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Clostridia: The Impact of Genomics on Disease Control

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6th ClostPath International Conference

Rome, 19 - 23 October 2009

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Istituto Superiore di Sanità
Hall Pocchiari

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The Organisers are grateful to the following sponsors for their generous support to the 6th ClostPath International Conference

Dear Colleagues

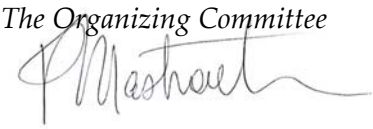
It is a great pleasure to welcome you to Rome and to the 6th ClostPath International Conference.

As many of you know the First ClostPath International Conference took place 14 years ago in Arizona. In the mind of the organizers it intended to be a venue for the presentation and discussion of the latest scientific news of the genus Clostridium. Its importance has increased over the years and we are not too bold if we say it is now the leading forum for the international community to meet and discuss progress made in understanding pathogenic clostridia and their associated diseases. The evidence is the over 150 excellent abstracts submitted which made it really hard to select oral presentations and the long list of would-be participants we've been obliged to turn down for various reasons.

We are especially proud of the number of young researchers attending this meeting since, I'm sure we all agree, besides carrying out research it is our duty and pleasure also to contribute to training the next generation of Clostridia experts. Therefore, as this conference is part of the series of Marie Curie training conferences and workshops on Clostridia we would like to thank the EU through the project officer for having allowed us to increase to 75 the number of fully sponsored fellowships. At the same time we are grateful to ESCMID for having granted support to an extra 16 young scientists bringing the total to the incredible number of 90.

We hope the oral presentations, posters and interactions among participants will strengthen collaboration and bring forth new ideas and, needless to say, that you may enjoy the social events and your stay in the eternal city.

The Organizing Committee



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***Clostridia:* The Impact of Genomics on Disease Control**
Istituto Superiore di Sanità, Rome, Italy
19-23 October 2009

Monday 19th October 2009

15:30 - 18:00 Registration

16:30 - 17:00 Welcome Address

17:00 - 18:00 **Keynote Lecture** - The Impact of Genomics on Understanding Clostridial Pathogens
G. Gottschalk - Göttingen Genomics Laboratory, Germany

18:00 - 19:30 Welcome Cocktail at the Istituto Superiore di Sanità

Tuesday 20th October 2009

Session I: **Molecular Epidemiology & Laboratory Diagnosis**
Chairperson: *Paola Mastrantonio*

09:00 - 09:30 Application of Molecular Methods to Diagnose CDI and to Study Its Epidemiology
E. J. Kuijper - Leiden University Medical Center, The Netherlands

09:30 - 09:50 Novel Mass Spectral Approaches Based upon Surface – Associated and Stable Intra-Cellular Proteins for the High Throughput Identification of *C. difficile*.
L. Rajakaruna - HPA, London, UK

09:50 - 10:10 Antibody-Mediated Enhanced Cytotoxicity of *Clostridium difficile* Toxin: Application in Diagnosis
H. Feng - Tufts USA

10:10 - 10:30 The Enterotoxin Gene (CPE) is Plasmid-Borne in *Clostridium perfringens* Type A Isolates Related to Sudden Infant Death Syndrome
A. Heikinheimo - University of Helsinki, Finland

10:30 - 11:00 Coffee break

Session II: **Clostridial Toxins – Part 1**
Chairperson: *Maja Rupnik*

11:00 - 11:30 The Role of NetB toxin in Necrotic Enteritis
R. J Moore - Australian Animal Health Laboratory, Geelong, Australia

11:30 - 11:50 Studies of *C. perfringens* Enterotoxin: Claudin Interactions: Therapeutic Implications?
S.L.Robertson - University of Pittsburgh, USA

11:50 - 12:10 What Does the 3D-Structure of *Clostridium perfringens* Enterotoxin Reveal?
A. K. Basak - Birkbeck College, London, UK

12:10 - 12:30 TcsL is an Essential Virulence Factor in *Clostridium sordellii*
G.P. Carter - Monash University, Clayton, Australia

12:30 - 14:00 Lunch

Session III : **Clostridial Toxins – Part 2**
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I. Just - Hannover Medical School, Germany

14:30 - 14:50 Toxin B is Essential for Virulence of *Clostridium difficile*
D. Lyras – Monash University, Clayton , Australia

14:50 - 15:10 Deciphering the Role of TCDE in the Toxin Secretion of *Clostridium difficile*
B. Dupuy - Institut Pasteur, Paris, France

15:10 - 15:30 Tcde: The Silent Member in the Paloc of *Clostridium difficile*?
A. Olling - Hannover Medical School, Germany

15:30 - 15:50 A Novel Function of *C. difficile* Transferase and Related Actin-ADP- Ribosylating Toxins
K. Aktories - Albert-Ludwigs-Universität, Freiburg, Germany

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Session IV: **Genomics, Metabolomics & Transcriptomics – Part 1**
Chairperson: *Wolfgang Liebl*

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T.J.Smith - USAMRIID - Fort Dietrick, USA

09:30 - 09:50 An Online Resource for Clostridium Genetic Tools
J.T. Heap - University of Nottingham , UK

09:50 - 10:10 Cold Tolerance of *Clostridium botulinum*
M. Lindström - University of Helsinki, Finland

10:10 - 10:30 Global Gene Regulation in Response to Cysteine Availability in *Clostridium perfringens*
I. Martin-Verstraete - Institut Pasteur, Paris, France

10:30 - 11:00 Coffee break

Session V: **Genomics, Metabolomics & Transcriptomics – Part 2**
Chairperson: *Nigel Minton*

11:00 -11:30 Comparative Genome and Phenotypic Analysis of *Clostridium difficile* O27 Strains Provides Insight into the Evolution of a Hypervirulent Bacterium
R.A. Stabler - London School of Hygiene & Tropical Medicine, London, UK

11:30 - 11:50 Comparative Proteomic Analysis of *Clostridium difficile*
C. Chilton – HPA Centre for Infections, London, UK

11:50 - 12:10 Transcriptional Profiling of *Clostridium difficile* and Caco-2 Cells During Infection
T. Janvilisri - Cornell University, Ithaca , USA

12:10 - 12:30 Understanding the Pathogenesis of *Clostridium difficile* by an *in vivo* Transcriptomic Approach
C. Janoir - Faculté de Pharmacie, Chatenay-Malabry, France

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Chairperson: *Julian Rood*

14:00 -14:30 Complex Regulatory Networks for Virulence in *C. perfringens*
T. Shimizu - Kanazawa University – Japan

14:30 - 14:50 Regulation of NetB Production in *Clostridium perfringens*
J.K. Cheung - Monash University, Clayton, Australia

14:50 - 15:10 Impact of Carbon Catabolite Repression in the *C. difficile* Physiopathology
A. Antunes - Institut Pasteur, Paris, France

15:10 - 15:30 Elucidating the Mechanisms of Sporulation in *Clostridium difficile*
A.Faulds-Pain - University of Nottingham and HPA, UK

15:30 - 16:00 Coffee break

16:00 Networking Discussions

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Chairperson: *Glenn Songer*

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M. Wilcox - University of Leeds, UK

09:30 - 09:50 Passage of Botulinum Neurotoxin A through the Intestinal Barrier
M. R. Popoff - Institut Pasteur, Paris, France

09:50 - 10:10 Biogenesis of the *C. difficile* S-Layer
R. P. Fagan - Imperial College London, London, UK

10:10 - 10:30 Contact with Enterocytes Causes Vir S/Vir R-Mediated Upregulation of Beta Toxin Production by *C. perfringens* Type C Isolates
B. McClane - University of Pittsburgh, USA

10:30 - 11:00 Coffee break

Session VIII: **Veterinary Diseases and Animal Models**
Chairperson: *Miia Lindström*

11:00 - 11:30 Main Clostridial Diseases in Animals
V. Båverud - National Veterinary Institute, Uppsala, Sweden

11:30 - 11:50 Mouse Models for Studying *Clostridium perfringens* Type C Infections
F.A.Uzal - University of California, Davis, USA

11:50 - 12:10 Epidemiology of Enterotoxaemia in Flemish Veal Calves
B. Pardon – Ghent University, Belgium

12:10 - 12:30 Prevalence of *Clostridium difficile* in Piglets With and Without Diarrhoea in Australia
T.V. Riley - The University of Western Australia, Nedlands, Australia

12:30 - 14:00 Lunch

Session IX: **Treatment & Exploitation**
Chairperson: *Bruce McClane*

14:00 - 14:30 Clostridia Associated Infections: New Weapons From Emerging Vaccines?
I. Castagliuolo - University of Padova, Italy

14:30 - 14:50 A Live Recombinant Oral Vaccine Expressing *Clostridium perfringens* Antigens Confers Protection of Broiler Chickens Against Necrotic Enteritis
J. F. Prescott - University of Guelph, Canada

14:50 - 15:20 Clostridial Neurotoxins as a Platform for Novel Therapeutics
K. Foster - Syntaxin Limited, Abingdon, UK

15:20 - 15:40 Vaccination with Parenteral Toxoid B Protects Hamsters Against Lethal Challenge with Toxin A-Negative/B-Positive *Clostridium difficile* but does not Prevent Colonization
S. Johnson - Hines VA Hospital, Chicago,USA

15:40- 16:00 Antibody-Based Therapeutics for *Clostridium difficile* Infection
C. Shone - HPA, Porton Down, Salisbury, UK

16:00 - 16.30 Coffee break

16:30 - 18:00 Poster Sessions IV-IX

20:00 - 23:00 Farewell Dinner at "Taverna dei Mercanti", Trastevere, Rome

Friday 23rd October 2009

Special Public Session

Chairpersons: *P. Mastrantonio, N. Minton*

09:30 - 10:15	Clostridium Infections: Afflictions For All Seasons *Sherwood Gorbach* – Tufts University School of Medicine, Boston, USA
10:15 - 10:30	Training the next generation of European Clostridium Researchers *Nigel Minton* – Nottingham University, UK
10:30 - 11:00	Coffee Break
11:00 - 11:20	*Clostridium botulinum* in Italy *Paolo Aureli* - Istituto Superiore di Sanità, Italy
11:20 -11:40	*Clostridium difficile* in Italy *Paola Mastrantonio* - Istituto Superiore di Sanità, Italy
11:40 - 12:00	Questions & Answers

Abstracts of Oral Presentations

O1

THE IMPACT OF GENOMICS ON UNDERSTANDING CLOSTRIDIAL PATHOGENS

G. Gottschalk

Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Georg-August-University of Goettingen, Grisebachstr.8, 37077 Goettingen, Germany

Several clostridial genomes have been sequenced in the past including genomes of clostridia of biotechnological interest such as *C. acetobutylicum* and *C. kluyveri*, but also those of species exhibiting a pathogenic lifestyle, such as *C. botulinum*, *C. tetani*,
C. perfringens and *C. difficile*. What are the main lessons we learned from the large amount of new information? The energy metabolism of the clostridia is much more complex than known before, especially the presence of the Rnf-cluster has widened our view. This cluster allows the coupling of electron transfer with proton translocation. Clostridial genomes exhibit information for the synthesis of a large number of surface layer proteins and adhesins allowing new insights in their host/matrix interacting properties and colonization. Very distinct is the increase of our knowledge on the regulation and function of botulinum and tetanus neurotoxins as well as the enterotoxins and α-toxins. Finally, some general features of the genus *Clostridium* will be outlined applying comparative genomics.

O2

APPLICATION OF MOLECULAR METHODS TO DIAGNOSE *CLOSTRIDIUM DIFFICILE* INFECTION (CDI AND TO STUDY ITS EPIDEMIOLOGY.

Ed J. Kuijper and Renate J. van den Berg.

Reference Laboratory for *Clostridium difficile*, Medical Microbiology Department, LUMC, Leiden and The National Institute for Public Health and Environment, Bilthoven, The Netherlands.

Accurate diagnosis of *Clostridium difficile* infection (CDI) is essential for patient management and appropriate infection control measurements. ESGCD recently performed a systematic review to evaluate the available evidence on laboratory diagnosis of CDI and to formulate recommendations to optimize CDI testing. In comparison with cell culture cytotoxicity assay (CCA) and toxigenic culture (TC) of stools, commercial available enzyme immunoasssays (EIA) had low positive predictive value (PPV). Therefore, a two step approach is recommended, with a second test or a reference method in case of a positive first test. The application of real-time PCR as a first screening assay is an interesting option. Several commercially available real-time PCRs are now at the market, such as BD-GeneOhm, Prodesse ProGastro *C. diff* Assay, GeneXpert *C. diff* Assay of which most of them target TcdB gene and are now investigated in clinical studies.

Since the 1980s the epidemiology of *Clostridium difficile infection* (CDI) has been investigated by the application of many different typing or fingerprinting methods. All typing methods have certain advantages and disadvantages, but their ultimate contribution to knowledge is dictated by their performance according to typeability, reproducibility, stability, discriminatory power, and epidemiological concordance. It should also have technical advantages, such as ease of performance, relative low cost, and high throughput. A growing number of molecular methods have been applied to *C. difficile*. For the early and rapid detection of outbreak situations, methods such as restriction enzyme analysis, arbitrary primed polymerase chain reaction (PCR), and PCR ribotyping are commonly used. For long-term epidemiology, multilocus sequence typing, multilocus variable number of tandem repeats analysis, and amplified fragment length polymorphism are of interest. Currently, the PCR-ribotyping method and the library of PCR ribotypes in Cardiff are the benchmarks to which most typing studies around the world are compared. Multilocus variable number of tandem repeats analysis is the most discriminative typing method and will contribute significantly to our understanding of the epidemiology of this important nosocomial pathogen.

O3

NOVEL MASS SPECTRAL APPROCHES BASED UPON SURFACE-ASSOCIATED AND STABLE INTRA-CELLULAR PROTEINS FOR THE HIGH THROUGHPUT IDENTIFICATION OF C. DIFFICILE.

L. Rajakaruna[1], H. Shah[1], G. Hallas[1], G. Balls[2], M. Erhard[3] and W. Kallow[3].

[1]Molecular Identification Service Unit, Department for Bioanalysis and Horizon Technologies, HPA Centre for Infections, London, England NW9 5HT, [2]Nottingham Trent University Nottingham NG11 8NS and [3]AnagnosTec GmbH, Am Mühlenberg 11, D-14476 Potsdam-Golm, Germany.

C. dificile causes a spectrum of diseases ranging from nosocomial Antibiotic-Associated Diarrhoea (AAD) to pseudomembranous colitis (PMC), especially in hospitalized patients over 65 years, which may occur after exposure to a wide range of antibiotics. In order to gain a more holistic profile of proteins of this diverse species, comparative proteomics of strains that span a 30 year period were utilised. Intact cells were examined directly by MALDI-TOF-MS, from a pilot study carried out using the existing database of over 5,000 mass spectral profiles of intact cells of type and reference strains, built in a collaborative study with Waters Inc and Manchester Metropolitan University. Spectral profiles indicated a range of surface-associated mass ions that reflect its intraspecific diversity. But more recent work of 54 *C. difficile* clinical isolates including other *Clostridia* sp., using the far more extensive database of AnagnosTec (SARAMIS) containing “SuperSpectra” and slight modifications to the sample preparation have significantly improved the data and appears to be more universally applicable. The mass spectral database developed by AnagnosTec is now equivalent in size to the 16S rRNA database and contains mainly clinical isolates. This therefore offers for the first time in microbiology, the potential to develop a genomic-proteomic polyphasic system for the identification of microorganisms.

O4

ANTIBODY-MEDIATED ENHANCED CYTOTOXICITY OF CLOSTRIDIUM DIFFICILE TOXIN: APPLICATION IN DIAGNOSIS

X. He[1], X. Sun[1], J. Wang[2], Q. Zhang[1], S. Tzipori, and H Feng[1]

[1]Tufts Cummings School of Veterinary Medicine, North Grafton, MA, 01536, USA.
[2] School of Bioscience and Biotechnology, South China University of Technology, Guangzhou, China. hanping.feng@tufts.edu

We report that the cytotoxicity of *Clostridium difficile* toxin A (TcdA) for cultured cells can be substantially enhanced via an opsonizing antibody through Fc gamma receptor I (FcγRI)-medicated endocytotic pathway. An anti-TcdA monoclonal antibody, A1H3, significantly enhanced the cytotoxicity of TcdA of murine macrophages and human monocytes. In addition, the A1H3-dependent enhancement of glucosyltransferase activity, cytoskeleton disruption, and TNF-α production by TcdA was also demonstrated in macrophages. While blocking FcγRII/III with anti-CD16/32 antibodies did not affect TcdA-mediated Rac1 glucosylation and cytotoxicity in macrophages, pre-saturation of FcγRI with anti-CD64 antibodies significantly reduced these activities. Moreover, expression of FcγRI in Chinese hamster ovarian (CHO) cells strikingly enhanced its sensitivity to TcdA when A1H3 was present. Confocal imaging studies indicated that A1H3 facilitated the recruitment and internalization of TcdA, contributing to the antibody-dependent, FcγRI-mediated enhancement of cytotoxicity of TcdA. Finally, studies using chlorpromazine and endosomal acidification inhibitors revealed an important role of endocytotic pathway in A1H3-dependent enhancement of TcdA activity.
By utilizing the enhancing antibody, we established a cell-based immunocytotoxicity assay sensitive enough to detect less then 1 pg/ml of TcdA in samples, which is dramatically higher than the sensitivity of immunoassays and Cytotoxin B assay. Moreover, a real-time electronic cell sensoring (RT-ECS) system was used to monitor the cell rounding caused by toxins in a real time and automatic fashion. Finally, the method was optimized allowing defrosted cells from cryopreservation to be mixed with samples, obviating the need for cell culture facilities. This immunocytotoxicity assay is ultrasensitive for the detection of TcdA and highly sensitive for TcdB It is simple to perform and highly specific, with a short turnaround time of approximately 3 hours. Our assay may potentially provide a real solution for detecting *C. difficile* infection in clinic laboratory.

O5

THE ENTEROTOXIN GENE (cpe) IS PLASMID-BORNE IN CLOSTRIDIUM PERFRINGENS TYPE A ISOLATES RELATED TO SUDDEN INFANT DEATH SYNDROME

A. Heikinheimo[1], M. Nevas[1], M. Lindström[1], E. Vuori[2], P. Saukko[3], H. Korkeala[1]

[1] Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland
[2] Department of Forensic Medicine, University of Helsinki, Helsinki, Finland
[3] Department of Forensic Medicine, University of Turku, Turku, Finland

Enterotoxin gene (*cpe*) has a chromosomal location in most of the *Clostridium perfringens* type A isolates related to food poisonings, and is plasmid-borne in antibiotic-associated diarrhea and sporadic diarrhea isolates. *cpe*-carrying (*cpe*-positive) *C. perfringens* type A has been also linked to sudden infant death syndrome (SIDS), but the location of *cpe* in SIDS isolates has not been established. We studied the presence of *cpe* in the gastrointestinal tract of 17 SIDS infants and faeces of 20 healthy infants in Finland. *C. perfringens* was isolated and strains were further characterized. Results showed of 53% of SIDS infants carrying *cpe*. All *cpe*-positive *C. perfringens* type A isolates possessed plasmid-borne cpe, with prevalences of 6% and 18% for plasmid-borne genotypes IS1151-cpe and IS1470-like-cpe, respectively. *cpe* was not detected nor *cpe*-positive C. perfringens type A isolated from healthy infants. We conclude that *cpe* is plasmid-borne in *C. perfringens* isolates related to SIDS and that *cpe*-positive *C. perfringens* type A is commonly involved in Finnish SIDS cases. The study offers new knowledge on the molecular epidemiology of different subpopulations of *cpe*-positive *C. perfringens* type A.

O6

THE ROLE OF NetB TOXIN IN NECROTIC ENTERITIS

Robert J. Moore[1,2,3], Anthony L. Keyburn[1,2,3], Trudi L. Bannam[2], John D. Boyce[2], Xuxia Yan[2], Mark E. Ford[1], Filip Van Immerseel[4], Julian I. Rood[2,3]

[1]CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia. [2]Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Clayton, Victoria, Australia. [3]Australian Poultry Cooperative Research Centre, Armidale, New South Wales, Australia. [4]Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.

Clostridium perfringens is the major cause of necrotic enteritis in chickens. The disease causes substantial economic losses within the poultry industry. All types of *C. perfringens* strains encode alpha-toxin, which has phospholipase C and sphingomyelinase activity, and has been shown to be of central importance in the development of human gas gangrene. However, we have demonstrated that a virulent chicken strain of *C. perfringens* from which the alpha-toxin gene was deleted was still fully pathogenic in a chicken model of necrotic enteritis, indicating that alpha-toxin is not essential for virulence in this chicken disease. Subsequently, we have identified a new pore-forming toxin, NetB. When *netB* was deleted the resultant strain was completely avirulent in chickens; complementation with the wild-type *netB* gene restored virulence. These results demonstrated the importance of this toxin in the development of necrotic enteritis. A survey of chicken derived *C. perfringens* strains from three continents has shown that most isolates from necrotic enteritis diseased birds contained *netB* whereas the gene was relatively uncommon in isolates from healthy birds. Finally, *netB*-negative strains isolated from diseased birds have proven to be avirulent when tested in an experimental disease induction model. Based on the data it is concluded that NetB has a central role in the pathogenesis of necrotic enteritis in chickens.

O7

STUDIES OF *C. PERFRINGENS* ENTEROTOXIN: CLAUDIN INTERACTIONS: THERAPEUTIC IMPLICATIONS?

S.L.Robertson[1], C. M. Van Itallie[2], J.Saputo[3], J. M. Anderson[2], F. A, Uzal[3] and B. A. McClane[1].

[1]Dept. of Microbiology & Molecular Genetics, Uni. of Pittsburgh, Pittsburgh, PA, USA.
[2] Dept. of Cell & Molecular. Physics, Uni. of N. Carolina-Chapel Hill, Chapel Hill, NC, USA.
[3] CAHFS Laboratory System, School of Veterinary Medicine, Uni. of California, Davis, CA, USA.

Claudins, a 24 member protein family, help to maintain the structure and function of epithelial tight junctions. Previous studies with fibroblast transfectants and naturally-sensitive Caco-2 cells implicated certain claudins (e.g., claudin -4) as receptors for *Clostridium perfringens* enterotoxin (CPE). The current study specifically shows the importance of sequences in the second extracellular loop (ECL-2) of claudin receptors for CPE binding. Rat fibroblast transfectants expressing a claudin-4 chimera, where the natural ECL-2 was replaced by ECL-2 from claudin-2, showed no CPE-induced cytotoxicity. Conversely, CPE bound to, and killed, CPE-treated Rat transfectants expressing a claudin-2 chimera with a substituted ECL-2 from claudin-4. Site-directed mutagenesis (SDM) was then employed to alter an ECL-2 residue that consistently aligns as N in claudin CPE receptors, but as D or S in claudin non-CPE receptors. Switching this single ECL-2 residue from N to D or S completely abolished, the ability of transfectants expressing this mutant claudin-4 to bind or respond to CPE. Identifying the conserved N residue in ECL-2 of claudin receptors as being key for CPE binding and sensitivity explains why only certain claudins can serve as CPE receptors. Preincubating CPE with soluble claudin-4 blocked cytotoxicity on Caco-2 cells, while no protection occurred using soluble non-CPE receptor claudin-2. That result suggested soluble claudin receptors might be therapeutically useful as receptor decoys to neutralize CPE in vivo. This hypothesis was confirmed using rabbit ileal loops, where no pathology was observed after preincubation of CPE with claudin-4.

O8

WHAT DOES THE 3D-STRUCTURE OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN REVEAL?

A. K. Basak[1], C. E. Naylor[1], D.C. Briggs[2], J. G. Smedley (III)[2] and B.A. McClane[2]

[1]School of Crystallography, Birkbeck College, Malet Street, London, UK.
[2]Department of Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine, Pittsburgh, USA.

The Clostridium perfringens enterotoxin (CPE) is a cytotoxin and the causative agent of type-A food poisoning and other human gastrointestinal (GI) diseases. The toxin is also associated with certain other, e.g. community-acquired antibiotic-associated diarrhoea (AAD) and sporadic diarrhoea (SD) which are more severe than normal type-A food borne complications.
CPE is a 35 KDa membrane active toxin, which forms a series of complexes in mammalian plasma membranes, including an SDS-sensitive small and two other SDS-resistant large complexes. Despite many years of intense study, the mode of action of CPE is not yet clear at the molecular level. Recently we have been able to determine the 3D-structure of this toxin, by X-ray crystallography to 2.7Å resolution. This has revealed overall fold of the protein, which has some resemblance to other pore-forming toxins. Our structure, along with preliminary EM-study provided some valuable information about the larger oligomers of CPE. The atomic structure of CPE, EM-images and their functional implications will be discussed in this presentation.

O9

TcsL IS AN ESSENTIAL VIRULENCE FACTOR IN Clostridium sordellii

G.P. Carter[1], M.M. Awad[1], Y. Hao[2], D.M. Aronoff[2], J.I. Rood[1] and D. Lyras[1]

[1]Department of Microbiology, Monash University, Clayton 3800, Australia. [2]Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, MI, USA.

