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Renaissance Hotel • Long Beach, California USA
June 24-27, 2008

## SESSION VI—CLOSTRIDIUM DIFFICILE: PATHOGENESIS

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# ADHERENCE AND PROTEIN PROFILING STUDIES OF *CLOSTRIDIUM DIFFICILE* BI/NAP1/O27 ISOLATES

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*Clostridium difficile* is a leading cause of nosocomial infections, and newly-emerged "hypervirulent" (HV) *C. difficile* strains have been implicated as the cause of numerous diarrheal disease epidemics in the USA, Canada and Europe. These strains are designated BI/NAP1/027 using various molecular typing methods (restriction enzyme analysis, pulse-field analysis or PCR ribotyping, respectively). There is still only a limited understanding of *C. difficile* pathogenesis and the HV phenotype, as well as the cause for rapid geographic dissemination of HV isolates is even less understood. We have developed a completely anaerobic host-cell adherence assay and determined that independently isolated HV*C. difficile* strains have up to a 100% increased adherence to human intestinal epithelial cells, compared with their phylogenetically related but non-HV counterparts (comparator strains). HV strains also have altered total protein profiles in contrast to comparator strains, and a major surface-layer protein, SlpA, varies in both size and amount in HV strains. We have shown via adherence-inhibition and antibody-blocking assays that SlpA contributes to host epithelial cell adherence in *C. difficile.*

The requirement for antibiotic displacement of gut flora prior to establishment of *C. difficile* disease underscores the importance of bacterial adherence for infections caused by this species. Taken together, the results from our current studies indicate that that HV *C. difficile* isolates have increased adherence to human intestinal epithelial cells that is mediated in part by surface-layer protein A. Efficient adherence may be one factor contributing to the establishment and predominance of *C. difficile* strains in hospitals where outbreaks have occurred.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# ARE TOXIN A AND TOXIN B ESSENTIAL VIRULENCE FACTORS OF *CLOSTRIDIUM DIFFICILE*?

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*Clostridium difficile* is the causative agent of a spectrum of chronic gastrointestinal syndromes in humans, ranging from mild diarrhoea through moderately severe disease with watery diarrhoea, abdominal pain and fever to hypotension, sepsis, and fatal pseudomembranous colitis. Pathogenesis primarily involves the action of two large clostridial cytotoxins, toxin A (308 kDa) and toxin B (270 kDa), which are encoded by the *tcdA* and *tcdB* genes, respectively. Toxin A is an extremely potent enterotoxin that also has cytotoxic activity. Toxin B is a potent cytotoxin. These toxins have been purified and *in vitro* studies have been extensive. Based on studies of oral administration of toxin to hamsters, toxin A has been proposed as the major pathogenic toxin. However, the precise role of these toxins in the disease process has not been determined, primarily because of the lack of tools for the genetic manipulation of *C. difficile*. Using a genetic manipulation approach developed in our laboratory, which involves the use of recombination vectors designed for the specific inactivation of genes of interest, we have constructed separate chromosomal *tcdA* and *tcdB* mutants that are derived from the virulent *C. difficile* strain 630. Genotypic characterisation that involved PCR analysis and Southern hybridisations confirmed that the mutants had the expected genetic organisation. Phenotypic analysis of the mutants involved Western blotting, *in vitro* cell culture assays, adherence assays, and quantitative real-time PCR. These results confirmed that the *tcdA* and *tcdB* mutants did not produce toxin A or toxin B, respectively. Further analysis involved assessing the virulence of these strains by using the hamster model of disease. Unexpectedly, the presence of toxin A alone did not adversely affect the hamsters. Instead, the results provide evidence that toxin B is the major virulence factor of *C. difficile* since *tcdB* disruption led to an attenuated virulence phenotype, and *tcdA* mutants were fully virulent. The results also suggest that toxins A and B do not act synergistically. These mutants represent the first virulence gene mutants to be constructed in *C. difficile* and the results obtained from this study have significant implications for the future diagnosis and treatment of *C. difficile* infections.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# THE CODY REGULON OF *CLOSTRIDIUM DIFFICILE*

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The ability to sense excess or limiting nutrients is essential to the ability of *Clostridium difficile* to proliferate in the intestine and survive in the environment when not in the spore form. CodY, a global regulatory protein that monitors the nutrient sufficiency of the environment by responding to the intracellular levels of GTP and the branched-chain amino acids, likely plays an important regulatory role in this process. In previous work, we have shown that CodY is a potent repressor of toxin gene expression in *C. difficile*. CodY appears to repress toxin synthesis in nutrient rich conditions, releasing this repression when nutrients become limiting. In the intestine, such derepression presumably leads to increased synthesis of toxins A and B, lysis of intestinal epithelial cells, and the liberation of potential nutrients. CodY is likely to play an important role in regulating overall cellular physiology as well. When we compared the transcription profile of a *C. difficile codY* mutant to that of a wild-type strain using DNA microarray analysis, we found that the *codY* mutation caused 177 genes to be overexpressed and 12 genes to be underexpressed. In addition to the toxin genes, genes for amino acid biosynthesis, transport (amino acids, peptides, and sugars), and cell membrane and surface proteins were overexpressed in the *codY* mutant. Several genes were shown to be direct targets of CodY by gel mobility shift and DNase I footprinting assays. Full characterization of the CodY regulon will likely shed light on how growth, survival, and pathogenesis of *C. difficile* respond to nutrient availability.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# PARTIAL *tcdC* GENE DELETIONS IN *C. DIFFICILE* ISOLATES OF VARIOUS PCR-RIBOTYPES

