDAD, including NAP1/BI (TT III), were the most common TT isolated from presumed CA-CDAD. TT V, however, is a historically rare cause of human CDAD, but causes epidemic CDAD in food-producing animals. Further study is required to determine whether there is link between \textit{C. difficile} from animals and human CA-CDAD.
MULTICENTER STUDY OF PREVALENCE OF TOXIN A(+)/B(+) AND TOXIN A(-)/B(+) CLOSTRIDIUM DIFFICILE. IN KOREA

BM Shin¹, EY Kuak¹ SJ YOO¹, EC Kim², K Lee³, JO Kang⁴, JH Shin⁵

¹Department of Laboratory Medicine, Sanggye Paik Hospital, Inje University, Seoul, ²Seoul National University, ³Yonsei University, ⁴Hanyang University, Kuri, ⁵Busan Paik Hospital, Korea

The prevalence of toxigenic Clostridium difficile (C. Difficile) were reported about 35-80% in Korea. Although the prevalence of toxin A(-)/B(+) C.difficile strain was less than 5% before 2000, it became an emerging nosocomial pathogen in Korea. Therefore, we need to investigate the nationwide prevalence of toxin A(+)/B(+) and toxin A(-)/B(+) C. difficile for the epidemiologic study. These might provide the basic data for controversies about toxigenicity of toxin A(-)/B(+) C. difficile. We performed C. difficile culture from patients Admitted in 6 tertiary hospitals during 2001 to 2005. PCR assay for toxin A and toxin B genes was done in 724 unduplicated C. difficile isolates among culture positive cases. The proportion of toxin A(+)/B(+) and toxin A(-)/B(+) C. difficile were 30.2-61.1% and 9.3-54.7% in 6 hospitals respectively. The proportion of toxin A(-)/B(+) strains began to increase from 2003-2004 and was rapidly increased in 2005. The factors associated with antibiotic usage patterns might contribute the high prevalence of toxin A(-)/B(+) strains. We need further study to define the role of A(-)/B(+) C. difficile strains in Korea.
A CLUSTER OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DISEASE – CAUSED BY A RIBOTYPE OTHER THAN 027- IN AN UNIVERSITY HOSPITAL IN AUSTRIA, 2006

M. Hell¹, D. Schmid², A. Indra², S. Huhulescu³, M. Maass³ and F. Allerberger³

¹Department of hospital epidemiology and infection control, University hospital Salzburg, Austria, ²National agency for food and health safety, Vienna, Austria, ³Institute of clinical microbiology, University hospital Salzburg, Austria

Background: CDAD (*Clostridium difficile* associated disease) is changing in its clinical severity and epidemicity. Hypervirulent strains of *Clostridium difficile* are causing hospital outbreaks in Europe. Therefore surveillance programs should be implemented in hospitals.

The aims of our surveillance program are to monitor incidence and death rate of CDAD in our hospital, to describe cases by clinical course and therapeutic outcome, and to assess the efficacy of *C. difficile* infection control precautions in the hospital.

Methods: Since February 2006 an active clinically and laboratory based surveillance program has been in place. All patients with diarrhea are obligatory screened for *Clostridium difficile* infection. Case definitions according to the *Clostridium difficile* working group recommendations (ECDC Advisory Forum 2006) were used for case identification and classification.

Results: 277 patients fulfilled the definition criteria of a case of CDAD. Out of these, 129 cases were considered to have been acquired the CDAD at our hospital based on the CDAD onset and cases’ hospital admission date. The other 145 cases were either community acquired or acquired from a health-care facility other than our hospital. Of these 277 cases, 22 cases were selected, 15 cases because of the severe course of their CDAD episode and 7 cases because of having been related in time and/or space with the severe cases. All 22 cases were retrospectively subjected to extensive epidemiological and molecular biological investigation including PCR-ribotyping. There was no ribotype 027 found among the isolates from the 22 cases. The most prevalent ribotype found was ribotype 053 (45.5%), whereas six of these cases showed severe course of CDAD. Ribotype AI5 was observed as the second most frequent ribotype (5, 22.7%), cases of this ribotype had severe CDAD.

Conclusions: Based on ribotyping 2 major strains were identified in a surveillance period of 1 year. The preliminary findings indicate a good transmissibility of strain Ribotype 053 and suggest strain AI-5 to be hypervirulent and also epidemic. Further surveillance data are required for supporting these hypotheses.
RECENT INTERNATIONAL TYPE 027/NAP1 C. DIFFICILE ISOLATES HAVE UNCHANGED TcdC AND TOXIN PRODUCTION IN VITRO BUT EXTENDED RESISTANCE TO NEW FLUOROQUINOLONES COMPARED TO HISTORICAL SWEDISH ISOLATES

Åkerlund, T., Unemo, M., Norén T. and Burman, L.G.

Department of Bacteriology, Swedish Institute for Infectious Disease Control, SE-17182 Solna, Sweden. Department of Infectious Diseases and Department of Clinical Microbiology, Örebro University Hospital, SE-70185, Örebro, Sweden.

We identified three isolates of C. difficile of PCR ribotype 02 (SE10 according to the Swedish nomenclature based on a refined method) among >1500 isolates typed 1997 – 2001. None was part of an outbreak but all three belonged to the “historical” variant of this strain based on resistance to ciprofloxacin but sensitivity to moxifloxacin. The three isolates produced 360 (211 - 538) U toxin/mL culture compared to 340 U/mL of the moxifloxacin/gatifloxacin-resistant outbreak-related type 027/NAP1 first isolated from North America. Toxin production was optimized by using PY medium with no cysteine added and measured using the Ridascreen Toxin A/B kit. The corresponding level from the highly toxin producing reference strain VPI1463 was 2200 U/mL. Isolates representing common Swedish PCR ribotypes, SE20 (serogroup G), SE21 (serogroup H) and SE30 (unknown serogroup) produced 3, 28, and 125 U/mL, respectively. The tcdC gene of all three historical Swedish 027/SE10 isolates had an 18-bp deletion and a frame-shift mutation causing a truncated protein of 65 amino acids, most likely rendering the protein inactive. PCR ribotypes SE20 and SE30 had identical tcdC lacking the mutations found in type 027/SE10. Thus, despite a defective TcdC typical of PCR ribotype 027, its toxin producing capacity was not increased by more than three-fold compared to SE20 and SE30 isolates. Although this subtle increase may be contributing to the disease severity, previous studies have indicated that in vitro toxin levels do not necessarily correlate with those in vivo and that factors such as C. difficile counts and the competing microflora are critical for in vivo toxin levels. Our data also indicate that the new epidemic isolates may have an increased growth yield, different morphology and higher sporulation frequency in vitro compared to historic isolates. We propose however that the use of new antibiotics, e.g. moxifloxacin, highly active against colonic anaerobes promotes a more severe overgrowth of the resistant C. difficile 027/NAP1. Thus, changed clinical practice appears to be of importance to limit the selection for and nosocomial spread of this strain.
THE CHANGING EPIDEMIOLOGY OF *CLOSTRIDIUM DIFFICILE* IN QUÉBEC, CANADA

V.G. Loo

McGill University Health Centre, McGill University, Montréal, Québec

*Clostridium difficile* is an important cause of nosocomial diarrhea. In 2002, several hospitals in Québec, Canada noted a marked increase in the incidence of *C. difficile* associated diarrhea (CDAD) with increased morbidity and mortality. Hospitals in Québec had a higher incidence of CDAD compared with other Canadian provinces.

A predominant strain of *C. difficile* (NAP1/02) that is resistant to fluoroquinolones, binary toxin positive and has a deletion in the regulatory component of toxins A and B is responsible for the geographically dispersed outbreaks of CDAD.

A provincial *C. difficile* surveillance program was implemented in August 2004. Hospitals need to be vigilant and should conduct surveillance for CDAD. Early diagnosis and treatment are essential. Effective infection control measures coupled with judicious use of antibiotics need to be in place to prevent CDAD outbreaks.
A COMPARATIVE STUDY IN THE HOSPITALISED ELDERLY POPULATION IN EDINBURGH: WHAT MAKES A PATIENT SUSCEPTIBLE TO CLOSTRIDIUM DIFFICILE?

K. Sánchez-Hurtado¹, M. Corretge², E. Mutlu¹, R. McIlhone², J.M. Starr² and I.R. Poxton¹.

¹Centre for Infectious Diseases, University of Edinburgh, Edinburgh, UK, ²Royal Victoria Hospital, Edinburgh, UK

Clostridium difficile is a nosocomial pathogen with an increasing incidence worldwide. It has been widely reported that patients in the same ward and colonized by the same strain of C. difficile do not respond in the same way. Some will remain asymptomatic while others will develop disease. Among these, some will have mild symptoms that respond to treatment with no more symptoms and others will suffer multiple episodes and severe complications.

The ability to mount a specific immune response to C. difficile toxins or other cellular antigens has been key to explain why some patients suffer multiple recurrences while others simply get better after one treatment. But what makes the patient susceptible in the first place?

In this study, the systemic levels of antibodies, the strain of C. difficile colonizing and the antibiotic treatment that the patient was following prior to sampling were investigated in three groups of elderly patients: 1) C. difficile cases, 2) asymptomatic carriers of C. difficile and 3) matched controls. In total 69 patients were sampled.

Systemic levels of specific IgG and IgM against several antigens were measured by ELISA and no statistically significant differences were found between the groups. The presence of toxin-neutralizing antibodies in serum was investigated with Vero and Caco2 cells and no differences were found either. When Western blots were performed for serum antibodies against C. difficile surface proteins, the stronger signal was produced by those who developed symptoms – the cases.

The isolates were ribotyped, toxinotyped and S-layer typed, and were found to be too similar to explain the different outcomes after colonization.

A partial analysis to correlate antibiotics prescribed and the use of proton-pump inhibitors with symptoms has been done, but in the small sample no significant differences were evident. However, antibiotic restriction policies to stop outbreaks have proven very successful, which may well imply that the key to understand the variability of symptoms may not lie within the host’s immune response but within the stability of the gut flora.
EMERGING CASES OF CLOSTRIDIUM DIFFICILE INFECTION IN CHILDREN

Radsel A\textsuperscript{1}, Pokorn M\textsuperscript{1}, Rupnik M\textsuperscript{2}, Cizman M\textsuperscript{1}

\textsuperscript{1}Department for Infectious Diseases, University Medical Centre Ljubljana, Japljeva 2, SI-1525 Ljubljana, \textsuperscript{2}Institute of Public Health Maribor, Centre for Microbiology, Prvomajska ulica 1, 2000 Maribor

Compared to adults, Clostridium difficile infections are seldom reported in children and usually follow a mild course.

Eight cases of C. difficile infection in children occurring over a 6-month period (from November 2006 to April 2007) are presented. Children were aged 3 months to 13 years. The course of the disease in three cases was extremely severe, one child was moderately affected and the rest had a mild course. All of the severe cases needed ICU treatment, one of them required partial colectomy. 6 patients were treated orally with either metronidazole or vancomycin or both. The outcome was favorable in all cases. In 7 patients the infection was community-acquired, in one patient it was nosocomial. In all patients ELISA was positive for A/B toxin in stool, in two patients C. difficile was grown in stool culture.

Health professionals should be aware that C. difficile infections do occur in children and sometimes follow a severe course.
EPIDEMIOLOGICAL FEATURES OF PATIENTS WITH CLOSTRIDIUM DIFFICILE–ASSOCIATED DIARRHEA

B Levent¹, BE Citil¹, R Kayali¹, A Gozalan¹, H Arslan², T Togan², H Bayrak¹, F Aktras³, M Dizbay³, S Kacar⁴, N Sasmaz⁴, A Azap⁵, B Dokuzoguz⁶, T Cirkin⁶, DN Ozucelik⁷, A Karademir⁸, C Ustun⁸, B Esen¹

¹Refik Saydam National Hygiene Center, ²Faculty of Medicine, Baskent University, ³Faculty of Medicine, Gazi University, ⁴Yuksek Ihtisas Hospital, ⁵Faculty of Medicine, Ankara University, ⁶Ankara Numune Educational and Research Hospital, ⁷Faculty of Medicine, Hacettepe University, and ⁸Bayindir Hospital, Ankara, Turkey

In a prospective multi-centre study, stool samples and the epidemiological features of both inpatients and outpatients with diarrhea, suspected as CDAD were investigated. The study was carried out in collaboration with 11 hospitals and health centres located in Ankara, between January and December 200. Clostridium difficile–associated diarrhea (CDAD) was diagnosed by using cell cytotoxicity test or anaerobic culture followed by toxigenicity testing. Standard questionnaire was applied to determine the epidemiological features of the patients. The risk factors such as age, gender, period of diarrhea, current or former hospitalisation, antibiotic or anti-neoplastic treatment, history of abdominal surgery and other disease were compared among the toxin B and/or toxigenic culture positive and negative patients.

One hundred seventy eight stool samples were collected from the patients complaining of diarrhea shorter than two months after antibiotic or anti-neoplastic therapy. Twenty (11.2%) stool samples were positive either with the cell cytotoxicity test for toxin B or identified as toxigenic C. difficile by culture. One hundred fifty eight (88.2%) stool samples were negative for both test methods.

The mean patient age was 42.0 ± 20.2 years (1-86 years) and 102 (57.3%) of patients were male. 53.4% of total stool samples and 55.0% of positive stool samples were from the patients stayed in the same hospital. According to our results, statistically significant association was found between the patients older than 50 years and risk for CDAD (OR: 3.81, 95% CI=1.28-11.83).
C. DIFFICILE S-LAYER PROTEINS: CHARACTERISATION AND POTENTIAL IN NANOTECHNOLOGY

Neil F. Fairweather

Centre for Molecular Microbiology and Infection, Imperial College London, London SW7 2AZ, UK

Clostridium difficile, like many Bacteria and Archaea, produces an S-layer on the external face of the vegetative cell. S-layers are proteinaceous, two-dimensional paracrystalline arrays that surround the cell, and are attached to the underlying cell wall via non-covalent forces.

A remarkable feature of S-layer proteins is their ability to self-assemble in vitro to form regularly spaced arrays of defined morphology. As a consequence, there is now considerable interest in the exploitation of S-layers for nanotechnological applications, from construction of novel nano-surfaces incorporating metallic components or semi-conductors, to novel “S-liposomes” displaying defined arrays of active biological molecules. Our laboratory is a member of a European Union “NASSAP” consortium: Nano Arrayed Systems based on Self Assembling Proteins. This consortium aims to exploit bacterial S-layers and other self assembling bacterial systems to develop new materials for medical and industrial applications.

The C. difficile S-layer is composed of two proteins; the low molecular weight S-layer protein (LMW-SLP) and the high molecular weight S-layer protein (HMW-SLP). We have undertaken a series of experiments to question how the LMW SLP and HMW SLP proteins interact to form a structure capable of self-assembly. Our studies suggest that the two proteins interact via strong non-covalent forces to form a stable complex that is the basic subunit required for self-assembly. We have also identified a domain on the LMW SLP that is required for formation of the complex. These results, and those of preliminary structural analysis, will be discussed in the context of the extreme sequence variability of the C. difficile S-layer.
DETECTION OF THE TOXIN B GENE OF CLOSTRIDIUM DIFFICILE IN STOOL SPECIMENS BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) AND DIRECT TYPING OF THE SlpA GENE BY SEQUENCING

H. Kato and Y. Arakawa

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011, Japan.

A rapid, simple and sensitive laboratory testing is required not only for appropriate treatments of Clostridium difficile infection but for its infection control. To prevent nosocomial infection, monitoring the specific C. difficile types, which are more likely to cause nosocomial outbreaks and severe diseases, is necessary. We established and evaluated the molecular methods for detecting tcdB by loop-mediated isothermal amplification (LAMP) and typing C. difficile by sequencing slpA in stool specimens without culturing. A total of 151 stool specimens obtained from patients with intestinal symptoms were examined. The specimens were cultured for C. difficile and slpA sequence typing was performed on the recovered isolates. tcdB was detected by LAMP on DNA extracted directly from stool specimens, and slpA amplified from the LAMP-positive DNA samples by nested PCR was sequenced. Of the 151 stool specimens, tcdB-positive C. difficile was recovered from 79, of which 76 stool specimens were tcdB-positive by LAMP. Nine specimens were LAMP-positive but culture-negative. Of 85 specimens tested for direct typing, typing results were successfully obtained in 76; the results from direct typing and those from recovered isolates completely agreed on 69 specimens; the remaining 7 specimens were culture-negative. Primers used here for direct typing were feasible for typing of PCR ribotype 027 responsible for many outbreaks in North America as well as PCR ribotype smz, which has been reported to cause multiple outbreaks in Japan. One of the advantages of slpA sequence typing is that typing results can be compared with those of epidemic types via the Internet without obtaining reference strains. A combination of LAMP detecting tcdB and direct typing by sequencing slpA is reliable and valuable for detection and identification of the epidemic strains in stool specimens.
INTERNATIONAL MULTI-LABORATORY COMPARISON OF SEVEN CLOSTRIDIUM DIFFICILE TYPING TECHNIQUES


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Seven Clostridium difficile typing techniques were evaluated using 42 human clinical isolates contributed by laboratories in Canada, Netherlands, UK, and USA. Typing techniques included Multilocus Variable Number Tandem-Repeat Analysis (MLVA), Amplified Fragment Length Polymorphism (AFLP), surface layer protein A gene sequencing (slpA-Typing), PCR-Ribotyping (PCR-RT), Restriction Endonuclease Analysis (REA), Multilocus Sequence Typing (MLST), and Pulsed-Field Gel Electrophoresis (PFGE). Techniques were evaluated for discrimination, typability, and ability to cluster isolates into 1 of 6 allele profiles (AP) (A through F), defined by: toxinotype (TT), presence or absence of binary toxin gene (bin+/−), and tcdC sequence (wild type or deletion) (Cwild/Cdel). All isolates were typable by all techniques. Discrimination index (DI) scores ranged from 0.964 to 0.631 in the following order: MLVA, REA, slpA, PFGE, PCR-RT, MLST, and AFLP. Despite the wide range in DI scores, all the techniques differentiated the current epidemic strain BI/027/NAP1 (APB= TTIII,bin+,Cdel) from other isolates. All of the techniques also identified multiple types among the historic wild type profile i.e., APA (APA= TT0, bin−, Cwild). However, only REA and MLVA could subtype APB isolates obtained from geographically diverse outbreaks. While all techniques proved capable of detecting local outbreak strains, only REA and MLVA appear capable subtyping APB strains.
CwpV, A VARIABLE CELL WALL PROTEIN OF CLOSTRIDIUM DIFFICILE

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The surface of *C. difficile* is covered in a paracrystalline array of two surface layer proteins (SLPs) derived from a single gene, slpA, but there are also other proteins present on the cell surface layer. The high-molecular weight SLP incorporates three copies of the Pfam04122 cell-wall binding domain, as do twenty-eight other proteins encoded within the *C. difficile* 630 genome. One of these, designated CwpV as it is variable between strains, contains three Pfam04122 domains in its N-terminal domain, and has nine almost identical 120 amino acid repeats at its C-terminus. These repeats are present in six of twelve strains examined, and have highly conserved sequences, although the numbers of repeats varies from four to six. We have analysed *cwpV* in two hyper-virulent strains from the Quebec and Stoke Mandeville outbreaks, R2032 and R20921 respectively. In these strains, the N-terminal domain of CwpV is highly related to the other strains. However, the C-terminal domain has eight almost perfect 79 amino acid repeats which appear to be completely unrelated to those found in *C. difficile* 630 and in the other strains analysed, in both amino acid sequence and predicted secondary structure.