Clostridium sordelliis an important cause of disease in livestock and an emerging human pathogen. It is associated with disease in postpartum and postabortive women and in intravenous drug users. It is also an important contaminant in cadaveric tissues used in transplantation. *C.sordelliicauses* several infections including pneumonia, endocarditis, arthritis, peritonitis, myonecrosis, and more rarely sepsis and severe toxic shock syndrome. Infection with *C.sordelliis* associated with high rates of mortality that can approach 70%. *C.sordellii* produces at least three toxins: lethal toxin (TcsL) and hemorrhagic toxin, both members of the large clostridial monoglucosylating toxin family, and sordellilysin, a cholesterol dependant cytolysin, as well as several other putative virulence factors including phospholipases and neuramindases. The role of these factors in disease is not known, primarily due to a lack of tools for the genetic manipulation of *C.sordellii*.To address this issue we have used an RP4-mediated conjugation system to facilitate the transfer of autonomously replicating plasmids into *C.sordellii*. Furthermore, using this methodology and TargeTron technology we have constructed the first isogenic mutants of *C.sordellii*.Specifically, several independently derived tcsLmutants were generated in *C.sordellii* strain ATCC9714. Subsequent analysis showed that the mutants no longer produced any detectable TcsL protein and caused significantly less cytopathic effects in a Vero cell cytotoxicity assay. Finally, preliminary animal experiments have shown the mutants to be avirulent in a mouse infection model, providing evidence that TcsL is an essential virulence factor in *C.sordellii*.

O10

CLOSTRIDIUM DIFFICILE TOXINS A AND B: PROGRESS IN THE UNDER-STANDING OF THEIR MODE OF ACTION

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Clostridium difficile toxin A and B (TcdA and TcdB) are homologous single-chain protein toxins, which are co-produced and co-released. They recruit different membrane receptors for cell entry but the mode of cell-entry is comparable. Both toxins are up-taken by receptor-mediated endo¬cytosis and subsequently released from an acid endosomal compartment. Translocation into the cytoplasm is accompanied by autoproteolytic cleavage resulting in the release of the glucosyltransferase (GT) domain. Whereas cleavage is essential for the cellular activity of TcdB, cleavage of TcdA does only influence the potency of the cytopathic (reorganisation of the ctin cytoskeleton) activity and has almost no effect on the cytotoxic (cell death) activity.
Whereas inositol-hexakisphosphate (IP6) is potent stimulator for autocatalytic cleavage of TcdB based on binding of IP6 to the cysteine protease domain, additional pH-induced conformational changes seem to be important for TcdA self-cleavage. If autoproteolytic cleavage takes place prior to cell entry both toxins are biologically inactive as the GT domain must be covalently connected to the delivery domain to be correctly delivered to the cytoplasm.
There are some reports showing indirectly GT-independent effects of the toxins on cells for example on the mitochondria. Direct proof of this notion is the application of GT-deficient full-length toxin. Trans¬ferase-deficient (mutation DXD-motif) and transferase-reduced full-length TcdA show no and less biological activity, respectively, the latter correlates exactly with the reduced transferase activity. So far there are no data with enzyme-deficient toxins supporting cellular effects of GT-free TcdA.
TcdA and TcdB mono-glucosylate a subset of Rho family proteins, Rho, Rac, Cdc42, RhoG and TC10. The glucose moiety renders the GTPases functionally inactive to block down stream signalling. Based on these findings both toxins have been classified as “Rho-inactivators”. This notion, however, is not the full story. Inactivation of RhoA by glucosylation triggers up-regulation of RhoB. RhoB – an immediate early gene product – is a poor substrate for the toxins so that upregulation of RhoB is accompanied by its activation. Thus, repro-gramming of cellular signalling by TcdA and TcdB is more complex than thought as yet.

O11

TOXIN B IS ESSENTIAL FOR VIRULENCE OF Clostridium difficile

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Clostridium difficile is the leading cause of infectious diarrhoea in hospitals worldwide and 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. These toxins are members of the large clostridial glucosylating toxin family, which are monoglucosyltransferases that are proinflammatory, cytotoxic and enterotoxic in the human colon. Inside host cells, both toxins catalyse the transfer of glucose onto the Rho family of GTPases, leading to cell death. However, the role of these toxins in the context of a *C. difficile* infection was unknown. We have constructed isogenic toxin gene mutants of a virulent *C. difficile* strain and used these derivatives in the hamster disease model to show that toxin B is a key virulence determinant. Previous studies showed that purified toxin A alone can induce most of the pathology observed following infection of hamsters with *C. difficile* and that toxin B is not toxic in animals unless it is co-administered with toxin A, suggesting that the toxins act synergistically. Our work provides evidence that toxin B, not toxin A, is essential for virulence, which represents a major paradigm shift. Furthermore, it is clear that the importance of these toxins in the context of infection cannot be predicted exclusively from studies using purified toxins. These results reinforce the importance of using the natural infection process to dissect the role of toxins in disease, which is particularly important in studies of the epidemic toxinotype III NAP1/027 strains, which produce higher levels of both toxin A and toxin B.

O12

DECIPHERING THE ROLE OF TCDE IN THE TOXIN SECRETION OF CLOSTRIDIUM DIFFICILE

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In clostridia, most toxins are released into the extracellular environment, using the general secretion pathway for release. They possess a classical N-terminal signal peptide, which is generally cleaved during the translocation through the cell membrane, a prerequisite step before secretion. *C. difficile* toxins have no export signature and their secretion is not explained by cell lysis. This suggested that toxins might be secreted by an unusual secretion mechanism as suggested by Tan *et al.,* J. Med. Microbiol. (2001), involving TcdE a small hydrophobic protein of 19 kDa. The TcdE protein encoded by the third accessory gene of the *C. difficile* PaLoc locus, has structural features similar to those of bacteriophage proteins called holins. In most phage infections (such as infection by λ phage) host lysis is accomplished when an endolysin is exported to the surface of the bacteria through a pore formed by holin oligomerization, allowing the endolysin to cross the cytoplasmic membrane. We tested the hypothesis that TcdE acts as a holinlike protein to facilitate the release of *C. difficile* toxins to the extracellular environment. TcdE definitely has holin-like activity, since it functionally complemented a λ phage deprived of its holin. To study the role of TcdE in toxin secretion, we constructed a *C. difficile tcdE* mutant using the ClosTron technique. The mutant grew at the same rate as the wild-type strain, but secreted a dramatically reduced amount of toxin proteins into the medium. On the other hand, as cells approached stationary phase, more toxin accumulated in the cytoplasm of the *tcdE* mutant than in the wild-type. There was no difference in the levels of *tcdA* and *tcdB* gene expression and several cytoplasmic proteins were equally abundant when we compared the mutant and wild-type strains. Moreover, TcdE cause no or below detectable level of membrane permeabilization in *C. difficile*. All of these data indicated, unlike the phage holins, that TcdE pore aids secretion of toxin but does not cause the non-specific release of cytosolic contents. Thus, TcdE appears to be the first example of a bacterial protein that releases toxins into the environment by a phage-type system.

O13

TCDE: THE SILENT MEMBER IN THE PALOC OF *CLOSTRIDIUM DIFFICILE*?

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The small open reading frame *tcdE* within the PaLoc is located between the genes for the toxins A (TcdA) and B (TcdB). Whether the deduced 19 kDa protein TcdE participates in virulence of *C. difficile* is not yet known. Sequence and structure homologies to bacteriophage-encoded holins have led to the assumption that TcdE mediates the release of the toxins from *C. difficile* into the extracellular environment.
To investigate TcdE function, insertional inactivation of the *tcdE* gene was perfomed to generate a TcdE-deficient *C. difficile* strain. Data revealed that TcdE does not regulate *C. difficile* growth or sporogenesis. Furthermore, TcdE knockout neither altered kinetics of toxin release nor the absolute TcdA and TcdB protein levels, refuting the often discussed hypothesis of a TcdE-mediated release of the pathogenic toxins as a result of bacteriolysis.
However, expression of TcdE in *E. coli* correlated with cell death of *E. coli*. This bactericidal property was used to identify and characterize functional domains of the protein by analyzing the influence of full length and deleted TcdE on *E. coli* growth. Analyses showed an essential role of the three transmembrane domains with regard to bacteriolysis and a modulatory impact of the N- and C-termini. Furthermore, *tcdE* exhibits an alternative ribosome binding site combined with a dual start motif leading to expression of the 19 kDa full length TcdE and an N-terminal truncated 16 kDa product. The truncated TcdE was shown to be crucial for bacteriolysis whereas the full length form inhibited lytic function of the truncated form. *C. difficile,* however, exclusively produces the inhibitory, full length TcdE supporting the finding that TcdE was not involved in the delivery of the toxins.
The data indicate that TcdE might have antimicrobial impact on intestinal commensals or prevent the utilization of PaLoc by other bacterial species since the subsequent transcription under control of the ribosome binding site would lead to an excess of truncated TcdE resulting in lysis of the respective bacterium.

O14

A NOVEL FUNCTION OF *C. DIFFICILE* TRANSFERASE AND RELATED ACTIN-ADP-RIBOSYLATING TOXINS

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Besides the Rho GTPase-glucosylating toxins A and B, emerging hypervirulent *Clostridium difficile* strains additionally produce the ADP-ribosylating toxin CDT (*Clostridium difficile* transferase), which inhibits actin polymerization. CDT belongs to the family of binary actin-ADP-ribosylating toxins, also including *C. botulinum* C2 toxin and *C. perfringens* iota toxin.
The toxins ADP-ribosylate actin at arginine 177, a modification, which inhibits actin polymerization. Moreover, ADP-ribosylated actin acts like a capping protein to inhibit polymerization of unmodified actin.
Here we report that CDT and other binary actin-ADPribosylating toxins induce redistribution of microtubules and formation of long (>150 µm) microtubule-based protrusions at the surface of intestinal epithelial cells. The toxins increase EB1/3, CLIP-170 and CLIP-115 comet lengths at microtubule plus-ends and cause redistribution of capture proteins CLASP2 and ACF7 from microtubules from the cell cortex into the cell interior. The CDT-induced microtubule protrusions form a dense meshwork at the cell surface, which wrap and embed bacterial cells thereby largely increasing the adherence of clostridia.
This study describes a novel type of microtubulebased structures caused by less efficient microtubule capture and offers a new perspective for the pathogenetic role of CDT and other binary actin-ADP-ribosylating toxins in host-pathogen interactions.

O15

INSIGHTS GAINED FROM THE STUDY OF *CLOSTRIDIUM BOTULINUM* GENOMES

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The recent availability of multiple *C. botulinum* genomic sequences has initiated a new genomics era that strengthens our understanding of the host bacteria that express botulinum neurotoxins (BoNTs). Analysis of the genomes has reinforced the historical organism Group I-IV designations and provided evidence that the BoNT genes can be located within the chromosome, phage or plasmids. The sequences provide the opportunity to closely examine the components of the BoNT complex, their flanking regions and their specific locations within the different strains. These comparisons provide evidence of horizontal gene transfer, such as site-specific insertion and recombination events, that have contributed to the variation observed among the neurotoxins, toxin complexes, and the bacteria that house them.
This work was partially supported by NIAID cooperative agreement U01 AI056493. The opinions, interpretations and recommendations are those of the author and are not necessarily those of the US Army. The DOE Joint Genome Institute (JGI) at Los Alamos National Laboratory acknowledges the support of the Intelligence Technology Innovation Center (ITIC) for this research.

O16

AN ONLINE RESOURCE FOR CLOSTRIDIUM GENETIC TOOLS

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Directed mutagenesis and the use of shuttle plasmids are both important fundamental approaches in molecular microbiology, but both have historically posed difficulties in *Clostridium* research. Previously we developed the ClosTron, a group II intron-based mutagenesis system for *Clostridium*, which allows the direct selection of stable mutants in almost any gene. We also constructed a modular system for the quick and easy construction and modification of a large variety of *Clostridium – E. coli* shuttle plasmids. Recently we have greatly improved the ClosTron approach both in terms of scope (host range, multiple mutations, 'cargo' sequence delivery) and ease-of-use. We will describe perhaps the most important development, which allows us to rapidly obtain re-targeted ClosTron plasmids without PCR or cloning, making the improved ClosTron approach among the easiest to use and least labour-intensive directed mutagenesis methods available. To share these methods with the research community and maximise accessibility, we have established a new online resource (which will be made publicly available, free-of-charge) including protocols and other information, most importantly bioinformatics tools. Here we demonstrate how the online resource may be easily used by a non-specialist to: (1) automatically identify intron target sites in a gene of interest, design a suitable re-targeted intron, and obtain a corresponding re-targeted ClosTron plasmid, and; (2) automatically identify a standard pMTL80000-series modular shuttle plasmid (from 400 possible combinations) by picking from a 'menu' of 18 elements, and download the annotated sequence. Such a plasmid is easily constructed in a standard way from the 18 standard elements available from our lab. We hope these methods and the online resource will make genetic approaches in *Clostridium* more accessible.

017

COLD TOLERANCE OF *CLOSTRIDIUM BOTULINUM*

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The chill chain is the main tool to control bacterial growth and toxin production in modern foods. Consequently, bacteria have developed strategies to sense and adapt to low temperatures. Although *Clostridium botulinum* has long been known to cause a substantial safety hazard in refrigerated packaged foods, the cellular events behind the ability of *C. botulinum* to grow at low temperature have remained unknown. Understanding these mechanisms is a key to developing novel control measures against potentially lethal foodborne botulism.
Central mechanisms that bacteria utilize in sensing and adaptation to environmental changes include the two-component signal transduction systems (TCS). The membrane-located histidine kinases sense environmental stimuli with an extracytoplasmic sensor domain and send autophosphorylation signals to the cognate response regulators. The response regulators possess DNA-binding activity, ultimately resulting in a specific response in gene expression.
Using DNA microarrays and quantitative reverse transcription PCR, we have identified for the first time a TCS (CBO0365/366) that is activated in the *C. botulinum* strain ATCC 3502 upon exposure and adaptation to low temperature. Gene knockout experiments with the novel ClosTron tool have shown that an insertion mutation in either of the CBO0365/366 system gene impairs the growth of ATCC 3502 at low temperature. Genes under the regulation of the cognate response regulator are being analysed.

O18

GLOBAL GENE REGULATION IN RESPONSE TO CYSTEINE AVAILABILITY IN CLOSTRIDIUM PERFRINGENS

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Cysteine has a crucial role in cellular physiology and its synthesis is tightly controlled due to its reactivity. In *C. perfringens*, the two-component system, VirR-VirS, controls expression of several toxin genes and also the ubiG operon involved in methionine to cysteine conversion (1). Very little is known about sulfur metabolism and its regulation in clostridia. We performed a reconstruction of sulfur metabolism in *C. perfringens* and correlated these data with the growth of the strain 13 in the presence of various sulfur sources. Surprisingly, *C. perfringens* can convert cysteine to methionine by an atypical still uncharacterized pathway. By transcriptomic experiment, we further compared the expression profiles of strain 13 after growth with cysteine or homocysteine (cysteine starvation). 177 genes were differentially expressed. We found genes involved in sulfur metabolism controlled by premature termination of transcription via a T-box$_{cys}$ system (*cysK-cysE*, *cysP1* and *cysP2*) or a S-box motif (*metK* and *metT*). Several transporters for cystine (CysP1-CysP2) and methionine (MetT) were identified. We also showed that the *ubiG-mccBAluxS* operon was submitted to a double regulation by the VirR-VirS, VR-RNA and VirX network (1, 2) and by cysteine availability via a T-box system.
Interestingly, expression of genes involved in [Fe-S] biogenesis and in the maintenance of the cell redox status was induced during cysteine starvation. The synthesis of 2 cysteine desulfurases and 2 scaffold proteins for [Fe-S] assembly and the expression of genes encoding a ferredoxin and 2 rubredoxins were up expressed after growth with homocysteine. Additionally we identified a new regulator responding to cysteine availability, Cpe1786. This repressor shares similarities to CymR from *Bacillus subtilis* and IscR from *Escherichia coli*. Finally, the expression of pfoA (□ toxin), nagL (one of the five genes encoding hyaluronidases) and genes of the fermentation pathways was differentially expressed in response to sulfur availability. Phenotypic tests and metabolic profiles are currently performed to confirm the transcriptomic data.

1) Banu et al (2000). Microbiology 35, 854; 2) Ohtani et al FEMS lett, 2002. 209, 113

O19

COMPARATIVE GENOME AND PHENOTYPIC ANALYSIS OF *Clostridium difficile* O27 STRAINS PROVIDES INSIGHT INTO THE EVOLUTION OF A HYPERVIRULENT BACTERIUM

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The continued rise of *Clostridium difficile* infections (CDI) worldwide has been accompanied by the rapid emergence of a highly virulent clone designated PCR-ribotype 027. To understand more about the evolution of this virulent clone, we made a three-way genomic and phenotypic comparison of a “historic” non-epidemic 027 *C. difficile* (CD196), a recent epidemic and hypervirulent 027 (R20291) and a previously sequenced PCR-ribotype 012 strain (630). Although the genomes are highly conserved, the 027 genomes have 234 additional genes compared to 630, which may contribute to distinct phenotypic differences we observe between these strains relating to motility, antibiotic resistance and toxicity. The epidemic 027 has five unique genetic regions, absent from both the non-epidemic 027 and strain 630, which include a novel phage island, a two component regulatory system and transcriptional regulators. Comparison with a series of 027 isolates showed that some of these genes appeared to have been gained by 027 strains over the past two decades. This study provides genetic markers for the identification of 027 strains and offers a unique opportunity to explain the recent emergence of a hypervirulent bacterium.

O20

COMPARATIVE PROTEOMIC ANALYSIS OF CLOSTRIDIUM DIFFICILE

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The Applied Functional Genomics Unit at the Health Protection Agency Centre for Infections is currently completing genome sequencing of three different *Clostridium difficile* strains, including an original pathogenic strain from the 1970s, a low virulence strain from the 1980s and a newly emerging O27 ribotype strain from an outbreak in 2006. These genome sequences have provided limited insight into the factors causing the increased virulence of more recently emerging strains, suggesting that differences in gene expression and regulation may play an important role. Therefore, an understanding of the proteomes of these strains and the differences between them may be key in determining the reasons for the increased virulence of emerging strains.
Using 2D gel electrophoresis followed by Maldi-TOF MS, a proteome reference map for the three strains has been produced, and Differential In Gel Electrophoresis undertaken to allow the reference maps to be compared, and identify unique proteins and expression differences in the three strains. Furthermore, extracts were passed through combinatorial peptide libraries to search for low abundance proteins. Addition proteins have been identified using 1D gel electrophoresis followed by LC-MS-MS. In addition to identification of several novel proteins, this work emphasised the importance of using multiple techniques to improve the coverage of the proteome of microorganisms.
This study revealed the presence of a number of strain-specific proteins, or differentially expressed isoforms between strains, including a number of surface-associated proteins that may be involved in the pathogenicity of *C. difficile.*

021

TRANSCRIPTIONAL PROFILING OF *CLOSTRIDIUM DIFFICILE* AND CACO-2 CELLS DURING INFECTION

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Clostridium difficile is an anaerobic spore-forming rod-shaped gram-positive bacterium that can infect both humans and animals. Most studies on the pathogenesis of *C. difficile* have focused on its toxins and their effect on the host cells. Recently, we utilized microarrays to identify conserved and divergent genes associated with virulence in *C. difficile* isolates from humans and animals. Our data provided the first clue toward a complex mechanism underlying host adaptation and pathogenesis. Microarray technology offers an efficient high-throughput tool to study the transcriptional profiles of pathogens and infected host cells. Transcriptomes of *C. difficile* after exposure to environmental and antibiotic stresses and those of human epithelial colorectal Caco-2 cells upon TcdA treatment have been analyzed. To our knowledge, there are still no reports on the transcriptomic study of host-pathogen interactions for *C. difficile* infection (CDI). In vitro analyses of interplay between host and pathogen are essential to unravel the mechanisms of infection and to investigate the host response to infection. We therefore employed microarrays to study both bacterial and human cellular transcriptome kinetics during CDI to Caco-2 cells. Here we present a large-scale analysis of transcriptional profiles to reveal molecular determinants playing a role in *C. difficile* pathogenesis and the host response. We found that there were 254 and 224 differentially-expressed genes after CDI in *C. difficile* and Caco-2 cells, respectively. These genes are clustered according to their functional categories and their potential roles in pathogenesis and host response are discussed. Our results will not only increase our understanding on the host-pathogen interaction, but may also provide targets for drug development.

022

UNDERSTANDING THE PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE* BY AN *IN VIVO* TRANSCRIPTOMIC APPROACH.

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The virulence factors of *Clostridium difficile* have been studied since many years, but the main *in vivo* pathogenesis processes of this bacterium have to be investigated. Specially, data on the early mechanisms of *C. difficile* adaptation to the host are not yet available. The objective of our study was to improve the understanding of the pathogenesis of *C. difficile* by the analysis of the genome-wide temporal expression of *C. difficile* genes during the first hours of infection. Three groups of 4 axenic mice each were challenged by vegetative cells of the 630 *C. difficile* strain, and sacrificed at 8, 14, and 38 hours post-infection. Pure prokaryotic RNA was obtained from caecal bacteria. Comparative hybridizations on 630 microarrays were done using a cDNA issued from an 8-hours *in vitro* culture as control, with a dye swap protocol for each sample. Normalisation and statistical analysis of the data were done with different functions of the limma package. The pathogenesis of *C. difficile* could be seen as the result of the successful metabolic adaptation of the bacterium to its host, contributing to its persistence and multiplication, and the coordinate expression of virulence factors. Analysis of our data lightened some of these aspects. The results support strongly a two-steps infection model, since, during the course of infection, a significant increase in the toxins expression contrasted with a decreased expression of most of the putative colonization factors. Several paralogs of the High Molecular Weight Slayer protein are also down regulated, some of which could be strong candidates for colonization factors. Bacterial adaptation to the host microenvironment was assessed by the regulation of numerous metabolic pathways, such as the up regulation of the ethanolamine catabolic operon or the modulation of several PTS systems. Inactivation of some putative virulence factors identified by this methodology will complete this analysis.

023

COMPLEX REGULATORY NETWORKS FOR VIRULENCE IN CLOSTRIDIUM PERFRINGENS

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Clostridium perfringens produces numerous toxins and enzymes, which are responsible for the pathogenicity through synergestic actions of each toxin and enzyme on their specific targets in the tissue. Many genes for toxins and enzymes are regulated by the two-component VirR/VirS system, and many other secondary regulators including regulatory RNA molecules (ex. VR-RNA, *virT*, *virU* and *virX*) might be also involved in the regulation of virulence genes. Using genomic information of *C. perfringens* strain 13, we have designed DNA microarrays, which were used to compare the gene expression profiles between wild type and various mutants of regulatory genes. Total of >200 microarray data was obtained and analyzed using several bioinformatic methods, and numerous candidates for operons and regulons were obtained. One of the regulons was the VirR/VirS regulon including approx.150 genes regulated by VR-RNA, which implies that the VirR/VirS system regulates many genes involved not only in virulence but also in metabolism and energy production. Previously, we identified the *virX* gene as a global RNA regulator for toxin genes. From the microarray analysis, *virX* was also found to be involved in the negative regulation of virulence genes, pCP13 plasmid genes, and others, suggesting that *virX* is involved in another global regulatory network in *C. perfringens*.

O24

REGULATION OF NetB PRODUCTION IN Clostridium perfringens.

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Clostridium perfringens is the causative agent of several animal diseases, including necrotic enteritis, which results in significant economic losses to the global poultry industry. It has recently been shown that a novel pore-forming toxin, NetB, plays a crucial role in the disease process. In *C. perfringens* the production of several extracellular toxins and enzymes is regulated by the VirSR two-component system. In this regulatory network, the VirR response regulator directly controls the expression of target genes, such as the perfringolysin O gene, *pfoA*, by binding to a pair of imperfect direct repeats called VirR boxes. Sequence analysis of the region upstream of the *netB* gene has revealed the presence of potential VirR boxes. Gel mobility shift assays showed that the purified VirR protein was able to recognise and bind to the *netB* VirR boxes *in vitro*. Furthermore, analysis of the *netB* VirR boxes in a perfringolysin O reporter system demonstrated that this binding site facilitated the expression of the *pfoA* reporter gene, indicating that the VirR boxes located upstream of netB were functional. The results suggested that the production of the NetB toxin is regulated by the VirSR system. To test this hypothesis, the *virR* genes of two virulent chicken isolates, EHE-NE18 and 56, were insertionally inactivated by use of TargeTron technology. The results showed that for both strains culture supernatants from the *virR* mutants were no longer cytotoxic for LMH cells, and Western blot analysis showed that the *virR* mutations resulted in significantly reduced levels of NetB. Both effects were restored to wild-type levels when the *virR* mutant strains were complemented. These results provide good evidence that the VirSR system plays a critical role in the regulation of NetB production.

O25

IMPACT OF CARBON CATABOLITE REPRESSION IN THE C.DIFFICILE PHYSIOPATHOLOGY

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A correlation between carbon metabolism and virulence has been shown in several bacterial pathogens. The presence of glucose or other rapidly metabolizable carbon sources in a culture medium strongly represses *C. difficile* toxin synthesis suggesting a mechanism of Carbon Catabolite Repression (CCR). In Gram-positive bacteria, the CCR has developed mechanisms to take up carbon and energy sources in the most profitable and economical way. In *Bacillus subtilis,* CCR modulates expression of about 10% of all genes in response to the availability of carbon compounds. Furthermore the CCR mechanism is implicated in the regulation of virulent factors in several Gram-positive bacteria. The main regulator of CCR is CcpA. When sugars such as glucose are transported by the Phosphoenolpyruvate-dependent phosphoTransferase System (PTS), CcpA binds to a specific DNA region of the controlled target gene (the *cre* site), thereby regulating its expression. The DNA-binding activity of CcpA is enhanced by its interaction with HPr-Ser-P a phosphorylated form of HPr, a PTS component. In the *C. difficile* genome, all elements involved in CCR are present and sequences similar to *cre* sites have been found in toxin gene promoter regions. To elucidate *in vivo* the role of CCR in *C. difficile* toxin synthesis, we have recently constructed using the ClosTron technique, mutants of the major components of the CCR signal transduction pathway including PTS components (Enzyme I and HPr), the HPr-K/P and the CcpA in strain 630. Our *in vivo* results showed that the CCR is implicated in the regulation of the *tcd* genes in response to rapidly metabolizable sugars. Moreover, CcpA plays a major role in the regulation of toxin synthesis. Furthermore, we showed that purified *C. difficile* CcpA protein is able to specifically bind to the *tcd* promoter regions and that fructose-1, 6biphosphate (FBP) alone enhances CcpA binding to the *tcd* promoter in absence of HPr-Ser46-P. In order to better understand the global regulatory network of CcpA-dependent CCR and its influence on all processes implicated in the *C. difficile* physiopathology, we are currently comparing *in vivo* the global gene expression profiles between the wild type and the *ccpA* mutant in the axenic mice model. Several target functions co-regulated with toxin production by CCR and potentially involved in the different colonization steps are now investigating.

