

Anaerobe ♦ 2008

The 9th Biennial Congress of the
Anaerobe Society of the Americas
Renaissance Hotel ♦ Long Beach, California USA
June 24-27, 2008

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CLOSTRIDIUM SORDELLII AND PREGNANCY- ASSOCIATED TOXIC SHOCK CATS: CURRENT PATHOBIOLOGY AND UPDATED RECOMMENDATIONS

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Background: *Clostridium sordellii* (Cs) and other Clostridium spp are rare but well recognized causes of pregnancy associated infection morbidity and mortality (postoperative, postpartum, postabortal). Five cases of lethal toxic shock in association with administration of misoprostol and mifepristone (RU486) has prompted reexamination of both Cs pathobiology and obstetrical procedures in order to propose prevention and treatment strategies versus CATS.

Methods: Available literature regarding *C sordellii*, pregnancy, mifepristone, and inflammation/endocrine responses to infection were analyzed.

Results: Recent advances in the understanding of microbiologic/host interactions, especially innate immunity, provide insight into pathogenesis and possible prevention and treatment strategies for toxigenic strains of Cs (TcsL, others). Large clostridial and similar toxins possess multiple-step, multi-level pathogenic mechanisms, including inactivation of p38 MAPK, which can inactivate GTP ases (Rho, Ras, others) and p38 as well as interfere with glucocorticoid receptor (GR) phosphorylation (Tait A. Infect Immun. 2007.75.3935) Appropriate GR activation regulates immune/inflammatory responses and restores homeostasis. RU486 is a long acting (T1/2 @ 2d) progesterone receptor (PR) inhibitor which similarly blocks GR function, for up to 7 days. RU486 increases lethality in sepsis models by impairing glucocorticoid modulation of inflammatory responses.

RU486 combined with misoprostol has gained wide acceptance as a medical form of pregnancy termination, despite marginal increases in efficacy (88% misoprostol alone vs 93% completion for the combined agents).

Conclusion: Recent basic and clinical advances regarding Cs, TcsL, and similar toxins, and pregnancy related complications and treatments (RU486) informs: 1) molecular and innate immunity research, 2) clinical practice (use of misoprostol alone or surgery), and 3) public health approaches ("black box warning"), as well as, 4) underscoring need for prompt recognition and innovative treatments (extirpative surgery, Cs or TcsL antisera, adjunctive steroids) and novel other treatments for potentially lethal *Clostridium sordellii* infections complicating pregnancy.

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CLOSTRIDIUM NOVI-NT SPORES AS A TREATMENT FOR SOLID TUMOR MALIGNANCIES

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Strategies that successfully target and destroy human cancers must recognize differences between normal and malignant tissues. In this regard, an abnormal vasculature is being increasingly recognized as a consistent feature of solid tumors and a potential point of attack. Malignant solid tumors are generally comprised of a hypoxic, often necrotic core and a viable rim. The core has a poor vascular supply and is therefore deficient in nutrients and oxygen. Therapeutic interventions to date have focused on the well-vascularized outer shell of the tumor, as these are most susceptible to chemotherapeutic agents and radiation. In contrast, few therapies, target the inner, hypoxic core, which can make up a major part of the tumor's mass. For this purpose, we have investigated the potential of using live anaerobic bacteria. In particular, we developed a strain, termed *Clostridium novyi-NT* (*C. novyi-NT*), that is a spore-forming Gram-positive anaerobe whose toxicity was attenuated by eliminating a bacteriophage that carried its systemic toxin gene. *C. novyi-NT* has undergone pre-clinical efficacy and toxicity evaluations in several animal models. *C. novyi-NT* spores delivered through a single intravenous dose to mice results in substantial tumor regressions in most animal models. Cure rates of 20%-30% are common when spores are used alone in immunocompetent mice or rabbits. In nude mice bearing human tumor xenografts, complete regressions are generally observed, and cures are routinely obtained when the spores are administered with selected radiation and chemotherapeutic protocols. Toxicity studies demonstrated no clinical toxicity and minimal pathologic toxicity in healthy animals. While toxicity in tumor-bearing animals was evident in mice, it was manageable with hydration or antibiotics.