*cwpV* is transcribed and expressed throughout growth in *C. difficile* 630, as demonstrated by reverse-transcription PCR and Western immunoblotting of total cell lysates. The presence of CwpV at the cell surface of *C. difficile* 630 has been shown by immunostaining; FACScan analysis; immunogold transmission electron microscopy; and proteomic analysis of the cell wall. Although implicated by the observed repetitive structure, no adhesion or haemagglutination properties have yet been found and the function of this protein is still to be elucidated.
CLINICAL LABORATORY TESTING FOR CDAD: PITFALLS AND PROGRESS: A DISCUSSION

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Clostridium difficile-associated diarrhea (CDAD) is the major cause of healthcare-associated infectious diarrhea and pseudomembranous colitis. Of concern is the fact that it appears to be rapidly increasing in prevalence, more than doubling between 1996 and 2003 in the United States. Recently there have been two major clinical developments: \textit{i}) emergence of a hypervirulent form of the disease, and \textit{ii}) the suggestion that metronidazole is less effective. However, in most reports the description of diagnostic laboratory testing for detection of toxin-producing \textit{C. difficile} is minimal and overlooks the fact that current assays suffer from poor sensitivity and specificity. This raises the concern that the appearance of increased disease, disease severity, and therapeutic failure may be based upon an uncertain diagnosis of CDAD. The purpose of this discussion session is to decide the best testing approach and to suggest how to implement this in clinical laboratories. Anaerobic culture, properly performed, has sensitivity approaching 100%, but the false positive rate exceeds 10%. Similarly, while tissue culture cytotoxicity is considered to have good specificity, the sensitivity when compared to anaerobic culture in clinically defined CDAD can be as low as 71%. Confounding the current diagnostic approach to CDAD is the reported test performance of commercial EIA assays, with sensitivities of 50-99% and specificities of 70-100%, posing diagnostic challenges since the EIA accounted for nearly 95% of the toxin assay methods used in the USA for laboratory diagnosis of CDAD by 2003. Perhaps, most importantly for the accurate diagnosis of this infectious disease, is the fact that new rapid, real-time PCR (qPCR) assays can have the sensitivity of culture for detecting the presence of toxigenic \textit{C. difficile} while retaining the specificity of the direct cytotoxicity test. In our work we have found that detection of \textit{C. difficile} toxin B gene by qPCR assay is significantly more sensitive than commonly used EIA and cytotoxin methods ($p<0.05$). With a test turnaround time of under 4 hours, qPCR is an accurate method of detecting toxigenic \textit{C. difficile} for CDAD diagnosis.
IMMUNE RESPONSES AND NEURONAL EFFECTS MEDIATED BY C. DIFFICILE-DERIVED PROTEINS

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Clostridium difficile produces an array of molecules able to affect directly or indirectly the activity of the mucosal associated immune cells and the enteric nervous system. The effects that C. difficile toxins play during colitis have been extensively investigated whereas the effect of C. difficile-derived molecules on the healthy intestine are just becoming to be investigated.

The vast majority of the adult healthy population carries significant levels of humoral and mucosal antibodies against C. difficile toxin A and B as well as against surface layer proteins (SLPs), indicating that the microbe is present in the normal intestine at some point of over 80% of subjects and that C. difficile-derived products stimulate the adaptative immune system. Indeed C. difficile directly binds to the normal intestinal mucosa and eventually affect the activity of cells sensing the gut environment, such as epithelial cells, dendritic cells and possibly neurons. Thus, in normal subjects C. difficile derived proteins may influence the activity of the immune system and contribute to modulate host’s cytokine micro-environment. Furthermore, SLPs and a non-toxigenic fragment of the receptor binding domain of toxin A are able to modulate the activity of dendritic cells up-regulating the expression of co-stimulatory molecules and inducing the secretion of a pattern of cytokines compatible with the induction of a mixed Th1/Th2 response. During pathological conditions C. difficile toxins A and B orchestrate the development of a massive inflammatory response in the intestinal mucosa since they induce depolymerization of actin, enhance mucosal permeability and trigger the release of a variety of pro-inflammatory mediators either directly from mucosal epithelial cells or inducing direct inflammatory cells activation that recruit the inflammatory cells. Although during C. difficile-mediated colitis the enteric neurons are instrumental to amplify the inflammatory process, it is not clear whether C. difficile toxins directly modulate the activity of the enteric neuronal network.

In summary, C. difficile produces a variety of factors that are able to interact with and modulate the activity of key components of the intestine during pathological conditions. However, the ability of certain C. difficile-derived proteins to interact and modulate the activity of immune cells suggest that this microbe may influence the activity of the immune system also in physiological conditions.
ANTIBIOTICS INVOLVED IN *CLOSTRIDIUM DIFFICILE* – ASSOCIATED DISEASE INCREASE COLONIZATION FACTOR GENE EXPRESSION

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Pseudomembranous colitis (PMC) and diarrhea have long been recognized as important adverse effects of antimicrobial chemotherapy and *Clostridium difficile* has been established as their etiological agent in many cases. Antibiotics are presumed to disrupt the intestinal microbiota, which displays a barrier effect against colonization and subsequent infection by *C. difficile*. In addition, it is known that antibiotics could modify colonization factor expression of many human pathogenic bacteria. Consequently, we investigated in vitro the impact of various antibiotics (ampicillin, clindamycin, fluoroquinolones, kanamycin) on the expression of genes encoding known or putative colonization factors: the S-layer protein P47, the two adhesins Cwp66 and Fbp68 and the protease Cwp84; three of the four genes encoding these proteins, *slpA*, *cwp66* and *cwp84*, have been previously localized in a genetic locus carrying 17 open reading frames. This analysis was performed on seven toxigenic and non toxigenic *C. difficile* isolates, including the PCR-ribotype 027 epidemic strain, by real-time RT-PCR.

Presence of subinhibitory concentrations (½ MIC) of ampicillin or clindamycin increased the expression of genes encoding colonization factors of *C. difficile*; on the other hand, presence of ofloxacin, moxifloxacin or kanamycin did not significantly modify the colonization factor gene expression. In addition, no difference in gene expression was observed between toxigenic and non toxigenic strains, or even the epidemic strain.

In conclusion, ampicillin and clindamycin, which are antibiotics with high propensity to induce *C. difficile* infection, could play a role more important than a simple alteration of the gut microbiota: they increase colonization factors gene expression and could then play a direct role in enhancing colonization by *C. difficile*. In contrast, moxifloxacin, which is now recognized as a major risk factor for *C. difficile* infection, does not induce gene expression and might favour CDAD only by disrupting the barrier microbiota, due to its broad-spectrum activity against anaerobic bacteria.
HYPERVERULENT C. DIFFICILE STRAINS HAVE ALTERED SURFACE PROTEINS AND INCREASED ADHERENCE TO HUMAN INTESTINAL EPITHELIAL CELLS

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Background: Toxin-producing (toxigenic) Clostridium difficile (CD) strains are leading agents of nosocomial diarrhea. Since 2000, there have been epidemics of CD-associated-disease (CDAD) caused by hypervirulent toxigenic CD belonging to the restriction endonuclease type group BI. BI strains overproduce clostridial toxins A and B; however, since CDAD progression is accelerated upon BI infection, and since BI strains predominate in hospitals during epidemics, we hypothesized that non-toxin proteins were also dysregulated in these strains, and that some of these proteins influenced CD colonization and persistence.

Methods and results: We used anaerobic bacterial adherence assays protein profiling and binding interference studies to test our hypothesis. First, we optimized an anaerobic CD adherence assay. We found that three different BI strains (BI-6, BI-8 and BI-17) each exhibited at least 50% higher adherence to Caco-2 human intestinal epithelial cells than non-epidemic strains. Second, we found that BI strains had altered protein profiles compared to non-epidemic CD strains; one dysregulated protein was identified by mass-spectrometry as the CD Surface Layer Protein A (SlpA). Third, we found that SlpA from BI strains differed in both size and amount from SlpA of non-epidemic strains. Fourth, we showed that when a total Slp preparation from a CD strain was pre-incubated with Caco-2 cells, adherence of that CD strain to a Caco-2 monolayer was markedly reduced. Lastly, we found that pre-incubation of the epidemic BI-17 strain with an anti-SlpA antiserum reduced its Caco-2 adherence by 50%, clearly implicating SlpA in adherence.

Conclusions: Hypervirulent CD strains have dysregulated protein expression. They also have increased adherence to human intestinal epithelial cells mediated by altered surface-layer proteins in general and SlpA in particular. Efficient adherence may be one factor contributing to the severity of CDAD (and its recurrence) in patients, and in the establishment and predominance of hypervirulent CD in hospitals.
TcdC, THE NEGATIVE REGULATOR OF TOXIN SYNTHESIS IN C. DIFFICILE

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Toxin synthesis by C. difficile is growth dependent and is regulated by various environmental signals. Toxin genes tcdA and tcdB are encoded in a pathogenicity locus, which also includes three accessory genes tcdR, tcdC and tcdE. TcdR has been shown to act as an alternate sigma factor, which involves in positive regulation of both toxin genes as well as itself. Toxin genes tcdA, tcdB and their positive regulator tcdR are expressed during the stationary growth phase. However TcdC is expressed during the exponential stage of the culture growth. This expression pattern suggested that TcdC is a negative regulator of toxin gene expression. TcdC is a small acidic protein without conserved DNA binding motif. It is able to form dimmers and it presents on its N-terminal region a putative transmembrane domain. The sub-cellular localization of TcdC was determined using specific anti-TcdC antibodies confirming that TcdC is a membrane-associated protein. In addition, TcdC was found to be mainly present during exponential growth, which correlates well with the transcription pattern already observed by others and it strengthens the role of TcdC as negative regulator of the toxin synthesis. We present direct evidence that TcdC negatively regulates C. difficile toxin synthesis and show that TcdC acts as an antagonist of TcdR through its direct interactions with TcdR protein and RNA polymerase. Currently we are working on deciphering the regulatory aspects of tcdC gene in response to various environmental signals. Recently, high toxins producing epidemic strains have been characterized with tcdC mutations and we are investigating the role of tcdC mutations for their role in hyper virulence.
REPRESSION OF CLOSTRIDIUM DIFFICILE TOXIN GENE EXPRESSION BY CodY

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CodY, a global regulator of gene expression in low G + C Gram-positive bacteria, was found to repress toxin gene expression in Clostridium difficile. Inactivation of the codY gene resulted in derepression of all five genes of the C. difficile pathogenicity locus during exponential growth and stationary phase. CodY was found to bind with high affinity to a DNA fragment containing the promoter region of the tcdR gene, which encodes a sigma factor that permits RNA polymerase to recognize promoters of the two major toxin genes and its own promoter. CodY bound with low affinity to the toxin gene promoters, suggesting that the regulation of toxin gene expression by CodY occurs primarily through direct control of tcdR gene expression. Binding of CodY to the tcdR promoter region was enhanced in the presence of GTP and branched-chain amino acids, suggesting a link between nutrient limitation and the expression of C. difficile toxin genes.
STRUCTURE AND FUNCTIONS OF THE GLUCOSYLTRANSFERASE DOMAIN OF CLOSTRIDIUM DIFFICILE TOXINS

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Clostridium difficile toxins A and B are the cause of antibiotics-associated diarrhea and pseudomembranous colitis, occurring during treatment with antibiotics. The toxins are tripartite in structure with an N-terminal glucosyltransferase domain, a C-terminal receptor binding domain and a middle part, which is involved in translocation of the toxin into the cytosol. The toxins bind to cell membrane receptors, which are not well characterized. After endocytosis the low pH of endosomes causes conformational changes, which allow insertion of the toxin into membranes and subsequent translocation of the enzyme domain into the cytosol.

The glucosyltransferase domain has a structure of a GT-A type glucosyltransferase. Recently, we identified several residues important for catalysis, co-substrate and protein substrate interaction of glucosyltransferase domain. Introduction of helix alpha17 of toxin B into Clostridium sordellii lethal toxin inhibited modification of Ras subfamily proteins but enabled glucosylation of RhoA, indicating that helix alpha17 is involved in RhoA recognition by toxin B. The data allow the design of a model of the interaction of the glucosyltransferase domain of toxin B with its protein substrate RhoA.

Because only the catalytic domain of clostridial glucosylating toxins is translocated into the cytosol, we attempted to identify residues involved in processing and translocation of the toxin into the cytosol. These data are discussed.
TcdA-INDUCED APOPTOSIS IS P53-INDEPENDENT BUT DEPENDS ON GLUCOSYLATION OF RHO GTPASES

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Clostridium difficile toxin A (TcdA) is one of two homologous glucosyltransferases that mono-glucosylate Rho GTPases. Glucosylation of Rho GTPases interferes with effector coupling, and Rho-dependent signal transduction is abrogated. As a result, the actin cytoskeleton re-organizes and cells eventually become apoptotic. HT29 cells were challenged with wild-type and mutant TcdA to investigate the mechanism by which apoptosis is induced. The TcdA-induced re-organization of the actin cytoskeleton led to an increased number of cells within the G2/M phase. Depolymerization of the actin filaments with subsequent G2/M arrest, however, was not causative for apoptosis, as shown in a comparative study using latrunculin B. Thus, apoptosis is induced by another mechanism. Mutant TcdA (W101A) possessing 50-fold reduced glucosyltransferase activity was applied to check whether apoptosis is induced in a Rho GTPase-independent manner. We found that activation of caspase-3, -8, and -9 strictly depended on the glucosylation of Rho GTPases. Furthermore, Apoptosis measured by flow cytometry was completely abolished by a pan-caspase inhibitor (z-VAD-fmk). Interestingly, cleavage of procaspase-3 and Bid was not inhibited by z-VAD-fmk, but was inhibited by the calpain/cathepsin inhibitor ALLM. Cleavage of procaspase-8 was susceptible to inhibition by z-VAD-fmk and to the caspase-3 inhibitor Ac-DMQD-CHO, indicating a contribution to the activation of caspase-3 in an amplifying manner. Although TcdA induced mitochondrial damage and cytochrome c release, p53 was not activated or up-regulated. A p53-independent apoptotic effect was also checked by treatment of HCT 116 p53−/− cells. In summary, TcdA-induced apoptosis in HT29 cells depends on glucosylation of Rho GTPases leading to activation of caspase-3 by a non-caspase protease. Re-organization of the actin cytoskeleton is not causative for apoptosis.
AUTOCATALYTIC CLEAVAGE OF CLOSTRIDIUM DIFFICILE TOxin B

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Clostridium difficile, the causative agent of antibiotic-associated diarrhea and pseudomembranous colitis, is a major nosocomial germ in many health care institutions throughout the world¹. Recent reports indicate that its virulence is increasing and infections are detected also in populations previously considered at low risk²,³. The main virulence factors are two large protein cytotoxins, toxin A (TcdA) and toxin B (TcdB)⁴. It was proposed earlier that TcdB is cleaved by a cytosolic factor of the eukaryotic target cell during its cellular uptake, with solely the N-terminal catalytic domain reaching the cytosol⁵,⁶. Here we report that cleavage of TcdB is an intramolecular process and that host cytosolic inositolphosphates act as cofactors for protease activation. We show that the addition of inositol hexaphosphate (IP₆) activates the latent protease function not only in TcdB but also TcdA, Tcnα and TcsL, from C. difficile, C. novyi and C. sordellii. 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), a covalent inhibitor of aspartate proteases, was used to block the intrinsic aspartate protease function and allowed the identification of the catalytically active site of TcdB. The EPNP-derivative of TcdB does not show autocatalytic activation and loses cellular toxicity, but remains toxic when directly injected into the cytosol. This is the first report on a bacterial toxin that uses eukaryotic signals for induced autoproteolysis to deliver its toxic domain into the cytosol of target cells. Based on our data, we present an integrated model for the uptake and intracellular inositolphosphate-induced activation of TcdB.
ENTRY OF CLOSTRIDIAL BINARY TOXINS INTO CELLS


Some Clostridium difficile, specially emergent strains involved in recent outbreaks of nosocomial colitis, produce in addition to the large clostridial toxins a binary toxin (CDT) which is related to the iota and C2 toxin families of clostridial binary toxins. The precise role of CDT in natural diseases is not yet fully understood. Clostridial binary toxins are composed of a binding (B) protein (Ib, CDTb and C2-II, respectively) that recognizes distinct membrane receptors and mediates internalization of a catalytic (A) protein (Ia, CDTa and C2-I, respectively) with ADP-ribosyltransferase activity that depolymerizes the actin cytoskeleton. B components are activated by a proteolytic cleavage which removes a 20 kDa N-terminal propeptide. Mature B component recognizes specific receptor on target cells, and form oligomers which localize in detergent-resistant membrane microdomains and generate Na+/K+ permeable pores. B component bound to cell membrane traps A component into endocytic vesicles. Upon acidification of the endosomal compartment, A component is translocated into the cytosol, probably through the pore formed by oligomerized B components. C2 and Iota toxins exhibit differential sensitivity to inhibitors of endosome acidification. While the C2-I component of C2 toxin is translocated into the cytosol from early endosomes, translocation of the Ia component of Iota toxin occurs between early and late endosomes, is dependent on more acidic conditions, and uniquely requires a membrane potential gradient.
NEW PATTERNS IN *CLOSTRIDIUM DIFFICILE* RESISTANCE TO ANTIBIOTICS