O26

ELUCIDATING THE MECHANISMS OF SPORULATION IN CLOSTRIDIUM DIFFICILE

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Clostridium difficile are Gram-positive, spore forming bacteria that are a major and increasing cause of nosocomial infections. The spores are the infective agent of the disease and are highly resilient to harsh environments. Once favourable conditions are reached, such as in the human gut, they can then germinate into vegetative cells Despite the spores of C. difficile being of major importance in the spread of disease little is known of the mechanism by which spore formation is controlled. In the Bacillus paradigm, sporulation is initiated by Spo0A which sits at the top of the sporulation cascade. Sensor kinases detect alterations in environmental conditions and autophosphorylate, the phosphate then enters a phosphorelay which leads to the phosphorylation of Spo0A. However, as is the case in all clostridia examined to date, the pathway of the initiation of sporulation in C. difficile does not correlate with this system as the two genes encoding the phosphorelay proteins are absent. This has led to the hypothesis that phosphorylation of Spo0A is directly mediated by sensor kinases. The ClosTron was used to inactivate the five orphan kinases in the C. difficile genome most likely responsible for phosphorylation of Spo0A. These mutants were analysed for a range of phenotypes including sporulation, toxin production, growth and motility. The majority of the mutations had little effect on spore formation. Inactivation of one kinase, however, resulted in a null sporulation phenotype. The comparative effect of this mutation on the transcriptome, relative to the wild type and a spo0A mutant, has been evaluated. These results will be compared and contrasted with similar studies undertaken with Clostridium botulinum and Clostridium acetobutylicum.

O27

UNDERSTANDING THE ROLE OF ANTIBIOTICS IN INDUCING CLOSTRIDIUM DIFFICILE INFECTION (CDI)

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We have used a gut model primed with pooled human faeces to simulate CDI caused by epidemic *C. difficile* strains. We have shown that antibiotics, such as cefotaxime, ceftriaxone and clindamycin, known for their predisposition to CDI promote C. difficile germination and toxin production. Conversely, neither piperacillin-tazobactam nor tigecycline induces toxin production. All three fluoroquinolnes tested (ciprofloxacin, levofloxacin and moxifloxacin) promoted *C. difficile* germination and toxin production in the gut model. In the gut model *C. difficile* ribotype 027, which is associated with epidemic spread, increased clinical virulence and poor outcome, germinates for prolonged periods with consequent extended toxin production compared with another epidemic strain (*C. difficile* ribotype 001). Hence, there is good evidence that the gut model circumvents many of the problems encountered during in vivo studies – namely confounding factors, faecal specimen collection and ethical issues associated with animal/human testing. Moreover, greater experimental control affords the investigators a level of reproducibility, which would be difficult to achieve in vivo without substantial numbers of subjects/animals. The gut model has also been successfully used to investigate the therapeutic potential of novel agents in comparison with established CDI treatment options. In summary, our studies suggest the gut model predictably reflects CDI, and thus this approach can be used to evaluate the potential of antimicrobial agents to induce CDI. We are further developing the model to understand the interaction between *C. difficile*, gut flora and inducing agents at the mucosal interface.

O28

PASSAGE OF BOTULINUM NEUROTOXIN A THROUGH THE INTESTINAL BARRIER

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Botulism is mainly acquired by the oral route, and botulinum neurotoxins (BoNT) escape the gastrointestinal tract by crossing the digestive epithelial barrier prior to gaining access to the neuromuscular junctions where they cause an inhibition of acetylcholine release. We showed that biologically active BoNT/A crosses intestinal cell monolayers via a receptor-mediated transcytosis, including a transport inhibition at 4°C and a passage at 37°C in a saturable manner within 30-60 min. BoNT/A passage rate was about 10-fold more efficient through the intestinal crypt cell line m-IC_{cl2}, than through the carcinoma Caco-2 or T-84 cells, and was not increased when BoNT/A was associated with the non-toxic proteins (botulinum complex). Like for neuronal cells, BoNT/A binding to intestinal cells was mediated by the half C-terminal domain (Hc) as tested by fluorescence activated cytometry and by transcytosis competition assay. However, BoNT/A enters intestinal and neuronal cells via a different pathway. Indeed, BoNT/A uses a transcytotic pathway in epithelial intestinal cells which delivers whole BoNT across the intestinal barrier, whereas BoNT enters motoneurons through a pathway which permits the translocation of light chain into the cytosol. We found that BoNT/A Hc is trapped into endocytic vesicles, which progressively migrate to a perinuclear area in neuronal NG108-15 cells, and in a more scattered manner in intestinal cells. In both cell types, BoNT/A Hc enters through a dynamin- and intersectin-dependent pathway, reaches an early endosomal compartment labeled with EEA1. In neuronal cells, BoNT/A Hc enters mainly via a clathrin-dependent pathway, in contrast to intestinal cells where it follows a Cdc42-dependent pathway, supporting a differential toxin routing in both cell types.

O29

BIOGENESIS OF THE *C. DIFFICILE* S-LAYER

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Clostridium difficile, like many bacteria, possesses a paracrystalline protein surface layer (S-layer), which in *C. difficile* is comprised of two proteins, the high and low molecular weight S-layer proteins (SLPs). The *C. difficile* SLPs are derived from a single precursor, SlpA, by post-translational cleavage. Interestingly, purified SLPs can reassemble *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-train differences involve single amino acid substitutionswith occasional small insertions and deletions. In contrast, the lowmolecular 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 Slayer, is not calcium-dependent. We have also identified domains within both the low and high molecular weight SLPs that are required for the formation of this complex. We have generated a high-resolution crystal structure of the surface-exposed low molecular weight SLP and a low-resolution SAXS envelope of the entire SLP complex. This represents the first structural model of the morphological unit of the *C. difficile* S-layer and the first high-resolution structure of an S-layer protein from a pathogen.

O30

CONTACT WITH ENTEROCYTES CAUSES VIR S/VIR R-MEDIATED UPREGULATION OF BETA TOXIN PRODUCTION BY *C. PERFRINGENS* TYPE C ISOLATES

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C. perfringens type C isolates cause necrotizing enteritis in humans and enteritis and toxemias in domestic animals. *In vitro*, type C isolates often produce beta toxin (CPB), beta2 toxin (CPB2), alpha toxin (CPA), perfringolysin O (PFO), and TpeL during (or after) late-log phase growth. However, toxin production kinetics in the presence of host cells have not yet been evaluated for these isolates or for other pathogenic *Clostridium* spp. This study now reports that most, if not all, type C isolates respond to the presence of enterocyte-like Caco-2 cells by producing all toxins, except TpeL, much more rapidly than occurs during *in vitro* growth. This effect involves rapid transcriptional upregulation of the *cpb*, *cpb*2, *pfo*A and *plc* toxin genes (but not *tpe*L) and requires close contact between type C isolates and intact host cells. Rapid *in vivo* induction of CPB and PFO production by type C isolates was shown to involve the VirS/VirR two-component system since Caco-2 cell-induced upregulated transcription of the *pfo*A and *cpb* genes (but not the *plc* gene) was blocked by inactivating the *vir*R gene. This inhibition was reversible by complementation to restore VirR expression. However, the *lux*S quorum sensing system is not required for the rapid Caco-2 cell-induced upregulation of type C toxin production. Animal studies, using rabbit ileal loops, demonstrated that the VirS/VirR system is required for *in vivo* CPB secretion into the intestinal lumen and to induce intestinal damage. To our knowledge, these results provide the first indication of host cell: pathogen cross-talk affecting toxin production kinetics by any pathogenic *Clostridium* spp. Since enterocyte-induced upregulation of CPB appears to be important for disease, this process may represent a potential therapeutic target.

O31

MAIN CLOSTRIDIAL DISEASES IN ANIMALS

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Clostridium difficile, a well-known pathogen in humans, is also known to be an important pathogen in animals. In horses, the bacterium and its toxin is associated with antibiotic-associated diarrhoea. Often, the diarrhoea was developed when the horses were hospitalized and treated for diseases other than gastro-intestinal at animal hospitals. Despite intensive therapy, the mortality was high. Healthy foals may by asymptomatic carriers of toxigenic strains. The organism has frequently been found in soil samples at stud farms and at animal hospitals.
Recent studies question whether *Clostridium difficile* infection is an emerging zoonosis. *C. difficile* ribotype 027 was found in faecal samples from calves and also in retail ground meat in Canada. Further, isolates of ribotype 078 were found in pigs and in humans with diarrhoea in the Netherlands and these strains were found to be highly genetically related. It is important to study further whether animals can serve as a reservoir of toxin-producing *C. difficile* causing human infections.
Clostridium botulinum type C or C/D is an emerging pathogen that causes the serious and often fatal disease botulism in poultry and wild birds in several European countries. A chimeric C/D sequence of the gene has been reported. The toxin causes flaccid paralysis. There is a fundamental lack of knowledge in this area and there is an urgent need to increase understanding of the epidemiology of the bacterium and the pathogenesis in order to prevent the disease.
Clostridium perfringens causes enteritis and enterotoxaemia in domestic and wild animals. Necrotic enteritis and subclinical disease in poultry have become serious threats to poultry health due to the diminished use of growth-promoting antibiotics. Recently, a novel toxin (netB) has been found to be associated with avian necrotic enteritis caused by *C. perfringens.*

O32

MOUSE MODELS FOR STUDYING CLOSTRIDIUM PERFRINGENS TYPE C INFECTIONS

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Clostridium perfringens type C causes enterotoxaemia and necrotizing enteritis in humans and almost all livestock species. It is believed that lethality of type C disease involves absorption of toxin(s) from the intestines to the circulation, but this has never been proved. In response, we developed two mouse challenge models that mimicked *C. perfringens* type C lethality. When inoculated by intragastric gavage, Balb/C mice showed lethality using 7 of 14 pathogenic type C isolates. Inoculated directly into the duodenum, all 14 strains produced lethality. Prior to death, clinical signs of those challenged mice included respiratory distress, abdominal distension, neurological alterations and depression. At necropsy the small, and occasionally the large intestine, were dilated and gas-filled in most mice developing a clinical response. Histological changes in the gut were relatively mild and consisted mostly of attenuation of the mucosa with villus blunting. A type C Δ*cpb* mutant showed no lethality in the oral model, while its lethality was attenuated in the intraduodenal model. However, a type C Δ*plc or* Δ*pfo*A mutant showed only a slight decrease in lethality for mice. Mice could be protected against lethality by intravenous passive immunization with a beta toxin antibody prior to oral challenge. This study provides two new mouse models for studying the pathogenic mechanism of *C. perfringens* type C-induced lethality and suggests that beta toxin is the most important toxin contributor to the systemic effects of type C infection.

O33

EPIDEMIOLOGY OF ENTEROTOXAEMIA IN FLEMISH VEAL CALVES

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The veal calf industry is a relatively unknown niche market. Mortality and morbidity in this sector was studied with a longitudinal survey on 3873 calves on 10 farms (3 Belgian Blue, 5 dairy, 2 mixed breed). Global mortality was 5.5% (212 calves). Mortality due to enterotoxaemia was 10.4% of all losses. The Belgian Blue breed was significantly more susceptible to enterotoxaemia (19.5%) than dairy (3.7%) or mixed breeds (6.3%). In the dairy and mixed breeds enterotoxaemia cases occurred sporadic during production rounds. Larger outbreaks of enterotoxaemia were recorded on all Belgian Blue farms and accounted for 15 to 28.6% of the total mortality. In two farms the outbreak occurred near the end of the round (week 26 to 30), when daily milk powder (2500 g per calf a day) and concentrate uptake was at its highest. In the other farm cases occurred at week 5 to 9 and week 12-13 and were invariably associated with sudden feed changes. At necropsy macroscopic lesions varied between mild to pronounced haemorrhagic enteritis and mixed pathologies (acute ruminitis and moderate enterotoxaemia lesions) were also present. Ruminitis was overall the third most important cause of mortality (12.3%) and the disease occurred throughout the fattening period as individual cases on most farms. On two farms, clusters (3-4 calves within one week) occurred, which could be associated with accidental concentrate overload in one farm and with a high concentration of milk powder (3000 g per calf per day) and concentrates at the end of the production round in the other. In general 44% of ruminitis cases occurred in the last 9 weeks of production. On two of the three Belgian Blue farms cases of enterotoxaemia and ruminitis occurred in the same period. Non-perforating abomasal ulceration was often present in enterotoxaemia and ruminitis cases and *C. perfringens* could be isolated from the abomasal content, usually in lower numbers than in the intestine. This longitudinal survey demonstrates the importance of enterotoxaemia in veal calves and especially in Belgian Blue calves, who receive a milk powder diet substantially higher in protein than dairy calves. The feeding regime therefore seems to be a crucial eliciting factor both for enterotoxaemia as for ruminitis but additional risk factors may play a role.

O34

PREVALENCE OF *CLOSTRIDIUM DIFFICILE* IN PIGLETS WITH AND WITHOUT DIARRHOEA IN AUSTRALIA

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Clostridium difficile is the most common cause of hospital-acquired infectious diarrhoea in humans. It has also been found in both diarrhoeal and non-diarrhoeal pigs, horses, cattle and various other species, suggesting a potential reservoir for human infection. In Canada and the USA, *C. difficile* contaminates 20-40% of meat products (beef, pork and turkey), suggesting the possibility of food-borne transmission, although this has not been proven. Little is known about the prevalence of *C. difficile* in animals, and specifically pigs, in Australia. We surveyed 3 groups of piglets for *C. difficile*, one from a herd in which neonatal scours had been a problem (A), one from a herd on the same property in which scouring had not been a problem (B), and one from a herd at a different site where scouring had been variable (C). The respective isolation rates were: A, 51/101 (50%), B, 17/29 (59%) and C, 44/54 (81%). Overall, the isolation rate was 101/154 (61%). All isolates were tested for toxin genes by PCR and the majority were found to be A negative, B positive and binary toxin positive, a toxin profile uncommon in animals from other parts of the world. Given that Australia is an island continent with very strict import regulations, and importation of live production animals does not occur, it is likely that the epidemiology of *C. difficile* disease in animals in Australia will be different to the rest of the world.

O35

CLOSTRIDIA ASSOCIATED INFECTIONS: NEW WEAPONS FROM EMERGING VACCINES?

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The incidence of Clostridia-mediated infections is increasing world-wide posing novel challenges to the scientific and medical community. The development of successful vaccine strategies against Clostridia-mediated diseases is still facing diverse and numerous problems. The variable age of population at risk (such as newborns for *C. perfringens* or elderly subjects for *C. difficile* enterocolitis), the real amount of the population requiring protection, the necessity to stimulate strong mucosal immunity, the limited knowledge on the protective antigens, and the presence of multiple toxins with several serotypes produced from the same pathogen are all issues that require further studies. Thus, new strategies under investigations include humanized monoclonal antibodies to treat patients with *C. difficile*-associated diarrhoea or immunization with multiple peptide epitopes to neutralize different *C. botulinum* toxins serotypes. An intriguing opportunity is offered by genetically engineered bacteria used as antigen delivery method to stimulate the mucosal associated immune system and successfully used so far to protect laboratory animals. Finally, for Clostridia-mediated gastrointestinal infections accessory virulence factors that could play a role in intestinal colonization have been used to protect small laboratory animals. Thus, the scientific community is devoting a great deal of efforts to develop new vaccines to prevent/treat Clostridia-mediated infections and in this review we will attempt to evaluate the clinical prospective of these new therapeutics.

O36

A LIVE RECOMBINANT ORAL VACCINE EXPRESSING *CLOSTRIDIUM PERFRINGENS* ANTIGENS CONFERS PROTECTION OF BROILER CHICKENS AGAINST NECROTIC ENTERITIS

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Currently no effective vaccine is available to control necrotic enteritis (NE) in broiler chickens, a disease caused by C*lostridium perfringens*. We previously showed that protection in broiler chickens against NE can be achieved through intramuscular immunization with alpha toxin- toxoid combination (AT) and Hypothetical Protein (HP). In the current study, we identified immunoreactive linear B-cell epitopes in these protective antigens and the immunodominant regions were cloned into pYA3493 expression vectors and the resultant plasmid constructs were then introduced into an attenuated *Salmonella* Typhimurium vaccine vector, χ9352. Expression of *C. perfringens* proteins by recombinant *Salmonella* was confirmed by immunoblotting. Protection of broiler chickens against NE was assessed at two levels of severity of challenge.
Birds immunized orally with recombinant *Salmonella* expressing AT showed significant protection against a moderate NE challenge, whereas HP immunized birds were significantly protected against both severities of challenge. Immunized birds developed serum IgY and mucosal IgA and IgY antibody responses against *C. perfringens* and *Salmonella* antigens. The degree of protection against NE was lower than observed previously after intramuscular immunization which was in part attributed to the poor colonizing ability of the vaccine strain (χ9352) used. In conclusion, this study shows the immunizing ability of two important *C. perfringens* antigens when delivered orally using attenuated *Salmonella* as vaccine vectors.

O37

CLOSTRIDIAL NEUROTOXINS AS A PLATFORM FOR NOVEL THERAPEUTICS

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Clostridial neurotoxins are the most potent lethal toxins known, and yet botulinum neurotoxins are highly effective therapeutic products. The clinical application of neurotoxin is expanding into areas distinct from its initial neuromuscular use. The therapeutic success of the botulinum neurotoxins results from their highly specific and potent inhibition of neurotransmitter release combined with a duration of action measured in months. Understanding the structure-function relationship of the clostridial neurotoxins and how this relates to their unique clinical properties, has opened up an opportunity to engineer novel recombinant proteins that employ the specific pharmacological properties of the different neurotoxin domains in the generation of novel therapeutic proteins. This understanding of the biology of the neurotoxins and its application will make available a range of therapeutic proteins for the treatment of diseases which are not amenable to therapy with the native neurotoxins, but where the particular pharmacological properties of the neurotoxin sub-units provide clinical benefit and therapeutic advantage.

O38

VACCINATION WITH PARENTERAL TOXOID B PROTECTS HAMSTERS AGAINST LETHAL CHALLENGE WITH TOXIN A-NEGATIVE/B-POSITIVE *CLOSTRIDIUM DIFFICILE* BUT DOES NOT PREVENT COLONIZATION

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One of the first clues suggesting the importance of toxin B in the pathogenesis of *C. difficile* infection (CDI) was the recognition of bona fide human cases of CDI caused by toxin A-negative/B-positive (A-/B+) CD strains. Because these strains are also virulent in hamsters, we tested the hypothesis that immunization with a toxoid preparation of toxin B would protect against challenge with the A-/B+ CD strain, CF2. All 10 hamsters challenged with 10^6 spores of CF2 five days after receiving 30 mg/kg of clindamycin by oral gavage became colonized 2 days after challenge and 9 hamsters died a mean of 4.7 days after challenge. Next, 12 hamsters were vaccinated intraperitoneally with a toxoid preparation of standard toxin B mixed with RIBI adjuvant on day 0 (10 µg toxoid), followed by boosters on day 14 (5 µg toxoid), and day 21 (5 µg toxoid). Two additional hamsters were injected intraperitoneally with the adjuvant, alone. Oral clindamycin was administered 1 week later and all hamsters were challenged with 10^6 CF2 spores five days after the clindamycin administration. All 12 hamsters vaccinated with toxoid B became colonized a mean of 1.7 days after challenge with CF2, but all remained healthy until euthanasia 30 days after challenge. The two hamsters that received adjuvant, alone became colonized 1 day after challenge with CF2 and died 6 and 16 days, respectively after challenge. Fecal pellets from all 12 vaccinated hamsters (and 1 hamster that received adjuvant only) obtained 10 days after challenge with CF2 were highly cytotoxic to human fibroblasts and the cytotoxicity was neutralized by CD antitoxin sera. Sera from 6 hamsters immunized with toxoid B+RIBI as above all neutralized the toxicity of standard toxin B in the same cytotoxicity assay. In summary, parenteral immunization of hamsters with toxoid B prevented mortality, but not colonization or fecal cytotoxin expression after challenge with a naturally-occurring A-/B+ CD strain. These data lend additional support to the importance of toxin B in the pathogenesis of CDI.

O39

ANTIBODY-BASED THERAPEUTICS FOR CLOSTRIDIUM DIFFICILE INFECTION

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Clostridium difficile infection, CDI, continues to be a major problem as a healthcare associated infection. The bacterium produces two large protein toxins (Toxins A and B) which are the principal virulence factors and the primary cause of the symptoms of CDI which can range from mild, self-limiting diarrhoea to life-threatening colitis. A substantial body of evidence suggests that an immune response to Toxins A and B provides protection against CDI in animal models. Furthermore, it has been shown that a systemic immune response alone is sufficient to afford protection against the disease. In the present study, we describe the requirements of potential antibody-based therapeutics for CDI. A toolbox of assays and techniques has been established as well as the capacity to produce polyclonal antibodies to the toxins of *C. difficile* on a large scale and to GMP quality. In the present report, we describe data demonstrating the efficacy of these antibodies as prophylactics against CDI in passive immunisation studies using an animal model. The neutralising antibody requirements against Toxins A and B to provide protection against CDI will be described and the clinical potential of antibody-based therapeutics discussed.

Abstracts of Poster Presentations

P1

USE OF *E. COLI*-EXPRESSING THE GREEN FLUORESCENT PROTEIN AS A POSITIVE PROCESS CONTROL FOR DNA EXTRACTION AND PCR DETECTION OF *CLOSTRIDIUM BOTULINUM* IN COOKED MEAT BROTH.

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The polymerase chain reaction (PCR) has been used successfully for the detection of a wide range of pathogenic microorganisms. However, false negative results, i.e. failure to detect a pathogen when present, can occur and may be due to: interference with target-cell lysis necessary for nucleic acid extraction; nucleic acid degradation; direct inhibition of the PCR. Therefore, it is essential to include appropriate controls for the application of PCR for the detection of pathogens in order that results may be interpreted correctly. We describe the use of a strain of *E. coli* containing the *gfp* gene, to monitor the complete detection procedure, from DNA extraction through to amplification and detection. This 'positive process internal control' was developed for the application of real-time PCR assays for the detection of *Clostridium botulinum* in Cooked Meat Broth (CMB) cultures. DNA was extracted using Instagene© matrix from CMB inoculated with 1.3×10^6 to 13 cfu of *C. botulinum* type A plus 10^5 cfu of *E. coli-GFP* cells. The *C. botulinum* neurotoxin A and the *gfp* genes were subsequently detected using a duplex real-time PCR assay. Consistent Ct values (30-33) for the *E. coli-GFP* were obtained across the varying *C. botulinum* concentrations enabling the detection of PCR inhibition with no loss of sensitivity in the pathogen specific PCR assay. The results demonstrate that *E. coli* containing the *gfp* gene can be used as a valuable positive process internal control to improve the reliability and the interpretation of PCR detection assays.

P2

RELATEDNESS OF HUMAN AND ANIMAL *CLOSTRIDIUM DIFFICILE* PCR RIBOTYPE 078 ISOLATES BASED ON MULTI LOCUS VARIABLE TANDEM REPEAT ANALYSIS AND TETRACYCLINE RESISTANCE.

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Recently, we reported an increase in *C. difficile* infection (CDI) caused by PCR-ribotype 078 (Type 078) in the Netherlands. *C. difficile* Type 078 is the predominant PCR ribotype in cattle. To investigate the relatedness between human and animal strains we applied a Multi Locus Variable tandem repeat Analysis (MLVA) and investigated the tetracycline susceptibility.
In total 53 Type 078 strains were available, including 42 human and 11 porcine strains. The MLVA genotyping method and minimum spanning tree analysis of the MLVA types was performed as previously described (Goorhuis et al., 2008). In total we tested 36 Type 078 strains and 9 non type 078 strains for their susceptibility to tetracycline using the E-test method according to the most recent CLSI breakpoint (2007). Tetracycline resistance was further investigated by PCR. We indentified the mobile elements Tn5937-like and Tn916-like, harboring the gene tet(M), previously shown to be responsible for tetracycline resistance in *C. difficile* strains (Roberts et al., 2001, Spigaglia et al., 2006). We used previously described primer pairs (Agersø et al., 2006) to detect the tet(M) gene and the marker genes for the mobile elements Tn916 and Tn9537.
The minimum spanning tree analysis revealed that 40 (75%) strains, encompassing isolates from human and porcine origin, were genetically related with a summed tandem repeat difference (STRD) ≤10. Six clonal complexes (CC, defined by STRD ≤2) were recognized. One CC contained both human (n=4) and porcine (n=3) strains. Twenty of the 25 (80%) tested human type 078 strains and 6 of 11 (55%) porcine Type 078 strains were resistant to tetracycline. All tested non type 078 strains were susceptible to tetracycline. All tetracycline resistant *C. difficile* type 078 strains contained the mobile element Tn916, harboring the tet(M) gene, irrespective of human or animal origin.
The MLVA revealed that type 078 strains from human and animal origin are genetically related. This observation was confirmed by the finding that both human and animal tetracycline resistant type 078 strains harbored the tet(M) gene on the mobile element Tn916.

P3

COMPARATIVE STUDY OF A MONOCLONAL ANTIBODY BASED SANDWICH ELISA WITH THE MOUSE BIOASSAY FOR DIAGNOSIS OF BOTULISM IN CATTLE.