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A REVIEW OF TAXA AND RESEARCH FINDINGS ASSOCIATED WITH *CLOSTRIDIUM* CLUSTER IV: THE “*CLOSTRIDIUM LEPTUM* SUPRA-GENERIC rRNA CLUSTER GROUP”

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In 1994, Collins et al published a landmark paper describing phylogenetic relationships based on analyses of 16S rRNA gene sequences and that extreme heterogeneity among validly named *Clostridium* species existed. Many pathogens were assigned to *Clostridium* cluster I, which contains the type species of the genus, *Clostridium butyricum*. Many well known pathogenic *Clostridium* species however, were found to be only distantly related to this group, and so were assigned to provisional cluster groups numbered II to XIX. Each cluster contains at least 1 *Clostridium* species and most coclustered with taxa assigned to other genera except for clusters I and II. For improved taxonomic precision, members of these clusters will or should be excised from the genus *Clostridium* and assigned to new genera and families. One such provisional group was *Clostridium* Cluster IV. In 1994 this cluster included *C. orbiscindens*, *C. viride*, *C. leptum*, *C. sporosphaeroides*, *C. cellulosi*, various *Ruminococcus* spp, *Faecalibacterium* (formerly *Fusobacterium*) *prausnitzii* as well as other validly named taxa. This cluster was described as probably representing a coherent relationship at family level. Synonyms for this group in the literature include ‘*C. leptum* subgroup’, ‘*C. leptum* supra-generic rRNA cluster’ and ‘butyrate producers’. Detection /cloning studies suggest that these bacteria predominate in human fecal microflora and a decreased level of these taxa has been observed in patients with inflammatory bowel disease. Various reports describe remarkable diversity of taxon groups which were detectable, but not culturable from the bowel, suggestive of validly named families, genera and species as well as putative novel taxa. Therefore, we reviewed those bacteria, (all medically relevant) referrals to the Canadian federal reference center and identified using 16S rRNA gene sequencing/other characterization methods, as ‘closest to’ Cluster IV taxa as described in the Collins paper. We also reviewed taxa which had been validly described by 1994 but not included in the Collins et al paper, or those genera and species nova described after 1994, and where being ‘closest to Cluster IV’ members was used as a phylogenetic reference point.

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VACUOLIZATION AND APOPTOTIC ACTIVITIES OF *CLOSTRIDIUM HISTOLYTICUM* CYTOTOXINS

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Clinical reports concerning infections caused by *Clostridium histolyticum*—including infective endocarditis, necrotic infections in drug users, and necrotizing fasciitis—were published recently. Pathogenesis of these infections is always connected with tissue lysis caused by protein toxins and enzymes produced by *C. histolyticum*. Some strains of *C. histolyticum* produce lethal and hemolytic factors differing in heat stability and susceptibility to oxidation. Lethal factor disappears very rapidly after logarithmic phase of growth, being inactivated by concurrently produced proteolytic enzymes. Few publications suggest the important role of this microorganism as a member of fecal microbiota of autistic children. We studied toxin production potential of *C. histolyticum* ATCC 19401 strain cultured in BHI for further comparison with those isolated from fecal samples obtained from autistic children. *C. histolyticum* lethal cytotoxin was partially purified from culture broth by ammonium sulfate precipitation, gel filtration, and hydrophobic interaction chromatography. Two different activities were determined: the cytotoxin caused vacuolization of HeLa cells visible under light microscope already after 2h and distinct after 8h. Calcium ion influx was detected by flow cytometry. Vacuolization of HeLa cells was inhibited by bafilomycin A1, suggesting involvement of H⁺-ATPase in the formation of vacuoles. Lethal activity was detected after 24 h: practically all cells were detached from the culture vessel. Apoptotic activity was demonstrated using caspase activation and DNA fragmentation assays. In EM the cells displayed condensation of chromatin and fragmentation of nuclei with formation of micronuclei and apoptotic bodies. Differences in susceptibility of cell cultures to *C. histolyticum* vacuolization and lethal activity were observed. We concluded, that *C. histolyticum* produces cytotoxin with two different activities: first one causing vacuolization involving H⁺-ATPase, and the second causing lethal effect by apoptosis.

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NEURONAL CELL-BASED BOTULINUM NEURO-TOXIN (BONT) ASSAYS FOR HIGHLY SENSITIVE AND SPECIFIC DETECTION OF BoNT AND NEURO-INHIBITORS

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Clostridium botulinum produces a characteristic neurotoxin (BoNTs) that are responsible for the dreaded disease botulism. BoNTs are also widely used as pharmaceuticals to treat various neurologic disorders and in cosmetic applications. We have developed a primary neuronal cell-based assay using spinal cord and hippocampal cells from fetal rats that is capable of reproducibly detecting BoNT/A at concentrations as low as 1 picogram per milliliter (approximately 0.1 mouse LD50). Not only has this assay been useful in detection of BoNTs, it has also shown considerable utility in detection of antibodies and inhibitors to BoNT and neuronal cell viability.