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It has been suggested that an 18bp sequence deletion found in the toxin regulatory *tcdC* gene of *Clostridium difficile* strain BI/NAP1/027 may be relevant to the increased morbidity and mortality associated with this strain. The aim of this study was to look for similar deletions in isolates representing a wide range of PCR-ribotypes and toxinotypes.

A total of 220 *C. difficile* isolates representing 116 mostly toxigenic ribotypes were screened for *tcdC* deletion using a CEQ 8000 genetic analyser and primers described by Spigaglia and Mastrantonio (J Clin Microbiol 40:3470-5). Where deletions were detected, sequences of representative isolates were determined and compared with those of reference strains of ribotype 001 (no deletion) and ribotype 027 (18 bp deletion).

An identical 18 bp deletion was detected in 35 isolates representing 13 ribotypes including ribotype 027. Deletions in the same genetic location of 36bp, 39bp and 54bp were found in four, six, and four other ribotypes respectively. No deletions were detected in the UK 'epidemic' ribotypes 001 or 106 (eight isolates of each ribotype, including some from fatal cases).

Gene deletions were found in ribotypes belonging to toxinotypes 0, III, IV, VI, XIa, XIV and unknown types. Strain BI/NAP1/027 belongs to toxinotype III. Single representative isolates of three other ribotypes (034, 075, and 080) assigned to this toxinotype also had the 18bp deletion but these ribotypes are very rarely isolated in the UK and have not been associated with severe disease.

Gene deletions were detected in 14 (82%) of 17 ribotypes known to be binary toxin producers but in only four (6.1%) of 66 binary toxin-negative ribotypes. The binary status of 34 ribotypes (9 with deletions) was unknown. The 76.3% (95% CI 51.4-88.3) difference between binary toxin-positive and -negative types is statistically significant ($p$ value = 0.0001).

In total, 63 of 220 isolates representing 27 of the 116 ribotypes tested had deletions in the *tcdC* gene. Deletion was significantly associated with binary toxin production and occurred in several toxinotypes. Deletions were detected in the 'epidemic' strain BI/NAP1/027 and 26 other 'non-epidemic' toxigenic ribotypes. However, no deletion was detected in UK 'epidemic' ribotypes 001 and 106. This suggests that *tcdC* gene deletions *per se* are not associated with increased virulence and that other factors account for the epidemic nature of certain strains.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# TOXIN A AND B NEGATIVE, BINARY TOXIN POSITIVE *CLOSTRIDIUM DIFFICILE* ISOLATED FROM A CASE OF BACTERAEMIA

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The role of binary toxin production by *Clostridium difficile* has been brought into focus recently by the emergence and intercontinental spread of PCR ribotype 027 *C. difficile*, a strain that produces binary toxin. Some investigators suggest that binary toxin may be acting as an additional virulence factor in PCR ribotype 027 strains, resulting in more severe disease, although animal experiments have given equivocal results. We report here a case of bacteraemia from which a toxin A and B negative, binary toxin positive strain of *C. difficile* was isolated. A 40-year old Aboriginal male presented to a hospital in the north of Western Australia with a 1 day history of vomiting, diarrhoea, feeling unwell, and abdominal pain. He was conscious and oriented but appeared dehydrated with clinical jaundice. His previous medical history was extensive and included: alcoholism, liver dysfunction and failure, bone marrow suppression with severe pancytopaenia (probably due to alcoholism), pancreatitis, and recurrent pneumonia. It was felt that urosepsis was the likely diagnosis as he had not completed a recent course of cephalexin for a UTI caused by a susceptible strain of *Escherichia coli* (500 mg q.i.d orally given for 72 h). He had also received 9 days of cephalexin (500 mg q.i.d for 72 h), 1 month earlier for a chest infection. Blood cultures were obtained, and he was rehydrated with intravenous fluids. Ceftriaxone 1 g IV daily was commenced, and he settled quickly. He had 4 days of antibiotic therapy before he discharged himself. Both blood culture bottles were positive with the anaerobic bottle containing *C. difficile*. The aerobic bottle grew a *Staphylococcus epidermidis*. The *C. difficile* isolate was determined to be $A^-B^-Cdt^+$ by PCR detection of the toxin A, toxin B and binary toxin genes. The isolate was PCR-negative with the primer pair Lok1-Lok3 indicating that the Paloc was not completely absent. Amplifying the Paloc integration region by long-range PCR revealed the presence of an element approximately 6 kb long. Attempts to toxinotype the strain were unsuccessful. By PCR ribotyping the isolate was genetically distinct from 201 other Australian *C. difficile* clinical isolates. Although it is likely this isolate reflects skin contamination with *C. difficile*, the possibility that the organism was a pathogen should not be ignored and it is worthy of further investigation.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# BILE SALTS AND GLYCINE AS CO-GERMINANTS FOR *CLOSTRIDIUM DIFFICILE* SPORES