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The number of suspect *Clostridium botulinum* clinical cases submitted to the Veterinary Sciences Division for diagnostic investigation since 2001 has risen significantly, and the number of these that have been confirmed as botulism, largely from the testing of intestinal contents, has been 35 out of 138 cases in 2003 and subsequently an average of 12 out of 77 cases per year during the years 2004-2007. The mouse bioassay remains the 'gold standard' for confirmatory diagnosis of botulism in cattle and other species but has significant disadvantages, not least animal welfare considerations. The present study was undertaken to develop a MAb based sELISA as a potential replacement for the mouse bioassay. The sELISA detects both type C and D toxins and was used to test gastrointestinal samples from suspect field cases of cattle botulism. The samples were tested directly as received and also after heat shock at 80°C for 10 minutes followed by anaerobic culture for one and five days. The sELISA results were compared with those of the mouse bioassay. Although the basic sELISA lacked sufficient sensitivity for the direct detection of toxin from most of the mouse bioassay positive samples, the application of a procedure for the enrichment culture of any spores that may be present in the test samples greatly improved the detection rate of the toxin by increasing the sample toxin levels.The results indicate that the use of this procedure for screening samples for *C. botulinum* types C and D toxins, with the mouse bioassay being used for confirmation, would improve the diagnostic rate as well as significantly reducing the number of mice involved in diagnosis.

P4

MOLECULAR CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE CLINICAL ISOLATES IN THE UNIVERSITY-AFFILIATED HOSPITAL OF PARMA

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C.difficile variant strains have been frequently involved in C. difficile-associated disease (CDI) and outbreaks in hospital settings. The research of the genes encoding for the binary toxin (CDT) was described as a good marker to detect the *C. difficile* strains with variant PaLoc. In this study, we investigated 438 clinical strains, isolated in the Section of Microbiology-University of Parma from 334 patients with CDI over a seven year period (2000-2006). All these strains were analysed by PCR to detect the genes encoding for the toxins A, B and CDT. Out of 438 clinical isolates 390 resulted toxigenic: 265 (67.9%) were non variant strains (A+B+CDT-) and 125 (32.1%) were variant (A+B+CDT+). The in vitro production of toxin A by these strains was confirmed by the commercial immunoenzimatic assay (Triage, Biosite, U.S.A.). These 438 isolates, analysed by the PCR-ribotyping method described by Bidet et al. (1999), showed to belong to 76 different ribotypes, arbitrarily named. Variant strains showed ribotypes different from those found in non-variant or non-toxigenic isolates. In particular, 118 variant strains (94.4%) belonged to ribotype 1 (subtypes 1a-1d). The ribotype 1a was predominant among variant strains (82.4%), whereas the ribotypes 13, 17, 18 and 33 were the most frequent among the non variant strains. The 125 binary toxin positive isolates were also examined by the toxinotyping method, described by Rupnik M. (2007). The majority of the strains was typed as toxinotype V (29.6%). Interestingly, 97.3% of these strains were characterized as ribotype 1. This variant clone, appeared at the end of 2000 and was isolated throughout the whole examined period of the study, with a peak from 2001 to 2003 when it was responsible for a severe outbreak involving 15 geriatric patients (Dec. 2002-Sept. 2003). Differently, a wide outbreak concerning 42 patients hospitalised in Pneumology wards (April-Sept. 2006) was caused by a non-variant strain ribotype 17. The data highlight the need of a molecular monitoring to detect significant changes in *C. difficile* clinical isolates circulating in hospital settings.

P5

MOLECULAR CHARACTERISTICS STUDY OF 12 *CLOSTRIDIUM DIFFICILE* CLINICAL ISOLATES IN CHINA*

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C. difficile has been recognized as a common cause of nosocomial diarrhoea and animal enteric disease. As an anaerobic bacterium, *C. difficile* has a pathogenicity locus (PaLoc) including genes encoding enterotoxin A (tcdA) and cytotoxin B (tcdB). Though the research of *C. difficile* may ascend from the 1970's, outbreaks continue to occur in many US, Canadian and European hospitals. While, hypervirulent *C. difficile* strain BI/NAP/027 and BI/NAP/078 had been shown to be responsible for recent outbreaks. However, the related data from China hospitals is quite limited. Our study is to make an overview of molecule characteristics of *Clostridium difficile* isolated from hospital by toxin profiles, PCR ribotyping, antimicrobial susceptibility detection. We carry out conventional PCR to detect the gene *tcdA, tcdB* of toxin A and B, *cdtA, cdt B* of binary-toxin. Results show, 8 toxic *C. difficile* strains are found out of 12 clinical isolates, in which 5 strains are *tcdA*+ *tcdB*+, and 3 strains *tcdA*- *tcdB*+, accounting for 62.5% and 37.5% respectively. Binary-toxin gene detection is negative in all the strains. Genotyping of toxic *C. difficile* are conducted by means of analysis of 16s-23s internal spacer region polymorphism with conventional PCR. 8 toxic isolates are typed into 4 gene types, the dominant type is ZR I, accounting for 62.5%. We find no isolates belonged to ribotype 027 or 078. Furthermore, we conduct antibiotic resistance of toxic *C. difficile* to Ampiciline (AC), Clindamycin (CM), Metronidazole (MZ) and Vancomycin (VA) with E-test, and detect *erm* B for clindamycin resistance. Resistance rate of 8 toxic *C. difficile* strains against AC, CM, MZ and VA was 37.5%, 87.5%, 12.5%, and 0 respectively**.** Clindamycin resistance associated gene *ermB* was positive in 4 out of 8 toxic *C. difficile* strains. From our study, we conclude that **i**solation rate of toxic *C. difficile* is high to 66.7%, and there is obvious gene polymorphism in Chinese toxic *C. difficile* clinical isolates. Though there is some resistance to ampiciline, clindamycin, metronidazole, *C. difficile* still show certain susceptivity to vancomycin.

P6

DIAGNOSTIC PROCEDURE OF A CLOSTRIDIUM BOTULINUM TYPE D INTOXICATION IN A DAIRY COW STOCK IN SAXONY-ANHALT IN SPRING 2009

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Botulism in cattle is rare, but it can have a high mortality rate and may cause considerable financial losses to the animal owner. It is often associated with the finding of carrion in feed or drinking water and results from ingestion of botulinum toxin.
In an open pen cows of all ages showed a variety of symptoms including ataxy and sternal recumbency with aggravated breathing. In some cases a reduced tonus of the tongue was noted and particularly calves seemed weak. 105 animals died or had to be euthanized. The clinical signs and the exclusion of alternative diseases raised the suspect “botulism”.
Thus, organ material of several animals (liver, ruminal and intestinal contents) as well as feed was tested via polymerase chain reaction (PCR) and mouse bioassay. *Clostridium botulinum* toxin gene type D was found in various organ samples using PCR. These results allowed us a pre-selection of samples to be tested in the mouse bioassay to reduce the number of mice to be inoculated. The mouse bioassay was positive (wasp-waist) in the chosen organ samples and the neutralization test with type-specific antitoxin confirmed the presence of toxin type D.

P7

MOLECULAR CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE ISOLATED FROM ANIMALS IN ITALY

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Clostridium difficile (CD) is an established cause of antibiotic associated diarrhoea and pseudomembranous colitis in humans but, it was also reported as a cause of serious enteritis in animals including horses, dogs, hamsters, pigs, rabbits and calves. Some recent studies have established that there is a high degree of genetic overlap between human and animal isolates and this observation suggested that CD-associated infection could be a potential zoonotic disease. In order to investigate this hypothesis, 54 CD strains isolated from intestinal content and faeces of different animal species were tested by multiplex PCR for tcdA, tcdB and binary toxin genes. The isolates were subsequently analyzed by PCR-ribotyping to investigate their genetic relatedness. To this purpose the resulting patterns of PCR-fragments obtained from field strains were compared with those of 13 reference strains (4 Italian human isolates and 9 epidemic European strains). 87% (47/54) of CD strains carried the toxin coding genes whereas 13% (7/54) were not toxigenic. Two of the 30 strains isolated from swine and one isolated from a cat belonged to the PCR-ribotype 020, 12/30 strains of swine origin and 1/10 rabbit isolates belonged to PCR-ribotype 078, one strain isolated from cattle was PCR-ribotype 126, whereas 5/10 belonged to a ribotype that has also been isolated from human in Italy. No strains belonging to the epidemic PCR¬ribotype 027 were found. These results confirm that there is a substantial genetic match between animal and human isolates and that, consequently, animals could represent a reservoir for C. difficile, transmitted to humans directly or by meat consumption.

P8

COMPARISON OF A SEMI-AUTOMATED REP-PCR SYSTEM WITH PCR-RIBOTYPING FOR TYPING OF *CLOSTRIDIUM DIFFICILE* STRAINS

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Molecular typing methods (*i.e.* REA, PFGE and PCR-ribotyping) are frequently used to detect emergence of new clones of *C. difficile* and to investigate outbreaks. This study compared a semi-automated rep-PCR method (DiversiLab™ system, bioMérieux) with PCR-ribotyping. A collection of 77 *C. difficile* strains of well-defined PCR-ribotypes was studied. This collection included 20 Belgian PCR-ribotype 027 strains, 10 Italian isolates previously characterized by PCR-ribotyping and manual rep-PCR and 37 strains from 35 hospitals in 14 European countries. Additionally, 2 strains were tested for reproducibility studies. Each isolate was cultured and genomic DNA was extracted using the UltraClean™ Microbial DNA Isolation kit. After amplification, the amplified products were separated using microfluidic lab-on-a-chip technology and analyzed using a web-based data analysis software. The modified Kullbeck-Leibler similarity calculation and Pearson correlation were used to analyse data. Electrophoregrams were interpreted by manual examination and a rep-PCR fingerprint type was defined as having ≥1 band difference.
The reproducibility (intrarun and interrun) tests gave a similarity index ranging from 93.2% to 99.9% for strain 1 and from 97.4% to 99.7% for strain
2. The discriminatory power (using Hunter and Gaston index) was 0.997 and 0.959 for rep-PCR and PCR-ribotyping, respectively. Among 10 Italian strains, 9 rep-PCR fingerprints-types were defined; rep-PCR was more discriminatory than PCR-ribotype and manual rep-PCR. Among Belgian PCR-ribotype 027 strains, different subtypes could be distinguished by semi-automated rep-PCR. In conclusion, rep-PCR showed a high discriminatory power; however visual interpretation of rep-PCR fingerprint patterns may be fastidious.

P9

GENOTYPING OF *CLOSTRIDIUM DIFFICILE* STRAINS BY MULTILOCUS VARIABLE-NUMBER TANDEM-REPEAT ANALYSIS

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Many *Clostridium difficile* genotyping methods (*i.e.* PCR-ribotyping, restriction enzyme analysis, pulsed field gel electrophoresis) are currently used for epidemiological purposes. However some of these methods lack discriminatory power, more particularly when a clone of *C. difficile* is predominant within a hospital or a country (such as PCR ribotype 027). MLVA (Multi-Locus VNTR [Variable Number Tandem Repeat] Analysis) has been successfully used for investigating *C. difficile* outbreaks and seems interesting because of its high discriminatory power (Killgore *et al.*, J. Clin. Microbiol., 2008).
The objective of this study was to evaluate the usefulness of MLVA for typing strains of *C. difficile*: 27 strains were studied including 13 strains of toxinotype III (3 historical and 10 PCR-ribotype 027 isolates from 3 different hospitals), 9 strains of toxinotype V (including 3 PCR-ribotype 078 and 3 PCR-ribotype 126) and 5 strains of toxinotype 0 (including strains from a possible transmission from a patient to a nurse). After DNA extraction, 7 tandem repeats loci (A6, B7, C6', E7, F3, G8, H9 as previously described by Zaiss *et al*. BMC 2009) were amplified by PCR and sequenced. The distance between two strains was determined by calculating the STRD (summed tandem repeat difference). Genomic diversity was evaluated by using the minimum spanning tree available in the Bionumerics 5.1 software program. Among the 27 *C. difficile* isolates examined, 26 unique MLVA-types were identified suggesting a high discriminatory power. Four clusters (defined by a STRD ≤10) were identified including 7, 4, 3 and 2 strains, respectively. A good agreement was observed between MLVA types and toxinotypes. MLVA could distinguish clusters due to PCR ribotype 027 within different hospitals. Results of MLVA also indicated that strains from toxinotype V were closely related whatever their PCR ribotypes. This highly discriminatory method is useful to investigate outbreaks and to understand molecular evolution of *C. difficile*. MLVA remains however time consuming and expensive but is an interesting tool to subtype clones of *C. difficile*.

P10

SUCCESSFUL CONTROL OF *CLOSTRIDIUM DIFFICILE* 027 IN FRANCE

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In March 2006, a large outbreak of *Clostridium difficile* PCR-ribotype 027 infections was identified in Northern France. An active surveillance of *C. difficile* infections (CDI) was implemented with a mandatory notification of severe CDI cases and outbreaks to health authorities. For each notified case, the corresponding strain had to be sent for typing to one of the 6 reference laboratories.
From March 2006 to March 2007, 41 healthcare facilities from Northern France reported 515 CDI cases. Among the 410 strains typed, 266 (65%) belonged to the epidemic clone 027. Elsewhere in France, 118 healthcare notified 347 cases of CDI, only 7% of strains (11/161) were 027. During this epidemic period, a national active prevention campaign was launched by the health authority including guidelines for diagnosis and prevention, media-based information and support to healthcare facilities to improve care organization.
From April 2007 to December 2008, 747 cases of CDI from 176 healthcare facilities were notified from 21 out of the 22 regions of France; 13.4% of CDI cases were still from North of France. A total of 955 strains were characterized : the epidemic clone 027 accounted for 15.8% (n=151). This clone was mostly isolated in Northern France (n=121, 80.7%) whereas 29 strains were isolated from 5 other regions. The proportion of 027 significantly decreased from 22.7% during April-June 2007 to 6% during October-December 2008 ($p<0.01$).
These data suggest that the large outbreak of 027 in Northern France has been successfully controlled although this strain still remains at a low level of endemicity in a few healthcare settings. This strain has been sporadically detected from other areas but large outbreaks were not reported. Mandatory surveillance was effective to follow the spread of 027 clone in France. These data will be soon completed by a national survey of CDI.

P11

MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIUM DIFFICILE* IN AUSTRALIA

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Clostridium difficile is an important nosocomial pathogen and a major cause of infectious diarrhoea in hospitalised patients. Toxigenic strains typically produce both toxin A and toxin B, the primary virulence factors of *C. difficile*. Some strains produce an additional APD-ribosyltransferase known as binary toxin. Recent epidemics of *C. difficile* infections in North America and Europe have been linked to the emergence of a hypervirulent, fluoroquinolone-resistant strain of *C. difficile* (PCR ribotype UK 027). The objective of our study was to determine the types of *Clostridium difficile* circulating in Australia. A total of 563 isolates, collected from five Australian states between 2005 and 2009, were included in the study. Of the 563 isolates, 367 were from Western Australia and 196 from Eastern Australia. All isolates were screened for the toxin A, toxin B and binary toxin genes. Of the isolates, 437 (77.6%) were toxin A-positive, toxin B-positive and 29 of these (6.6%) were binary toxin-positive, while 109 (19.4%) were toxin A-negative, toxin B-negative, including 5 that were binary toxin-positive (4.6%). There were 17 toxin A-negative, toxin B-positive isolates and 12 of these (70.6%) were binary toxin-positive. Ninety-five distinct ribotypes were identified among the 321 isolates typed by PCR ribotyping. The most common type was AU31 (UK 014) to which 60 (18.7%) of isolates belonged. Seventeen toxin A-negative, toxin B-positive strains were distributed among nine ribotypes, with only one isolate belonging to UK 017 (AU15). There were no PCR ribotype UK 027 strains identified among the isolates typed. Clinical isolates of *C. difficile* in Australia are diverse with respect to both their toxin genotype and PCR ribotype. The high level of diversity seen among Australian toxin A-negative, toxin B-positive strains is unusual.

P12

RAPID DETECTION AND DIFFERENTIATION BY MULTIPLEX PCR REAL TIME OF *Bacillus anthracis*, *Clostridium chauvoei* AND *Clostridium septicum.*

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In the present study a multiplex PCR real time with melting curves analysis is described as diagnostic, differential tool to detect anthrax, blackleg and malignant oedema. Sudden deaths of grazing ruminants such as cattle, sheep and goats that show a symptomatology related to hyperacute infective processes, made necessary the study and the setting of a rapid and precise diagnostic molecular method. Specific primers for protective antigen (PA) of *Bacillus anthracis*, for alpha toxin (haemolysin) gene of *Clostridium septicum* and for the spacer region 16S-23S rRNA of *Clostridium chauvoei* were used. The multiplex PCR was tested on a total of 22 DNAs including 4 *Clostridium chauvoei* strains, 5 *Clostridium septicum* strains, 4 *Bacillus anthracis* strains, 7 other *Clostridium spp*. and 2 *Bacillus spp*. strains. Amplifications were obtained just for DNAs of *Bacillus anthracis*, *Clostridium chauvoei* and *Clostridium septicum* strains. Amplifications of other DNAs were not observed. The authors confirmed the efficacy and the specificity of this test by the analysis of melting curves, showing the presence of 3 highly different temperatures: 72,5°C for *Bacillus anthracis*, 76,5°C for *Clostridium septicum*, 81,5°C for *Clostridium chauvoei*.

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P13

CLOSTRIDIUM DIFFICILE INFECTIONS IN A UNIVERSITY HOSPITAL IN THE CZECH REPUBLIC

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Currently, CDAD is of great clinical importance due to its increasing incidence involving a more severe course and higher mortality. However, the data regarding *C. difficile* infections in the Czech Republic are rather fragmentary. The only paper referring CDAD in a regional hospital was published as early as in 2003 (Zemanova *et al*., 2003). In our study we aimed to characterize *C. difficile* strains isolated in a General Teaching Hospital in Prague, one of the biggest health-care institutions in the Czech Republic.
During a one-year period (from April 2008 to March 2009) we isolated 27 *C. difficile* strains. The presence of toxin A and B in stool samples was determined by using the Xpect *Clostridium difficile* toxin A/B test (Remel, USA). The toxin-positive samples were cultured onto selective media with cefoxitin and cycloserin and incubated under anaerobic conditions at 37°C for 48 – 72 hours. The isolates were identified based on morphological criteria, Gram staining and characteristic odour. The identification was further confirmed by amplification of *C. difficile* specific gene *cdd3*. All of 27 strains isolated were separated into three distinct toxinotypes. The majority of the isolates, 18 (66.7 %), were toxin A negative. Our preliminary data confirmed the necessity to detect both toxins A and B in the stool sample instead of using kits based on only toxin A detection.

P14

EVALUATION OF AN ENZYME IMMUNOASSAY FOR DIRECT DETECTION OF TOXINS FROM *C. DIFFICILE* COLONIES.

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Toxigenic culture is considered as a very sensitive method for the diagnosis of *Clostridium difficile* infections. However this technique is labor-intensive when using the cytotoxicity assay from a filtrate of a five-days broth culture.
Objective: To evaluate a rapid enzyme immunoassay (ImmunoCard® Toxin A&B, Meridian, France) (ICTAB), for toxins detection directly from colonies.
Methods: In the first part of the study, we used a collection of 70 toxigenic strains including 47 strains from hospitalized patients and 23 strains previously characterized by toxinotyping (3 toxinotypes 0, 5 toxinotypes III, 3 toxinotypes V, and 12 variant A^-B^+ including toxinotype X and 11 toxinotypes VIII). These strains were subcultured on taurocholate cycloserine cefoxitin agar and colonies were tested according to the manufacturer's recommendations. In addition, titration of toxin B was performed for the 23 strains previously toxinotyped, using a quantitative *in vitro* cytotoxicity assay (IVCT) on MRC-5 cells.
In a second part, we conducted a prospective study on 105 positive fresh stool cultures. The ICTAB was performed from a suspension of at least 5 colonies and results were compared with the IVCT.
Results: Among the 70 toxigenic strains, all the toxinotypes VIII were negative by ICTAB. Interestingly, one of the strains from a hospitalized patient was also negative and was subsequently characterized as toxinotype VIII. The IVCT for these 12 strains were positive. However, the 11 strains of toxinotype VIII produced a significant lower amount of toxin than the other toxinotypes (p=0,0002). During the prospective study, ICTAB performed on colonies displayed a sensitivity and specificity of 100%.
Conclusion: ICTAB on colonies is a rapid and easy-to-perform test. It might be an alternative to IVCT for determining toxin production from isolates, except for strains of toxinotype VIII. Considering the very low prevalence of toxinotype VIII in France, ICTAB can be used for toxigenic culture.

P15

DEVELOPMENT OF A REALTIME PCR ASSAY FOR DETECTION OF CLOSTRIDIUM CHAUVOEI AND CLOSTRIDIUM SEPTICUM

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Clostridium chauvoei is the causative agent of blackleg in cattle and sheep. This severe disease is very similar to malignant edema caused by *Clostridium septicum*, other clostridia species belonging to the gas edema complex, and *Bacillus anthracis*. *C. chauvoei* infected animals are often co-infected with *C. septicum*, making it difficult to distinguish both closely related taxa using traditional microbiological methods. Thus, there is a need for a fast and reliable identification method that allows the specific detection of both species in samples. We have developed a multiplex realtime PCR-assay based on sequence data from the *spo0A*, that allows the specific detection of *C. chauvoei* and *C. septicum*. Assay validation was performed using a collection of 27 *C. chauvoei* and 46 *C. septicum* strains. Authenticity of these strains was previously confirmed by conventional PCR using published oligonucleotide primer pairs. The realtime PCR-assay was successfully tested on DNA extractions from tissue samples from blackleg cases in Austria and Germany.

P16

MULTILOCUS SEQUENCE TYPING (MLST) OF *CLOSTRIDIUM DIFFICILE* : ESTABLISHMENT OF A PUBLICLY ACCESSIBLE WEB DATABASE HOSTED AT THE PASTEUR INSTITUTE (PARIS, FRANCE).

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Clostridium difficile (CD) infection is a leading cause of pseudomembranous colitis and antibiotic associated diarrhea. Epidemiological or phylogenetic analysis are limited by the lack of availability of a robust and portable typing scheme. Sequence based typing methods, such as Tandem Repeat Sequence Typing, MLVA, or MultiLocus Sequence Typing (MLST), offer an promising solution. From our initial MLST analysis (Lemée et al., J. Clin. Microbiol. 2004), we extended the MLST analysis to a large European collection of CD isolates . The MLST scheme, targeting 7 housekeeping genes (*aroE*, *dutA*, *gmk*, *groEL*, *recA*, *sodA* and *tpi*), was applied to well characterized strains, corresponding to 57 PCR-ribotypes (PR). We identified 50 sequence types among the 358 strains and confirmed the low recombination rate in CD populations. Phylogenetic analysis revealed several divergent lineages : one corresponding to A+B+CDT+/Tox. V/PR 078 or PR 126 strains, another one to A-B+CDT-/Tox. VIII/PR 017 strains, and another one to A+B+CDT+/Tox. IV/PR 023 strains. All the A+B+CDT+/Tox. III/PR 027 strains clustered together, in a lineage closely related to the main population of CD. Binary toxin-encoding genes were found only in very divergent strains (except the PR 017 lineage) and in the "027" cluster. MLST provides unambiguous sequence data, that can be generated from various laboratories and shared in a common web database. For this purpose, a public database was made available at http://www.pasteur.fr/mlst. MLST data reflect the phylogeny of strains and lineages and will be useful for evolutionary questions as well as for global epidemiology of CD.

P17

CLOSTRIDIUM DIFFICILE RESISTANCE AND RIBOTYPES IN NORWAY AND ESTONIA.

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C. difficile is the most frequently recognized agent of antibiotic associated diarrhoea in hospitalised patients. During resent years some new emerging hypervirulent ribotypes (027 and 078) have been recognized in several countries. These strains are also associated with higher resistance to newer fluoroquinolones. Despite of overall sensitivity of *C. difficile* to metronidazole and vancomycin (antibiotics used for treatment of *C. difficile* infection) several reports describe strains with elevated MICs to these agents. The aim of our study was to detect possible regional variations in resistance and ribotype distribution and correlation between strain type and resistance patterns. *C. difficile* strains (N= 138) from adult patients with antibiotic-associated diarrhoea were collected in Estonia (4 hospitals) and Norway (1 hospital) during July – December 2008. Minimal inhibitory concentrations (MIC, 92 strains) and PCR ribotypes (98 strains) were investigated. Ribotypes were determined by gel and capillary electrophoresis and compared with ECDC reference strains. MIC ranges, medians and percentages of non-susceptible strains (defined as non wild-type population) were following: Metronidazole 0.01-1; 0.094; 6,6%; Vancomycin 0.125-1.5; 0,5; 0%; Erythromycin 0.064-246; 1; 10%; Clindamycin 0.064-246; 6; 10%; Moxifloxacin 0.25-32; 1; 11%. Strains isolated from Estonian patients as compared with Norwegian ones were more resistant to Metronidazole (17 vs 0%; p= 0.001), Erythromycin (21 vs 3,5%; p= 0.008) and Clindamycin (21 vs 3,5%; p= 0.007). Out of 98 strains 36 were characterized as known ribotypes. Ribotype 077 was most frequent (n=9). One probable 027 and one 078 ribotypes were detected. We did not find correlation between resistance pattern and ribotype. The most resistant strains belonged to different unknown ribotype groups. Out of 5 typed fluoroquinolone resistant strains, 2 were identified as ribotypes 020 and 077.