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In severe CDAD cases, a specific treatment is necessary and typically metronidazole or vancomycin are used. Currently, both of these treatments show serious limitations, including incomplete response and recurrence rates as high as 47% and some reports have rung an alarm on the emergence of strains with decreased susceptibility to both drugs. This led to investigate the activity of new compounds especially among the new generation fluoroquinolones of the group IV, mainly moxifloxacin which, contrary to the older quinolones shows good in vitro activity. Unfortunately, also for this antibiotic decreased susceptibility has rapidly appeared. The resistance has been associated with chromosomal mutations in gyrA and gyrB whereas no sequence homology with parC has ever been found in the C.d. genome suggesting it is missing in this bacterium. Interestingly, different mutations seem associated to circulating clones. Moreover, fluoroquinolone resistance is frequently linked with resistance to erythromycin and/or clindamycin. The presence of these resistances may be important for the selection of a population of multiple resistant strains which may be involved in the spread of epidemic strains in hospitals. The resistance to MLS is mainly due to the specific dimethylation of the 23S rRNA coded by an erm(B) gene that is located on the mobile transposon Tn5398. Recent studies demonstrated heterogeneity in the genetic arrangement of this element among different clones and some resistant strains, such as the current epidemic 027 ribotype, lack erm(B), or other known erm genes or efflux mechanisms. Resistance to tetracycline is known to be transferable between strains and the transfer has been found to be independent of the transfer of other antibiotic resistances. *C. difficile* may harbour tet genes coding for ribosomal protection proteins. In particular, tet(M) gene is commonly detected in resistant strains harbored on the conjugative Tn 5397. More recently, in some resistant strains the presence of Tn916-like elements have also been demonstrated and another class, tetW, has been detected, too. A very recent study conducted in Europe under the coordination of the ESGCD on 406 *C. difficile* strains collected in 14 countries during 2005 offered the opportunity to analyse different patterns of resistance and characteristics of the genetic elements.
SECOND INTERNATIONAL CLOSTRIDIUM DIFFICILE SYMPOSIUM
Session VI  ANTIBIOTIC RESISTANCE

EMERGENCE OF A CLINDAMYCIN RESISTANT CLONE OF CLOSTRIDIUM DIFFICILE PCR-027 IN DUBLIN IRELAND

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Since 2003 outbreaks of severe Clostridium difficile associated disease associated with PCR ribotype 027 have been identified in Canada and the United States. More recently this strain has been identified in healthcare facilities in several European countries. The aims of this study was to investigate the presence of hypervirulent C. difficile PCR-027 in Dublin and to investigate the rates and mechanisms of antimicrobial resistance in any PCR-027 C. difficile isolates. C. difficile isolates were screened from 6 hospitals in Dublin. 16-23S PCR ribotyping and toxinotyping were used to identify PCR-027. Amplification and sequencing were used to identify the Binary toxin gene (cdtB) and an 18 bp deletion and a frameshift mutation in tcdC. Antimicrobial susceptibilities to five fluoroquinolones, clindamycin and erythromycin were determined using E-tests. The quinolone-resistance-determining-region (QRDR) of gyrA and gyrB were amplified by PCR and characterised. The presence of ermB was determined by PCR. PCR ribotyping profiles identified one major cluster of C. difficile PCR-027. All PCR 027 isolates were categorized as toxinotype III. All isolates were positive by PCR for cdtB and contained an 18 bp deletion and a frameshift mutation at nucleotide 117 in tcdC. These strains were universally resistant to the fluoroquinolones tested (MIC >32 mg/ml). Fluoroquinolone resistance was associated with a single transition mutation (C to T) resulting in the amino acid substitution Thr-82-Ile in gyrA. No amino acid substitutions were identified in the QRDR of gyrB. Isolates from four of the six hospitals investigated demonstrated high-level resistance to Clindamycin and Erythromycin (MIC >32 mg/ml respectively) and contained the ermB gene. C. difficile PCR 027 was identified in the six Dublin hospitals investigated. High-level resistance to fluoroquinolones in C. difficile PCR-027 is associated with a transition mutation in gyrA. This study identifies the first Clindamycin resistant PCR 027 clone reported in Europe. Antimicrobial resistance may provide a selective advantage allowing specific strain types to emerge and predominate in the health-care environment.
PHAGE-MEDIATED TRANSFER OF ANTIBIOTIC RESISTANCE GENES IN CLOSTRIDIUM DIFFICILE

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The recently sequenced temperate phage of Clostridium difficile, φC2, was investigated for transduction of antibiotic resistance genes. A phage sensitive and antibiotic resistant C. difficile strain, CD80, was found to be a suitable donor. φC2 was propagated in CD80 and used to infect five randomly picked antibiotic sensitive strains or recipients. φC2-dependent transfer of erythromycin resistance (ermr) and clindamycin resistance (clinr) to recipient strains was observed. Transfer frequencies were dependent on multiplicity of infection (moi), where moi of 0.1 resulted in frequencies of 1.6 × 10⁻⁷– 1.8 × 10⁻⁶ for ermr-transfer and 3.7 × 10⁻⁸ – 1.6 × 10⁻⁷ for clinr-transfer. The frequency of transfer decreased slightly when DNaseI and RNaseA-treated phage suspensions were used. Transducing phage and transductants were tested for presence of ermB genes by PCR. Sequence analysis of the PCR products revealed identical ermB sequences in transducing phage and transductants. Although the mechanism of transfer is not yet determined, this is the first report that phages may be implicated in transfer of antibiotic resistance genes between strains of C. difficile.
LONGITUDINAL MONITORING OF CLOSTRIDIUM DIFFICILE IN SWEDEN IN THE CONTEXT OF ANTIBIOTIC RESISTANCE AND EMERGENCE OF EPIDEMIC STRAINS

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A high susceptibility of C. difficile (CD) isolates is preferred in the evaluation of all antimicrobial agents for potential use in treatment and of the risk of developing C. difficile associated diarrhea (CDAD).

Currently, the epidemic surge of CDAD and failures of metronidazole therapy reported from US, Canada and Europe have not been conclusively explained by metronidazole resistance of the CD strains tested in vitro. Instead, the emergence of the epidemic strain (NAP1/027) involved a selection mechanism possibly due to fluoroquinolone resistance. In Örebro county, Sweden, we have analyzed the antibiotic susceptibility of 505 CD isolates from 1993-2004 (consecutive isolates during each year). All isolates were highly resistant to cefuroxime and imipenem. The susceptibility to Penicillin V and piperacillin substantially varied during these years and MIC90 ranged between 4-6 mg/L and 8->256 mg/L respectively. Piperacillin-tazobactam would in this perspective be favorable in the treatment of abdominal infections rather than cephalosporins and carbapenems. Clindamycin resistance, was extensive through a local spread of ribotype 012 up to 2002, but this has now reverted to non-epidemic conditions. This has also been the case for rifampicin since 2002, with approximately 80% of isolates displaying high susceptibility and the remaining isolates showing full resistance. Most isolates during the studied years were highly resistant to ciprofloxacin but this has been a standard historical finding not apparently selecting for virulent CDAD strains. However, the use of modern quinolones is still limited in Sweden, possibly reflecting the fact that yet only one isolate of ribotype 027 (from 1999) has been documented in Örebro catchment area. Thus, moxifloxacin resistant CD strains are extremely rare in Örebro county and in Sweden, not yet related to excess morbidity. This fact together with the high prevalence of macrolide resistance in clinical strains (90%) can promote the use of inexpensive susceptibility testing of erythromycin and moxifloxacin as phenotypic markers for detection of emergence and clustering of epidemic NAP1/027 in Sweden. Finally, newly introduced antimicrobials like linezolid, daptomycin and tigecycline presented low MIC’s and should presumably not select for C. difficile during therapy.
CLOSTRIDIUM DIFFICILE IN ANIMALS AND FOOD

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While most of the attention towards C. difficile is focused on humans, this organism can infect a wide range of animal species. Infection or colonization has been reported in diverse animal species internationally, including household pets, food-producing animals, zoo animals, laboratory animals and wildlife. Among companion animals, C. difficile has been best explored in horses where C. difficile is a leading cause of enterocolitis and may be an important cause of duodenitis/proximal jejunitis. Colonization of healthy horses is uncommon (<10%), but the prevalence is higher in foals and horses treated with antimicrobials. Higher colonization rates may be present in pigs, where C. difficile associated disease (CDAD) is also an important problem. The role of C. difficile in enteric disease of dogs and cats is less clear, although CDAD has been reported in these species and high rates of colonization have been identified in some groups of dogs. C. difficile is commonly found in healthy and diarrheic calves. An association between C. difficile toxins in the feces of dairy calves and diarrhea has been reported, but CDAD could not be reproduced experimentally. An interesting observation in animals in that CDAD is that an antimicrobial association is only identified in minority of cases. Some studies have reported that isolates from animals are commonly indistinguishable from those from humans. This includes ribotype 027/NAP1/B1 and raises concerns about the potential for interspecies transmission. There have also been reports of a predominance of toxintype V strains in food animals. Broader studies are required to clarify this. There are also anecdotal reports of people and their household pets being colonized with indistinguishable strains. Because of the presence of C. difficile in food animals, concern has been raised about contamination of food products. Recent studies have identified C. difficile in retail food. Isolates recovered from meat are often indistinguishable from those found in people with CDAD, and include ribotype 027/NAP1/B1. The clinical relevance of this is unclear. Clostridium difficile is clearly an important pathogen in many animal species and may be an under-recognized cause of disease in others. From a public health standpoint, the greatest concern about animals and C. difficile may be the potential for household pets to transmit C. difficile to their human contacts. Broader studies of C. difficile in animals and incorporation of animal and food contact into community based human CDAD studies is required to better understand this potential problem.
ISOLATION OF CLOSTRIDIUM DIFFICILE FROM FOOD ANIMALS IN SLOVENIA

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Clostridium difficile is an emerging cause of enteric disease in animals. So far, it is not clear whether the food animals are the possible source of human infections. However, recently published data suggest an overlap between C. difficile populations in humans and food animals.

Faecal samples from 257 piglets from three different farms and 56 calves from two different farms were analyzed for the presence of C. difficile. The samples were cultured onto standard selective medium with cefoxitin and cycloserine under standard conditions. The isolates were identified based on morphological criteria and the identification was confirmed by amplification of C. difficile specific gene cdd2 using the primer pair Tim6/Struppi6. Calves samples were additionally tested with a commercial enzyme immunoassay (Premier™ Toxins A & B, Meridian Bioscience, Inc.).

One hundred thirty four C. difficile isolates were obtained, 133 from the pigs and one from the calf. Toxin test was positive in 25 culture negative calves samples, suggesting the need for changes in isolation procedure. The strains were characterised by toxinotyping and binary toxin genes were detected by PCR. All piglet isolates from farm 1 and 2 (102 strains) were binary toxin-positive and belonged to toxinotype V. Isolates from farm 3 (31 strains) belonged to toxinotype 0. The ribotyping of representative strains from farm 3 shows that all strains belong to identical ribotype. The calf strain was typed as toxinotype XIa, the variant strain which is positive for binary toxin and negative for TcdA and TcdB (A·B·CDT+). A high prevalence of binary toxin positive strains among food animals was comparable to previous reports. Toxinotype V was reported in piglets in USA and toxinotype XIa in calves from Canada. Certain C. difficile types are therefore more likely associated with animal hosts. However, toxinotype V is also one of the most prevalent variant strains isolated from humans.
NATURAL AND EXPERIMENTAL INFECTION OF NEONATAL CALVES WITH CLOSTRIDIUM DIFFICILE

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The presence of Clostridium difficile toxins in feces was associated with calf diarrhea in a recent retrospective study, however, a causal relationship has not been prospectively investigated. This experimental infection study tested whether the oral inoculation of neonatal calves with a toxigenic strain of C. difficile (PCR-ribotype 077) could produce enteric disease. Fourteen 6-to-24 hour-old male colostrum-fed Holstein calves, received either three doses of C. difficile (combined vegetative cells and spores, 1.4x10⁸±3.5x10⁸ cfu) (n=8) or sterile culture broth (n=6). Calves were euthanized on day 6 or after the onset of diarrhea, whichever came first. Fecal and intestinal samples were blindly cultured for C. difficile, and tested for toxins A and/or B by ELISA. PCR-ribotyping was used to compare inoculated and recovered isolates. Diarrhea was observed in all control calves and 3/8 of inoculated calves (p=0.03). Fecal toxins were identified only from 2 controls. C. difficile was isolated from the feces of all inoculated calves, and PCR-ribotyping confirmed that it was the inoculated strain. 5 other PCR-ribotypes were isolated from 3/8 (37.5%) and 2/6 (33.3%) of inoculated and control calves, respectively, indicating early natural infection (≤24h of age). C. difficile was also cultured from 6/42 (14.3%) intestinal samples representing 6 calves; 2/6 ceca of controls, and 3/8 ceca and 1/8 duodenum of inoculated calves. Isolation of C. difficile was significantly more frequent from cecum (5/14) than collectively from duodenum (1/14) and ileum (0/14) (p=0.01)

In conclusion, the oral administration of C. difficile PCR-ribotype 077 to neonatal calves resulted in fecal/intestinal colonization but not in detection of toxins, or signs of enteric disease. This study was unable to experimentally reproduce disease, in contrast to a similar study in neonatal foals. At this point, there is inadequate information to conclude that C. difficile in a pathogen in calves, however further studies are indicated.
ISOLATION OF CLOSTRIDIUM DIFFICILE FROM RETAIL MEATS

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Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, Arizona USA (Songer, Trinh); Division of Health Quality Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Thompson, Killgore, McDonald, Limbago).

Clostridium difficile has emerged in recent years as a cause of enteric disease in food animals. It is likely the most important uncontrolled cause of enteritis in neonatal pigs, and is commonly found in stools of diarrheic calves. Many strains from food animals are ribotype 078, and there is increased detection or recognition of this type in human community-associated disease. Thus, it is possible that food animals are a source of infection for humans, and that meats may be the vehicle for transmission. We purchased meats at retail from three local grocery stores, sampling each store monthly over a three-month period. We selected ground or processed meats, representing beef (ground beef, summer sausage), ground turkey, and pork (ground pork, pork sausage, chorizo, braunschweiger). Duplicate samples (5 g each) were suspended in 45 ml brain heart infusion with 0.1% taurocholate, one of which was heat-shocked at 80°C for 10 min. After incubation at 37°C for 72 h, an aliquot from each tube was subjected to EtOH shock and then subcultured onto TCCFA. A total of 85 meat products were examined, 38 (44.7%) of which yielded C. difficile. Fourteen of 32 (43.8%) beef products, 21 of 45 (46.7%) pork products, and 3 of 8 (37.5%) turkey products were culture positive. One isolate was ribotype 012, but the majority (68.4%) were ribotype 078. Perhaps most striking was the isolation of the current human epidemic strain, NAP1/ ribotype 027/ toxinotype III, which represented 31.4% of isolates from beef and pork. These findings raise concerns about pre- and/or post-harvest food safety and lead to new lines of inquiry regarding the ultimate source of C. difficile for infection of humans in the community.
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ABSTRACTS
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P1

RISK FACTORS, INCLUDING ANTIBIOTIC USE, AT HOSPITAL LEVEL FOR OUTBREAKS WITH CLOSTRIDIUM DIFFICILE PCR RIBOTYPE 027

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1National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. 2Leiden University Medical Center, Leiden 3ECDC Stockholm 4Erasmus Medical Center, Rotterdam on behalf of the Dutch Foundation of the Working Party on Antibiotic Policy (SWAB).

Epidemics with C. difficile PCR ribotype 027, toxinotype III were first reported in The Netherlands in June 2003. National surveillance and typing of strains was started. Fluoroquinolone use has been recognized as an important risk factor at patient level for CDAD due to type 027. This study aimed at identifying risk factors at hospital level for outbreaks with type 027.

Data from 8 hospitals with outbreaks of type 027 (group A), 5 hospitals with sporadic cases (group B) and 10 hospitals from a random selection without known type 027 (group C) were included. Quarterly data for 2004-2005 were collected on CDAD-incidence, inpatient antibiotic use and hygienic and other preventive measures. The first 6 quarters were deemed the ‘pre-epidemic’ phase. The association of AB use and hygiene policy with the incidence was analysed with multilevel linear regression.

Mean pre-epidemic incidence in group A was 3.3 per 10,000 patient days, in group B 3.2 and in group C 2.4. During the epidemic phase this was 5.3, 3.3 and 2.8, respectively. The total use of the investigated AB pre-epidemically was 3384 DDD/10,000 patient days for the affected hospitals, 3 for group B and 346 for group C. In the epidemic phase, group A hospitals had reduced their use of fluoroquinolones (from 682 to 477) whereas this rose in group C from 757 to 937, significantly higher than in group A. In multivariate analysis, the use of 2nd generation cephalosporins (regression coefficient (r.c.) 0.0028, p = 0.08), wearing an apron when caring for diarrhoea patients (r.c. -2.97, p=0.017) and special instructions on disposal of bed linen in case of CDAD (r.c. -2.35, p=0.09) were (close to) significantly associated with the CDAD incidence.

Hospital wide use of most antibiotics known to be a risk factor for CDAD at patient level such as fluoroquinolones, are not significantly associated at institution level with higher incidences of type 027-associated CDAD in our study. Possibly, investigation at ward-level might correlate better. Wearing an apron when caring for diarrhoea patients appeared to be protective.
P2

EVALUATION OF PATIENTS WITH *CLOSTRIDIUM DIFFICILE* ASSOCIATED DIARRHEA

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*Clostridium difficile* associated diarrhea (CDAD) is the most important cause of nosocomial diarrhea. The aim of this study was to detect *C. difficile* toxin and bacteria by using ELISA test and anaerobic culture in stool samples obtained from the patients with diarrhea and to evaluate administrative data of patients, risk factors and clinical information.

Eighty stool samples were collected from the 72 patients (30 female and 42 male), aged 0-86 with diarrhea from January 2006 to February 2007. As 70 patients (97.2%) were hospitalised in six different hospitals, only two patients were outpatients. Out of 72 patients, 51 (70.8%) were on internal medicine wards, 5 (6.94%) were on surgery wards, 3 (4.2%) were on intensive care units, and 2 (2.8%) were on paediatrics clinics.

All stool samples were tested for *C. difficile* toxin A and B by using ELISA and cultured on CCFA. Six (7.5%) of 80 stool samples were toxin positive by ELISA and among *C. difficile* isolated samples (n=11) only nine isolates were toxigenic by cytotoxicity assay. Three of the isolates have been detected to be positive for *C. difficile* toxin A and B as well. As a result, 12 samples were detected as toxigenic either by ELISA or cytotoxicity assay followed by anaerobic culture. Only one patient was identified as community acquired CDAD. Five of 12 patients were hospitalised in stem cell transplant unit in different periods.
INFECTION CONTROL MEASURES TO LIMIT THE SPREAD OF CLOSTRIDIUM DIFFICILE – CONCLUSIONS FROM THE LITERATURE

R. P. Vonberg¹, E. J. Kuijper², M. H. Wilcox³, P. Tull⁴, P. Gastmeier¹, on behalf of the European C. difficile-Infection Control Group and the European Centre for Disease Prevention and Control (ECDC).

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Clostridium difficile-associated diarrhea (CDAD) is a nosocomial infection and usually presents after antimicrobial therapy. Many outbreaks have been associated with this pathogen. During CDAD large numbers of C. difficile (CD) spores may be excreted by affected patients. Spores then survive a long time in the environment; they cannot be destroyed by standard alcohol-based hand disinfection, and persist despite environmental cleaning. All these factors increase the rush of CD transmission. A new and hypervirulent strain of CD (REA type B1 / PFGE type NAP1 / ribotype 027 / toxinotype III) has recently been identified in many European and North American countries. This strain shows enhanced toxin production in vitro and may lead to a more severe course of disease and a poor patient outcome.