Two applications are described:

(1) Antibodies to BoNT treatment in patient sera. The major adverse effect BoNT treatments in medical use has been resistance to treatment after multiple injections. Currently, patients receiving BoNT therapies and patients enrolled in clinical trials for new applications and/or new formulations of BoNTs are not routinely monitored for the formation of neutralizing antibodies, since no assay is commercially available that reliably tests for the presence of such antibodies. This report presents a highly sensitive and specific neuronal cell based assay that provides sensitive and specific detection of neutralizing antibodies to BoNT/A.

(2) Neuronal toxicity of culture supernatants of *Clostridium* cultures isolated from autistic children. Nineteen *Clostridium* sp. cultures were isolated from the feces and small intestine of children suffering from late onset autism symptoms. The cultures were propagated in TPGY medium under anaerobic conditions, and the culture supernatants were tested for *in vivo* toxicity to mice by intraperitoneal injection and for cytotoxicity in primary rat hippocampal and cortical cells. Of the 19 culture supernatants tested in mice, 6 were highly lethal, while 4 caused death of the mice in only subset of the mice tested. All of the culture supernatants that had resulted in death of all mice also caused significant cytotoxicity in both primary hippocampal and cortical cells. Additionally, one supernatant resulted in minor cytotoxicity in hippocampal cells only. This supernatant was derived from culture WAL16351, also known as *C. bolteae*. The supernatants from control cultures of the non-toxic *Clostridium clostridioforme* (ATCC25537) did not cause any toxicity in either the mouse or the neuronal cells assay. The nature and biologic significance of these toxins remains to be elucidated. These data indicate that neuronal cells may be an excellent model for detection of neuronal toxins from clostridia.

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FeoB—A VIRULENCE-ASSOCIATED DETERMINANT THAT PLAYS A MAJOR ROLE IN IRON ACQUISITION IN *CLOSTRIDIUM PERFRINGENS*

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Since iron is essential for bacterial growth and survival, pathogens have adopted several mechanisms for acquiring iron from host proteins, including direct acquisition of ferric iron from haem-associated proteins or from iron-scavenging siderophores. Ferric iron, regardless of the source, requires transport into the cytosol, where it can be utilized by the bacterial pathogen. Under anaerobic conditions, bacteria can transport ferrous iron using the transmembrane transporter, FeoB. The overall aim of this project was to characterize iron acquisition systems of *Clostridium perfringens*. Bioinformatic analysis of the *C. perfringens* strain 13 genome sequence revealed that it has seven potential iron acquisition systems: three siderophore-mediated systems, one ferric citrate uptake system, two haem-associated acquisition systems, and one ferrous iron uptake system (FeoAB). Relative expression of these systems was determined using quantitative real-time RT-PCR on one gene from each system. The results showed that each gene was expressed, with *feoAB* generating the most abundant iron-uptake related transcripts in strain 13. To examine the role of these systems in growth and virulence, allelic exchange was used to isolate chromosomal *fepD*, *fhuBG*, and *feoB* mutants. Growth of these mutants in the presence and absence of iron revealed that only the *feoB* mutant had altered growth properties compared to the wild-type. Similarly, only the *feoB* mutant had a markedly reduced total iron content. Preliminary virulence data accumulated using a mouse myonecrosis model indicated no loss of virulence with either the *fepD* or *fhuBG* mutant, however, mice injected with the *feoB* mutant had reduced virulence when compared to the wild-type strain. These studies suggest that FeoB is required for the uptake of ferrous iron into the cell and may play an important role in virulence in *C. perfringens*.

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SIMULTANEOUS PRESENCE OF *CLOSTRIDIUM DIFFICILE* AND *CLOSTRIDIUM BOTULINUM* IN PATIENTS WITH INFANT BOTULISM

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Background: Infant botulism (IB) results when ingested *Clostridium botulinum* spores germinate and temporarily colonize the infant large intestine, where botulinum neurotoxin is produced and absorbed. *Clostridium difficile* is also known to colonize the infant large intestine. In 2004, an IB patient co-infected with nosocomially-acquired *C. difficile* succumbed to colitis and a perforated bowel, the first such fatality to occur in an IB patient. It is not known how frequently *C. botulinum* and *C. difficile* may coexist in the flora of the infant colon.