P18

INVESTIGATION OF A MINITN*10* TRANSPOSON DELIVERY SYSTEM IN *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile is the leading cause of nosocomial antibiotic-associated diarrhoea causing a spectrum of diseases from mild-self limiting diarrhoea to life threatening pseudomembranous colitis. The ability of this Gram-positive organism to produce potent cytotoxins and form spores is known to play a significant role in pathogenesis. Recently, some progress has been made in our ability to genetically manipulate this organism; this includes targeted gene disruption systems by derivatisation of the targetron technology and homologous recombination to generate gene knock outs. However there remains a need to generate random mutants in the *C. difficile* genome and work in our laboratory has shown that the conjugative transposon Tn*916* will insert apparently at random in the *C. difficile* genome. As the frequency of insertion is low (approximately 1 transconjugant per 10^7 cells), we investigated the use of a derivative of the composite transposon Tn*10*, miniTn*10.* This has been used to generate stable, high frequency insertion mutants in *Bacillus subtilis*. Here we describe the construction and testing of a delivery vector for miniTn*10* for *C. difficile.*

P19

DETECTION AND DIFFERENTATION OF CLOSTRIDIUM PERFRINGENS IN DIFFERENT KIND OF FOOD AND OUTBREAK SITUATIONS

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The incidence of toxiinfections caused by *C. perfringens* enterotoxin in Germany is largely unknown, as most cases of foodborne *C. perfringens* diseases are self-limited with mild and temporary symptoms and the affected people do not seek medical treatment. But
C. perfringens outbreaks are often associated with the catering in hospitals and nursing homes and therefore the risk of a severe progress of the toxiinfection for immunocompromised people in such institutions is given. For a well-founded risk assessment and a protective consumer protection policy it is important to have information about the prevalence of enterotoxigenic *C. perfringens* strains in different kinds of food of animal or vegetable origin and also in outbreak situations. Therefore qualitative and quantitative cultural methods in combination with real-time-PCR-assays for the detection of the *cpa*, *cpe*, *etx*, *cpb1*, *cpb2* and *iap* gene and the differentation of the *cpe*-gene (chromosomal or plasmid-located) were designed in our laboratory for a efficient screening of large numbers of samples and for differentiation of the *C. perfringens* isolates of food and human origin in the different toxinotypes. Enterotoxigenic (*cpe*-gene positive)
C. perfringens strains were not only found in food samples of animal origin but also e. g. in dried mushrooms or in tea. But the number of potential human-pathogenic strains in food samples of vegetable origin always was very low compared with numbers found in food of animal origin or associated with foodborne outbreaks. The results of our investigations in the last two years will be presented.

P20

PREVALENCE OF ENTEROTOXIN GENE CARRYING CLOSTRIDIUM PERFRINGENS FROM RETAIL MEAT PRODUCTS IN JAPAN

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Clostridium perfringens is an important anaerobic pathogen causing food-borne gastrointestinal (GI) diseases in humans and animals. It is thought that *C. perfringens* food poisoning isolates typically carry the enterotoxin gene (*cpe*) on their chromosome, while isolates from other GI diseases, such as antibiotic-associated diarrhea, carry *cpe* on a transferable plasmid. However, food-borne GI disease outbreaks associated with *C. perfringens* isolates carrying plasmid-borne *cpe* (plasmid *cpe* isolates) were recently reported in Japan and Europe. To investigate whether retail food can be a reservoir for food poisoning generally, we evaluated Japanese retail meat products for the presence of two genotypes of enterotoxigenic *C. perfringens*. Our results demonstrated that, approximately 70% of Japanese retail raw meat samples were contaminated with low numbers of *C. perfringens*, and 4% were contaminated with *cpe*-positive *C. perfringens*. Most of the *cpe*-positive *C. perfringens* isolates obtained from Japanese retail meat carried *cpe* on a plasmid. The plasmid *cpe* isolates exhibited lower spore heat resistance than did chromosomal *cpe* isolates. Collectively, these plasmid *cpe* isolates might be causative agents of food poisoning when these isolates contaminate foods from equipment and/or the environment after cooking or they may survive in food that has not been cooked at a high enough temperature.

P21

PREVALENCE OF *CLOSTRIDIUM* SPP. ISOLATED FROM DIARRHEOIC AND HEALTHY DOGS IN THE AREA OF PARMA (ITALY)

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The aim of this epidemiological study was to evaluate the prevalence of *Clostridium* spp. among diarrhoeic and non-diarrhoeic dogs in the area of Parma (Italy). Particular attention was addressed to *C. perfringens* and *C. difficile* isolation, since these microorganisms have been associated with acute and chronic large and small bowel diarrhoea, and acute hemorrhagic diarrhoeal syndrome in the dog.
Several dogs were from shelters (n=38), others were privately-owned dogs (belonging to students or staff of the Veterinary Medicine Faculty of Parma) (n=47), and another 10 dogs were patients at the Faculty Veterinary Hospital.
Ninety-five faecal samples (36 diarrhoeic and 59 non-diarrhoeic dogs), collected over an 8 month period were analysed by culture assay. Sixty-two (65.3%) were positive for one or more *Clostridium* spp. A total of 89 different *Clostridium* strains were identified. *C. perfringens* was the most common *Clostridium* detected (15.7%), followed by *C. bifermentans* (13.5%), *C. clostridiiforme* (13.5%), *C. fallax* (12.4%), *C. beijerinckii/butyricum* (11.2%), *C. difficile* (11.2%), and *C. septicum* (9.0%). Other *Clostridium* species were isolated in lower percentage, ranging from 2.3% to 1.0%.
Concerning *C. perfringens* isolation, 6 of the 14 positive faecal samples (42.9%) belonged to diarrhoeic dogs. In the case of *C. difficile*, 80% (8 of 10) of faecal samples, positive by culture assay, belonged to dogs with enteritis. In particular, the majority of *C. difficile* isolates (6/10, 60%) were toxigenic (*tcdA*+/*tcdB*+) and possessed *cdtA* and *cdtB* genes. All faeces tested by EIA were negative. On the contrary, all PCR-positive strains were positive for *in vit*ro toxin production.
Further investigation (detection of toxin production by immunological assays and/or the genes revelation by PCR assays for *C. perfringens* strains; ribotype profile determination for *C. difficile* isolates) should be carried out to assess properly the full implications and the epidemiological meaning of these findings in faecal samples of diarrhoeic and non-diarrhoeic dogs.

P22

RAPID DETECTION OF *CLOSTRIDIUM DIFFICILE* USING THE BD GENEOHM SYSTEM

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With the increasing prominence of *Clostridium difficile* as a cause of gastrointestinal illness world-wide, there has been greater emphasis given to improving laboratory diagnostic algorithms. Initially there were some technical issues with detecting *C. difficile* in faecal samples using PCR. Now these have been overcome and several products are available commercially for the first time. In this study, we compared one such product, the BD GeneOhm Cdiff system, with toxigenic culture. A total of 305 faecal samples submitted for investigation for *C. difficile* were tested. Approx. 20% of samples came from Royal Perth Hospital and another 20% from Sir Charles Gairdner Hospital, the two major teaching hospitals in Perth, WA. The remaining 60% of samples came from patients in the community and/or regional hospitals in WA. The BD GeneOhm method was performed as per the manufacturer's recommendations and culture was done using traditional CCFA. An enrichment broth culture was also used to enhance detection of *C. difficile*. Overall there were 31 (10%) positives by any method. Comparing BD GenOhm to toxigenic culture, the sensitivity, specificity, PPV and NPV were 86.4%, 99.3%, 90.5% and 98.9%, respectively. Seven additional isolates were recovered using enrichment culture versus direct plating on CCFA. Comparing BD GenOhm to enrichment broth culture, the sensitivity, specificity, PPV and NPV were 65.6%, 99.3%, 90.5% and 96.5%, respectively. There were no "unresolved", "invalid assay run" or "not determined" results. We concluded that the BD GeneOhm Cdiff system was rapid, sensitive and specific compared to toxigenic culture.

P23

MOLECULAR CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE STRAINS RESPONSIBLE FOR INFANT INTESTINAL COLONIZATION

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Clostridium difficile is often present in the infant intestinal tract during infancy. However, this colonization is rarely associated with Clostridium difficile-infection. The aim of this study was to analyse the biodiversity and the virulence factors among these *C. difficile* strains.
C. difficile strains were isolated from stools in healthy infants less than one year old from two French hospitals. The strains were characterized 1/ phenotypically according to the toxigenic culture, the antibiotic susceptibility profile and the cytotoxicity assay on MRC5 cells; 2/ genotypically by PCR-ribotyping, multilocus sequence typing (MLST: *aroE, dutA, gmk, groEL, recA, sodA*, and *tpi*) and PCR detection of toxin genes (*tcdA, tcdB, cdtA, cdtB*).
Forty-one *C. difficile* strains were isolated from 108 infant. Ten strains were toxigenic by both toxigenic culture and cytotoxicity assay. This was confirmed by the presence of both *tcdA* and *tcdB* genes among these ten strains. No *tcdA-/tcdB+* strain was detected. Among the toxigenic strains only one was binary toxin positive.
Concerning the genotypic characterization, the 41 strains were divided into five different PCR-ribotypes which differed from the most common ribotypes found in adult isolated strains. The phylogenetic analysis was done by MLST. Sequence types of infant strains were compared to those isolated in adult and animal populations.

P24

VARIABLE FLUOROQUINOLONE (FQ) RESISTANCE BETWEEN EMERGING NORTH AMERICAN (NA) AND EUROPEAN ISOLATES OF *CLOSTRIDIUM DIFFICILE* (CD) REA GROUP DH (PCR RIBOTYPE 106)

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Recent CD outbreaks in Europe and the United Kingdom (UK) have been linked to CD strains characterized as PCR ribotype 106, PFGE type NAP11, and restriction endonuclease anaylsis (REA) group DH. Isolates collected in 2008 and 2009 have shown the first emergence of CD REA Group DH detected by our laboratory in North American sites. REA Group DH has been isolated from diarrheal patients in the East, West, and Central NA. The predominant subtype in NA is REA Type DH7 (12/19 unique patient samples), whereas the predominant subtypes isolated from samples from the UK are REA types DH2 and DH4. There is currently no overlap between DH subtypes isolated in Europe and in North America. FQ use (specifically ciprofloxacin) has been shown to be a risk factor for type 106 infection in the UK. REA subtypes DH1 and DH2 from the UK are resistant (defined as MIC>32ug/ml) to the FQs levofloxacin (LV), and moxifloxacin (MX), and PCR ribotype 106 isolates from Sweden are resistant to MX. North American subtypes DH7 and DH10 have moderate MICs to levofloxacin (12ug/ml) and are susceptible to MX (2ug/ml). *gyrA* and *gyrB* gene mutations have been linked to FQ resistance in CD. Amplification of the quinolone resistance-determining regions (QRDR) of *gyrA* and *gyrB* (Dridi et al, AAC 2002; 46: 3418-3421) in North American REA types DH7 and DH10 showed no *gyrA* and *gyrB* sequence differences between REA type DH10 and Strain 630 (REA type R23 susceptible to MX at $\leq$4 ug/ml). Single point mutations in the *gyrA* and *gyrB* genes of type DH7 were found; however, there were no alterations of the extrapolated amino acid sequences compared to strain 630. REA group DH strains have now appeared in patient populations across NA but thus far lack high-level FQ resistance. The emergence of DH types in NA where they were previously not found bears continued vigilance for possible emergence of a new epidemic strain in NA that is currently present in Europe and the UK.

P25

DIFFERENTIATION OF CLOSTRIDIUM BOTULINUM PHYLOGENETIC GROUPS BASED ON THE SEQUENCE OF rnpB

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Clostridium botulinum strains express different neurotoxins i. e. serotype (A-G). Furthermore, strains are grouped into the so called phylogenetic groups I-IV. Traditionally, only the identification of the toxin type is done by mouse bioassay or PCR. However, for the differentiation of the phylogenetic background a specific and simple diagnostic method covering all groups is still lacking. Recent developments enable the PCR identification of proteolytic strains (group I) using the fldB gene, and differentiating between group I and II strains using the flagellin gene FlaA. Additional targets that may be useful for the differentiation of the groups I-IV are conserved regions and genes that are ubiquitous to bacteria. The 16S rDNA has been widely used for phylogenetic analysis of bacterial species and strains by sequencing. Since there are several copies per genome which may represent different alleles, PCR amplification and sequencing of parts of 16S rDNA harbours uncertainties and sequencing of at least 500 bp are recommended. A possible alternative target to the 16S rDNA is the rnpB gene which is conserved in bacteria. rnpB is the RNA component of ribonuclease P, an enzyme complex that processes tRNA. Its advantage compared to the 16S rDNA is its length of only 350 bases and the fact that it is a single copy gene.
We sequenced the rnpB locus of strains of all four phylogenetic groups Sequence alignments revealed only slight differences among serotypes, but greater differences between the phylogenetic groups. The obtained results are in agreement with data obtained by sequencing the 16S rDNA. Therefore, we conclude that sequencing of the rnpB gene easily allows the identification of the phylogenetic group of an isolate of *C. botulinum*.

P26

CLOSTRIDIUM DIFFICILE – VARIANT MORPHOTYPES AND CAPSULE PRODUCTION

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The formation of a bacterial capsule is thought to contribute to the virulence of a range of pathogenic bacteria by inhibiting phagocytic uptake into cells of the innate immune system. In this study we characterised the ability of *Clostridium difficile* to produce capsule positive and negative variants and found that capsule status was linked to colony morphotype.

A collection of twenty-one isolates of *C. difficile* of varying ribotypes taken from spore stocks were cultured onto Brain Heart Infusion Agar, Columbia Horse Blood Agar and Cycloserine Cefoxitin Fructose Agar and incubated at 37°C for 48 h under anaerobic conditions. Variant colony morphotypes were subjected to Gram and capsule staining and were sub-cultured to determine the stability of the phenotype.

Culture on all media yielded two colony morphotypes, large 4-6 mm in diameter, ground glass, grayish-white, irregular colonies comprised the predominant morphotype while the minor morphotype consisted of smaller <2 mm diameter round colonies with a glossy appearance. Microscopic examination revealed that the predominant colonial type contained encapsulated Gram positive rods. In contrast colonies of the minor morphotype contained Gram positive, filamentous rods with no visual evidence of capsule formation. Colonies were further characterised by electron microscopy. Subculture of the minor morphotype resulted in reversion to the conventional morphotype whereas the major morphotype bred true. The conventional morphotype represented the commonest colony type accounting for 60-90% of the population depending on the culture medium.

Capsule expression is common to all of the strains examined in this study. Occasional capsule deficient variants can occur, the frequency of which appears to be affected by the composition of the culture media.

P27

PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICILE* RIBOTYPES IN IRELAND, MARCH 2009

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A one-month national surveillance, typing and antimicrobial susceptibility study of *C. difficile* infection (CDI) was performed in Ireland in March 2009. *C. difficile* was isolated from toxin-positive faecal samples provided by 139 patients in 28 hospitals nationwide. Isolates were characterised by ribotyping and presence of virulence factor specific genes (*cdtB* and *tcdC*). Ribotypes were assigned by comparison to representatives in the LUMC library, Leiden. Antimicrobial susceptibility of 12 antibiotics was measured by the E-test method. The minimum inhibitory concentration of each isolate for all antibiotics was assessed, the MIC_{50} and MIC_{90} calculated and sensitivities of different ribotypes compared.

Ribotyping analysis showed that cases of CDI in hospitalised patients in Ireland were due to a wide variety of ribotypes. The most predominant, Type 027, comprised 21% of all isolates. Other common Types; 106, 078, 044, 014 and 001 were also represented (in ascending order of most prevalent respectively). Vancomycin susceptibility was similar across all ribotypes (MIC Range 0.19-1.5µg/ml). A ribotype-specific response to metronidazole was seen, with Types 106 exhibiting higher MIC's than the other common types. Fluoroquinolone resistance was also common in all ribotypes although Types 014 and 078 exhibited lower MIC_{50} values to moxifloxacin and levofloxacin than other types.

Thirty-three percent of ribotype 027 isolates demonstrated resistance to clindamycin. Clindamycin resistance was also found in ribotypes 044 (11%), 001 (11%) and 078 (7%).

In Ireland, circulating *C. difficile* strains are susceptible to metronidazole and vancomycin, but fluoroquinolone resistance is common. Clindamycin resistance was found in certain ribotypes, but most common in Type 027.

P28

COMPARISON OF FOUR TYPING METHODS FOR *CLOSTRIDIUM DIFFICILE*

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C. difficile infections continue to spread worldwide and appear to be increasing in severity. Accurate strain typing is critical for understanding the changing epidemiology of this organism and, if rapid, could be valuable for tracking hospital outbreaks in real time. 350 isolates of *C. difficile* from patients in 7 clinical centers in the U.S. and Canada were collected, confirmed biochemically, and typed using PCR-ribotyping (PCR-R), pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA), and the Cepheid Xpert C. diff test (Xp). Xp typed strains as "presumptive 027/NAP1/BI" (Xp+) based on the binary toxin gene and a deletion at base 117 in *tcdC*; results were reported in 47 minutes directly from stool. Other methods were as previously described. Of 350 isolates, 244 (70%) were assigned PCR-R types; 235 (67%) REA types; 171 (49%) PFGE types; and 97 (28%) were presumptive 027/NAP1/BI by Xp. The remaining isolates were unknown types (PCR-R, PFGE, Xp) or non-designated patterns (REA). There were 18 different PCR-R types, 8 REA types (with multiple subtypes), and 6 PFGE (NAP) types. 027/NAP1/BI isolates were detected in all 7 centers. There was complete agreement among the 4 typing methods for 83 027/NAP1/BI isolates. Xp 027/NAP1/BI results matched at least 2 of the 3 other methods 92% of the time. Four additional strains were 027/NAP1/Xp+ but not BI, and 2 were 027/BI/Xp+ but not NAP1. Only two 027 strains were not NAP1, but both were BI. There was overlap of strain types among the 3 methods. Five PCR-R types contained multiple REA types, and three NAP profiles contained multiple REA and PCR-R types. Among the more consistent profiles were 001/NAP2/J, 078/NAP7/BK, and 106/NAP11/DH. In summary, 027/NAP1/BI strains were widespread in North America. PCR-R, REA, and PFGE are all useful for typing, although they each provide a different pattern of strain clustering. Using >1 method increases strain differentiation and may help illuminate the changing epidemiology of *C. difficile.* The Xp assay was the only test that reported strain types directly from specimens. Rapid detection of 027/BI/NAP1 strains may be useful for tracking in-hospital or between-hospital outbreaks of *C. difficile* in real time.

P29

TYPING CLOSTRIDIUM DIFFICILE STRAINS BASED ON TANDEM REPEAT SEQUENCES

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Genotyping of epidemic *Clostridium difficile* strains is necessary to track their emergence and spread. However, current *C. difficile*-genotyping approaches often suffer from lack of interinstitutional reproducibility and can therefore obscure epidemiologic investigations. This report presents results from a systematic screen for variation in repetitive DNA in the genome of *C. difficile*. We describe two tandem repeat loci, designated 'TR6' and 'TR10', which display extensive sequence variation that may be useful for sequence-based strain typing. Based on an investigation of 154 *C. difficile* isolates comprising 75 ribotypes, tandem repeat sequencing demonstrated excellent concordance with widely used PCR ribotyping and equal discriminatory power. Moreover, tandem repeat sequences enabled the reconstruction of the isolates' largely clonal population structure and evolutionary history. DNA sequencing provides inherently portable data that may be stored in databases accessible over the internet, obviating the necessity for the exchange of reference strains. We therefore conclude that the analysis of the two repetitive loci may be highly useful for epidemiological studies on *C. difficile*.

P30

MOLECULAR EPIDEMIOLOGY OF C. difficile ISOLATED FROM RIVERS

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The most important reservoir for *Clostridium difficile* are hospitals and hospitalized symptomatic patients, but bacterium can be present also in animal hosts and in soil, water and food. Increasingly reported community-associated cases suggest that environment other than hospital can be potential source of infection. The aim of our study was to screen for the presence of *C. difficile* in Slovenian rivers, to characterize isolates with PCR ribotyping and toxinotyping and to compare genotypes with human and animal types. Altogether 55 water samples from 25 rivers were collected from November 2008 to May 2009 from various parts of Slovenia. Sampling points are included in the national surface water monitoring scheme ensuring the proper geographic coverage. Water was filtered using 0,45 µm pore size membrane filter (MicroPlus-21 ST, Whatman) and then cultured on *Clostridium difficile* selective agar plates with addition of 5mg/l of lysozyme and 0,1 % sodium cholate to improve the spore germination. After incubation one or more colonies were transferred to a fresh blood agar plate and pure cultures were subsequently toxinotyped and PCR ribotyped. Remaining bacterial growth was swabbed from the filter, used for DNA isolation and tested with Real-Time PCR for the presence of tcdB gene. Out of 55 river water samples 18 tested positive for *C. difficile* and altogether 52 isolates were obtained. Only 52 water samples were screened also with Real-Time PCR. The results of molecular analysis correlated with culture only in 38 samples (both tests positive or both tests negative), while 9 samples tested positive only with Real-Time PCR and 5 samples were positive only in the culture. Most of 52 isolates were toxinogenic and belonged to toxinotypes 0 and IV. Only 10 isolates were nontoxinogenic. Nineteen PCR ribotypes were found, 9 of them were identical to PCR ribotypes found in humans and/or animals in Slovenia. Only 6 samples contained more than one genotype. Our results show that *C. difficile* can be often isolated from rivers and that genotypes prevalent in patients seem to prevail also in the environment.

P31

SEQUENCING OF ALPHA- AND BETA2- TOXIN FROM C. PERFRINGENS STRAINS ISOLATED FROM RABBITS

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Rabbit enterocolitis is a gastrointestinal syndrome appeared in France since 1996, has high mortality rates (30-80%) and spreads very rapidly. There is clear experimental evidence that this disease is produced by C. perfringens. 71 strains strains, isolated from the intestine of sick rabbits, were collected from different rabbit livestocks throughout Germany (including 3 from Italy). All contain the α-gene and 25 also the β2-gene. Therefore all belong to type A. The α-toxin genes were isolated and sequenced, including their immediate upstream and downstream surroundings. The upstream region contains all elements necessary for expression but no transcriptional termination signals were found, suggesting the α-toxin gene to be member of an operon. The deduced amino acid differences within the 71 α -toxins are low and restricted to certain areas. All the functionally important amino acid residues are conserved in all the 71 α–toxins. Therefore, it can be concluded that all α-toxins are equally well active. Gilbert et al., 1997, showed that β2 toxin-producing strains are associated with animal diseases, therefore, the β2-toxin gene, cpb2, was analyzed as well. After finding the correct PCR primers for the gene amplification, it could be shown that from the 25 β2-toxin genes 16 belong to the so-called “porcine”- and 9 to the “non-porcine”-type. All the “porcine”- and 5 of the “non-porcine”-type genes were sequenced. They contain all regulatory elements necessary for transcription. 15 of the 16 “porcine”-type β2-toxin genes display an A-deletion within the putative signal peptide region, leading to a stop codon after 9 amino acids. Only one sample has an undisturbed ORF and should therefore be expressed as a complete protein. This is for the first time, that a “porcine-type” β2-toxin gene isolated from Clostridia grown in non-porcine animals. Only 5 of the 9 non-porcine β2-toxin genes were sequenced completely. All display an undisturbed open reading frame. The deduced amino acid differences within each β2-toxin-type are very low, although being marked between the two groups. From only the nucleotide sequence data no prediction can be made for the expression of the two β2-toxin genes. Therefore, they were cloned into a bacterial expression vector, expressed as fusion proteins offering the possibility to purify them. After immunizing animals, anti-β2-toxin-specific antibodies should become available to clarify the problem of the expression of the β2-toxin genes. Summarizing all the data on all toxin-specific genes, due to their low sequence variability, the development of a toxoid vaccine against rabbit enterocolitis should become feasible.

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IS α-CLOSTRIPAIN REQUIRED FOR THE PATHOGENESIS OF MYONECROSIS MEDIATED BY *Clostridium perfringens*?

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α-clostripain is an extracellular cysteine protease initially discovered in *Clostridium histolyticum.* A similar protease has also been identified in *Clostridium perfringens*, and has been shown to function in a biochemically analogous manner to its *C. histolyticum* homologue. Although previous studies have shown that mutations in the α-clostripain structural gene (*ccp)* altered the turnover of secreted extracellular proteins in *C. perfringens*, the role of α-clostripain in virulence was not characterized. We insertionally inactivated the *ccp* gene using TargeTron technology to construct a strain that is phenotypically non-proteolytic on skim milk agar. Quantitative protease assays confirmed the loss of protease activity. This activity was restored when the mutation was complemented with a wild-type *ccp* gene. These results reinforce the conclusion that α-clostripain is the primary extracellular protease produced by this *C. perfringens* strain. In addition, the role of α-clostripain in virulence was assessed in a mouse myonecrosis model. Preliminary data suggested that mutation of the *ccp* gene did not alter the progression and development of the infection, implying that α-clostripain is not essential for the virulence of *C. perfringens*. We have previously observed similar results with both collagenase and sialidase mutants. These results contrast with studies on other bacterial cysteine proteases, such as SpeB and YopJ from *Streptococcus pyogenes* and *Yersinia pestis* respectively, which have been shown to be essential for virulence. It is possible that other putative spreading factors secreted by *C. perfringens*, such as sialidases, hyaluronidases and collagenase, in combination with each other and/or α-clostripain are required for virulence.