Once CDAD is diagnosed in a patient immediate implementation of appropriate infection control measures and review of antibiotic policy is mandatory in order to prevent further spread within the hospital. We collected data from the medical literature on evidence-based measures that are useful to limit the spread of CD. These include patient isolation, environmental cleaning, antibiotic control, hand hygiene, and personal protective equipment. Current local protocols and practices for the control of CD should carefully be reviewed and become adjusted if necessary.
CLOSTRIDIUM DIFFICILE-ASSOCIATED DIARRHEA IN PEDIATRIC CANCER PATIENTS

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Background. Pediatric cancer patients during the period of treatments with antineoplastic chemotherapy often experience diarrhea and abdominal pain. The role of \textit{Clostridium difficile} as an agent of diarrhea in children has not been well studied. The purpose of this study is to clarify intestinal colonization and nosocomial spread of \textit{C. difficile} in children undergoing antineoplastic chemotherapy and effective methods of excretory care for the prevention of \textit{C. difficile} infection in pediatric cancer patients.

Methods. Stool specimens were obtained from 9 children (5-15 years of age) who were admitted to a pediatric ward for antineoplastic chemotherapy. \textit{C. difficile} was cultured and toxin-producing type of recovered isolates was determined by PCR detecting the genes encoding toxin A, toxin B and binary toxin. The isolates were analyzed by PCR ribotyping. Also, we investigated by whom and how these children were cared after each bowel movement.

Results and discussion. \textit{C. difficile} was isolated from 8 of the children examined, and 2 were given the diagnosis of \textit{C. difficile}-associated colitis and treated by vancomycin. \textit{C. difficile} was not detected at the time of admission in 6 of the 9 children, and was later detected as antineoplastic chemotherapy. Typing analysis showed that 2 children had the identical type of \textit{C. difficile}, suggesting nosocomial transmission. The types of 7 isolates from 6 children were unique. Excretory care was done by the children themselves or their mothers, and by short time-hand washing under running water or wiping with wet tissue. This study suggests that the children hospitalized for their cancer are more likely colonized by \textit{C. difficile}, and careful contact precautions, especially hand hygiene, should be required for such the children.
P5

AN EPIDEMIC HOSPITAL STRAIN AS A CAUSE OF COMMUNITY-ASSOCIATED CLOSTRIDIUM DIFFICILE-ASSOCIATED DISEASE: FOODNET PILOT STUDY, 2006


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Background: Clostridium difficile (CD) is the leading infectious cause of antibiotic-associated diarrhea and colitis among inpatients of healthcare facilities but is a historically rare cause of illness in the community. The epidemic CD strain, NAP1, produces binary toxin, has a deletion in the toxin regulatory gene tcdC, is rapidly transmitted in hospitals, and has caused outbreaks across the United States. Sporadic CD-associated disease (CDAD) may be increasing among persons in the community; it is not known if the epidemic strain causes community associated CDAD.

Methods: Community-associated CDAD (CA-CDAD) was defined in patients who had a CD toxinpositive stool specimen and no hospitalization in the preceding three months based on a medical record review. Toxin-positive stools were cultured anaerobically and available CD isolates were characterized by pulsed-field gel electrophoresis (PFGE), and PCR detection of binary toxin and tcdC deletions.

Results: Of 1573 cases of CDAD screened, 174 (11%) fulfilled criteria for CA-CDAD. Of 60 available isolates characterized, 13 (22%) were indistinguishable from the epidemic CD strain, NAP1, by PFGE and contained binary toxin and a tcdC deletion. NAP1 was identified in five states (range: 10–43% of isolates per state). 29% of CA-CDAD case-patients with NAP1 did not received antibiotics within three months preceding infection.

Conclusion: The epidemic CD strain, NAP1, caused community-associated CDAD in at least five states. Such community-associated infections suggest a shift in epidemiology of CD infections, and imply a mechanism for dissemination of NAP1 in the community. Preventing further transmission of NAP1 may rely, in part, on explaining the rapid geographic dissemination of this strain and conducting a prospective study to identify modifiable factors associated with transmission.
P6

EPIDEMIOLOGY OF Clostridium difficile STRAINS WITH BINARY-TOXIN GENES AMONG CLINICAL ISOLATES IN AN ITALIAN HOSPITAL

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Clostridium difficile variant strains have been increasingly involved in severe C. difficile-associated disease (CDAD) and outbreaks in hospital settings. The presence of binary-toxin genes was described as a good marker to detect the C. difficile strains with variant PaLoc. In this study, we investigated 435 clinical strains, subsequently collected in the Section of Microbiology-University of Parma and isolated from 310 patients with CDAD over a seven year period (2000-2006) to detect the presence of the binary-toxin genes. In 99 out of 435 isolates (22.8%) we identified both cdtA and cdtB genes by PCR, mainly in the period 2001-2003. All strains harboring these genes were PCR positive for tcdA and tcdB, encoding for the two large toxins A and B. The in vitro production of toxin A by these strains was confirmed using the commercial immunoenzymatic assay (Triage, Biosite, U.S.A.). The 99 variant strains were analysed by the PCR-ribotyping method described by Bidet et al. (1999) and 80% were found to have the same PCR-ribotype, arbitrarily named I. Forty-two percent of the 99 binary-toxin positive isolates were also examined by the toxinotyping method, consisting in the PCR amplification of two PaLoc fragments (B1 and A3) and subsequent digestion with specific restriction enzymes. Thirty-three isolates were typed as toxinotype V, 4 as XXIV and two as IV and VI, respectively. Interestingly, 18 variant strains ribotype I and toxinotype V were isolated from 15 patients hospitalized in three geriatric wards, sharing the Intensive Rehabilitation Service and all located in the same building over a ten-months period (December 2002 – September 2003). Five of the 15 patients suffered recurrent episodes of CDAD within 1 to 3 months from the first one, likely representing relapses of the original strain. The data indicate that a variant clone of C. difficile, appeared at the end of 2000, is still circulating in the hospital even if its detection, after a peak from 2001 to 2003, is currently decreasing and highlight the need of a molecular monitoring to detect future changes in C. difficile clinical isolates.
**P7**

**EVALUATION OF CLOSTRIDIUM DIFFICILE TESTING AND GENETIC DETERMINATION OF TOXIN POSITIVE STRAINS FROM ST. JAMES’S UNIVERSITY HOSPITAL, DUBLIN, IRELAND.**

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*Clostridium difficile* is the most common cause of bacterial nosocomial diarrhoea. Laboratory diagnosis is frequently made using ELISA’s which detect the presence of *C. difficile* toxin directly in faecal specimens. 1500 faecal specimens were investigated over a 4 month period in 2005. The objectives of this study were to compare the results obtained with the Premier™ ELISA AB kit with faecal culture followed by two separate methods of toxin detection, using the Premier™ ELISA AB kit and toxin detection by PCR. The toxin positive isolates were further investigated using 16-23S ribotying to identify clonal diversity amongst toxigenic strains within the hospital. The positivity rate, sensitivity and specificity of the direct method were 8.5%, 63.7% and 98.7% respectively. The culture method followed by toxin detection using the Premier™ ELISA AB kit produced 11.7% 94.9% and 100% positivity, sensitivity and specificity respectively, whereas the molecular method produced respective values of 12.2%, 97% and 100%. The direct method failed to detect the presence of toxin from 34 different patients that tested positive by the 2 other methods. Ribotyping was performed on 134 toxin positive isolates, producing 85 genetically unrelated strain types. Sixty seven (50%) of the strains could be categorized into 18 clusters based on 80% similarity of DNA banding patterns. The number of isolates found in these clusters ranged from 2 to 12. The isolates that had presented as false negatives on direct testing were spread throughout the clusters, and no particular cluster was attributing significantly to these results. The method currently in operation in this laboratory performed poorly compared to the other two methods. This method produced a very significant proportion of false negative results. This study would recommend the introduction of faecal culture in addition to direct toxin detection by the Premier™ ELISA AB kit. All isolates of *C. difficile* that tested negative using the direct method should be further tested for toxin production using Premier™ ELISA AB kit.
P8

MOLECULAR TYPE, TOXIGENIC STATUS AND ANTIMICROBIAL SUSCEPTIBILITY OF AUSTRALIAN ISOLATES OF CLOSTRIDIUM DIFFICILE

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Clostridium difficile is an important nosocomial pathogen usually producing toxins A and B, which are the primary virulence factors. Some strains produce a binary toxin CDT, the role of which in pathogenicity is unknown. There has been concern about the recent emergence of a hypervirulent fluoroquinolone-resistant strain of C. difficile (PCR ribotype 02) in North America and Europe. Our aims were to determine the circulating types of C. difficile in Australia and the extent of antimicrobial resistance. A total of 205 recent WA clinical isolates and 155 clinical isolates from Eastern Australia (EA) was examined. PCR was used to detect C. difficile toxin genes and PCR ribotyping to type isolates. Of the isolates, 284 (79%) were A-positive, B-positive and 21 of these (7%) were CDT-positive, while 64 (18%) were non-toxigenic, including 3 that were CDT-positive (5%). There were 12 isolates that were A-negative, B-positive and 9 of these (75%) were CDT-positive. There were no statistically significant differences between east and west Australia. Computer analysis of ribotyping patterns divided 95 Australian isolates into 51 PCR ribotypes. No isolate with a ribotyping pattern matching PCR ribotype 027 was found. Little antimicrobial resistance was found in WA C. difficile isolates. Fluoroquinolone resistance was detected in 3 WA and 1 EA isolates. High-level clindamycin resistance was detected in 8% of isolates. No metronidazole or vancomycin resistance was detected. Clinical isolates of C. difficile in Australia are diverse with respect to both their toxin genotype and PCR ribotype. Antimicrobial resistance, including fluoroquinolone resistance, is low.
P9

EVALUATION OF ELISA, CULTURE AND REAL TIME PCR FOR THE DETECTION OF CLOSTRIDIUM DIFFICILE IN AN IRISH UNIVERSITY HOSPITAL.

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*Clostridium difficile* is the leading cause of infectious diarrhoea in hospitalised patients. Specific strains of *C. difficile* are responsible for nosocomial outbreaks. Laboratory diagnosis of infection is usually based on direct detection of toxins A and/or B in faecal samples. The objectives of this study were to compare three assays for the detection of *C. difficile* toxin, develop a real time PCR assay and investigate all isolates by PCR.

278 faecal samples were collected for this study. All samples were examined using the Premierª AB ELISA, cell cytotoxicity assay and cultured on CCFA. 138 faecal samples were investigated using real time PCR which targeted tcdB. All isolates were investigated by PCR for the following genes (tpi, tcdA, tcdB and cdtB). 48, 42 and 61 positive samples were detected by the AB ELISA, cell cytotoxicity assay and culture and the sensitivities of the three methods were 54, 86 and 86% respectively. Real time PCR was positive for ten of the 138 samples tested. 61 isolates were recovered on culture (17 A–B, 20 A+B and 24 A–B+). All isolates were positive for tpi and negative for cdtB. The real time PCR assay was positive for all 44 toxigenic isolates using genomic DNA as template. This study highlights the poor sensitivity of the Premierª AB ELISA. The real time PCR assay requires further evaluation due to the small number of positive samples tested. The high percentage of A B+ isolates in this study highlights the importance of using a diagnostic approach which detects both toxins A and B.
P10

SPREAD AND EPIDEMIOLOGY OF CLOSTRIDIUM DIFFICILE PCR-RIBOTYPE 027/TOXINOTYPE III IN THE NETHERLANDS.

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Background. Following reports on emerging outbreaks in Canada and USA, Clostridium difficile-associated disease (CDAD) due to PCR ribotype 027 (type 027) was detected in two medium large hospitals in The Netherlands in 2005. A national surveillance was initiated to investigate the spread and the epidemiology of CDAD. Methods. Microbiologists were requested to send strains from patients with a severe course of CDAD or when an increased incidence was noticed. A standardized questionnaire was used to collect demographic, clinical and epidemiological patient data. Strains were characterized by PCR ribotyping, toxinotyping, presence of toxin genes and antimicrobial susceptibility. Results. Between February 2005 and November 2006, 1175 faeces samples from 863 patients were received from 50 healthcare facilities. Of these patients, 218 (25.3%) had CDAD due to type 027, and 645 (74.7%) had CDAD due to other types, mainly 001 (17.8%) and 014 (7.2%). Type 027 was more frequently present in general hospitals than in academic hospitals (OR 4.38, 1.60-12.0). Outbreaks were observed in 10 hospitals and in 1 nursing home. Patients with type 027 were significantly older (OR 2.18, 95% CI 1.43-3.33) and used significantly more fluoroquinolones (OR 2.88, 1.01-8.20). Clear trends were observed for more severe diarrhoea (OR 1.99, 0.83-4.73), higher attributable mortality (6.3% vs. 1.2%, OR 3.30, 0.41-26.4) and more recurrences (OR 1.44, 0.94-2.20). Conclusions. Type 027 was found in 20 (18.3%) of 109 hospitals in The Netherlands with a geographic concentration in the Western and central part. The clinical syndrome and affected patients differ with CDAD due other types and can be of benefit for an early recognition.
P11

AN EMERGING CLOSTRIDIUM DIFFICILE TOXINOTYPE IN HUMANS AND FOOD ANIMALS

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Background: Multiple recent hospital-associated Clostridium difficile-associated disease (CDAD) outbreaks in North America and Europe have been associated with a hyper-virulent toxinotype (Tox) III strain, possessing binary toxin genes and a deletion in a toxin-regulatory gene, \textit{tcdC}. Tox V strains have been identified as pathogens in food-producing calves and neonatal pigs. We examined Tox V as a cause of human disease and its potential for increased virulence.

Methods: Case finding was conducted by examining >650 recent (2001-2006), and >6,000 historic (before 2001) isolates from two \textit{C. difficile} clinical isolate collections in the U.S. Case-patients had CDAD caused by Tox V. Fourteen Tox V isolates from symptomatic animals were obtained from diagnostic laboratories. Isolates were characterized by pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA) and PCR detection of binary toxin and \textit{tcdC} deletions. \textit{In vitro} toxin A & B production was compared for Tox V, Tox III, and historic human strains (Tox 0).

Results: Seven human cases were identified in eleven years prior to 2001. Seven additional cases were identified from 2001-2006; 6 of 12 (50%) cases with epidemiologic information available had community-associated CDAD. All human and food animal Tox V isolates contained both binary toxin and a 39 base-pair \textit{tcdC} deletion and all isolates belonged to REA Group BK. One human-animal isolate pair was indistinguishable by PFGE and by REA. Tox V strains produced more toxins A and B than Tox 0 (p<0.001), but less than Tox III (p<0.001) strains.

Conclusions: Tox V \textit{C. difficile}, a pathogen in food animals, is a rare cause of disease in humans, but when found are often community-associated. Tox V strains produce more toxins than historic Tox 0 strains and share genetic characteristics with hyper-virulent Tox III strains. Further study is needed to understand the epidemiology of Tox V CDAD, including the potential of food borne transmission to humans.
P12

INVESTIGATION OF CLOSTRIDIUM DIFFICILE ISOLATES FROM PRINCE EDWARD ISLAND

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Clostridium difficile is an important cause of human disease in Canada however little information is available about the disease in the Maritime Provinces, including Prince Edward Island (PEI). Unpublished data have suggested that the Maritime Provinces have a lower prevalence than other provinces of the internationally recognized outbreak strain ribotype 027, also known as NAP1. The objective of this study was to characterize C. difficile isolates obtained from people hospitalized with CDAD in PEI. One hundred twenty-six C. difficile ELISA toxin positive isolates were obtained and cultured using an enrichment protocol. Isolates were typed by PCR ribotyping, and PCR was used to detect genes for toxins A, B, and CDT (binary toxin). C. difficile was isolated from 102/126 (81%) samples. Thirteen different ribotypes were identified. The most common ribotype represented 22% of isolates. The next common ribotype accounted for 17% of isolates and was also the most common isolate in Ontario (unpublished data). Ribotype 027 was present in 4 (3.9% of isolates). Thirty-three percent of isolates possessed binary toxin, which was higher than expected especially since the presence of ribotype 027 is low. The low prevalence of ribotype 027 compared to other provinces needs to be evaluated further in conjunction with clinical data. The possibility of the island providing a geographical barrier from more virulent outbreaks needs to be investigated. The high prevalence of CDT was surprising and may represent a diverse group of variant strains or evolution of the ribotype 027/NAP1 group.
P13

CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE FROM PATIENTS IN ONTARIO HOSPITALS

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Clostridium difficile is an important cause of human disease in Ontario yet there is little information regarding characteristics of such isolates. The objective of this study was to characterize C. difficile isolated from people from Ontario healthcare facilities. 21 diagnostic laboratories were recruited to submit consecutive stool swabs from specimens with positive C. difficile toxin ELISA results. Enrichment culture for C. difficile was performed. Isolates were typed by PCR ribotyping, and PCR was used to detect genes for toxins A, B, and CDT (binary toxin). Representatives from each ribotype were tested for deletions in the tcdC gene. Susceptibility to metronidazole (MZ), vancomycin (VA), levofloxacin (Levo) and clindamycin (Clinda) was evaluated by Etest. C. difficile was isolated from 1080/112 (94%) samples; 89% from inpatients. 6 different ribotypes were identified. Three ribotypes accounted for 56% of isolates. Ribotype 02, an international outbreak strain, was the second most common ribotype (14% of isolates), found widely distributed across the province and was more commonly associated with inpatients and older individuals (P<0.05 for each). Each healthcare facility had two or three predominant ribotypes, as well as a variety of less common types. Isolates with toxin profile A+B+CDT-, A+B+CDT+, A-B+CDT-, and A-B+CDT+ accounted for 62%, 34%, 3%, and 0.4% of isolates respectively. A-B+ isolates appeared to occur in temporal clusters in individual hospitals. Deletions in tcdC were detected in 5 different ribotypes, including ribotype 027. 1079/1080 (99.9%) and 1077/1080 (99.7%) of isolates were susceptible to MZ and VA respectively. MZ resistance in the one isolate has not been consistently reproducible. No isolates were susceptible to Levo or Clinda. The high prevalence and wide distribution of ribotype 027 needs to be evaluated further in conjunction with clinical data. The high prevalence of CDT was surprising as was the presence of multiple ribotypes with a tcdC gene deletion. Whether this represents a diverse group of variant strains or evolution of the ribotype 027/NAP1 group is unclear.
P14

MOLECULAR EPIDEMIOLOGY OF CLOSTRIDIUM DIFFICILE IN CANADIAN HOSPITALS, 2004-05

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Background: This study examined the molecular epidemiology and antimicrobial susceptibilities of \textit{Clostridium difficile} strains collected through the Canadian Nosocomial Infection Surveillance Program over a 6 month period spanning 2004-2005.