Methods: For this reason, we performed a 2-year prospective study to identify the presence of *C. difficile* in all fecal specimens submitted to our laboratory for diagnosis of IB. Specimens were cultured on Cycloserine-Cefoxitin Fructose Agar (CCFA), and the identity of colonies suspected to be *C. difficile* was confirmed by standard biochemical methods. *C. difficile* toxin (CdT) was identified by neutralization with CdT-specific antitoxin B (goat) using a mouse bioassay. Duration of excretion of *C. difficile* and *C. botulinum* was also studied in a pair of twin IB patients.

Results: Among 85 patients with confirmed IB, 6 (7%) also had *C. difficile* in their feces, four of whom were also positive for CdT. Among 20 patients who did not have infant botulism, 8 (40%) had *C. difficile* in their feces, one of whom was also positive for CdT. Infants colonized with *C. botulinum* were less likely to be colonized with *C. difficile*, and conversely ($p < 0.001$, Fisher's exact test). In Twin patient X, *C. difficile* replaced *C. botulinum* in the fecal flora after 13 weeks and maintained its presence until week 61 of the excretion study. Twin Y became colonized with *C. difficile* at week 15, which was sustained until week 58. To the best of our knowledge none of the patients were clinically symptomatic from *Clostridium difficile*.

Conclusion: *C. botulinum* and *C. difficile* appear to compete for the same ecological niche in the intestinal flora of the hospitalized infant because colonization with one species resulted in significantly less frequent intestinal colonization by the other species. Nonetheless, IB patients may be co-colonized with *C. difficile* and may or may not also have CdT in their feces. Some IB patients are therefore at risk of *C. difficile* disease, which may be severe, and clinicians should be especially alert to this possibility in antibiotic-treated IB patients.

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MOLECULAR EPIDEMIOLOGY AND SPATIAL-TEMPORAL CLUSTERING OF INFANT BOTULISM CASES IN CALIFORNIA, 1976-1994

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Background: Infant botulism (IB) is the intestinal toxemia form of botulism first recognized in 1976 that occurs when ingested spores of *Clostridium botulinum* germinate in the large intestine and produce botulinum toxin in it. The purpose of this ongoing study was to characterize strains of *C. botulinum* isolated from California IB patients with molecular methods and to evaluate the possible spatial-temporal clustering of genetically-related isolates.

Methods: DNA purified from 567 patient isolates was characterized by amplified-fragment length polymorphism (AFLP) analysis. PCR-specific primers for the type B toxin gene were used to identify unexpressed type B genes in some type A strains, designated type A(B). Possible clustering of AFLP-related isolates was evaluated for the 19-year period 1976-1994 using the Kulldorff spatial-scan statistic and a case-control (Bernoulli) model within a maximum time-window of 5 years.

Results: AFLP analysis enabled grouping of genetically-related isolates into at least 14 clades. Two A(B) clades were identified, one with isolates located mainly in coastal areas and the other with isolates located mainly in the Central Valley and Sierra Nevada foothills. Neither clade showed spatial-temporal clustering. However, 2 temporal-spatial clusters were identified, one containing 11 type A isolates in the San Diego area in 1984-87 ($P=0.04$) and one containing 12 type B isolates in Los Angeles County in 1988-91 ($P=0.006$).

Conclusion: These studies report the first recognition in California of *C. botulinum* type A(B). AFLP analysis of California patient *C. botulinum* isolates distinguished heretofore unknown genetic differences among them that enabled their grouping into clades. Two clades in Southern California clustered in both time and location, consistent with their being unrecognized outbreaks. These findings demonstrate the value of applying molecular methods to a large clinical *C. botulinum* collection. Additional insights from analysis of the remaining ~450 strains are anticipated.

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EFFECT OF ANTIMICROBIAL AGENTS ON THE PRODUCTION OF *CLOSTRIDIUM SORDELLII* CYTOTOXIN

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Objective: *Clostridium sordellii* is an anaerobic gram-positive organism, which causes rare but fatal septic shock. Especially, shock-like illness in postpartum and postabortion patients is serious problem. *C. sordellii* has reported to produce several toxins including lethal toxin (LT), which is known to have cytotoxic to Vero cells and other mammalian cells, and cross-reactive with *Clostridium difficile* toxin B (cytotoxin). We have focused on treatment for *C. sordellii* infection, especially for administration of antibiotics. While there have been some reports on immunomodulation of clindamycin (CLDM) and ciprofloxacin (CPFX). We examined the effect of antimicrobial agents on cytotoxin production.