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DETECTION OF *NETB* GENE IN *CLOSTRIDIUM PERFRINGENS* FIELD STRAINS ISOLATED FROM FARMED ANIMALS

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NetB toxin is a *Clostridium perfringens* (CP) β1-like toxin that was reported to be critical to the development of necrotic enteritis (NE) in chicken. The aim of this study was to investigate the diffusion of N*etB* gene among CP strains isolated from some farmed animal species. 418 CP strains were analyzed: 106 isolated from chickens (30 affected by NE, 54 affected by other enteric diseases and 22 healthy subjects) 99 from cattle, 112 from pigs and 100 from rabbits. All strains were toxinotyped and *NetB* gene was detected by means of PCR protocols. In addition β2 (*cpb2*) and enterotoxin (*cpe*) coding genes were investigated. All CP strains isolated from chickens resulted toxinotype A and 26.17% of these was positive also for β2 toxin gene. No strains were positive for enterotoxin coding gene. 27.10% of CP was *NetB* positive and 93.10% (27/29) of these strains were isolated from birds affected by intestinal disorders. 16 *NetB* positive strains were obtained from chickens affected by NE (16/30), 9 from subjects affected by other intestinal disorders (9/54) and 4 from healthy birds (4/22). A significant difference between the number of *NetB* positive strains isolated from birds affected by NE and healthy chickens was demonstrated (p=0.014). The difference was more evident (*p*=0.0079) if NE was produced without the synergism of coccidia. However, the percentage of positivity in NE isolates was only 53.3% and, in addition, even if in low percentage (17.4%), *NetB* gene was also detected in CP obtained from animals which did not have evidence of NE at necropsy. All CP strains isolated from cattle, swine and rabbits resulted type A and *NetB* negative. In conclusion, the examination of the occurrence of *NetB* among strains isolated from healthy and NE affected chickens revealed that there is a significant correlation between the presence of this gene and the appearance of NE even if the isolation of *NetB* positive strains also from healthy chickens, allow to consider that also other pathogenic mechanisms could be involved in the pathogenesis of NE. The absence of *NetB* genes in CP strains isolated from other animal species seems to confirm that this toxin is not involved on the appearance of enteric diseases in cattle, swine and rabbits. Furthermore the presence of *NetB* positive CP only in chickens sustains the hypothesis that these strains are strictly adapted to this animal species.

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CLOSTRIDIUM PERFRINGENS EPSILON TOXIN IS CYTOTOXIC FOR HUMAN RENAL TUBULAR EPITHELIAL CELLS

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Natural disease due to epsilon toxin (ETX) is most frequently seen in ruminant. However, different animal species as mice, rats, sheep, goats and cattle are, at some extent, equally susceptible to the lethal effects of ETX. In humans, there is not information about the susceptibility to ETX although the extreme potency of this toxin also makes it a bioterrorism concern. Since the few cell lines shown to be susceptible to ETX were originally obtained from kidneys, primary cultures of epithelial cells obtained from human kidney were examined for ETX cytotoxicity. Human renal tubular epithelial cells (HRTEC) were isolated from kidneys removed from different adult patients undergoing nephrectomies, characterized and used between three and five passages. The incubation of HRTEC with activated ETX produced a reduction of cell viability in a dose- and time-dependent manner. The binding of ETX to a specific antibody completely inhibited the cytotoxic effect on HRTEC viability. Cells exposed to ETX showed a marked swelling with subsequent large blebs surrounding most cells. The blebs appeared translucent, containing no cell organelle. The pulse with ETX for only 1 min did not change the cell viability compared with control cells, after 1 h of incubation. When cells were pulsed for 3 and 5 min with ETX the cell viability decreased to 63 ± 2% and 55 ± 1% respectively. Samples with ETX-treated and control non-treated HRTEC were analyzed by SDS-PAGE. A ladder of bands was observed with a major band at about ~160 kDa and a smaller at ~30 kDa. Human umbilical vein endothelial cells (HUVEC) were not sensitive to ETX. Levels of ETX necessary to induce changes in human cells were in the range of concentrations necessary to induce morphological alteration in MDCK cells. These results show that human kidney derived epithelial cells are sensitive to ETX and that cytotoxicity is probably produced by the mulitmeric ETX forming a membrane pore as it was previously described in other cell types.

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ULTRASOUND INDUCED EFFECT ON HEMAGGLUTININ-FREE BOTULINUM NEUROTOXIN TYPE A

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Susceptibility of the botulinum neurotoxins (BoNTs) to different chemical and physical agents has been widely investigated, both in relation to the potential applications in food safety and to the promotion of efficient defence strategies. However, a few data exist on the ultrasound (US) susceptibility of BoNTs. US refers to sound waves with a frequency of □20 kHz; they may affect biological systems by thermal effects and/or by cavitation, i.e. the implosion of gas bubbles resulting from regions of pressure change within a medium. Because of these effects, US is currently applied in a number of processes, including food technologies and clinical applications.
The main objective of this study was to analyse the US susceptibility of BoNT/A, which is one of the toxin serotypes more commonly involved in human botulism, and the most frequently used for therapeutics. A commercially available preparation of hemagglutinin-free BoNT/A was subjected to increasing times of sonication, using a sonicator system operating at 20 kHz. The temperature of each BoNT/A sample was measured during the US treatment. Samples were then tested for residual toxicity by the standard mouse bioassay. Our preliminary results show that 50 U of hemagglutinin-free BoNT/A were effectively inactivated after 20 sec exposure to US waves delivered at 0.375 W/cm2 intensity, pulsed-mode 1ON-15OFF, 31% amplitude, and 90 J/cm2 energy density. The temperature of the US-treated BoNT/A samples ranged from 27.3°C to 31°C. The modest temperature increase indicates that the US inactivation of BoNT/A under the experimental conditions used in this study was likely due to cavitation more than to heat generation. Further experiments with hemagglutinin-complexed BoNT/A preparations are ongoing.

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AUTOPROTEOLYTIC CLEAVAGE OF *C. DIFFICILE* TCDA IS NOT ESSENTIAL FOR ITS BIOLOGICAL FUNCTION

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Clostridium difficile Toxin A (TcdA) and B (TcdB) show autoproteolytic activity to release their glucosyltransferase domain into the cytosol of target cells. The present study shows that extracellular cleavage of the catalytic domain leads to functional inactivation of TcdA and TcdB. TcdA, however, was less susceptible to inositol hexakisphosphate induced cleavage than TcdB. MALDI-MS analysis defined the cleavage site of TcdA between Leu^{542} and Ser^{543}, which is in accordance with TcdB. To analyze the impact of cleavage in more detail, we generated mutant TcdA (Ala^{541} Gly^{542} Ala^{543}) that was resistant to autoproteolytic cleavage. Although the specific glucosyltransferase activity of mutant TcdA was identical to that of wild type TcdA, the EC_{50} regarding cytopathic effect was about 75fold reduced, whereas the cytotoxic effect, i.e. activation of caspase-3, was identical. Thus, extracellular cleavage inactivates TcdA and TcdB, whereas intracellular cleavage, at least for TcdA, is not a prerequisite for its biological activity. Since the non-cleavable mutant did not release the glucosyltransferase domain into the cytosol, we observed a recovery of cells in pulse experiments. These pulse experiments revealed that complete recovery of cytopathic effect without induction of the cytotoxic effect was achieved as long as the pool of non-glucosylated Rac1 was not less than 20% of total Rac1.
In addition, we found that conformational changes of TcdA induced at pH 5.0 accelerated the poor autoproteolytic cleavage of full length toxin. In contrast, the cysteine protease activity of only the cytosolic part of TcdA (aa 1-1065), however, is enhanced at pH 7.4. From these data it can be concluded that TcdA is more resistant to autoproteolytic cleavage under environmental conditions in the intestine than TcdB. Efficient release of the glucosyltransferase domain of TcdA can only be achieved after translocation into the cytosol of host cells, hence separated from the C-terminal part of the protein.

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EXPRESSION OF RECOMBINANT CLOSTRIDIUM PERFRINGENS BETA-TOXIN FOR CELL TOXICITY ASSAYS.

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C. perfringens beta-toxin has been identified as the major virulence factor in *C. perfringens* type C and B induced necrotizing enteritis. Based on pathomorphological and immunohistochemical findings we hypothesized that beta-toxin targets intestinal endothelial cells to induce vascular necrosis and subsequent hypoxic tissue damage, which in turn would lead to ideal growth conditions for the causative agent itself. Research in the field of beta-toxin target cell interaction is hampered by the lack of suitable *in vitro* systems which represent target cells from naturally affected host species and organ systems. Additionally, beta-toxin is rapidly degraded in bacterial culture supernatants and has been proven difficult to purify. Most common sources of beta-toxin are *C. perfringens* type C or B strains, which however secrete additional toxins that could interfere with cell culture based toxicity assays. Thus, knowledge on cellular and sub-cellular effects of beta-toxin on potential target cells is still largely lacking. To be able to exclude synergistic or confounding effects of additional, co-purified, clostridial toxins from *C. perfringens* type C culture supernatants we used the pET System (Novagen) for cloning and expressing recombinant beta-toxin in *E. coli*. This system allowed expression of inactive beta-toxin as a Nus•Tag™ fusion protein. After thrombin induced cleavage of the Tag protein, full length recombinant beta-toxin was purified and used in subsequent cell toxicity assays. Recombinant beta-toxin showed marked cytotoxicity towards porcine endothelial cells *in vitro.* Toxic effects, as measured by histomorphology, cell viability assays, and immunofluorescent staining were comparable to those induced by semi-purified beta-toxin derived from *C. perfringens* type C culture supernatants. Our results indicate full biological activity of recombinant beta-toxin and demonstrate the usefulness of this system for the expression of highly active which are otherwise difficult to purify, such as *C. perfringens* beta-toxin. Our system will allow further mechanistic studies on cellular effects of beta-toxin.

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CLOSTRIDIAL TOXINS INDUCE CHANGES OF PROTEINPROFILES IN COLONOCYTES

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Toxin A and B are the causative agents of *Clostridium difficile*-associated diseases (CDAD) like diarrhoea and pseudomembranous colitis. These toxins glucosylate and inactivate Rho-GTPases leading to reorganization of the actin cytoskeleton and cell rounding. Toxin-induced morphological changes are accompanied by only few alterations in mRNA level. Hence, we started to analyse the proteomes of toxin-treated and untreated colonic cells. Proteins were labelled using the ICPL technique that is based on heavy and light stable isotopes and enables the relative quantification of proteins. Toxin A-treated and untreated Caco-2 cells were lysed and extracted proteins were labelled with the heavy or the light ICPL reagent. Labelled proteins were combined, separated by SDS-PAGE and the whole gel lane was cut into 35 pieces which were processed by tryptic digestion. Formed tryptic peptides were separated by nanoscale RP-LC and directly fractionated onto a MALDI target. Peptides were analyzed off-line in an Ultraflex MALDI-TOF/TOF MS (Bruker Daltonics) and identified and quantified using WARP-LC software (Bruker Daltonics) and the MASCOT search engine. Proteins were extracted from untreated and toxin A-treated colonic cells separated at protein and peptide level and subsequently identified and quantified by LC-MALDI-MS. Overall 495 different proteins were identified and for 286 proteins (58%) regulation factors were calculated. In control experiments similar protein extracts were labelled 1:1 with ICPL and regulation factors were clearly below 1.2 showing the capability of the ICPL approach. Thus, proteins exhibiting a regulation factor exceeding 1.4 were termed regulated. Nine proteins were up-regulated and only one protein was down-regulated after toxin A treatment. Among these proteins Fructose-biphosphate aldolase A, Tubulin beta-2A and Actin-related protein 2 play a role in actin filament organisation and regulation of cell shape. Fructose-biphosphate aldolase A is also involved in glycolysis. The up-regulation of these proteins may be linked with the effects of toxin A-induced cytoskeletal reorganisation. Applying the ICPL technique we were able to identify several proteins that have not yet been recognized to be involved in toxin A-triggered cellular changes.

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THE FUNCTION OF THE H_{CN}-DOMAIN OF CLOSTRIDIAL NEUROTOXINS

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The family of clostridial neurotoxins (CNTs) consists of tetanus neurotoxin (TeNT) and the seven botulinum neurotoxin serotypes (BoNT/A-G). They are composed of four functionally independent domains that perform individual tasks in the intoxication process. The functions of the 50 kDa catalytically active light chain, the 50 kDa translocation domain H_N and the C-terminal 25 kDa H_{CC}-domain binding to two neuronal receptors are resolved but the role of the 25 kDa H_{CN}-domain remains unclear.
Deletion or substitution of the H_{CN}-domain may provide insight into its function. Crystal structures of BoNT/A, BoNT/B and TeNT holotoxins and biochemical data were used to define the sequence comprising the H_{CN}-domains. At first, mutants of BoNT/A, BoNT/B and TeNT were generated either lacking their H_{CN}-domains or containing H_{CN}-domains from one of the other two serotypes. Their residual biological activity was tested using the mouse phrenic nerve hemidiaphragm (MPN) assay. Replacement of the H_{CN}-domain in TeNT by H_{CN}A and H_{CN}B or its deletion similarly reduced the biologic activity by about 95 %, whereas BoNT/A and B deletion mutants displayed less than 0.3 % activity. Swapping H_{CN}-domains between BoNT/A and B does not impair their activity, but substitution with H_{CN}T clearly decreased the biological activity. Additionally, the H_{CN}-domain of BoNT/A was substituted with non clostridial peptides of similar size like the 26 kDa green fluorescent protein (GFP), the 21 kDa dihydrofolate reductase (DHFR) or a 12 amino acid long linker. Whereas the 12 amino acid linker construct behaves like the BoNT/A deletion mutant, the substitution of the H_{CN}-domain by the rigid structure of GFP or the flexible structure of DHFR harmed the activity of BoNT/A much more.
In conclusion, the exchange of H_{CN}-domains between different BoNT serotypes does not influence their functionality indicating that serotype specific neuronal receptor interactions are not mediated via this domain. Although structurally similar, the H_{CN}-domain of TeNT cannot equally substitute those of BoNT and vice versa, leaving the possibility that H_{CN}T plays a different role in the intoxication mechanism of TeNT. Proteins of similar size but different folding which could act as rigid spacer do not harmonize with the surrounding translocation domain H_N and the H_{CC}-domain suggesting that H_{CN} is more than just a luxury spacer molecule.

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EFFECTS OF *CLOSTRIDIUM PERFRINGENS* NETB ON THE SMALL INTESTINES OF CHICKENS.

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Necrotic enteritis is one of the most common bacterial diseases that affects the poultry industry worldwide. The disease occurs when the bacterium, *Clostridium perfringens* proliferates to high numbers in the small intestine of birds and produces extracellular toxins that damage the gastrointestinal epithelium. Our previous studies demonstrated that NetB is necessary for a type A isolate to cause necrotic enteritis in chickens. We have now used chicken intestinal ligated loops to investigate the effects of purified toxin in disease. Treatment of ligated loops for 6 h with purified NetB, in the presence of trypsin inhibitor (TI), resulted in significant luminal fluid accumulation and characteristic microscopic lesions, while no damage was observed in loops containing NetB or TI alone. The damage caused by NetB was prevented by pre-incubating purified NetB with a NetB polyclonal antibody. In addition, log-phase vegetative cultures of wild-type EHE-NE18 and isogenic *netB* and *plc* mutants were also tested in this model. The results provide further evidence that NetB alone is sufficient to induce necrotic lesions in chickens and advances our current understanding of *C. perfringens* virulence factors in avian necrotic enteritis.

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THE ROLE OF TOXINS IN *CLOSTRIDIUM DIFFICILE* INFECTION

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Two toxins, TcdA and TcdB, have been described as the main virulence factors in *Clostridium difficile* infection. They are members of the clostridial glucosylating toxin family, which comprises some of the largest known bacterial toxins. The toxins enter the cytosol of target cells via receptor-mediated endocytosis and then act by transferring glucose onto Rho GTPases, inactivating them. This causes the disruption of various signalling pathways, leading amongst others to changes in the organization of the actin cytoskeleton and ultimately to cell death. We constructed stable single and double mutants of *tcdA* and *tcdB* to investigate the relative importance of each of these two major toxins in pathogenesis of *C. difficile* infection. In recent years so-called 'hypervirulent', epidemic strains of *C. difficile* have emerged, which reportedly produce more toxin A/B than the 'non-epidemic' strains. In addition to their individual roles it is of interest to compare toxin activity *in vitro* and *in vivo* between these two types of strains. Many of the epidemic strains carry a third toxin, a two-component ADP-ribosyltransferase (CDT). Little is known about the importance of this toxin in infection. Combinations of toxin mutants, including a complete toxin knock out (triple mutant, *cdtA* or *cdtB, tcdA, tcdB*) will help to elucidate its role. Generated toxin mutants are being characterised, analysed and tested *in vitro* (including cytotoxicity assays) and *in vivo* using the hamster infection model.

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CONJUGATIVE PLASMIDS IN CLOSTRIDIUM BOTULINUM

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Clostridium botulinum produces seven distinct serotypes of the most lethal neurotoxins called botulinum neurotoxins (BoNTs). Five neurotoxin subtypes have been identified within serotypes A, B, E and F. The genes encoding different subtype neurotoxins of serotypes A, B and F were recently discovered to reside on large, highly homologous virulence plasmids in several proteolytic strains of *C. botulinum* including dual neurotoxin producing strains, suggesting that intra-species transfer of the toxin bearing plasmids may occur. To test this hypothesis, the virulence plasmids pCLK (strain Loch Maree) and pCLJ (strain 657Ba) were 'tagged' with the erythromycin resistance gene (*ermB*) using the ClosTron mutagenesis system. The BoNT/A3 gene of pCLK and the BoNT/B gene of pCLJ were inactivated by integration of the intron element containing the ErmB Retrotransposition-Activated Marker (RAM), resulting in the 'tagged' plasmids pCLK-Erm and pCLJ-Erm. Transfer of pCLK-Erm and pCLJ-Erm from the donor strains Loch Maree and 657Ba to a nontoxigenic recipient *C. botulinum* strain (LNT01) was evaluated in mating experiments. The nontoxic *C. botulinum* strain LNT01 was chosen as the recipient because the presence of a tetracycline-encoded conjugative transposon Tn*916* in this strain provides an excellent means for the selection of transconjugants. The plasmid transfer to the transconjugants was confirmed by pulsed-field gel electrophoresis and Southern hybridization analyses. Transfer was shown to require cell-to-cell contact and was DNase resistant. This suggests that transfer of these plasmids occurred via a conjugation mechanism. This is the first evidence supporting conjugal transfer of two virulence plasmids in *C. botulinum*, and provides a probable mechanism for the widespread distribution of virulence plasmids in other *C. botulinum* strains. The potential transfer of *C. botulinum* virulence plasmids to other bacterial hosts in the environment or within the human intestine is of great concern for human pathogenicity and necessitates further characterization of these plasmids.

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SUB-LETHAL DOSES OF EPSILON TOXIN INDUCES NEURODEGENERATIVE CHANGES, IN THE MOUSE BRAIN

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Clostridium perfringens type D enterotoxaemia is a disease that affects predominantly sheep and goats . The main histological changes observed in the brain are perivascular oedema which, occasionally, progresses to focal symmetric encephalomalacia. In both, experimental models and spontaneous outbreaks, lethal concentrations of this toxin affects the endothelial cells in the brain vasculature, inducing oedema and sudden death. It has been reported that sub-lethal doses of this toxin induces neurological changes through a massive neurotransmitter release. However, data on the effects of sub-lethal epsilon toxin doses is still scant. This study characterizes the histological and ultrastructural changes in the brain of rats and mice after sub-lethal doses of epsilon toxin. Groups of 4 mice were inoculated intravenously with sub-lethal doses of epsilon toxin (5, 0.5, 0.25, 0.125, 0.06 LD_{50}) or vehicle and four Sprague Dawley rats were injected via the right lateral ventricle, with either epsilon toxin (0.05 LD_{50}) or vehicle. In mice, the experiments lasted to 96 hours, in which some animals were given a toxin booster. Rats were euthanized 4h after inoculation. All animals were kept under standard housing condition throughout the experiment. In some animals, behaviour parameters were recorded. Animals were either perfused intracardiacally with 10% formalin or 2% glutaraldehyde, and the brains were removed and processed for haematoxylin and eosin stainings, Fluorojade-B, immunohistochemistry to detect active caspase-3 and transmission electron microscopy (TEM). Several toxin-inoculated mice –but no control- exhibited sporadic convulsive episodes after the inoculation until the euthanasia. Both, haematoxylin & eosin and Fluorojade-B stainings revealed shrunken degenerative neurons, with chromatolysis and axon swelling. These changes were seen predominantly in the hippocampus, cortex, striatum, and brain stem. At the ultrastructural level, increased neurofilaments in neurons and glial processes between pre- and post-synapses were observed. These results provide evidences of synaptic disruption observed at the EM level, which are in concordance with previous morphologic observations in CNS after sublethal epsilon toxin doses. The presence of glial processes between pre and post synapses may be an attempt of the CNS to block a massive neurotransmitter release.

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MOLECULAR ANALYSIS OF AN EXTRACHROMOSOMAL ELEMENT CONTAINING THE C2 TOXIN GENE DICOVERED IN *CLOSTRIDIUM BOTULINUM* TYPE C

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Clostridium botulinum cultures are classified into seven types, types A to G, based on the antigenicity of the botulinum neurotoxins (BoNTXs) produced. Of these seven types, only types C and D produce C2 toxin in addition to the neurotoxin. The C2 toxin consists of two components designated C2I and C2II. The genes encoding the C2 toxin components have been cloned, and it has been stated that they might be on the cell chromosome. The present study confirmed by using pulsed-field gel electrophoresis (PFGE) and subsequent Southern hybridization that these genes are on a large plasmid. The complete nucleotide sequence of this plasmid was determined by using a combination of inverse PCR and primer walking. The sequence was 106,981 bp long and contained 123 potential open reading frames (ORFs), including the *c2I* and *c2II* genes. The 57 products of these ORFs had sequences similar to those of well-known proteins. It was speculated that 9 of these 57 gene products were related to DNA replication, 2 were responsible for the two-component regulatory system, and 3 were sigma factors. In addition, a total of 20 genes encoding proteins related to diverse processes in purine catabolism were found in two regions. In these regions, there were 9 and 11 genes rarely found in plasmids, indicating that this plasmid plays an important role in purine catabolism, as well as in C2 toxin production.

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ESTABLISHMENT OF MOLECULAR METHODS FOR THE DETECTION OF CLOSTRIDIUM BOTULINUM AND TOXINS DIRECTLY IN FOOD STUFF

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Clostridium botulinum are gram-positive, spore-forming anaerobic bacteria occasionally responsible for food intoxication. The disease botulism is a rare paralytic illness caused by botulinum neurotoxins (BoNT). Seven major and serologically distinct BoNT's (BoNT type A to G) have been identified up to present. Three of these toxins are considered to be pathogenic for humans (A, B and E) the others are discussed to cause botulism of animals e.g. of horses, cattle and chicken. BoNT's are able to block the acetylcholine release at the neuromuscular nerve endings, which leads to flaccid paralysis and can end with death. BoNT's are the most poisoning microbial toxins for humans with a LD_{50} of 0,001 µg/kg body weight of concentrated toxin after intravenous application. Under natural conditions *Clostridum botulinum* is able to contaminate different meat and fishery products, cans and other food stuffs. To improve cultural and molecular detection methods milk, minced meat and beans where artificially contaminated with 10^2until 10^4cfu/g respectively ml. After homogenisation of the food samples blood agar and egg-yolk agar plates were incubated for 24 hours at 37°C under anaerobic conditions. Enrichment of contaminated food samples in Selzer bouillon for 24 hours at 37°C was performed in parallel. DNA extraction and Multiplex PCR for the detection of toxin types A, B, E and F was performed according to Lindström et al. [2001]. Additionally a second single PCR for the detection of toxin types A to F was performed according to Takeshi et al. [1995]. A detection limit of 100 cfu in 100 ml of suspension could be achieved by cultural and molecular PCR methods. Further research is needed to improve DNA extraction procedures directly from food stuff without enrichment.

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PHOSPHORYLATION OF RAC1 AND CDC42 AT SERINE-71 ATTENUATES THE TOXIN-INDUCED CYTOPATHIC EFFECT

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Clostridium difficile toxin A and B (TcdA and TcdB) and lethal toxin (TcsL) from *Clostridium sordellii* belong to the family of clostridial glucosylating toxins (CGTs). These toxins are single chain proteins glucosylate GTPases of the Rho family. Whereas TcdA and TcdB glucosylate RhoA/B/C, Rac1 and Cdc42, TcsL modifies Rac1 and H-/K-/N-/R-Ras. Glucosylation leads to functional inactivation and subsequent breakdown of the actin cytoskeleton. Since Rac1 and Cdc42 are phosphorylated by the Akt1 kinase, the question was adressed whether phosphorylation of at serine-71 affects toxin-catalyzed glucosylation.
We generated the phosphomimetic mutant of Rac1 and Cdc42 (Rac1 S71E/ Cdc42 S71E) to study the effect phosphorylation at serine-71 on the TcdA- and TcsL-catalyzed glucosylation. Whereas wild type Rac1 and Cdc42 were glucosylated in a time-dependent manner, the phosphomimetic mutants of both GTPases were about 5-fold poorer substrates for TcdA. Alike, Rac1 S71E was a poor substrate for TcsL although only about 2-fold reduced compared to wild type Rac1. A possible protective effect against glucosylating toxins was analyzed in transfection experiments. Hep-2 cells transfected with Rac1 S71E were partially protected against the TcdA-induced cytopathic effect compared to Rac1 transfected cells. Hep-2 cells transfected with the constitutive active Rac1 (Rac1 Q61L), which is not substrate for TcdA, were also partially protected. In addition, Cdc42 S71E reduced the effect of TcdA on cell morphology although to a lesser extent than Rac1 S71E. Similar results were observed when Rac1 S71E transfected cells were treated with either TcdB or TcsL.
In conclusion, toxin-catalyzed glucosylation of phosphorylated Rac1 and Cdc42 is attenuated, resulting in an increased pool of functional competent Rac1 and Cdc42 for maintaining signal transduction.