Methods: Toxin positive stools were submitted to the NML from 34 hospitals representing 8 provinces from Nov. 1, 2004 to April 30, 2005. \textit{C. difficile} was isolated from the stools and PCR was used to confirm species (\textit{tpi}), toxigenic status (\textit{tcdA}, \textit{tcdB}), detect \textit{tcdC} gene variations, and to detect for the binary toxin (\textit{cdtB}). PFGE was used to type the strains. Antimicrobial susceptibilities to 12 antimicrobials were determined using agar dilution following CLSI guidelines.

Results: Toxigenic \textit{C. difficile} was isolated from 1800 toxin positive stools (recovery rate 83%). Over 200 unique PFGE types were identified. The NAP1 strain was observed in every region under study and comprised 27% of all isolates. 60% of all NAP1 strains were identified in Quebec. The next most frequent cluster was NAP2 which comprised 21% of isolates and was also found in every region. 59% of all NAP2 strains were identified in Ontario. The third most prevalent strain was NAP4 comprising 5% of all isolates. No strains were resistant to metronidazole, vancomycin or teicoplanin. NAP1 and NAP2 strains were much more likely than other strains to be resistant to moxifloxacin and gatifloxacin.

Conclusions: The NAP1 strain is geographically wide spread in Canada but the majority of isolates are seen in Quebec. This is the first description of the molecular epidemiology of \textit{C. difficile} across Canada. Ongoing studies are planned to determine if the molecular epidemiology is changing in our hospitals across Canada over time.
P15

CLOSTRIDIUM DIFFICILE ISOLATED IN A TEACHING HOSPITAL IN MARIBOR, SLOVENIA (2006-2007)

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Institute of Public Health Maribor provides microbiological diagnostic for General Hospital Maribor as well as for two smaller hospitals, GPs and Health care facilities in Maribor region. C. difficile diagnostics was introduced in 1996. Since then the number of analysed samples per year ranges from 166 to 293 of which 14 to 45 samples per year are culture positive. At the time there is no increase in the number of requested laboratory diagnostic detected.

From June 2006 to March 2007 we collected strains and selected clinical data for all samples send to the microbiology lab from the General Hospital Maribor. From 274 fecal samples 29 strains were isolated. Most of them belong to the toxiotype 0 (A+B+CDT-). The number of variant strains is low and includes 1 strain of toxino- type XII (A+B+CDT-), 1 strain of toxino- type V (A+B+CDT+) and 2 strains of toxino- type IV (A+B+CDT+; isolated from the same patient within 2.5 months time interval). Most of the patients had clasical risk factors: age >65y, hospitalization, antibiotic and/or chemotherapy and gastric acid inhibitors.

Our results indicate that Maribor region at the time is not affected by the outbreaks and no strains of the type 027 or 017 (A-B+) were found.
P16

PRESENCE OF CLOSTRIDIUM DIFFICILE IN PATIENTS WITH NOSOCOMIAL DIARRHEA, IN A TERTIARY GREEK HOSPITAL

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Objective. This study investigates the frequency of toxinogenic C. difficile presence among patients hospitalized in a tertiary Greek hospital. Furthermore, it is conducted a research in some of Clostridium difficile-associated diarrhea (CDAD) epidemiological features. This is the first relevant study in Northern Greece.

Methods. The presence of C. difficile was investigated in 80 fresh diarrheic stool specimens, collected from 70 inpatients in AHEPA University Hospital, between 1/1/2006 and 30/6/2006. For the stool culture CCEY agar and Anaerobe Blood agar were used. Alcohol shock was performed prior the specimens’ inoculation on nutrient media. Preliminary identification of C. difficile colonies was made on the basis of their typical morphology, odor and Gram stain image, and final identification was performed by a biochemical identification system. C. difficile toxin A in stools and isolated colonies was investigated by a specific ELISA test. Demographic and clinical information were collected, for every examined patient.

Results. C. difficile strains producing toxin A were isolated in 8 specimens (10%). In one case a toxin A(-) strain was obtained. Toxinogenic strains were not place or time clustered. Statistical analysis showed significant differences (p<0.05) between patients suffering from CDAD and diarrhea of other cause, in duration of hospitalization (p:0.03) and treatment with more different classes of antibiotics (p:0.01). The CDAD group also presented higher mean age, longer duration of antibiotic therapy and later initiation of diarrhea, although non statistically significant.

Conclusions. C. difficile was found to be the causative agent of nosocomial diarrhea in 10% of tested samples. Patients that developed CDAD were hospitalized for longer periods of time and had received much more different classes of antibiotics. These findings agree with literature. The value of combination of different laboratory tests in diagnostic approach of CDAD was clearly demonstrated, regardless of time and financial burden. Toxinogenic culture proved to be particularly important.
P17

PREVALENCE OF PCR RIBOTYPES 017 AND 027 CLOSTRIDIUM DIFFICILE IN A CENTRAL PUBLIC HOSPITAL IN WARSAW, POLAND

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The recent emergence of the epidemics Clostridium difficile strains ribotype 017 and ribotype 027 in Europe could lead to important changes in the epidemiology of C. difficile-associated diseases (CDAD). A two years study (2005/2006) on C. difficile was conducted in an Central Public Hospital (CPH) of Medical University of Warsaw, Poland on the phenotypic and genotypic characterization of isolates. One hundred seventy five C. difficile strains isolated from patients with suspected CDAD were characterized by PCR ribotyping and toxin profile. Susceptibility to metronidazole (MZ), vancomycin (VA), erythromycin (EM), clindamycin (CM), ciprofloxacin (CI), moxifloxacin (MX), gatifloxacin (GA), tetracyclin (TC) and imipenem (IP) was investigated using E-test method. Of 175 strains, 166 (95%) were toxigenic. Prevalence of the 017 strain and 027 were 35% (n=61) and 0.6% (n=1), respectively. Resistance to erythromycin and clindamycin, ciprofloxacin, moxifloxacin, gatifloxacin, tetracyclin, imipenem was found in 45%, 42%, 97%, 33%, 33%, 23% and 79% respectively. All strains were fully susceptible to metronidazole and vancomycin. High level resistance to CM, EM, MX and GA was tightly associated. Strains belonging to PCR ribotype 017 were highly resistant to CM, EM, CI, MX and GA. One strain was classified as PCR ribotype 027/toxinotype III. This isolate was positive for binary toxin genes, has an 18-bp deletion in tedC gene and was resistant to erythromycin and fluoroquinolones.

Conclusion: between 2005-2006, multiresistant C. difficile strains belonging to PCR ribotype 017 were the most frequently found type among hospitalized patients with antibiotic-associated diarrhoea in CPH, Warsaw. Only one patient was found with CDAD due to C. difficile PCR ribotype 027.

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CDAD INCIDENCE AND SEVERITY IN SPAIN IN THE AGE OF 027 STRAIN OUTBREAKS

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Background: Data from the USA, Canada and some European countries suggests a widespread increase in incidence and severity of CDAD. Objective: To evaluate the evolution of incidence and recent severity of CDAD in Spain where the epidemic 027 strain is not yet present. Methods: To determine the incidence of CDAD in our hospital we used the data available in the Microbiology department. We also obtained information about the population and hospital admission during the study period. To determine the current spectrum of the disease, we prospectively studied clinical significance of CDAD over the course of 4 months, from April 2005 to July 2005. Results: The mean incidence of CDAD during the whole period (2000-2006) was 8.9 per 1,000 patient admissions. The highest incidence was in 2003, 10.8/1,000 admissions. From 2004 we observed a significant decreased in incidence until 2006 with 6.5/1,000 admissions. From April 2005 to July 2005 we visited 111 patients that had an episode of CDAD. Of these, 68 were male (61.3%), the median age was 70.1 years. The first department of admission was internal medicine with 34 cases. The medium McCabe index was 2. A total of 41.4% of the patients had a hospital admission during the previous six months. During the month before the onset of diarrhea, 101 patients (91.0%) had received antibiotics; the most frequent class was quinolones (49.5%). None treatment to cure the diarrhea was necessary in 28% of the cases, of the rest 72 patients received metronidazole and 8 vancomycin. Recurrence of CDAD was found in 14 cases (12.6%) and severe CDAD was described only in 4 patients (3.6%) and not colectomy was necessary. Global mortality was of 16% but related mortality was not observed. Conclusion: Although our incidence of CDAD is high, we do not observe any increase, in fact, in the last two years we describe an opposing tendency. Besides, the morbidity and severity of the disease in our series do not reveal any change comparable with those described in recent outbreaks related to 027 strain.
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SURVEILLANCE OF CLOSTRIDIUM DIFFICILE NAP1/O27 SINCE 2006 IN BELGIUM

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The European prospective study (Barbut et al. 2005) revealed the presence of Clostridium difficile NAP1/O27 strains in the Netherlands, Ireland and Belgium. Since January 2006 an official reference centre was created in Belgium. The aim of the center is to help laboratories in managing Clostridium difficile outbreaks, in identifying and typing their strains.

Objective: We organised two surveillance systems of Clostridium difficile associated disease in Belgium. The first surveillance, which started in January 2006, is to detect Clostridium difficile clusters in the different healthcare institutions in Belgium on a voluntary basis. The second surveillance study was a prospective study from July till December 2006, aiming at establishing the baseline incidence of Clostridium difficile – associated disease (CDAD) in acute care hospitals. Each participating laboratory was asked to collect clinical data and to send the first five Clostridium difficile isolates.

Methods: Strains were analyzed, after species confirmation, for TcdC deletion (RT-PCR), binary toxin (RT-PCR), toxinotyping (PCR B1-A3 + RFLP) or ribotyping (RT-PCR). On 92 strains typed as NAP1/O27 we determined minimal inhibitory concentrations of Ciprofloxacin and Moxifloxacin by use of E-test.

Results: In the first study from January 2006 until end of March 2007 we analysed 603 strains from 47 centers, 567 were confirmed as Clostridium difficile; 194 strains (34 %) from 22 centers were typed as belonging to ribotype NAP1/O27. For the prospective study we received 342 strains from 65 centers, 316 were confirmed as Clostridium difficile. Seventy one strains (22.4 %) from 25 centers belonged to ribotype NAP1/O27. In total, in 46 (50.5 %) centers on 91 at least one NAP1/O27 strain was found. Of the 165 NAP1/O27 strains, 92 were tested for Moxifloxacin and Ciprofloxacin susceptibility and all of them were resistant to both drugs.

Conclusions: Since the participation to the surveillance was not mandatory, we have no data from all centers, but geographical analysis showed that ribotype NAP1/O27 was spread all over the country. In some centers, outbreaks were obvious according to the large number of NAP1/O27 strains, however a lot of sporadic cases caused by NAP1/O27 strain were also observed.
P20
PREVALENCE OF VARIANT STRAINS IN HOSPITAL-ASSOCIATED C. DIFFICILE FROM GERMANY

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The prevalence of variant C. difficile strains is increasing, mainly due to the spread of toxinotypes VIII (ribotype 017) and III (ribotype 027) in EU countries and elsewhere. Both types as well as other binary toxin positive variants are often associated with increased disease severity. It is therefore important to monitor the local prevalence of C. difficile infections. Equally important is to isolate and characterize the strains to obtain the data on strain types distributed in the local environment.

In routine diagnosis of the Institute for Medical Microbiology, University Hospital of Goettingen, stool samples are usually only tested for the presence of toxin A (VIDAS CDA). In two time intervals, cultivation of C. difficile was additionally performed from stool samples to obtain information about the presence of different toxinotypes. The first time interval was from July 2003 to April 2004 and the second from June to December 2006. Because the Institute provides microbiological service also to several external hospitals from the suburban areas of Goettingen, the obtained C. difficile strains originated from 8 different wards of 11 different hospital settings.

The total number of strains characterized was 164 from the period 2003/2004 and 124 from 2006. Of 164 strains isolated in 2003/2004, 23 (14.0 %) were variants. Binary toxin genes were present in 19 strains (11.5 % of all tested strains). The proportion of variant strains was similar also in 2006 as 18/124 belonged to variant toxinotypes (14.5%). However, in 2006, only 10 of 18 variants had binary toxin genes (8.0 % of all tested strains). A large proportion of C. difficile strains in both time periods were toxin negative (A-B-CDT-); 8.5 in 2003/04 and 15.3% in 2006.

The toxinotypes found were I, III, IV, V, VIII, IX, XI, XII. Toxinotypes III, V, VIII and XII were present in both time intervals. The same types were also the most prevalent ones in a recent study performed in 12 EU countries. Ribotyping results suggest that already in the year 2003, a patient in Goettingen was infected with a 027-like strain.
P21

CLOSTRIDIUM DIFFICILE FLUOROQUINOLONE RESISTANT MUTANTS SELECTED “IN VITRO” WITH MOXIFLOXACIN

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Objectives: Decreased susceptibility to fluoroquinolones (FQ) in Clostridium difficile has been recently described. Since no data are available on the development of FQ resistance in C. difficile, we performed “in vitro” experiments to evaluate the mutagenic potential of moxifloxacin on this bacterium. Methods: Three FQ susceptible C. difficile clinical isolates, were selected: C253 and CD, isolated in two different Italian hospitals, and the reference strain 630. MIC values were determined using the E-test method. The used breakpoints were: >=16 mg/L for ciprofloxacin (CP), >=4 mg/L for moxifloxacin (MX), and >=8 mg/L for both gatifloxacin (GA) and levofloxacin (LE). The quinolone resistance-determining regions (QRDRs) of gyrA and gyrB were amplified and sequenced. Selection of MX-resistant mutants were performed by plating bacteria on Blood Agar plates with a twofold increases in concentrations of MX (from 1 to 16 mg/L) for 24-48 h. For each concentration, mutants grown for three repeated passages were further analysed. Results: Before induction, all C. difficile strains had MICs for MX, LE and GA between 0.25 - 1, 0.5 - 3 and 0.5 - 0.75 mg/L, respectively, and MIC = 6 mg/L for CP. C253 and 630 showed the same QRDR sequences, whereas CD had three silent mutations in gyrA, 11 silent mutations and one amino acid substitution (Ser416 - Ala) in gyrB. Resistant mutants isolated from plates containing 4 and 16 mg/L of MX were selected and examined for susceptibility to the 4 antibiotics and for the QRDR status. Their MICs for MX and LE increased and were comprised between 8 and 32 mg/L, whereas MICs for CP and GA were >= 32 mg/L. C253 and CD5 mutants selected at the first antibiotic concentration showed the same amino acid substitution in gyrA (Ala 118 – Ser), whereas 630 showed an amino acid substitution in gyrB (Asp426 – Val). At the second concentration, only CD5 showed a further substitution in the QRDR of gyrA (Thr82-Ala). Conclusions: This results demonstrate that MX is able to generate C. difficile FQ resistant mutants “in vitro”. The apparent easiness in developing resistant mutants may explain the circulation of highly resistant strains, such as C. difficile 027, in hospital environment.
P22

INTEREST OF THE DISK DIFFUSION METHOD FOR SCREENING ANTIBACTERIAL RESISTANCE IN CLOSTRIDIUM DIFFICILE

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Antibacterial susceptibility testing of Clostridium difficile is not routinely performed. The aim of this study was to evaluate the performance of the disk diffusion method for screening Clostridium difficile isolates with decreased susceptibility to antibiotics. Forty two C. difficile isolates were studied by disk diffusion method on Mueller-Hinton agar supplemented with sheep blood (Biorad) with 16 antibiotics. Clostridium perfringens ATCC 13124 reference strain was used as control. The plates were incubated in anaerobiosis during 48h and were read with Osiris (Biorad) according to CA-SFM and EUCAST recommendations. To check the results obtained with the disk diffusion method, MIC were performed respectively with the E-test for glycopeptides and metronidazole and with the agar dilution reference method and E-test for new molecules with a potential activity against anaerobes : ertapenem, linezolid and moxifloxacin. The decreased susceptibility observed was 40% for amoxicillin/clavulanate, 60% for piperacillin/tazobactam, 100% for ceftriaxone, 81% for imipenem, 61% for ertapenem, 2% for chloramphenicol, 5% for tetracycline, 34% for erythromycin, 90% for lincomycin, 26% for telithromycin, 2% for linezolid, 98% for levofloxacin, 17% for moxifloxacin and 0% for vancomycin, teicoplanin and metronidazole. No major discrepancy was detected between the disk diffusion method and MICs for glycopeptides, metronidazole, linezolid and moxifloxacin. The disk diffusion method appears to be a low cost and easy to perform method to detect isolates suspected to have a decreased susceptibility such as the epidemic PCR ribotype 027 strain. However, when a decrease susceptibility is suspected MIC must be determined.
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FLUOROQUINOLONE RESISTANT CLOSTRIDIUM DIFFICILE STRAINS ISOLATED DURING THE “EUROPEAN PROSPECTIVE STUDY OF CLOSTRIDIUM DIFFICILE STRAINS”.

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Objectives: Recent outbreaks due to the epidemic C. difficile 027 strain have been associated with fluoroquinolone (FQ) exposure. A large number of strains from 14 EU countries was collected during 2005 within the European prospective study of Clostridium difficile strains. We performed a phenotypic and molecular analysis on a selection of these strains resistant to FQ. Methods: 149 C. difficile isolates were found resistant to moxifloxacin (MX) (MICs from 4 to 32 mg/L) during the European study. Sixty six of these strains (44%) were examined in this study for gatifloxacin (GA) and levofloxacin (LE) susceptibility. Twenty of these 46 strains were recognized as 027 during the European study. MICs were determined by E-test and the breakpoint used was >=8 mg/L for both GA and LE. Seventy percent of the 66 selected strains were also analysed for the amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of both gyrA and gyrB by PCR and sequencing. Results: The 66 strains selected had MICs range for GA ranging from 2 to >=32 mg/L and were high resistant to LE (MICs >=32 mg/L). Forty strains, including the 20 C. difficile 027 strains, showed an amino acid substitution in gyrA (Thr82-Ile), with MICs between 6 and >=32 mg/L for MX and highly resistance to GA (MIC >=32 mg/L). Three strains showed an amino acid substitution in gyrA (Thr82-Ile) and one of the following in gyrB: Asp 426-Val, Ser416-Ala, Asp481-Asn. These strains had lower MICs for MX (4-12 mg/L) and MICs for GA between 6 and >=32 mg/L. One C. difficile isolate showed two amino acid substitutions in gyrA (Thr82-Ile and Gly113-Glu) and one in gyrB (Ser416-Ala), with MIC=12 mg/L for MX and >=32 mg/L for GA. Finally, two strains showed only one amino acid substitution in gyrB (Arg447-Lys) and were resistant to MX and LE, but susceptible to GA. Conclusions: This study indicates that not only the epidemic strain 027, but the majority of the C. difficile clinical isolates currently circulating in Europe and resistant to FQ are characterized by the amino acid substitution Thr82-Ile in gyrA, that is often associated with high levels of resistance to these antibiotics.
SUBTYPING OF PCR-RIBOTYPING 027 CLOSTRIDIUM DIFFICILE ISOLATES BY MULTI-LOCUS VARIABLE NUMBER OF TANDEM REPEAT ANALYSIS (MLVA).