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Spore formation by *Clostridium difficile* is a significant obstacle to overcoming hospital acquired *C. difficile*-associated disease. Spores are resistant to heat, radiation, chemicals, and antibiotics, making a contaminated environment difficult to clean. To cause disease, however, spores must germinate and grow out as vegetative cells. Germination of *C. difficile* spores has not been examined in detail. In an effort to understand the germination of *C. difficile* spores, we characterized the response of *C. difficile* spores to bile. We found that cholate derivatives and the amino acid glycine act as co-germinants. Deoxycholate, a metabolite of cholate produced by the normal intestinal flora, also induced germination of *C. difficile* spores, but prevented the growth of vegetative *C. difficile.*

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# SPORULATION IN *CLOSTRIDIUM DIFFICILE*

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*Clostridium difficile* is the sporogenic, anaerobic bacillus that can cause disease ranging from mild self-limiting diarrhoea to pseudomembranous colitis, collectively referred to as *Clostridium difficile*-associated disease (CDAD). Most CDAD is associated with treatment using broad spectrum antibiotics that disrupt the normal flora of the gut. The loss of colonisation resistance allows *C. difficile* spores to germinate into vegetative cells and produce the exotoxins A and B. Though the main virulence factors of *C. difficile* are its toxins, the main factor involved in the transmission of the disease are its spores. Sporulation is an important feature of most bacilli. And the gene *spo0A*, which codes the stage 0 sporulation protein, is the key regulator of the sporulation process. In *Bacillus subtilis*, the protein Spo0A is a transcription factor belonging to the response regulator family. It has a DNA binding domain and is responsible for the initiation of sporulation. It has been previously hypothesised that sporulation and toxin production may be related processes in *C. difficile*. Sporulation was studied in *C. difficile* strain 630 by performing viable counts every 4 h using serial dilutions of culture heated at 80ºC for 20 min to destroy all viable cells. It has been observed that sporulation begins at approximately 6 to 8 h and then steadily increases through the exponential and stationary phases till 48 h. Thus, we can hypothesise that there would be a similar increase in the transcription of the *spo0A* gene over time. In order to quantify this transcription, Real Time RT-PCR experiments will be performed till 24 h as it is difficult to obtain RNA from cells beyond 24 h. Further, the sporulation will be correlated to the production of toxins and the transcription of *spo0A* will be correlated to that of the toxin genes in order to relate the two processes.

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## CLOSTRIDIUM DIFFICILE: PATHOGENESIS

# THE MUCOSAL IMMUNE RESPONSE TO *CLOSTRIDIUM DIFFICILE*

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**Introduction:** Infection with *C. difficile* results in a spectrum of disease ranging from asymptomatic carriage to life-threatening conditions such as toxic megacolon and pseudomembranous colitis (PMC). Histological changes in the colonic mucosa range from minimal inflammation to PMC. Host immune response is important in determining the outcome of infection; it has been shown that low levels of serum IgG to Toxin A are associated with recurrent disease. Furthermore the presence of the AA genotype in the IL-8 promoter at position -251 is associated with increased susceptibility to *C. difficile*-associated disease (CDAD) and with increased faecal IL-8 in diarrhoeal stools.

**Aim:** To determine whether the mucosal immune response to *C. difficile* infection and IL-8 genotype differ between asymptomatic carriers, patients with CDAD, and controls.

**Methods and Results:** Study subjects are being recruited from amongst patients attending a colorectal outpatient clinic at a local hospital. Faecal samples are cultured to identify asymptomatic carriers. Specimens of colorectal tissue taken during biopsy or resection are being obtained from 10 carriers, 10 symptomatic individuals and 20 controls. To date we have screened faecal samples from 102 individuals and identified five carriers and five CDAD patients. Immunohistochemical studies of mucosal immune system cells are being performed using antibodies to T cells (CD3), macrophages (CD68), B/plasma cells (CD20), and to IgA, IgM, and IgG. Labelled cells in the lamina propria are being quantified. T cell responses to certain *C. difficile* antigens, including Toxin A, will be measured by proliferation assay and analysis of IL-8 gene expression by RT-PCR. Subjects' IL-8 genotype will be identified using allele-specific PCR primers.

**Discussion:** A previous study has shown that B/plasma cell, in particular IgA producing cells, and macrophage counts are significantly reduced in biopsies from patients with CDAD compared with controls. Our hypothesis is that asymptomatic carriers mount a protective immune response and that comparable cell counts will be seen in carriers and controls, with both being significantly higher than those seen in CDAD patients.