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GENETIC MANIPULATION OF *C. DIFFICILE* USING A SUICIDE VECTOR

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Several studies have reported transfer of genetic material to the clostridia using shuttle vectors. However, this approach results in a detectable frequency of gene knock-out phenotype reversion due to instability of the disrupting construct. The aim of this study was thus to generate stable gene disruptions in *C. difficile*. To achieve this, we constructed a suicide vector which successfully integrates into the chromosome of *C. difficile*. We genetically engineered pJIR1456, which has a chloramphenicol/ thiamphenicol resistance gene and a replication gene from *C. prefringens (repCP)* by inactivating *repCP* with a 2.6kb internal fragment of the *tcdB* gene from a ribotype 027 strain of *C. difficile* (BI-1/NAP1). This construct was introduced into the *E. coli* strain CA434, which was then mated with the *C. difficile* BI-1 strain using conjugations assays. A crossover homologous recombination event resulting in *tcdB* gene disruption was assessed by selecting transconjugants on tetracycline and thiamphenicol.

Antibiotic resistant transconjugants were then further characterized using multiple tests. PCR amplification results for junctional fragments indicated successful chromosomal recombination of the suicide construct. Further, multiple, independently isolated transconjugants were grown to saturation, and culture supernates tested in cytotoxicity assays that also confirmed that *tcdB*-disrupted strains produced significantly less toxin B compared to the non-manipulated parent strain. Importantly, all transconjugants tested were found to retain the integrated suicide plasmid in the chromosome after several passages under antibiotic pressure. This new suicide vector can, therefore, be added to existing tools to stably and efficiently disrupt *C. difficile* genes.

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CELL ENTRY OF BOTULINUM NEUROTOXIN TYPE C IS DEPENDENT UPON TWO CARBOHYDRAT BINDING SITES

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The seven botulinum neurotoxin (BoNT) serotypes cause muscle paralysis by selectively cleaving core components of the vesicular fusion machinery inside motoneurons. Their extraordinary activity relies on the highly specific uptake by neuronal cells. Initially they bind to complex gangliosides via a conserved binding pocket which has so far been characterized in detail only for BoNT/A, B, E, F and G. An additional interaction with a protein receptor is required for neurotoxin uptake as shown for BoNT/A, B, E, F and G. The BoNT/B and G protein receptor binding site is located in the neighborhood of the ganglioside binding pocket at the tip of their cell binding domain. Information about corresponding sites is lacking for the remaining BoNTs. Here, we report the identification and characterization of the canonical ganglioside binding pocket and of a second receptor binding site for BoNT/C. Substitution of W1258 and Y1259 as well as of Y1179, A1200, and L1203, the latter three residues being predicted to be located at a surface region that corresponds to the protein receptor binding sites of BoNT/B and G, drastically decreased toxicity at mice phrenic nerve preparations. Results of CD-spectroscopic analyses evidenced that these effects were not due to mutation evoked structural alterations. Interaction studies employing immobilized gangliosides revealed that not only mutation of the canonical ganglioside binding pocket but also of the second interaction site considerably impaired ganglioside binding. Furthermore, the activity of BoNT/C-W1258L, a mutant exhibiting a largely deactivated canonical ganglioside binding pocket, at phrenic nerves from mice deficient in ganglioside biosynthesis was about 400-fold lower compared to its activity at phrenic nerves from wildtype mice. Only the existence of a second physiologically relevant ganglioside interaction site in BoNT/C explains this observation. Thus, this study delineates a variant double receptor scenario without a direct neurotoxin protein - protein receptor interaction.

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INVOLVEMENT OF MAP KINASES IN CLOSTRIDIUM DIFFICILE TOXIN A-MEDIATED TNF-α PRODUCTION BY MACROPHAGES

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Clostridium difficile toxins A and B (TcdA and TcdB) are the causative agents of antibiotic-associated pseudomembranous colitis and elicit the production of immune mediators in epithelial and immune cells. In *C. diffcile* colitis, tissue macrophages may well become exposed to TcdA and TcdB. Macrophages are key sources of inflammatory mediators and TNF-α is one of the central mediators of inflammation and plays a critical role in host response to infection and injury. Previously, we demonstrated a crucial role of the glucosyltransferase activity of *C. difficile* toxins in the induction of TNF-α in macrophages. In this study we further investigated the mechanism of TcdA-induced TNF-α production by Raw 264.7 macrophages. Both p38 and ERK were activated within 3 hr of exposure to TcdA. Exposure of cells with either MEK kinase inhibitor (PD98059) or p38 kinase inhibitor (SB 203580) resulted in a partial reduction of TNF-α synthesis, whereas inhibition of both kinases simultaneously abrogated TNF-α production. PD98059 and SB 203580 did not affect TcdA-mediated Rac1 glucosylation, but inhibited toxin-induced ERK and p38 activation, respectively. SP600125, a JNk inhibitor, had no effect on TcdA-induced TNF-α production. These findings suggest that TcdA-mediated TNF-α production in macrophages is dependent on both MEK/ERK and p38 kinase signaling pathways. Immunofluorescence staining of p65 showed that TcdA induced nuclear translocation of NF-kB in macrophages. Moreover, sulfasalazine, an NF-KB inhibitor, inhibited TcdA-induced expression of TNF-α, indicating that NF-KB signaling events are involved in TcdA-induced TNF-α synthesis. Finally, knock down of RhoA, but not Rac1 with small interference RNAs stimulated TNF-α synthesis in macrophages. Consistent with this result, the exposure of Raw 264.7 cells to c3-exoenzyme (Rho inhibitor) led to TNF-α production. In summary, our data demonstrated that the inactivation of RhoA by *C. difficile* toxin A induced the activation MEK/ERK and p38 signaling pathways and NF-κB translocation, leading to the production of TNF-α in macrophages.

P50

BIOLOGICAL EFFECTS OF C-TERMINAL REPETITIVE DOMAIN OF CLOSTRIDIUM DIFFICILE TOXIN B

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Clostridium difficile produces three toxins, two of them, namely toxin A (enterotoxin, TcdA) and toxin B (cytotoxin, TcdB) are recognized as the main virulence factors responsible for diarrhea and antibiotic associated colitis. TcdB and TcdA are 63% homologous and are large (270 and 308 kDa) single chain proteins with three functional domains. Early and more recent studies have shown that the C-terminal repetitive domain is responsible for binding to host cell receptors, while cytotoxic effect were associated with the N-terminal enzymatic region. Recombinant N-terminus TcdB microinjected into cells had comparable effects as holotoxins and glycosylated small GTPases leading to changes in cell cytoskeleton and cell death. Although the C-terminal repetitive region of TcdA was recently shown to stimulate immune cells, it is not known whether TcdB C-terminal repetitive region has any significant effect on epithelial cells. We have cloned and expressed 74,080 and 73,104 kDa fragments representing the C-terminus domain from two *C. difficile* strains carrying TcdB with a 2 % difference in the aminoacid sequence in this region. Human intestinal epithelial cells were treated with holotoxins or recombinant purified TcdB fragments for 24 to 120 hours and we evaluated a) cytotoxicity by the MTT colorimetric assay, 2) cytoskeleton integrity by fluorescein-labeled phalloidin staining in epithelial cells, 3) tight-junction integrity by measuring transepithelial resistance. Both TcdB repetitive domains induced cell death after 3 days, that further increased after 5 days exposure, although their effects were significantly lower than in cells treated with holotoxins. By confocal microscopy we observed changes in cytoskeleton structure following 3 days exposure to recombinantTcdB repetitive domains. These changes were associated with decrease in transepithelial resistance. In summary, we report for the first time cytopathic effects of C-terminal repetitive region in TcdB which was so far implicated only in the enzymatic domain, suggesting that different domains of these large clostridial toxins may exert several biological effects.

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AN INTENSIVE YET ACCURATE PIPELINE FOR THE IDENTIFICATION OF PROTEIN MARKERS IN CLOSTRIDIUM BOTULINUM USING MASS SPECTROMETRY AND BIOINFORMATICS

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Clostridium botulinum is one of the most toxic species mankind has ever witnessed. It causes a rare form of food poisoning called Botulism that is caused by the ingestion of foods containing the neurotoxin formed during growth of the bacteria. *Clostridium botulinum* and its spores are widely distributed in nature, they occur in both cultivated and forest soils, bottom sediments of streams, lakes, and coastal waters, and in the intestinal tracts of fish and mammals, and in the gills and viscera of crabs and other shellfish. Because of its severe toxic effects, it has also been used as a biological warfare agent which intensifies the need for its rapid and accurate identification.

In mass spectrometry, rapid identification of bacteria is achieved by detecting the masses of unique biomarkers for each bacterium. Currently, proteins are the most reliable form of biomarkers for the detection of these bacteria. In this study, we have used mass spectrometry together with detailed *in silico* techniques to create an intensive yet accurate pipeline for the detection of protein biomarkers in *Clostridium botulinum*.

After the bacterial samples have been prepared and the proteins digested, the proteins are identified by mass spectrometry. The list of peptides is then analysed using various *in silico* techniques for the identification of unique peptides.

After parsing the data and importing it to the database, a separate non-redundant reference database (NCBI NR and in house database) is created. Each of our query sequence is searched against the reference database using the sequence matching algorithm BLAST. Using scripts, all query sequences that are genus or species markers are selected. A comparative proteomics tool is then used to compare the different unique peptides identified from different *Clostridium botulinum* strains and biological replicates. The peptides that are present in all strains and replicates are then selected as the biomarkers.

We have implemented a full script that performs the *in silico* procedures automatically. We hope to optimize this even further for a more rapid identification.

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GLOBAL GENE REGULATION IN RESPONSE TO CYSTEINE AVAILABILITY IN CLOSTRIDIUM PERFRINGENS

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Cysteine has a crucial role in cellular physiology and its synthesis is tightly controlled due to its reactivity. In *C. perfringens*, the two-component system, VirR-VirS, controls expression of several toxin genes and also the ubiG operon involved in methionine to cysteine conversion (1). Very little is known about sulfur metabolism and its regulation in clostridia. We performed a reconstruction of sulfur metabolism in *C. perfringens* and correlated these data with the growth of the strain 13 in the presence of various sulfur sources. Surprisingly, *C. perfringens* can convert cysteine to methionine by an atypical still uncharacterized pathway. By transcriptomic experiment, we further compared the expression profiles of strain 13 after growth with cysteine or homocysteine (cysteine starvation). 177 genes were differentially expressed. We found genes involved in sulfur metabolism controlled by premature termination of transcription via a T-boxcys system (*cysK-cysE*, *cysP1* and *cysP2*) or a S-box motif (*metK* and *metT*). Several transporters for cystine (CysP1-CysP2) and methionine (MetT) were identified. We also showed that the *ubiG-mccBAluxS* operon was submitted to a double regulation by the VirR-VirS, VR-RNA and VirX network (1, 2) and by cysteine availability via a T-box system.
Interestingly, expression of genes involved in [Fe-S] biogenesis and in the maintenance of the cell redox status was induced during cysteine starvation. The synthesis of 2 cysteine desulfurases and 2 scaffold proteins for [Fe-S] assembly and the expression of genes encoding a ferredoxin and 2 rubredoxins were up expressed after growth with homocysteine. Additionally we identified a new regulator responding to cysteine availability, Cpe1786. This repressor shares similarities to CymR from *Bacillus subtilis* and IscR from *Escherichia coli*. Finally, the expression of pfoA (□ toxin), nagL (one of the five genes encoding hyaluronidases) and genes of the fermentation pathways was differentially expressed in response to sulfur availability. Phenotypic tests and metabolic profiles are currently performed to confirm the transcriptomic data.

1) Banu et al (2000). Microbiology 35, 854; 2) Ohtani et al FEMS lett, 2002. 209, 113

P53

TRANSCRIPTOMICS OF A SIGD MUTANT STRAIN OF CLOSTRIDIUM BOTULINUM

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The alternative sigma factor σ^D has been shown to be responsible for the transcription of multiple genes in many bacterial species. These genes include late flagellar structural and regulatory genes and chemotaxis genes. The ClosTron method of insertional gene inactivation was successfully performed on the $\square^D$ encoding gene *sigD* in *Clostridium botulinum* Hall A. The resulting mutant was shown to be deficient in swarming motility when compared to the wild type strain indicating that the gene has an effect on flagella mediated motility. RNA samples were collected and purified from both the wild type and *sigD* mutant strains at four growth points. These samples underwent comparative microarray to determine the effects of the insertional inactivation on the transcription of every gene in the *Clostridium botulinum* genome. After analysis of the data it would appear that the biggest differences in transcription between the wild type and mutant strains occurred at late exponential growth phase. The genes downregulated in the *sigD* mutant when compared to the wild type were similar to those reported to be transcribed using $\square^D$ in *Bacillus subtilis*. In addition to these flagellar and chemotaxis genes several other genes of interest appear to be transcribed at lower levels when $\square^D$ is absent. These include the *sag* cytolysin encoding genes, the alternative sigma factor *sigF* and *botR*, the alternative sigma factor responsible for the transcription of the botulinum neurotoxin genes.

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CREATING A MUTANT LIBRARY USING MARINER-BASED RANDOM MUTAGENESIS IN *CLOSTRIDIUM DIFFICILE.*

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Because of limited genetic tools for *C. difficile* manipulation, our ability to understand the molecular mechanisms controlling sporulation and pathogenesis of *C. difficile* has been hindered. We have developed a new genetic approach allowing random inactivation of *C. difficile* genes based on the extensively used *Mariner* eukaryotic transposition system. The *Mariner* system is composed of a DNA fragment that encodes a transposase and two inverted terminal repeat regions (ITRs) necessary for mobility. In our approach, we used the transposon Tn*916* to introduce the mobile element of the *Mariner* into the *C. difficile* chromosome. This mobile element is composed of an antibiotic gene flanked by the two ITRs and has been inserted into Tn*916* by recombination with the tetracycline gene. The modified transposon was then transfer to *C. difficile* by conjugation. Next, we introduced the *Mariner* transposase under the control of different promoters (Pgdh or PtcdR, glutamate dehydrogenase or toxin gene regulator promoter, respectively) on a *E. coli-C. perfringens* shuttle vector which is known to be unstable in *C. difficile*. Instability of the plasmid is essential to create stable insertions in the *C. difficile* genome, since the loss of the *Mariner* transposase locks the mobile element at a specific site. Experiments are ongoing to determine the frequency of transposition and to generate a mutant library that could be used to identify genes that are required for important aspects of *C. difficile* physiology, virulence and sporulation.

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INVESTIGATION INTO THE CONJUGATIVE TRANSPOSONS OF *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile strain 630 contains many putative and some proven mobile genetic elements many of which appear to be conjugative transposons. In this work the mobility and epidemiology of some of the putative conjugative transposons was investigated.
Excision from the genome is a prerequisite for conjugative transposition to recipient cells and 6 out of 7 of the putative conjugative transposons are able to excise producing a circular form and leaving behind an empty target site.
As the putative conjugative transposons do not contain any obvious selectable markers, we have marked the conjugative transposon CTn*1* with the ClosTron, resulting in a CTn*1* derivative encoding for erythromycin resistance. Conjugation studies have been carried out on the recombinant CTn*1* to determine its host range.
In addition to this, we have looked for CTn1 homologues in the sequences of strain R20291 (responsible for an outbreak at Stoke Mandeville Hospital, UK) and other strains that have (partially) been sequenced. All of these strains contain a conjugative transposon related to CTn*1*, although the accessory genes differ between strains. Interestingly, all the accessory regions in elements from strains of the epidemic ribotype 027 are conserved.

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CHARACTERIZATION OF A NEW PEPTIDOGLYCAN HYDROLASE INVOLVED IN *CLOSTRIDIUM PERFRINGENS* VEGETATIVE GROWTH AND STRESS-INDUCED AUTOLYSIS.

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Peptidoglycan hydrolases, also called autolysins, are implicated in the physiology (growth and cellular division, sporulation) of bacteria. They could also contribute to the virulence of some pathogenic species, by facilitating toxins releasing, generating muropeptides with proinflammatory activity or by harbouring adhesive properties. We characterized *in silico* the sequence of an *acp* gene encoding a putative autolysin of *Clostridium perfringens*. We cloned this gene and expressed the corresponding protein in order to study the properties and functions of this putative autolysin. Acp displayed a lytic activity on the walls of several bacterial species in SDS-renaturing gels. Acp hydrolytic specificity was established by analyzing *Bacillus subtilis* peptidoglycan digestion products by coupling RP-HPLC analysis and MALDI-TOF MS, and revealed *N*acetylglucosaminidase activity, confirming *in silico* analysis predictions. The study of *acp* expression (qRT-PCR and Western blot analyses) during various growth phases of *C. perfringens* showed a constant expression, which suggests a role in cell separation or division of *C. perfringens*. Acp cell localisation by indirect immunofluorescence staining revealed the targeting of Acp at the septum of vegetative cells during exponential growth phase. Selective mutants of *acp* were obtained by using insertion of mobile Group II introns (ClosTron) strategy. Microscopic examination of wild type and mutant strains revealed a lack of vegetative cell separation in the mutant strain. The response of wild-type and mutant strains to stresses induced by Triton X-1OO, bile salts and vancomycin revealed a critical role of Acp in the autolysis induced by such stresses. Overall, Acp appears as the major autolysin expressed in vegetative growth and stress-induced autolysis of *C. perfringens*.

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DEVELOPMENT OF RANDOM MUTAGENESIS SYSTEM FOR Clostridium difficile

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C. difficile infection (CDI) is the leading cause of nosocomial diarrhoea in Europe and North America. The role of the *C. difficile* Pathogenicity Locus (PaLoc) in the clinical outcome of CDI is well established. Likewise, the importance of spores in the dissemination of CDI is also widely recognised. However, the identity and role of virulence factors other than those encoded on the PaLoc remain largely elusive, as do a number of genes involved in the formation and germination of spores. With this in mind, we have developed a random mutagenesis system for use in *C. difficile* by derivatising the *Himar1* transposon of the *mariner* family of transposable elements. This new technology opens the way for construction and forward genetic screening of a *C. difficile* random mutant library. This will enable us to identify genes involved in the virulence and dissemination of CDI which have thus far remained elusive.

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TRANSCRIPTIONAL RESPONSE OF CLOSTRIDIUM DIFFICILE TO DEFINED IRON SOURCES

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To establish intestinal colonization prior to either a commensal or pathogenic interaction with the host, *C. difficile* will encounter iron-limited niches where there is likely to be intense competition from the host and normal microbiota for iron. To gain a better understanding of iron homeostasis in *C.difficile*, growth of *C. difficile* was investigated under defined growth conditions and whole-genome DNA microarrays were used to monitor global gene expression in iron-rich and iron-limited growth conditions. The growth results showed that *C. difficile* 630 was not using haemoglobin, lactoferrin or transferrin but FeSO4, FeCl3, ferritin and ferric citrate as iron sources in the presence of an iron chelator. Microarray analysis identified significantly regulated genes and operons that were unique to or common between different conditions. The majority of these genes encoded *C. difficile* proteins that were involved in cell surface and architecture, metabolism and oxidative stress responses, DNA/RNA synthesis, transport and binding. Only two genes seemed to be expressed at elevated levels under all of the conditions tested. The transcriptional profiles also show that genes with increased expression in the presence of FeSO4 and FeCl3 did not respond to ferric citrate or ferritin as iron sources. In contrast, genes with increased expression in the ferric citrate and ferritin experiments were expressed at reduced levels when FeSO4 was supplied as the sole iron source. The data suggest that different pathways respond to inorganic or organic sources of iron in *C. difficile*. Full characterisation of these unique subsets of genes will be presented.

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GENOMIC LOCATION OF BOTULINUM NEUROTOXIN GENES IN CLOSTRIDIUM BOTULINUM BIVALENT STRAINS

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Clostridium botulinum bivalent strains harbour two distinct botulinum neurotoxin (*bont*) genes in their genome. Some of these strains are capable of producing both neurotoxins, though in different quantities: to date, a few of such dual toxin-producing strains have been identified, and they are referred to as *C. botulinum* type Ab, Af, Ba, and Bf, respectively, the capital letter indicating the neurotoxin serotype that is produced in major amounts. Other bivalent strains, referred to as *C. botulinum* type A(B) strains, only produce BoNT/A, as the *bont*/B gene remains unexpressed due to nonsense mutations leading to premature stop codons. Plasmid-encoded *bont* genes have recently been detected in several *C. botulinum* strains, a few of which were bivalent dual toxin-producing strains. The purpose of the present study was to determine the genomic location of the *bont* genes in a large number of *C. botulinum* bivalent strains: most of them were type A(B) strains, and the remaining ones were dual toxin-producing strains of type Ab and Bf. To this end, pulsed-field gel electrophoresis and Southern blot hybridization with specific *bont* gene probes were performed. Our results show that both *bont*/A and /B genes were chromosome-encoded in all *C. botulinum* type A(B) strains tested. On the other hand, the genomic location of the *bont* genes in the dual toxin-producing strains was variable, i.e. the genes were placed either in the chromosome or in the same plasmid, depending on the strain. These results provide novel data that might help elucidating the lateral transfer of *bont* genes and the evolution of neurotoxigenicity in *C. botulinum*.

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REDUCTION OF TAUROBILIRUBIN BY INTESTINAL BACTERIUM *CLOSTRIDIUM PERFRINGENS*

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Bilirubin, the product of heme catabolic pathway, is in the intestinal lumen naturally degraded by the intestinal microflora to urobilinoids, the heterogenous group of bilirubin reduction products. It is generally believed that only unconjugated bilirubin (UCB) is the natural bile pigment reduced. In our study we aimed to identify whether the bilirubin-reducing strain of *Clostridium perfringens* isolated from neonatal stools is capable of reducing also taurine-conjugated bilirubin (BDT) that naturally occurs in the bile of several marine fishes, such as yellowtail *Seriola quinqueradiata*.
C. perfringens was incubated with synthetic BDT (50 μM) under anaerobic conditions. The reduction products of BDT were isolated from the culture medium using a C8 column and consequently separated on the thin layer chromatography (TLC RP-18 silica gel plates; solvent system CH_3OH:H_2O:CH_3COOH = 250:250:1, by vol.). Separated pigments were examined under visible and UV light. Based on comparison with the UCB catabolic products, three possible reduction products of BDT were identified: mesobiliviolin ditaurate, urobilin ditaurate and urobilinogen ditaurate.
Our preliminary results indicate that *C. perfringens* is capable of reducing of also BDT without prior deconjugation. Such a broad substrate specificity of bilirubin-reducing enzyme(s) may serve for effective disposal of electrons produced by fermentolytic processes within these anaerobic bacteria.

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PROTEOMIC AND GENOMIC CHARACTERIZATION OF HIGHLY INFECTIOUS *Clostridium difficile* 630 SPORES

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Clostridium difficile, a major cause of antibiotic-associated diarrhea, produces highly resistant spores that contaminate hospital environments and facilitate efficient disease transmission. We purified *C. difficile* spores using a novel method and show that they exhibit significant resistance to harsh physical or chemical treatments and are also highly infectious, with <7 environmental spores per cm^2 reproducibly establishing a persistent infection in exposed mice. Mass spectrometric analysis identified ~336 spore-associated polypeptides, with a significant proportion linked to translation, sporulation/germination and protein stabilization/degradation. In addition, proteins from several distinct metabolic pathways associated with energy production were identified. Comparison of the *C. difficile* spore proteome to other clostridial species defined 88 proteins as the clostridial spore “core” and 29 proteins as *C. difficile* spore-specific, including proteins that could contribute to spore-host interactions. Thus, our results provide the first molecular definition of *C. difficile* spores opening up new opportunities for the development of diagnostic and therapeutic approaches.

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FUNCTIONAL INACTIVATION OF CAP0129 IN *CLOSTRIDIUM ACETOBUTYLICUM*

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The cell cycle of the strict anaerobic Gram-positive *Clostridium acetobutylicum* is characterized by some interesting metabolic and morphological differentiations. For example cells are able to produce the polysaccharide granulose in form of unsoluble granules which are expected to serve as energy source during sporulation. Analyses of purified granulose granules revealed two proteins to be associated to the granules: Cap0129 with two carbohydrate binding modules which is encoded by the megaplasmid (pSol1) and glycogen synthase (GlgA). Here we present results dealing with the functional inactivation of the *cap0129* gene based on the integration of a group II intron using the ClosTron-System. Molecular analyses (PCRs, Southern Blots, Northern Blots) clearly proved the correct integration of the intron into the *cap0129* gene and strongly indicate functional inactivation of protein production. However, the mutant-cells were still able to produce granulose granules similar in shape and size to the wild type. Preliminary data might indicate a reduced amount of granules in the cells, but, contradictory the same effect seemed to be occur in wild type cells in which the amount of Cap0129 protein is increased (plasmid based homologous overexpression).

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COMPARATIVE GENOMICS OF CLOSTRIDIUM PERFRINGENS ISOLATES ASSOCIATED WITH NECROTIC ENTERITIS IN POULTRY

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Necrotic enteritis (NE) is an enteric disease of chickens caused by *Clostridium.perfringens*, and characterized by necrosis of the small intestinal mucosa, causing mortality and losses in production. The bacteria produces extracellular toxins that are implicated in the disease, in particular αtoxin and NetB, however the exact disease mechanism is still poorly understood. While environmental factors such as a proteinrich diet and coccidiosis clearly predispose to NE, several recent studies underscore the importance of strain, suggesting the critical involvement of strainspecific virulence factors.

We have determined the genome sequence of two unrelated *C. perfringens* poultry isolates, JGS4143 and CP4, that are able to consistently cause NE in a challenge model. An estimated 27x and 340x coverage has been achieved for JGS4143 and CP4, respectively. Comparative genome sequence analysis against the three fully annotated *C. perfringens* genomes that are publicly available has revealed a number of openreading frames (ORFs) unique to each of the NE strains, including previously identified virulence factors such as NetB, as well as a number of novel sequences. For example, JGS4143 has 401 ORFs not found in the other annotated published sequences. While analysis is continuing, several of these unique ORFs are present in both strains, including a two-component regulatory system, several antibiotic resistance genes and a putative hemolysin.