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Objectives. To study the epidemiology of Clostridium difficile, a typing method with a high discriminatory power and reproducibility has been established: Multi-Locus Variable Number of Tandem Repeat Analysis (MLVA). The subtyping ability of MLVA within different PCR-ribotypes has been shown previously. Therefore, the ability of MLVA to subtype isolates belonging to the new emerging hypervirulent PCR-ribotype 027 strain, from different regions and countries, was further investigated.

Methods. Short tandem repeat loci (3-9bp) were identified and amplification was performed using a single PCR-protocol. PCR-fragments were analysed using multi-coloured capillary electrophoresis on an ABI3100. The number of repeats per fragment was subsequently determined. The subtyping ability of PCR-ribotype 027 isolates was tested on 37 strains from 15 different regions from 7 different countries.

Results. 17 MLVA-clusters were recognized among 37 PCR-ribotype 027 strains at 71% similarity. Among the Dutch strains (n=25), 6 clusters could be identified. Pairs of strains from Japan and 5 Dutch regions were 100% identical. One strain from Canada clustered with an USA-strain. All type 027 strains were completely identical for markers CdF3 and CdH9. Marker CdE7 showed 10 repeats in all Dutch strains (9 regions) and 5 other strains. Markers CdC6 and CdG8 were identical for 20 Dutch strains and differed from all other (international) strains.

Conclusions. MLVA is a highly discriminatory genotyping method for C. difficile and is capable to identify country-specific and region-specific PCR-ribotype 027 strains. Therefore, MLVA is an important new tool to study the epidemiology of this strain.
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COMPARATIVE EVALUATION OF THE PERFORMANCE OF THE NEW VIDAS C. DIFFICILE TOXIN A&B ASSAY


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Objective: Establish the performance of the new assay VIDAS C. difficile Toxin A&B (bioMérieux) compared to Meridian Premier Toxins A&B and the Gold Standard cytotoxicity assay (CTA). Half of the samples found positive or equivocal with VIDAS were further cultured and ribotyped.

Methods: Performance of VIDAS C. difficile Toxin A&B and Meridian Premier Toxins A&B was assessed in comparison with the Gold Standard method (CTA). They were determined in the course of a clinical trial where over 382 prospective samples were collected from one site: Addenbrokes Hospital in Cambridge, UK. This was done in order to obtain a minimum collection of 40 positive samples.

Fresh stool specimens sent to the laboratory for suspicion of diarrhoea due to C. difficile were tested in parallel using VIDAS C. difficile Toxin A&B, Meridian Premier Toxins A&B and CTA according to the recommendations of the manufacturer.

Ribotyping was performed in Anaerobe Reference Laboratory in Cardiff according to Pr Brazier methodology.

Results: The sensitivity of VIDAS C. difficile Toxin A&B and Meridian Premier Toxins A&B compared to CTA was 88.4 % and 83.3 %, respectively. The specificity of VIDAS C. difficile Toxin A&B and Meridian Premier Toxins A&B compared to CTA was 100 % and 98.5 %, respectively.

Of 24 samples found either positive (18) or equivocal (6) with VIDAS C. difficile Toxin A&B and further culture for ribotyping, 18 were successfully ribotyped while 6 (equivocal ones) didn’t yield C. difficile in culture. Ribotyping of positive samples has shown a lot of type 106 which is the predominant strain in the UK.

Conclusion: The VIDAS C. difficile Toxin A&B test enables rapid and reliable detection of C. difficile toxins with good performance compared to the gold standard CTA. Being currently the only automated test on the market adapted to single testing per patient as well as series, it offers a “load and go”, easy-to-use alternative to existing techniques. In addition, the VIDAS system is convenient for the routine use as it enables traceability of the analysis and immediate access to validated results through LIS connectivity.
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MONOCLONAL ANTIBODIES SPECIFIC FOR TOXIN A + B OF CLOSTRIDIUM DIFFICILE

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Preparation and characterisation of monoclonal antibodies (mabs) recognizing Clostridium (C.) difficile A + B toxins have been performed by immunization of Balb/c mice with recombinant toxin A* and toxin B*. 25 µg of protein was given to the animals subcutaneously 3 times, using Freund adjuvants (FCA, ICA) and without adjuvant, respectively. One hybridoma was selected from TCDA and two from TCDB immunized mice. They belong to IgM subclass type. They bound to the recombinant and the native C. difficile A and B toxins in ELISA.

The HB-8712 hybridoma (ATCC) producing C. difficile A toxin specific mab and own developed hybridomas producing A+B specific mabs give a chance, using them in sandwich ELISA, to specifically quantify the A and B toxins produced by different C. difficile strains.

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IMMUNOREACTIVE CELL WALL PROTEINS OF C. DIFFICILE IDENTIFIED BY HUMAN SERA

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Clostridium difficile is a leading cause of nosocomial infection in the developed world, causing antibiotic-associated disease in susceptible populations. The identity of immunogenic proteins is important in designing future vaccines against this disease. In this study we have analysed the sera of 6 patients infected during a hospital outbreak of a C. difficile ribotype 17 strain. Using a proteomics based approach proteins, cell wall proteins were separated by 2 dimensional gel electrophoresis and immunoreactive proteins revealed by reaction with patient sera. The identity of immunoreactive proteins was established by mass spectrometry. The serum responses of patients to the cell wall proteins differed markedly as judged by western blot analysis. 42 different proteins were identified in total. Five patient sera reacted with the high - and low - molecular weight S-layer proteins, and with Slp paralog 2, suggesting these are immunodominant antigens. The role of these proteins as potential vaccine candidates deserves further study.
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CARBON CATABOLITE REGULATION OF CLOSTRIDIUM DIFFICILE TOXIN GENES EXPRESSION

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Toxin synthesis in \textit{C. difficile} is dependent on the growth phase, and is responsive to several kinds of environmental signals, including amino acids composition of the medium, the level of biotin, the growth temperature and the nature of the carbon source, such as glucose. However, little is still known about the underlying molecular mechanisms that mediate these influences on toxin production.

In Gram-positive bacteria, the Carbon Catabolite Repression (CCR) is one of the main system that regulate genes expression in the presence of sugars transported by the phosphotransferase system (PTS). The Catabolite Control Protein A (CcpA), a member of the LacI/GalR family of transcription regulators, plays a major role in this mechanism. In presence of a PTS-sugar, for instance glucose, CcpA binds to a catabolic-responsive element (cre), a specific DNA region in the gene target, thereby modulating the target gene transcription. CcpA binding to the cre site is enhanced by the interaction with its cofactor, HPr-Ser46-P, a key element of the PTS system that is phosphorylated in the Serine-46 residue by the HPr kinase/phosphorylase (HPr K/P) in presence of fructose-1,6-biphosphate (FBP). In \textit{C. difficile}, all major players of CCR can be found in the genome sequence.

Preliminary \textit{in vivo} studies suggest that CCR is implicated in the \textit{C. difficile} toxin genes expression. We have shown, that \textit{in vitro}, \textit{C. difficile} CcpA is capable to bind specifically to the \textit{tcdB} promoter where a putative cre site can be found ('AGA-CAACGTCTTTA\textsuperscript{3}). \textbf{Moreover, FBP seems to increase \textit{C. difficile} CcpA affinity to the \textit{tcdB} promoter, without the presence of HPr-Ser46-P.} This observation raises the question of FBP role and its ability to interact with CcpA, thus increasing the CcpA DNA-binding affinity. Furthermore, we are currently testing the contribution of the other CCR elements on the regulation of the toxin genes expression in presence of PTS-sugar in the medium.
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BEHAVIOUR OF THE CONJUGATIVE TRANSPOSON TN5397 IN CLOSTRIDIUM DIFFICILE 027

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Tn5397 is a tetracycline resistance encoding conjugative transposon that was originally isolated from Clostridium difficile 630. Work from our lab has shown that this element can be transferred between C. difficile strains and to and from Bacillus subtilis. Previous work has also shown that the element has a preferred insertion site into which it will always insert. However if the site is absent then it will insert into other sites in the genome that contain a GA dinucleotide at the target site with no other obvious sequence requirements. In the current work we wanted to determine if the preferred insertion site in the hyper virulent strain 027 were blocked by a copy of Tn5397 if a chloramphenicol resistant derivative of the element, Tn5397Δcm would then insert into the genome at random. The results showed that Tn5397Δcm inserted into a second preferred site. This work showed that vectors derived from Tn5397 will be useful for gene cloning at a specific site in C. difficile 027.
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COMPARATIVE GENOMICS OF THE EPIDEMIC CLOSTRIDIUM DIFFICILE STRAINS

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C. difficile strains belonging to PCR ribotype 027, PFGE type NAP1, REA type B1 and toxinotype III, termed NAP1/027, have been implicated in the increased frequency of outbreaks in North America and Europe. The NAP1/027 strains appears to be more virulent with an increased mortality and frequency of relapse. It has been suggested that the heightened virulence of this strain is due to an increased production of the toxin as a result of an internal deletion in the \textit{tcdC} gene that encodes a transcriptional repressor of the toxin. However, the molecular basis of the increased virulence of this strain is unknown.

The genome sequence of the Canadian strain (R20352) is available. The genome of a United Kingdom isolate (R20291), isolated from Stoke Mandeville Hospital, has been recently sequenced at the Sanger Institute using the 454 sequencing method. The genomes of these two epidemic strains were compared to that of the previously sequenced \textit{C. difficile} strain 630. Preliminary comparative analysis revealed a certain degree of variation in both gene content and sequence, and enabled us to identify genes that are shared between the three strains as well as genes that are unique to each strain. These strain-specific genes may provide clues on the increased virulence of the epidemic strains, and may be exploited as diagnostic markers.
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ANTIBIOTICS AND THE EXPRESSION OF THE CLOSTRIDIUM DIFFICILE PALOC GENES: IS THERE ANY CHANGE?

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The relationship between antibiotics and Clostridium difficile-associated disease is clear and well-established. Antibiotics, particularly broad spectrum ones, play a significant role in the disruption of the gut flora without which C. difficile cannot compete for the colonization of the gut. However, the effect that antibiotics have on the expression of C. difficile genes has not been thoroughly studied.

We’ve previously showed (Drummond et al. J Med Microbiol 2003; 52: 1033-1038) that sub-inhibitory concentrations of antibiotics trigger the over expression of toxins. In this study we aimed to correlate these changes to changes in the expression of the genes in the pathogenicity locus (PaLoc). The PaLoc is a 1.6 kb region of the C. difficile genome that encodes for toxins A and B (the main virulence factors) as well as three regulatory genes tcdC, tcdR and tcdE. TcdC has been established as the negative regulator, TcdR is an alternative sigma factor needed for the transcription of the toxins and TcdE is a holin-like protein most likely involved in release of toxins.

Despite the existence of other virulence factors, the total absence of the PaLoc leads to the non-virulence of the strain.

Reference strain 630 was grown in the absence and the presence of sub-inhibitory concentrations of amoxicillin and the total RNA extracted at different time points over a period of 24 h. cDNA was produced by reverse transcription and by means of real-time qPCR with SYBR green the levels of cDNA of six different genes: tcdA, tcdB, tcdC, tcdR, tcdE and tpi (triose phosphate isomerase: a housekeeping gene) were compared.

Some differences were observed, particularly involving the earlier transcription of the tcdE gene in the presence of amoxicillin. We believe that the differences observed in toxin levels in the presence of antibiotics is mainly due to a more effective release of toxin out of the cells which is very probably enhance by the damage to the cell wall caused by amoxicillin itself.

This study is currently being repeated with other antibiotics, particularly with ones that do not compromise the cell wall.
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COMPARATIVE ANALYSIS OF BI/NAP1/027 HYPERVIRULENT STRAINS REVEALS NOVEL TOXIN B GENE SEQUENCES

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In recent years the reported incidence and mortality due to Clostridium difficile associated disease has increased significantly which in part is likely to be due to the emergence of a new highly virulent strain in North America and Europe. This epidemic strain is known as toxinotype III, North American PFGE type 1, REA group BI, or PCR ribotype 027 (referred to as BI/NAP1/027). Increased virulence within these strains has been attributed to the presence of an 18 bp deletion in the negative regulator (tcdC) resulting in over expression of the two toxin genes tcdA and tcdB.

Whole genome comparisons using microarray analysis of 75 documented strains demonstrated that 21 out of 22 of the outbreak BI/NAP1/027 strains formed one of four distinct lineages, called the hypervirulent clade. A further observation was that the hypervirulent clade strains also appeared to either have a deletion or sequence divergence in the receptor binding 3’ end to toxin B gene (tcdB). Three regions at the 3’ of tcdB, responding to the microarray reporters, were sequenced from seven independent BI/NAP1/027 strains. PCR and sequenced data confirmed that the 3’ end of tcdB was present, but demonstrated sequence divergence from the sequenced strain C. difficile 630. BI/NAP1/027 tcdB consensus sequence has not been reported before. The divergent C-terminal binding domain may affect the binding capability of toxin B. Potentially the increased virulence observed in these strains may be due an altered binding avidity and/or alteration in host cell specificity of toxin B, in addition to increased toxin production through TcdC.
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ANALYSIS OF THE PALOC OF CLOSTRIDIUM DIFFICILE BY REAL TIME RT-PCR

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The main virulence factors of *C. difficile* are its exotoxins, A and B, which are responsible for the characteristic pathology of *C. difficile*-associated disease. The 19.6kb Pathogenicity Locus (PaLoc) in the *C. difficile* genome consists of five genes: *tcdA* and *tcdB* which code for these toxins together with three accessory genes *tcdC*, *tcdR* and *tcdE*. *tcdC* is a putative negative regulator of toxin production and *tcdR* is a putative positive regulator. *tcdE* codes for a holin-like protein that may be involved in release of the toxins. Previous experiments suggest that the expression of these genes varies over time; *tcdC* being expressed most highly in the exponential phase of growth, and the other genes being maximally expressed in the stationary phase. The transcription of the five PaLoc genes over the exponential and early stationary phases was studied using Real Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). *tpi* (the gene coding for triose phosphate isomerase) was selected as a control housekeeping gene. The growth curve over a 24 h period was followed by measuring optical density at 600nm. Every 4h during the exponential and early stationary phase of the growth curve - when toxins were being elaborated and sporulation was beginning - samples of culture were taken for RNA extraction. Real-time RT-PCR was performed with specific primers for each gene and SYBR Green. It was found that the levels of expression of all of the genes did not vary significantly over time, indicating their almost steady-state expression during the period of the growth curve investigated. This suggests that release of toxins, rather than their rate of production, might be associated with the high toxin levels observed in the stationary phase of growth. This indicates a possible role for the products of the accessory genes in toxin release rather than in the regulation of transcription of toxin genes. A slight decrease in the transcription of all the genes was seen between 12 and 20h. As this is the time sporulation begins, there might be a correlation between toxin and spore production.
GLIP/ DELETION ANALYSIS AND GENOMOTYPING OF C. DIFFICILE STRAINS ISOLATED IN CORK, IRELAND.
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Clostridium difficile is a well established hospital pathogen which has recently caused concern because of an apparent phenotypic change associated with increased virulence and increased antimicrobial resistance. Strains of this type have caused hospital outbreaks in North America and the UK. The increased virulence has been linked to a genetic change resulting in increased toxin production. Recognition of the presence of these strains requires culture of the organism and genetic testing - they cannot be detected by toxin assay alone. There is evidence of this strain's presence in Dublin but not elsewhere in Ireland. We have isolated infecting and environmental strains of C. difficile at Cork University Hospital (CUH), and carried out antimicrobial sensitivity testing. Strains isolated at CUH are being typed locally by PCR assays on pathogenicity locus. Genomotyping data will be available from DNA extracted and hybridised on the C. difficile array at the London School of Hygiene and Tropical Medicine in collaboration with Professor Brendan Wren. Information from genomotyping and the C. difficile genome 3 will be used to devise novel PCR assays (Genome level informed PCR) to type local strains.
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STUDIES ON THE EFFECT OF TOXIN A ON INTESTINAL EPITHELIAL CELLS AND ANIMAL ENTERITIS

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The incidence and severity of Clostridium difficile–associated disease (CDAD) are increasing and this increase may be associated with the emergence of a new strain of C. difficile with augmented virulence and/or resistance to antimicrobial therapy specially fluoroquinolone. This newly described C. difficile strains produce an additional toxin, a binary toxin designated C. difficile toxin (CDT) with a two-component ADP-ribosyltransferase toxin and bear a deletion in the pathogenicity locus gene, tcdC, that results in increased production of toxin A and B. As a consequence, the treatment has more progressively been associated with failure and recurrence and some cases of CDAC has become less responsive to the treatment with metronidazol, resulting in augmented use of oral vancomycin. All these facts have raised the need for additional clarification of the pathogenesis of CDAC and for search of new therapeutic strategies. In this direction, our group has been working on the mechanism of toxin A-induced experimental enteritis. We have demonstrated that TxA induces apoptosis by a mechanism dependent on inactivation of Rho, activation of caspases 3, 6, 8, 9 and Bid, and mitochondrial damage followed by cytochrome c release, inhibited epithelial cell migration, and decrease epithelial resistance. Some of these effects of toxin A are prevented by glutamine or alanyl-glutamine which showed to be effective to prevent caspase 8 activation, enhance epithelial repair, prevent epithelial the drop of epithelial resistance, and reduce the inflammatory response to toxin A. We have also demonstrated an increased adenosine deaminase activity following toxin A and that a new A²A adenosine receptor agonist, ATL 313, reduces tissue injury and inflammation in mice with toxin A-induced enteritis. Our data contribute to elucidation of the mechanisms involved on C. difficile toxin A-induced mucosal damage and shall lead to improved control of life-threatening colitis and diarrhea induce by this bacterium.
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TITLE: USE OF ATOMIC FORCE MICROSCOPY TO STUDY THE EFFECT OF CLOSTRIDIUM DIFFICILE TOXIN A ON RAT INTESTINAL EPITHELIAL CELL MORPHOLOGY