Materials and Methods: *C. sordellii* GAI60203 was isolated from vaginal specimen and identified in our laboratory. Minimum inhibitory concentration (MIC)s of this strain against CLDM and CPFX were determined by Etest (AB BioDisk) according to the manufacturer instruction. Presence of LT gene in this strain was detected by PCR. Cytotoxin production of this strain was detected by cytotoxicity to Vero cells. Vero cells were maintained in Ham's F-12K medium (Gibco) supplemented 5% bovine serum at 37 degree of centigrade in 5% CO₂. Serial dilution samples (20 µl well⁻¹) of culture supernatant were added to a 96-well plate containing 5 x 10⁴ cells per well. Cells were monitored for 3, 6, 24h posttreatment for cytopathic effects (CPE). Bacterial number at the point of culture supernatant collected and protein concentrations in applied supernatants were also detected.

Results: *C. sordellii* GAI60203 was cultured in BHI broth under the different concentrations (2MIC, 1MIC, 1/2MIC, 1/4MIC, and 1/8MIC) of CLDM and CPFX individually. CPE begun to be observed after 3h incubation in the control wells with drug-negative culture supernatant, and cell rounding, indicative of LT-like CPE was observed in those wells. Intensity of CPE was decreased in the well of culture supernatant treated with higher-concentration of drugs. CPE-positive culture supernatant showed positive-reaction in TOX A/B QUIK CHEK (TechLab), which implied the supernatant contains LT. The result showed that viable bacterial cells and cytotoxin production in culture supernatant decreased in concentration-dependent manner.

Discussion: Our results suggested that CLDM and CPFX would be effective in patients with infection by *C. sordellii*, from the viewpoints of not only control of viable cells but cytotoxin production.

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DETECTION AND CHARACTERIZATION OF AN ABC TRANSPORTER IN *CLOSTRIDIUM HATHEWAYI*

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Extrusion of antimicrobial agents from bacteria by multidrug transporters is one of the mechanisms by which bacteria resist therapeutic drugs. In a fluoroquinolone-resistant strain of *Clostridium hathewayi*, the efflux of the drugs appears to be one reason for resistance. An ABC transporter gene from this bacterium is characterized. Its deduced amino acid sequence has conserved functional domains with the ATPase components of the multidrug efflux pump genes of several bacteria that have duplicated ATPase domains in addition to a transmembrane protein. Cloning the transporter gene into *C. perfringens* and *E. coli* resulted in decreased sensitivities of these bacteria to fluoroquinolones. It also decreased accumulation and increased efflux of ethidium bromide from the cells containing the cloned gene, both of which were also inhibited in the presence of the ATPase inhibitor CCCP. The ATPase mRNA was overexpressed in the fluoroquinolone-resistant strain exposed to ciprofloxacin. This is the first report of an ABC transporter in *C. hathewayi*.

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BETA TOXIN IS ESSENTIAL FOR THE VIRULENCE OF *CLOSTRIDIUM PERFRINGENS* TYPE C IN EXPERIMENTAL CAPRINE ENTEROTOXEMIA

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Clostridium perfringens type C isolates, which cause enteritis necroticans in humans and enterotoxemias of domestic animals, typically produce (at minimum) beta toxin (CPB), alpha toxin (CPA), and perfringolysin O (PFO) during log-phase growth. We evaluated the contribution of CPB to the virulence of type C isolate CN3685 in an intraduodenal challenge model in goats. Similar to natural type C infection, late log-phase vegetative culture of wild-type CN3685 caused abdominal pain, hemorrhagic diarrhea, necrotizing enteritis and colitis, pulmonary edema, hydropericardium and death in 2 goats within 24 h of inoculation. Isogenic CPB toxin null mutants were prepared using TargeTron[®] technology. These mutants were completely devoid of virulence in this animal model; 2 goats inoculated with the null mutants remained clinically healthy during 24 h after inoculation and no gross or histological abnormalities were observed in the tissues of either animal, after euthanasia and post-mortem examination. Complementation of a *cpb* mutant restored its CPB production and virulence; 2 goats inoculated with these complemented mutants presented clinical and pathological changes similar to those observed in goats inoculated with the wild type strain. These results indicate that CPB is both required and sufficient for CN3685-induced disease, supporting a key role for this toxin in type C disease pathogenesis.

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