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Recent reports suggest increased incidence and severity of Clostridium difficile–associated disease (CDAC) linked with emergence of a new strain producing additional virulence factors toxin A and B and binary toxin. These facts have raised the need for additional clarification of the pathogenesis of CDAC. The atomic force microscope (AFM) is a high-resolution scanning probe microscope and one of the most powerful tools for determining the surface topography as well as the cells cytoskeleton. The purpose of this study was to investigate the morphological alterations induced by toxin A, the main responsible for the enterotoxic activity of C. difficile in animals, on intestinal epithelial cells by using AFM. A rat epithelial cell line named IEC-6 was plated in 6-well tissue culture plates at 37°C in 10% CO₂ atmosphere. When the cultures reached the confluence of 75%, the test group was incubated with toxin A (100 ng/mL) for 1 hour. After the incubation period, the cells were fixed with formaldehyde solution 10% for 14h and then observed at atomic force microscope. The images revealed that toxin A treated cells displayed a strong cell retraction with aggregation of the cytoplasm around the nucleus associated with multiple extensions resulting in a “multiarmed” shape. The control cells kept the original characteristics, where the limits of the nucleus and the cytoplasm are well defined. The cytoplasm of control cells presented the normal network corresponding to the cytoskeleton and the nucleolus was easily identified inside the nucleus. The network aspect of the cytoskeleton and the nucleolus disappeared after the incubation with TxA. In conclusion, AFM is a powerful tool to study the detailed effects of C. difficile virulence factors on cell morphology as well as to investigate the potential of protective substances on these morphological effects.
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QUICK AND EASY; THE USE OF BIOLUMINESCENCE TECHNOLOGY IN DESIGNING MORE EFFICIENT IN VIVO VIRULENCE EXPERIMENTS

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Animal models of infection continue to provide valuable data regarding the pathogenesis of human diseases. There is, however, increasing ethical and regulatory pressure to reduce the numbers of animals used in such experiments. The recent emergence of bioluminescence technology offers a significant means to achieve this aim. The translational fusion of constitutively expressed bacterial promoters to the luxABCDE operon (encoding LuxCDE, a fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction catalyzed by the luciferase LuxAB) allows us to estimate relative pathogen burden in animal organs without the need for obtaining direct bacterial counts. Altering this model to place luxABCDE under the control of specific virulence genes generates data on the locational and temporal expression of virulence genes. An exciting new field in the application of bioluminescence is the use of three dimensional imaging, yielding data on the internal localization of bacterial gene expression in the live animal. Combining these advances with anaesthesia technology allows us to use individual animals for repeated observations, leading to an overall reduction in numbers of animals used. We have developed these technologies for a number of bacterial pathogens and believe that this approach will also be of benefit in the study of Clostridium difficile pathogenesis.
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**CLOSTRIDIUM DIFFICILE TOXIN A C-TERMINUS PROMOTES DENDRITIC CELL MATURATION AND ACTIVITY**

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The lectin-like structure of the C-terminal region receptor binding domains of the of *Clostridium difficile* toxin A resembles the structure of the most potent mucosal adjuvants, such as cholera toxin, E. coli heat labile toxin and plant lectins. Indeed, a non-toxigenic peptide corresponding to amino acids 2394 to 2706 (TxAC314) showed potent mucosal adjuvant activities inducing mixed Th1/Th2 type responses toward co-administered mucosal antigens. Since dendritic cells (DCs) are the most potent antigen-presenting cells, to elucidate the mechanism(s) of TxAC314–mediated effects on immune response we analysed its activity on murine bone marrow-derived DCs. *In vitro* incubation of bone-marrow derived DCs with TxAC314, dose-and time-dependently, induced the up-regulation of MHC class II, of co-stimulatory (CD80 and CD86) and adhesion (CD40) molecules, as assessed by FACS analysis. Furthermore, TxAC314 also enhanced the secretion of cytokines (IL-1β, IL-6, IL-2, IL12) and chemokines (MCP-1) in the culture medium, quantified by capture ELISA. In addition, TxAC314 enhanced DCs antigen-presenting capacity that resulted in increased T-cell proliferation and cytokines (IFN-γ, IL-2, IL-12) release following exposure to the specific antigen. Furthermore, adoptive transfer of DCs pulsed with a specific antigen in presence of TxAC314 induced a robust antigen specific antibody production in vivo and cytokine release *in vitro* following antigen challenge. Thus, TxAC314 seems to exert its adjuvant activity at least in part by stimulation of DCs leading to an improved antigen presentation to T-cells.
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DIFFERENCE IN THE PROTEIN SUBSTRATE SPECIFICITY OF CLOSTRIDIUM DIFFICILE TOXIN B FROM SEROTYPE F STRAIN 1470 AND CLOSTRIDIUM SORDELLI LEthal TOxin

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The clostridial glucosylating toxins mono-glucosylate and thereby inactivate low molecular weight GTP-binding proteins of the Rho and the Ras families. Toxin B from the C. difficile serotype F strain 1470 (TcdB-F) has been characterised as a functional hybrid of the Clostridium sordellii lethal toxin (TcsL) and the C. difficile toxin B from strain VPI 10463 (TcdB). TcdB glucosylates Rho, Rac, and Cdc42, while TcdB-F and TcsL glucosylate Rac and Ras.

In this study, we re-investigated the substrate specificity of TcsL and TcdB-F using recombinant Rho and Ras proteins. TcdB-F and TcsL shared most substrate proteins, namely Rac, RhoG, TC10, Cdc42, Rap(1,2), and R-Ras. In contrast, H-/K-/N-Ras were only glucosylated by TcsL. Both toxins caused actin re-organization (“cytopathic effect”) of rat basophilic leukaemia (RBL) cells. The cytopathic effect correlated with the glucosylation of Rac1. Both toxins, however, differed in their ability to regulate the pro-apoptotic immediate-early gene product RhoB that was strongly up-regulated in TcsL- but not in TcdB-F-treated cells. Furthermore, TcsL caused apoptosis in rat basophilic leukaemia cells (“cytotoxic effect”), as determined by activation of caspase-3 and phosphatidyl serine exposure. In contrast, TcdB-F failed to do so. TcsL also reduced the viability of RBL cells, whereas the viability remained unchanged in TcdB-F-treated cells.

Our data show that TcdB-F fails to induce the cytotoxic effect. This finding is likely due to the fact that it does not glucosylate RhoA. The cytotoxic effect of TcsL is likely based on the glucosylation of H-/K-/N-Ras, as it fails to inactivate RhoA, as well.
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THE GLUCOSYLATION OF Rac1 IS CRITICAL FOR THE CYTOPATHIC EFFECT OF THE CLOSTRIDIAL GLUCOSYLATING TOXINS

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Glucosylation of Rho proteins by clostridial toxins results in re-modelling of the actin cytoskeleton (“cytopathic effect”). The Rho family proteins RhoG and TC10 exhibit a similarity to Rac1 of 70% at amino acid level. Rac1 is glucosylated by all members of the toxin family. In this study, the substrate specificity of the glucosylating toxins is re-analysed using a subset of recombinant Rho proteins including RhoG and TC10.

Clostridium difficile toxin B (TcdB) glucosylated Rho(A,B,C), Rac1, Cdc42, RhoG, and TC10, as determined by $[^{14}C]$glucosylation of recombinant Rho proteins. Toxin B from the variant C. difficile strain 1470 serotype F (TcdB-F) and lethal toxin from C. sordellii strain 6018 (TcsL) share these substrates except for Rho(A,B,C). The glucosylation of RhoG and TC10 was analysed in a toxin concentration-dependent manner. Rac1 and RhoG were modified by TcdB and TcdB-F with comparable kinetics, while TC10 turned out to be a minor substrate. TcsL glucosylated Rac1, RhoG, and TC10 with comparable kinetics. To show that RhoG and TC10 were involved in the cytopathic effect of the glucosylating toxins, a “protection assay” exploiting the inhibition of RhoGAP proteins by ectopic expression of constitutively active Rho proteins was applied. The inhibition of the GAP proteins results in elevated levels of the active, GTP-bound form of the respective endogenous Rho protein which are less efficiently glucosylated than the GDP-bound forms. Cells with an elevated level of active endogenous Rac1 were protected from the cytopathic effect of TcdB and TcdB-F, suggesting that glucosylation of Rac1 mainly regulated the cytopathic effect. Cells with an elevated level of active RhoG or TC10 were only partially protected whereas an elevated level of active RhoA did not protect cells from the cytopathic effect at all. In conclusion, glucosylation of Rac1 is the major impact on the cytopathic effect. The glucosylation of RhoG and TC10 further contributes to the cytopathic effect of clostridial glucosylating toxins.
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REFINEMENT OF THE SYRIAN GOLDEN HAMSTER MODEL OF Clostridium difficile.

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Clostridium difficile is a spore forming, anaerobic bacteria that causes gastrointestinal infections in humans with symptoms that range from simple colonisation with no adverse effects to severe diarrhoea, pseudomembranous colitis and death. Currently no vaccine exists and treatment is difficult, especially for susceptible groups, and the relapse rate is high (up to 55%) so improved treatments are desperately required.

Oral infection of the Syrian golden hamster is widely recognised as a reproducible model of CDAD (Clostridium difficile associated diarrhoea) in which many of the characteristics associated with the infection in humans can be observed. These include diarrhoea, histological damage and relapse of the condition following removal of antibiotic treatment.

In order to minimise the suffering of animals infected with this organism, which without intervention is fatal, we have used a number of measurements (both qualitative and quantitative) to establish the kinetics of infection. When assimilated with information on levels of colonisation and histological structure of different regions of the gut, this type of analysis can provide much information about the behaviour of individual strains. In particular, we have observed that the two strains B1 and 630 show significantly different kinetics of infection in this model this includes an extended time to established endpoints (drop in body temp of 2°C) for strain 630 and a significant change to the profile of gut colonisation. Characterisation of the model is important in this context as such differences will be key, not only to the analysis of genetically altered organisms that may only demonstrate subtle changes to their infection profile, but also in the identification of new gene targets. This could be achieved by the comparison of the genomes from strains that naturally display differences in their infection profiles.
ACTIVE RhoB IS REQUIRED FOR THE CYTOTOXIC EFFECT OF CLOSTRIDIUM DIFFICILE TOXIN B

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Clostridium difficile toxins A (TcdA) and B (TcdB) are a mono-glucosyltransferase that inactivate RhoA, Rac1, and Cdc42. By these means, TcdB causes actin reorganisation (“cytopathic effect”) and apoptosis (“cytotoxic effect”). The cytotoxic effect of TcdB has generally been attributed to the inactivation of RhoA. The RhoA homologue RhoB, whose expression is reportedly suppressed by active RhoA, is physiologically up-regulated during S-phase; RhoB is generally implicated as a regulator of apoptosis.

First, we analysed the intracellular level of RhoA, RhoB, Rac1, and Cdc42 in TcdB-treated cells. While glucosylated Rac1 was stably present in the TcdB-treated cell, RhoA was degraded in a toxin concentration dependent manner. The degradation was partially inhibited by the proteasome inhibitor MG132 but completely prevented by application of both MG132 and a pan caspase inhibitor (Z-VAD-FMK), indicating that treatment with TcdB caused activation of caspases. RhoB was up-regulated in a TcdB concentration dependent manner. This up-regulation was abrogated by actinomycin D, indicating that it was due to transcriptional activation. Accordingly, the rhoB promoter activity increased in a TcdB concentration dependent manner. Up-regulation of RhoB was due to inactivation of Rho, as it was also observed in cells treated with exoenzyme C3 from C. limosum that specifically ADP-ribosylates Rho(A/B/C). Rho(A/B/C) are described to be equal substrates of TcdB. We re-analysed the kinetics of Rho(A/B/C) glucosylation and found that RhoB was less efficiently glucosylated by TcdB compared to RhoA. Furthermore, up-regulated RhoB was susceptible to ADP-ribosylation by exoenzyme C3 and active as determined by Rhotekin pull-down assay in TcdB-treated cells. In contrast, RhoB from C3-treated cells was completely inactive, confirming the notion that C3 is an efficient inhibitor of Rho(A/B/C). Synchronized fibroblasts were susceptible to the cytotoxic effect of TcdB as analysed in terms of annexin V staining. The cytotoxic effect was responsive to inhibition by either Z-VAD-FMK or exoenzyme C3, suggesting that active RhoB is required for the cytotoxic effect of TcdB. Apoptosis induced by the C. difficile toxins has been generally attributed to “Impaired Rho signalling”. Our data suggest that the target cell responds to glucosylation of RhoA by degradation of RhoA and up-regulation of RhoB. In cells treated with TcdA or TcdB, RhoB escapes its inactivation and is required for the cytotoxic effect.
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RAC1 PHOSPHORYLATION PROTECTS AGAINST CLOSTRIDIUM DIFFICILE TOXIN A-INDUCED CYTOPHATHIC EFFECT

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Toxin A from Clostridium difficile is a single-chain protein, that monoglucosylates proteins of the Rho GTPase family. Glucosylation leads to functional inactivation of Rho GTPases and causes disruption of the actin cytoskeleton. In this study, we investigated the effect of Rac1 phosphorylation on the glucosylation by Clostridium difficile toxin A (TcdA).

Stimulation of CaCo-2 cells with epidermal growth factor (EGF) resulted in a maximal activation of Akt1 kinase as well as in a phosphorylation of Rac1 at Ser-71. Preincubation of CaCo-2 cells with EGF also delayed the TcdA-induced decrease in transepithelial electrical resistance. Accordingly, glucosylation of Rac1 by TcdA was reduced in EGF-treated cells compared to control cells. To check whether phosphorylated Rac1 was substrate for TcdA we generated the phosphomimetic mutant Rac1 S71E. [14C]-Glucosylation assay showed that Rac1 but not Rac1 S71E was substrate for TcdA. However, Rac1 S71E competed concentration-dependently with Rac1 and reduced glucosylation of Rac1.

To analyse the function of the phosphorylated Rac1 we performed pull down experiments. Rac1 as well as Rac1 S71E bound to their effector protein (Pak) and inhibitor (GDI) in a nucleotide-dependent manner. Interestingly, phosphorylated Rac1 from EGF-treated cells preferentially bound to Pak-Crib domain but not GDI in a pull down assay indicating that phosphorylation rather results in activation than in inactivation as reported for RhoA.

In sum, phosphorylation of Rac1 attenuates the cytopathic effect of TcdA. Phosphorylation at Ser-71 protects Rac1 from glucosylation but allows Rac downstream signalling.
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MICROARRAY ANALYSIS OF THE RESPONSES OF CLOSTRIDIUM DIFFICILE TO STRESS CONDITIONS

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In order to be able to adapt to the intestinal environment, C. difficile must react to the many stresses involved with colonisation. To investigate the response of C. difficile to various stresses, the C. difficile 630 microarray developed by the Bacterial Microarray Group at St. George’s, University of London (BµG@S) was utilised. C. difficile 630 was grown to logarithmic phase and then subjected to heat shock, oxidative shock, acid pH shock or alkaline pH shock; or was grown in the presence of amoxicillin, clindamycin or metronidazole at sub-inhibitory levels. RNA was extracted and gene expression was analysed using DNA/RNA competitive hybridisations. The data was normalised and then genes with statistically changing expression were identified.

As expected, the classical stress response is observed after heat shock and also after acid shock. Exposure to atmospheric oxygen induces a large number of electron transporters, including components of the alternative oxidative stress protection system. Metronidazole affected very few genes, probably due to the very low concentration necessary to allow C. difficile to grow. Heat shock and both clindamycin and amoxicillin exposure resulted in the regulation of many biochemical pathways. All three antibiotics resulted in an increase in the levels of ribosomal protein transcripts.

In this study, regulated genes and potential operons have been identified which are both unique to and common between different stresses. It is hoped that this information will allow us to further understand expression of C. difficile genes within the gut.
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CHARACTERIZATION OF SPORULATION IN CLOSTRIDIUM DIFFICILE

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Spores of Clostridium difficile are the infectious form of the organism and the form most likely to survive in hospital environments. Thus, identifying the conditions that induce sporulation and the factors that control its progression is critical to understanding and potentially intervening in the process. Sporulation has been extensively studied in the model organism, Bacillus subtilis. In B. subtilis, sporulation begins with polar septation that divides the cell into two unequal compartments. One of two copies of the chromosome is rapidly pumped into the smaller compartment. Through signaling between the mother cell and the forespore, the smaller compartment is engulfed by the mother cell, matured with the addition of coat proteins and released into the environment as a resistant spore. While many B. subtilis sporulation proteins are present in C. difficile, others are not conserved. We have taken a microarray approach to study the timing of gene expression during sporulation of C. difficile and to identify sporulation genes unique to C. difficile and. Initial results suggest that known sporulation genes are temporally transcribed in a cascade involving the four sporulation specific sigma factors (F, E, G and K). As an internal control for growth stage dependent transcription, the tcdA toxin transcript increases upon entry into stationary phase. Further analysis needs to be completed to identify hypothetical genes involved in sporulation. To confirm these initial results, we are utilizing the β-glucuronidase reporter system. Putative promoter elements of spoIIR, spa, spoIVA and cor/PC will be PCR amplified and fused to gusA. Constructs will be introduced into Tn916 within the B. subtilis chromosome and mobilized via conjugation into C. difficile strain 196.
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PREVENTION OF CLOSTRIDIUM DIFFICILE ADHERENCE TO HUMAN INTESTINAL EPITHELIAL CELLS WITH LACTOBACILLUS SPP

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Clostridium difficile is the most common cause of infectious diarrhoea in hospitalised patients. Antimicrobial therapy disrupts normal colonic flora allowing C. difficile to colonise and cause severe diarrhoea. Probiotic organisms may help prevent C. difficile colonisation. The aim of this study was to examine the ability of Lactobacillus spp to inhibit C. difficile binding to human intestinal epithelial cells. Differential fluorochromes were used to label Lactobacillus salivarius and Clostridium difficile. Using flow cytometry we assessed first whether L. salivarius bound to primary intestinal epithelial cells and intestinal epithelial cell lines. We then examined if pre-incubation of epithelial cells with Lactobacillus spp could reduce C. difficile adherence. Both C. difficile and L. salivarius could adhere to primary cultures of colonic and small intestinal epithelial cells and to epithelial cell lines. L. salivarius bound to all four intestinal cells used in this study in a dose dependent manner. Preincubation of intestinal cells with L. salivarius reduced subsequent C. difficile adherence in all cell types. This decrease in C. difficile binding was observed for toxin positive as well as toxin negative C. difficile with greater than 80 % reduction in adherence for both strains. Treatment with Lactobacillus spp may be useful for prevention of C. difficile diarrhoea in high-risk patients.
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PATHO-BIOTECHNOLOGY; USING BAD BUGS TO DO GOOD THINGS

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Given the increasing commercial and clinical relevance of probiotic cultures, improving their stress tolerance profile and ability to overcome the physiochemical defences of the host is an important biological goal. Pathogenic bacteria have evolved sophisticated strategies to overcome host defences, interact with the immune system and interfere with essential host systems. We coin the term ‘patho-biotechnology’ to describe the exploitation of these valuable traits in biotechnology and biomedicine. This approach shows promise for the design of more technologically robust and effective probiotic cultures with improved biotechnological and clinical applications as well as the development of novel vaccine and drug delivery platforms.

Our probiotic of choice, *L. salivarius* UCC118, exhibits significant therapeutic potential: stimulating the mucosal immune system, attenuating inflammation and reducing neoplastic lesions associated with auto immune disease states such as Crohn’s disease and ulcerative colitis, as well as providing an alternative for the treatment of chronic gut associated bacterial infections particularly *Clostridium difficile*.

Despite the potential health and nutritional benefits associated with *L. salivarius* UCC118, it is not a particularly robust culture. The aim of this project is to improve the stress tolerance profile of *L. salivarius* UCC118, both inside and outside the host, using gene systems isolated from the more physiologically robust pathogenic strains. Improving *L. salivarius* UCC118 stress tolerance will ultimately impact positively on the strain’s clinical relevance.
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REDUCTION OF *Clostridium difficile* SPORES IN THE ENVIRONMENT WITH HYDROGENPEROXIDE VAPOUR

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*Clostridium difficile* – associated disease (CDAD) is known as a common nosocomial infection following antimicrobial therapy and many outbreaks have been reported. During CDAD, large numbers of *Clostridium difficile* spores may disseminate in the room of the patient. These spores show very long survival time in the environment and cannot be destroyed by standard disinfection measures. Hydrogenperoxide is a known bactericidal and sporicidal agent.

**Objective:** The objective of our study was to compare the sporicidal activity of different hydrogenperoxide-brumisation machines. We also compared the sporicidal activity of hydrogenperoxide-based disinfectant and hypochlorite at different concentrations. We compared the sporicidal activity on NAP1/O27 spores and on non-NAP1/O27 spores.

**Methods:** We developed a method to coat a well-defined surface with a reproducible number of *Clostridium difficile* spores, which we exposed or not, to the different devices and disinfectants.

**Results:** We tested 5 hydrogenperoxide-brumisation machines. One of them was eliminated due to recurrent mechanical problems. We needed to readapt the parameters of all others to become effective in our experimental system. Under these conditions, they were able to decrease more than 6 log the number of spores in a very short time. There was no significant difference between the sporicidal activity towards NAP1/O27 spores and non NAP1/O27 spores. Hydrogenperoxide-based disinfectant for surface were even more effective than hypochlorite at usually recommended concentrations.

**Conclusions:** Hydrogen-peroxide-based brumisation machines could be very useful after usual room cleaning, to eliminate spores more efficiently where they are difficult to reach, in a quick and inoffensive way. Hydrogenperoxide-based products for surface cleaning are also very effective but their corrosive effect on different surfaces needs a prudent use and further investigation.
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DETECTION OF CLOSTRIDIUM DIFFICILE IN RABBITS OF ITALIAN COMMERCIAL FARMS

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Naturally acquired or antibiotic induced enteropathy due to C. difficile has been reported in rabbit, but the impact of this pathogen in commercial breeding losses has never been investigated in Italy where rabbit meat production represents the 44% of the whole European one. Intestinal contents were collected from the small intestine and the caecum of 317 rabbits with enteric lesions and from 78 healthy rabbits in 125 Italian commercial farms. Samples were stratified by age (< 3 days, 35 - 55, >55, breeders) and enteric lesions (caecal constipation or fluid-filled caecum). To isolate the micro-organism ethanol treated intestinal contents were cultured in a pre-reduced C. difficile selective medium and plates were incubated at 37 °C in anaerobic conditions for 5 days. C. difficile was identified using a commercial biochemical panel kit and a PCR targeting a species specific tpi gene internal fragment. Isolates were tested by multiplex PCR to assess the presence of tcdA and tcdB genes encoding for toxin A and toxin B respectively. Intestinal contents were screened for C. difficile toxins A and B by using a commercial ELISA. C. difficile was recovered from the intestinal content of 10 rabbits with enteric disease (10/317, 3.1%); 5 subjects were 35-55 and 4 were > 55 aged, 1 subject was a breeder. At necropsy 6/10 of these positive subjects showed a watery intestinal content and 4/10 showed caecal constipation. C. difficile was never isolated from the intestinal content of healthy rabbits. Eight C. difficile strains were tcdA+ /tcdB+, one strain was tcdA- /tcdB+ and one strain was tcdA-/tcdB-. The C. difficile toxins were found in the intestinal contents of 10 rabbits with enteric disease (10/317, 3.1%) and 1 healthy rabbit (1/78, 1.28%), in 5 of the former C. difficile was isolated. Results from this survey pointed out the low prevalence (3.1%) of C. difficile and its toxins in rabbits sampled in enteric disease outbreaks.
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CLOSTRIDIUM DIFFICILE: EVIDENCE FOR ZOONOTIC POTENTIAL

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Clostridium difficile-associated diarrhea is an increasing problem in human and veterinary medicine. While interspecies transmission of C. difficile has yet to be demonstrated, recent studies have shown that isolates from humans and animals are often indistinguishable. We evaluated the prevalence of C. difficile colonization in dogs that interact with hospitalized people through therapeutic visitation programs. In the summer of 2004, feces was collected from 102 healthy dogs in Ontario and selectively cultured for C. difficile. Isolates were characterized by PCR-ribotyping, toxin gene detection, and toxinotyping. Ribotype patterns were compared to a collection of human and canine isolates archived previously by the investigators. C. difficile was isolated from 8 (8%) of the fecal specimens. Of these, 1% (41/8) were toxigenic: 16 (39%) produced toxins A and B, but not cdt (A+/B+/cdt-), 24 (59%) were A-/B+/cdt- and one (2%) was A+/B+/cdt+. Ten of the 8 isolates (17%) were indistinguishable from the authors’ library of human CDAD ribotypes. One of these 10 (the A+B+/cdt+ strain) was classified as NAP1/ribotype 027. In a follow up study, 2 groups of dogs were enrolled from Ontario and Alberta: 100 dogs that visit people in healthcare facilities (the “exposed”) and 100 dogs that participate in other types of therapeutic activities but do not visit healthcare facilities (the “unexposed”). Between May 2005 and November 2006, fecal specimens were collected from each dog every 2 months for 1 year, and submitted to researchers for selective culture, along with a log of places visited during the previous 2 months. Results of the latter study showed that 28 (28%) exposed dogs acquired C. difficile over the period under surveillance, compared to only 1 (1%) unexposed dogs (p = 0.025). Further molecular work is required to appreciate the significance of these findings. In the meantime, we recommend that future studies consider the zoonotic potential of C. difficile when investigating risk factors for human infection, and that hospitals implement infection control policies to protect patients and dogs from transmission of C. difficile through visiting programs.
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PREVALENCE OF CLOSTRIDIUM DIFFICILE TOXINS A AND B IN PIGS WITH COLITIS IN SWITZERLAND.

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Clostridium difficile has been associated with clinically significant colitis in suckling piglets in the U.S.. In Switzerland we observed a rise in colitis but so far, data about the prevalence and role of C. difficile in these cases is lacking. To evaluate the role of Clostridium difficile in outbreaks of colitis in swiss pig farms we recently started a collaborative project between the porcine clinic, the Institute of veterinary pathology and the Institute Veterinary Microbiology at the University of Bern. Our study is designed to investigate the prevalence of C. difficile toxins A and B in cases of pigs with pathological lesions of colitis. As this is an ongoing study preliminary results will be presented on a poster during the conference.
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**CLOSTRIDIUM DIFFICILE IN RETAIL MEAT: CANADA**

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Isolation of *Clostridium difficile* was recently reported from retail ground meat in Canada in a preliminary study. The current study was performed to determine the prevalence of *C. difficile* contamination of meat using a broader sampling scheme and to compare different culture protocols. Meat samples were collected from Ontario, Quebec and Saskatchewan, Canada using the infrastructure of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program. 214 meat samples (149 ground beef, 65 veal chops) were processed by: enrichment of meat rinse in CDMN broth followed by alcohol shock (AS) and inoculation onto CDMN agar, enrichment of meat in CDMN broth followed by AS and inoculation onto CDMN agar, and enrichment of meat in CCF broth followed by AS and inoculation onto blood agar. The 2⁰ protocol was performed in duplicate. Overall, the prevalence of *C. difficile* was 6.1% (13/214); 6.7% (10/149) in ground beef and 4.6% (3/65) in veal chops, respectively. The proportion of meat samples harboring *C. difficile* was similar in the 4 cultures (1.9% with Rinse/CDMN, 2.3% and 1.4% with Meat/CDMN, and 1.4% with Meat/CCF; p>0.7), however there was poor agreement between the different methods as *C. difficile* was only isolated by 1 of the 4 methods in 11/13 (85%) positive samples. PCR ribotyping revealed 8 distinct ribotypes; 7 (88%) of which were toxigenic. Five of these toxigenic ribotypes have been isolated from humans in Ontario. Ribotype 027, containing genes encoding toxins A, B and CDT (A’B’CDT⁺) plus an 18 bp deletion in tcdC and of toxinotype III was isolated from 3 samples. Another strain was also A’B’CDT⁺/18 bp tcdC deletion/toxinotype III and classified as NAP1 but had a different ribotype pattern. Three ribotypes were A’B’CDT⁻/toxinotype 0. One of these was classified as NAP4, while the other 2 did not fit current NAP designations. One strain was A’B’CDT⁻/toxinotype VIII. Further studies are needed to determine the epidemiological relevance of these findings and to determine optimal culture methodology.
STRUCTURE AND ASSEMBLY OF THE CLOSTRIDIUM DIFFICILE S-LAYER

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Clostridium difficile, like many bacteria, possesses a paracrystalline protein surface layer (S-layer). Unusually in C. difficile this S-layer is comprised of two proteins derived from a single precursor, SlpA, by post-translational cleavage. Interestingly, purified S-layer proteins (SLPs) can be reassembled in vitro but this self-assembly requires calcium. The high molecular weight SLP contains putative cell-wall binding motifs and is highly conserved among C. difficile strains; most inter-strain differences involve single amino acid substitutions with occasional small insertions and deletions. In contrast, the low molecular weight SLP shows an extremely high degree of sequence divergence between strains. The function of the S-layer in C. difficile is still a subject of some debate but it has been suggested that the layer may act as an adhesin by binding to collagen I, thrombospondin, and vitronectin. Little is known about the structure of the S-layer or the mechanism of its assembly on the cell surface. We have isolated a protein complex containing both the high and low molecular weight SLPs that appears to be the basic subunit of the C. difficile S-layer. This complex can be formed in vitro using recombinant proteins and, unlike the assembly of an ordered S-layer, is not calcium-dependent. We have also identified a domain within the low molecular weight SLP that is required for the formation of this complex. The presence on this separate interaction domain is consistent with the low-resolution structure as observed by small angle X-ray scattering.
ASSESSING THE ROLE OF P-CRESOL IN VIRULENCE OF CLOSTRIDIUM DIFFICILE

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Clostridium difficile is an important nosocomial infection, resulting in antibiotic associated disease, ranging from mild diarrhoea to the life threatening pseudomembranous colitis. Although pathogenesis is largely linked to the production of toxins, designated A and B, clinical isolates have been identified which lack toxin A or contain deletions in the toxin sequences. Therefore the virulence and ability to cause disease is not limited to the toxin production. The life-style of C. difficile, and its ability to colonise the gut after antibiotic treatment, makes it an intriguing and complex bacteria. Significantly, it is among only a few bacteria able to ferment tyrosine to p-cresol. Tyrosine is reduced to para-hydroxyphenylacetate (pHPA), which is decarboxylated into p-cresol; a phenolic compound, toxic to other microbes, via its ability to interfere with metabolism. Indeed p-cresol is included in some disinfectants. We hypothesise that p-cresol may give C. difficile a metabolic advantage over commensal gut bacteria, resulting in proliferation after antibiotic treatment and subsequent C. difficile-associated disease CDAD.

A number of different clinical outbreak strains of C. difficile have been characterised as hypervirulent (eg O27 and A-B+ strains). Analysis of these strains by micro-array linked these different outbreak strains into clades; therefore the phenotypic differences related to virulence in these clades, will be assessed. Significantly it appears that the BI strains, including the Stoke Mandeville outbreak strain (R20291) are more tolerant to p-cresol than other 027 strains (including Popoff) and the sequenced strain 630. We will further analyse the potential physiological differences between these strains and construct and evaluate a defined p-cresol mutant in the hamster model of infection to ascertain the role of p-cresol in CDAD.
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IDENTIFICATION OF PUTATIVE CELL WALL ASSOCIATED PROTEINS WHICH MAY AID IN THE ADHERENCE OF CLOSTRIDIUM DIFFICILE TO THE COLONIC EPITHELIUM

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Clostridium difficile is an increasingly predominant nosocomial enteric pathogen causing severe pseudomembranous colitis (PMC) and antibiotic associated diarrhea (AAD). While disruption of the human colonic mucosa results from the activity of exotoxins, toxins A and B produced by C. difficile, colonization of the gastrointestinal tract is undoubtedly an important aspect of infection.

The unfinished genome sequence of Clostridium difficile 630 was mined using the PEDANT database to identify loci expressing putative cell wall-associated proteins, which could aid in establishment of C. difficile, thus infection. Following genomic DNA extraction, candidate genes were amplified and cloned into E. coli, and subsequently expressed in L. lactis using the nisin-controlled gene expression system (NICE). Flag Tags were fused to the C-terminal of the candidates to aid in the tracking of cellular localization of each protein using anti-Flag antibodies. Candidates found to be expressed within the cell wall (insoluble) fraction were examined for their ability to adhere to the human colonic epithelial cell line (C2Bbe1). The expression of an internalin-like protein and a cell wall-associated hydrolase in the surrogate host L. lactis increased adherence to the mucosal epithelial cells suggesting a putative role in adherence of C. difficile to the colonic epithelium.
P56
CHARACTERIZATION OF Acd, A FIRST PEPTIDOGLYCAN HYDROLASE (AUTOLYSIN) OF CLOSTRIDIUM DIFFICILE
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Autolysins, enzymes that hydrolyse the cell wall peptidoglycan, are implicated in different cellular functions of the bacteria that require cell wall remodeling (growth, cellular division and sporulation). They could also contribute to the virulence of some pathogenic species, by mediating cellular adherence, toxin release or by generating peptidoglycan released peptides with proinflammatory activity. We have characterized in silico a gene encoding a putative autolysin of Clostridium difficile (acd). Then we expressed and purified this putative autolysin in order to study its properties and functions.

The Acd protein is an autolysin with N-acetylglucosaminidase activity able to lyse the glycan chains of the wall of several bacterial species. The hydrolytic specificity of Acd was established by RP-HPLC analysis and MALDI-TOF MS using Bacillus subtilis cell wall extracts. Muropeptides generated by Acd hydrolysis demonstrated that Acd hydrolyses peptidoglycan bonds between N-acetylglucosamine and N-acetylmuramic acid, confirming that Acd is an N-acetylglucosaminidase.

We showed that Acd is mainly expressed during the exponential growth phase, suggesting that Acd has a role in cellular physiological functions of C. difficile such as peptidoglycan turnover or cell separation.

The Acd protein appears highly conserved within C. difficile strains and particularly in the C-terminal part that exhibits the enzymatic domain.

The role of Acd in the virulence of C. difficile is currently investigated. We are studying its implication in adherence of C. difficile to the eucaryotic cells and in release of inflammatory mediators by the peptidoglycan degradation products.
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SURFACE-LAYER AND TOTAL PROTEIN PROFILING OF 51 C. DIFFICILE ISOLATES REVEALS INTRA-GROUP VARIATIONS

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Background: C. difficile (CD) strains are typed using various approaches, one of which is restriction endonuclease analysis (REA). Isolates within an REA group cannot be further differentiated based on DNA patterns alone. Our recent studies of hypervirulent CD strains have revealed that isolates within an REA group exhibit different characteristics. The aim of this work was thus to assess if members of a single REA type were functionally identical. This was achieved by examining the protein profiles (total and surface-associated) of multiple CD strains belonging to defined REA types.

Methods and results: We performed total-protein and surface-protein profiling experiments on 1 non-toxigenic and 4 hypervirulent CD isolates, and surface protein profiling of 32 toxigenic CD isolates belonging to 20 REA groups. Isolates were grown to exponential phase, harvested, lysed and fractionated to obtain total soluble proteins, or subjected to an acid-pH/glycine treatment to extract surface-layer proteins (SLP). Equalized amounts of proteins were electrophoresed using SDS-PAGE, and protein banding patterns compared. First, we found that total protein profiles varied within isolates of a single REA type. Second, SLP protein profiles - especially SlpA - greatly varied among CD isolates. We also found that the hypervirulent CD strains had a SlpA profile different from those previously published. Third, mass-spectrometry analyses of 13 protein bands in a SLP preparation from a hypervirulent strain established identities of the surface proteins, and confirmed that SlpA was indeed dysregulated. Further western blotting experiments revealed that an anti-SlpA antiserum generated against SlpA from the sequenced CD strain 630 reacted strongly with the altered SlpA proteins from hypervirulent strains but not with SlpA from some of the other CD strains.

Conclusions: Within a single REA type, CD isolates show marked protein profile differences. Hypervirulent CD isolates have dysregulated SLP expression. The varying ability of CD strains to cause disease is thus likely not limited to toxin production alone, but related to altered expression of other proteins as well. Strain variations may thus become an important consideration when treating CDAD.
ENHANCEMENT OF THE CYTOTOXICITY OF CLOSTRIDIUM DIFFICILE BY SURFACE ANTIGENS

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Clostridium difficile is a potent nosocomial pathogen. Its main virulence factors are two large exotoxins, A and B, encoded in the pathogenicity locus (PaLoc). However, the virulence of the strain is not strictly correlated with the amount of toxin produced by the strain in vitro. Other virulence factors are therefore considered to be not essential, but very significantly involved in pathogenesis.

Among those virulence factors surface proteins, and more in particular the two surface layer proteins (SLPs) stand out. Also, consideration needs to be taken of the natural habitat of C. difficile, the gut, which contains Gram negative bacteria and their very biologically active lipopolysaccharides (LPS: endotoxin). It is not known if LPS contributes to the pathogenesis of C. difficile, but since it is present in every human gut, it is not unreasonable to believe the existence of some sort of synergy between C. difficile exotoxins and endotoxin.

Vero cells in culture were challenged with different concentrations of toxin A purified by affinity chromatography to bovine thyroglobulin. Fixed concentrations of four different types of surface antigens were added: 1) pool of surface proteins extracted with EDTA; 2) SLPs extracted with GHCl; 3) the surface lipocarbohydrate of C. difficile (LC) and 4) E. coli LPS.

Cell viability was measured by an MTT assay. After checking that none of the antigens affected cell viability on their own, it was found that at very low concentrations of toxin A, the addition of any of the four antigens tested reduced the cell viability. However, at higher levels of toxin A, the only antigen able to reduce cell viability was LC which is analogous to the lipoteichoic acid of other Gram positive bacteria.

The mechanism by which these surface antigens (innocuous on their own) increase the cytotoxicity of toxin A in vitro is not known, but it might help to explain the inconsistencies seen in vivo.
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