Based upon these two genome sequences and the other published genomes, we have developed a *C. perfringens* 70mer oligonucleotide microarray, which will be used to investigate the presence of these putative NErelated genes in a broader range of isolates, as well as expression of these genes under different conditions, including during infection.

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DETERMINING HYPERVIRULENT MARKERS FOR CLOSTRIDIUM DIFFICILE BY ARRAY COMPARATIVE GENOMIC HYBRIDISATION

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Clostridium difficile is now recognised as the main cause of nosocomial diarrhoea in the developed world. With emerging resistance to the penultimate antibiotic of choice in these strains and the emergence of community-acquired infections, it is imperative that better diagnostics and typing methods be developed. The aim of the current study was to use a comparative genomic approach for the identification of hypervirulent markers in *C. difficile* in an effort to aid quicker identification of these strains. A high density oligonucleotide microarray was designed to the *C. difficile* 630 sequence and constructed by Oxford Gene Technology (UK). Extra probes were also designed to regions of divergence in the un-annotated sequences of two PCR ribotypes O27 strains, *C. difficile* R20291 (WTSI, UK) and QCD-32g58 (McGill University, Canada) when compared to C. difficile 630 and regions of interest, such as the agr operon. A set of 85 clinical strains comprised of the most commonly represented PCR ribotypes throughout Europe, including type O27 was hybridised to the microarray. These included 30 ribotype 027 strains originating from Europe, the USA and Canada, and 10 strains representing the subtypes of ribotype 001. Strains representing the new emerging ribotype 078 have also been hybridised to the microarray. Analysis of the microarray data was performed using GeneSpring GX v7.3 (Agilent, USA). Microarray analysis revealed distinct regions of divergence amongst the strains analysed in comparison to *C. difficile* 630 across all PCR ribotypes tested. No specific probes were identified as markers for ribotype 027 strains. Comparison of O27 strains from North America showed genetic differences or divergence from R20291 specific probes, yet probes designed specifically to the Canadian strain QCD-32g58 appear to hybridise consistently to all European O27 strains. aCGH successfully identified regions of divergence that could be used as markers for *C. difficile* 630.

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COMPARATIVE GENOME AND PHENOTYPIC ANALYSIS OF *CLOSTRIDIUM DIFFICILE* 027 STRAINS PROVIDES INSIGHT INTO THE EVOLUTION OF A HYPERVIRULENT BACTERIUM

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The continued rise of *Clostridium difficile* infections (CDI) worldwide has been accompanied by the rapid emergence of a highly virulent clone designated PCR-ribotype 027. To understand more about the evolution of this virulent clone, we made a three-way genomic and phenotypic comparison of a “historic” non-epidemic 027 *C. difficile* (CD196), a recent epidemic and hypervirulent 027 (R20291) and a previously sequenced PCR-ribotype 012 strain (630). Although the genomes are highly conserved, the 027 genomes have 234 additional genes compared to 630, which may contribute to distinct phenotypic differences we observe between these strains relating to motility, antibiotic resistance and toxicity. The epidemic 027 has five unique genetic regions, absent from both the non-epidemic 027 and strain 630.Comparison with a series of 027 isolates showed that some of these genes appeared to have been gained by 027 strains over the past two decades. This study provides genetic markers for the identification of 027 strains and offers a unique opportunity to explain the recent emergence of a hypervirulent bacterium.

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COMPARATIVE GENOMIC ANALYSIS OF *CLOSTRIDIUM DIFFICILE*

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The association of *C. difficile* associated diseases (CDAD) with high exposure to antibiotics, within hospital settings has been well documented Cases of CDAD continue to rise, in 1990, 1172 cases were reported; by 2007 this figure had risen sharply to 57,247. Based upon PCR-ribotyping, more than 150 *C. difficile* types have been characterised, of which types 01, 106 and 027 comprised about 25% of all isolates. At present many isolates within, but not exclusive to, strain type 027 have been implicated as the cause of many outbreaks. These newer and more virulent *C. difficile* isolates have emerged as a cause of antibiotic resistant diarrhoea and has led to a dramatic increase in frequency and severity of *C. difficile* related infections.

The genetic changes that have occurred during the evolution of *C. difficile* have been difficult to determine due to the lack of genome sequence information. Recent advances in genome sequencing and genome comparison tools have now made it possible to perform high throughput sequencing as well genome wide, experimental, profiling with greater accuracy and speed. New genome sequence information has been made available, through in-house sequencing of virulent *C. difficile* strains from the 1970s and 2000s, as well as a less virulent strain from the 1980s. Such studies generate vast amounts of data, which need to be analysed and interpreted efficiently. It is impossible to perform such comparative analysis without the utilisation of a variety of bioinformatic tools and algorithms.

An integrated approach utilising genomic and bioinformatic techniques have been applied to determine the genetic changes that have occurred during the evolution of these *C. difficile* strains. The identification of these changes will greatly aid our understanding of this pathogen; in addition it will also help to develop more accurate typing strategies.

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GENETIC ANALYSIS OF ENTEROTOXIN GENE CARRYING TYPE A CLOSTRIDIUM PERFRINGENS STRAINS

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Clostridium perfringens type A is both a ubiquitous environmental bacterium and a major cause of human gastrointestinal disease, which usually involves strains producing *C. perfringens* enterotoxin (CPE). The gene (*cpe*) encoding this toxin can be carried on the chromosome or a large plasmid. Interestingly, strains carrying *cpe* on the chromosome and strains carrying *cpe* on a plasmid often exhibit different biological characteristics. In this study, we investigated the genetic properties of *C. perfringens* by PCR-surveying 21 housekeeping genes and genes on representative plasmids and then confirmed those results by Southern blot assay (SB) of five genes. Furthermore, sequencing analysis of eight housekeeping genes and MLST analysis were also performed. Fifty-eight *C. perfringens* strains were examined, including isolates from: food poisoning cases, human gastrointestinal disease cases, foods in Japan or the USA, or feces of healthy humans. In the PCR survey, eight of eleven housekeeping genes amplified positive reactions in all strains tested. However, by PCR survey and SB assay, one representative virulence gene, *pfoA*, was not detected in any strains carrying *cpe* on the chromosome. Genes involved in conjugative transfer of the *cpe* plasmid were also absent from almost all chromosomal *cpe* strains. MLST showed that chromosomal *cpe* isolates, i) assemble into one definitive cluster ii) lack *pfoA* and iii) lack a plasmid related to the *cpe* plasmid. Similarly, strains carrying a *cpe* plasmid also appear to be related, but are more variable than chromosomal *cpe* strains, possibly because of the instability of *cpe*-borne plasmid(s) and/or the conjugative transfer of *cpe*-plasmid(s) into unrelated *C. perfringens* strains.

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GLOBAL GENE EXPRESSION ANALYSIS DURING *C. DIFFICILE* INFECTION.

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Gene expression *in vitro* is not always comparable with that observed *in vivo* during infection (Mahan, *et al.,* 2000). For example in *C. difficile*, no correlation was found between *C. difficile* toxin yields *in vitro* and fecal toxin levels (Akerlund *et al,* 2006). To identify genes that are preferentially expressed or regulated in the susceptible host by *C. difficile* strains, we compared *in vitro* and *in vivo* the transcriptional profiles of *C. difficile* strains. We also studied the kinetics of the genes expression in vivo, in order to understand the initial steps of the colonization process of this bacteria and the relationship with toxins production. Axenic mice were challenged by vegetative cells of *C. difficile* strain 630, and were sacrificed at 8, 14, and 38 hours post-infection. Then *C. difficile* microarrays (St George's microarray group) were hybridized with total RNA extracted either from bacteria obtained from mice intestinal lumens or from bacteria grown *in vitro*. Normalisation and statistical analysis of the transcriptomic data were done according to the functions of limma package. In parallel, we are performing the functional annotation and the reconstruction of the metabolic pathways of *C. difficile* using platform MaGe (http://www.genoscope.cnrs.fr/agc/mage) a graphical interface used for expert annotation. All transcriptomic data generated are managed by the Genoscript database (http://feu.sis.pasteur.fr/cgi-bin/WebObjects/i386GenoList) allowing the comparative studies across all experiences. We showed that 563 genes are differentially regulated and more than 200 genes are expressed only *in vivo* and distributed in more than ten functional families. Among genes temporarily expressed *in vivo* we showed: 1) PaLoc genes are over-expressed at 36 hours. 2) Genes responsible for the fermentation are expressed at 14 hours when those involved in energetic carbon metabolism are regulated down. 3) Genes encoding cell processes (mobility, amino acids and carbohydrates transport) are down regulated at 14 hours whereas genes of the differentiation processes are up regulated. 4) Expression of cell envelope encoding genes is highly increased at 14 hours. We are inactivating genes highly regulated *in vivo* to be then evaluated for their role in the pathogenicity process of *C. difficile.*

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BIOLOGICAL INSIGHTS OF PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* FROM A WIDE-RANGING COMPARATIVE GENOMIC INDEXING STUDY

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Proteolytic *Clostridium botulinum* is the causative agent of botulism, a severe neuroparalytic illness. Given the severity of botulism, surprisingly little is known of the population structure, biology, phylogeny or evolution of *C. botulinum.* We have carried out a comparative genomic indexing study of 61 widely-representative strains of proteolytic *C. botulinum* and the closely related *C. sporogenes*. Whole genome microarray analysis revealed that 63% of the coding sequences (CDSs) present in a sequenced reference strain (ATCC 3502, Hall A) were common to all 61 strains tested. This indicates a relatively stable genome. There was, however, evidence for recombination and genetic exchange, in particular within the neurotoxin gene and cluster and the flagellar glycosylation island (FGI). These two loci appear to have evolved independently from each other, and from the remainder of the genetic complement. A number of strains were atypical; for example, while 10 out of 14 strains that formed type A1 toxin gave almost identical profiles in whole genome, neurotoxin cluster and FGI analyses, the other four strains showed divergent properties. Furthermore, a new neurotoxin sub-type (A5) has been discovered in strains from UK heroin-associated wound botulism cases. This is most closely related to type A1 neurotoxin, but has amino acid differences in regions known to be important for targeting the neuron. These strains also contained a partial type B neurotoxin gene. A finding of great interest was that for the first time, differences in flagella glycosylation profiles, as obtained by mass spectrometry analysis, could be linked to differences in FGI gene content.

Carter *et al.* (2009). BMC Genomics 10, 115
Peck (2009). Advances Microbial Physiology 55, 183-265

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OXIDATIVE STRESS RESPONSE IN *CLOSTRIDIUM ACETOBUTYLICUM*

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The reaction of microbial organisms to changing environmental conditions or stress factors does often result in genetic responses. Such genetic regulation mechanisms are for instance induced by oxidative stress. Clostridia belong to those bacteria that are considered as obligate anaerobe. In consequence survival of such organisms is impaired by oxidative stress
e.g. in the form of O2 or H2O2. Nevertheless, recent studies revealed that Clostridia are able to cope with microoxic conditions indicating protection mechanisms to defend against oxidative stress.
Recently we were able to identify the genes responsible for O2, O2 and H2O2 detoxification in *Clostridium acetobutylicum* by *in vivo* and *in vitro* assays on the respective proteins. Analyses of the promoter sequences led to the identification of PerR, the responsible regulator of the oxidative stress response. Its mutation showed a high tolerance to oxidative stress as a result of the upregulation of oxidative stress genes. Another protein that is considered to be involved in the regulation of the defence against oxidative stress in *C. acetobutylicum* is SoxR which is encoded by the open reading frame *cac2451*. To confirm this function for SoxR we manipulated the *soxR* gene and analyzed the mutants with regard to the reaction to oxidative stress. Here, we present first results respecting the regulation of the oxidative stress response in *C. acetobutylicum*.

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THE GENOME SEQUENCE OF BACTERIOPHAGE CpV1 LYTIC FOR *CLOSTRIDIUM PERFRINGENS*

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Application of bacteriophages and their lytic enzymes to control *Clostridium perfringens* is one potential approach to reduce the pathogen on poultry farms and in poultry-processing facilities. We have established a collection of 30 bacteriophages lytic for *C. perfringens*. These were isolated from sewage, feces and broiler intestinal contents. Phage CpV1 one of the more virulent phages was classified in the family *Podoviridae*. The phage had an icosahedral head and collar of approximately 42nm and 23 nm in diameter, respectively with a structurally complex tail of about 37nm lengthwise and a basal plate of 30 nm. To date the phage double-strand DNA genome was sequenced up to 16.3 kb pairs with a net GC composition of 31 %. Twenty-four open reading frames (ORFs) coding for putative peptides containing 30 or more amino acid residues were identified and analyzed. Amino acid sequences of the putative proteins from phage CpV1 were compared with those from the NCBI database and potential functions of 10 proteins were determined by sequence homology. Five putative proteins were similar to hypothetical proteins with unknown functions, whereas nine proteins did not have similarity with any phage or bacterial proteins. Identified ORFs form at least four genomic clusters accounted for replication of the phage DNA, its folding, production of structural components and lytic properties. One lysin was predicted to share homology with N-acetylmuramoyl-L-alanine amidases and a second lytic enzyme was predicted to be a lysozyme-endopeptidase. These enzymes digest peptidoglycan of the bacterial cell wall and are considered potential therapeutics to control *C. perfringens*.

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tcdC-SUBTYPES IN CLINICAL ISOLATES OF *CLOSTRIDIUM DIFFICILE* AT HEIDELBERG UNIVERSITY HOSPITAL

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tcdC is the negative regulator of toxin production in *Clostridium difficile*. *tcdC* mutations such as the single basepair deletion 117delA in Ribotype 027 are thought to result in higher levels of toxin production and increased pathogenicity. To identify potentially hypervirulent strains at Heidelberg University, *tcdC* sequence analysis was performed in *C. difficile* strains isolated from samples that tested positive for *C. difficile* toxin, glutamatdehydrogenase or both.
During a 13 month period 226 strains from 164 patients were isolated. 38 of these 226 strains (31 patients) were isolated from samples that tested positive for glutamatdehydrogenase only. PCR for *tcdC* was performed in one isolate from each of the 164 patients. 5 isolates tested negative for *tcdC*, all of which have had a negative toxin test initially. Sequence analysis was performed in the remaining 159 isolates.
12 different *tcdC* subtypes could be retrieved. 108 isolates (67.9 %) were of subtype *tcdC-sc3* (148G>T). 14 isolates (8.8 %) showed subtypes with nonsense mutations expected to result in severely truncated proteins. One isolate had a previously not described single basepair deletion at position 387. Another isolate did show a newly recognized 18 basepair duplication. Only one isolate with an 117delA mutation was discovered and was confirmed as Ribotype 027 by PCR ribotyping.
tcdC-sc3 was the most frequently observed subtype. *tcdC* subtypes that are expected to result in considerably truncated amino acid sequences were a common feature. These strains could prove to be of increased clinical relevance since they are suspected to produce higher levels of toxin. Investigation of *tcdC* mutations seems to be a valuable tool in helping to identify such strains as was shown by the first isolation of a Ribotype 027 in the Heidelberg region.

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IMPACT OF ENVIRONMENTAL CONDITIONS ON SURFACE PROTEINS OF *Clostridium difficile*

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The pathogenicity of the enteropathogenic bacteria *Clostridium difficile* is mainly mediated by toxins A and B; the colonization process involves various surface proteins, including the high molecular weight S-layer protein (HMW-SLP), the adhesins Cwp66 and Fbp68, the flagellar proteins FliC and FliD and the protease Cwp84 that may play a role in the dissemination of the infection. The expression of genes encoding the virulence factors in pathogenic bacteria could be influenced by environmental conditions such as pH, CO2, glucose and antibiotics. It has been previously shown that the expression of toxins A and B of *C. difficile* is negatively regulated by different environmental factors, particularly the glucose. Therefore, we analysed *in vitro* the effect of glucose and the accompanying decrease in pH that results from glucose metabolism on the expression of colonization factors of *C. difficile.* The proteome analysis of the cell wall extracted from cultures of *C. difficile* grown in tryptone yeast (TY) or TY supplemented with 0.5% glucose (TYG) media showed no striking difference. This result was confirmed by the identification of the most important spots by tandem mass spectrometry. However, analysis of some surface proteins by immunoblotting using specific antibodies revealed that the expression of Cwp84, HMW-SLP, Cwp66, and FliC was increased in media containing glucose. This was correlated with the up regulation of the *slpA* gene while expression of the 16S RNA gene was similar with or without glucose in the culture media. When the pH of the TYG media was maintained constant during growth of *C. difficile*, the expression of the surface proteins was even more increased. Effect of pH was further explored, independently of the anaerobic metabolism of glucose. We observed that a short exposure to an acidic pH led to a significant decrease in the amount of surface proteins. These results suggest that expression of some surface proteins involved in the colonization process is positively regulated by glucose and is also under the control of pH; this could lead *in vivo* to modulation of their expression.

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DETECTION AND FUNCTIONAL ANAYSIS OF PARA-CRESOL PRODUCTION IN CLOSTRIDIUM DIFFICILE

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Clostridium difficile is a unique nosocomial pathogen, responsible for *Clostridium difficile* infection (CDI), as a result of changes in the gut microflora brought about by antibiotic treatment. The transcontinental spread of virulent clones, including designated PCR-ribotypes 017, 027 and 078, suggests that pathogenesis not solely attributed to the production of toxins, designated TcdA and TcdB; as 017 ribotypes lack TcdA (A-B+). The life-style of *C. difficile* and its ability to colonise the gut after antibiotic treatment, makes it an intriguing and complex pathogen. A potentially important trait of *C. difficile* is its' ability to ferment tyrosine via para-hydroxyphenylacetate (pHPA) to p-cresol; a phenolic compound, which interferes with microbial metabolism. The production of p-cresol may give C. difficile a metabolic advantage over commensal gut flora, resulting in proliferation and disease.

Mutants in the decarboxylase regulon (*hpdA, hpdB* and *hpdC*), which converts *p*HPA into p-cresol have been constructed using the clostron system in both Δ630erm and R20291, an 027 outbreak strain from Stoke Mandeville. It has been demonstrated that R20291 is more tolerant to p-cresol than the sequenced strains 630. However, both R20291 and 630 only produce *p*-cresol in minima Yeast-Peptone (YP) media, or in BHI media supplemented with *p*HPA, indicating that tyrosine is only used as a carbon source in the absence of an alternative. Both Z-nose and Mass Spectroscopy data indicate that p-cresol is not produced in any of the *hpdA, hpdB* or *hpdC* mutants. The *hpdA* and *hpdC* mutants in R20291 grow better in YP than wild-type R20291, suggesting the production of p-cresol slows growth in R20291, but not in 630. This may be attributed to the differences in production of p-cresol, whereby R20291 may produce more p-cresol than 630.

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REGULATION OF VIRULENCE BY THE RevR RESPONSE REGULATOR FROM Clostridium perfringens

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Clostridium perfringens produces several extracellular hydrolytic enzymes and toxins, of which α-toxin and perfringolysin O have been shown to be involved in the development of gas gangrene. The expression of these toxins is regulated by the VirSR signal transduction system. RevR (regulator of extracellular enzymes and virulence) from *C. perfringens* is a putative orphan response regulator that has a high degree of similarity to YycF and PhoB from other Gram positive bacteria. The RevR protein appears to have two domains, an N-terminal response regulator receiver domain and C-terminal domain with a putative helix-turn-helix DNA binding region. A *revR* mutant was constructed by allelic exchange and examined by microarray analysis. The results showed that more than 100 genes were differentially expressed, including several genes involved in cell wall metabolism. The *revR* mutant had an altered cellular morphology, the cells formed long filaments with no apparent septa. By comparison, the wild-type cells formed consistent short rods. Cellular morphology was restored back to the wild-type phenotype by complementation with a plasmid that carried the wild-type *revR* gene. Furthermore, several genes encoding extracellular enzymes (sialidase, hyaluronidase and α-clostripain) were shown to be differentially expressed in the *revR* mutant. Complementation of the *revR* mutation restored the expression of these genes to levels similar to wild-type. Quantitative enzyme assays confirmed that these changes led to altered enzyme activity in culture supernatants and cell wall extracts. Finally, when mice were injected with the *revR* mutant virulence was attenuated in the mouse clostridial myonecrosis model when compared to injection with the wild-type strain. Complementation of the *revR* mutation restored wild-type virulence. These results provide evidence that a novel orphan response regulator, RevR, regulates virulence in *C. perfringens*. It represents the first response regulator other than VirR to be shown to be involved in virulence in *C. perfringens*.

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COMPARATIVE PROTEOMIC ANALYSIS OF CLINICALLY RELEVANT HEAT STRESS IN CLOSTRIDIUM DIFFICILE.

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Clostridium difficile is the leading cause of hospital acquired diarhoea worldwide and represents a significant social and financial burden. C. difficile infection (CDI), usually following antibiotic treatment in hospitals, results in a number of symptoms ranging from mild self limiting diarrhoea to potentially fatal pseudomembranous colitis, with associated mortality rates of between 6 and 15% of patients. C. difficile produces toxins that are responsible for the pathology of the disease, in addition to a number of cell-wall associated virulence factors.
It is notable that despite its obvious importance in human health, C.difficile remains relatively poorly characterised, and our primary research interest is in understanding more fully the response of C. difficile, at a systems level, to clinically relevant stresses. In our laboratory we have carried out iTRAQ driven comparative proteomics analysis of a clinically relevant heat stress in C. difficile strain 630 (37°C control, with 41°C heatshock). ProQUANT analysis generated a list of some 83 proteins, out of a total of ~600 identified, whose expression is significantly changed and we have validated a proportion of this data using RT-Q-PCR.
In this paper we present the main findings of our research, thus giving insight into how C. difficile responds to heat stress such as might been countered in feverish patients, in addition to comparing the robustness of the iTRAQ workflow with other, semi-quantitative, proteomics methods.

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BINDING OF THE α SUBUNIT OF RNA POLYMERASE TO THE PHASED A-TRACTS UPSTREAM PHOSPHOLIPASE C GENE OF CLOSTRIDIUM PERFRINGENS

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There are three phased A-tracts (-66 to -40) upstream the promoter of the phospholipase C (*plc*) gene of *Clostridium perfringens*. The phased A-tracts are thought to be curved intrinsically and enhance the *plc* gene expression. The C-terminal domain of the α subunit (αCTD) of the *Clostridium perfringens* RNA polymerase (CpRNAP) binds to the phased A-tracts. To determine which groove of the phased A-tracts DNA is involved in the binding, gel shift assay was performed with groove-specific DNA-binding drugs. The result of this assay showed that the α subunit bound to the minor groove of the DNA. To identify the amino acid residues involved in the binding, 27 amino acid residues in the αCTD of CpRNAP were substituted to Alanine. The bindings of the wild-type α subunit and the mutated α subunits to the phased A-tracts were examined with both gel shift assays and kinetic analyses using Biacore. Among the mutated α subunits, the affinity of [R261A]α, [N264A]α, and [K294A]α to the phased A-tracts decreased drastically. The positions of these animo acid residues assembled on the three-dimensioned structure of the αCTD of CpRNAP predicted, based on the structure of *Bacillus subtilis* αCTD, using the SWISS-MODEL program. This region was thought to be the binding site to the phased A-tracts. The position of this DNA binding site appear to be similar to that of the *Escherichia coli* α subunit reported previously. The affinity of the α subunit of CpRNAP to the phased A-tracts was examined at different temperatures (25ºC, 30ºC, and 37ºC) using Biacore. The result of this kinetic analysis indicated that this affinity increased at lower temperatures than 37ºC.

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CWP84, A SURFACE-ASSOCIATED, CYSTEINE PROTEASE, PLAYS A ROLE IN THE MATURATION OF THE S-LAYER OF CLOSTRIDIUM DIFFICILE

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Clostridium difficile is a major problem as a hospital-associated infection which can cause severe, recurrent diarrhoea. Over the past 8 years the appearance of more virulent strains of bacterium and changes in antibiotic usage and have led to a significant increase in morbidity and mortality. The mechanism by which the bacterium colonises the gut during infection is poorly understood but undoubtedly involves protein components within the surface layer (S-layer) which play a role in adhesion. In *C. difficile*, the S-layer is composed of two principal components, the high and low molecular weight S-layer proteins, which are formed from the post-translational cleavage of a single precursor, SlpA. In the present study, we demonstrate that a recently characterised cysteine protease, Cwp84 plays a key role in maturation of SlpA. Using a gene knock-out approach, we show that inactivation of the *cwp84* gene in *C. difficile* 630 ΔErm results in a bacterial phenotype in which only immature, single chain SlpA comprises the S-layer. The Cwp84 knock-out mutant (CDΔCwp84) displayed significantly different colony morphology compared to the wildtype, grew more slowly in liquid medium and sporulated with reduced efficiency. Using the hamster model for *C. difficile* infection, CDΔCwp84 was found to be competent at causing disease with a similar pathology to the wildtype strain. The data show that, while Cwp84 plays a role in the cleavage of SlpA, in the animal model it is not an essential virulence factor and that bacteria expressing immature SlpA are able to cause disease.

P79

GENE REGULATION BY INTRA-AND INTER-CELLULAR SIGNALING IN *CLOSTRIDIUM PERFRINGENS*

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Gram-positive anaerobic bacteria, *Clostridium perfringens* produces many enzymes and toxins to degrade host tissues. Since *C. perfringens* has very few genes to synthesize amino acids, it is suspected that *C. perfringens* needs to produce enzymes and toxins quickly and effectively, especially in infectious conditions. To pull off it, a well-coordinated regulation system must exist in toxin production system in *C. perfringens*. But until now the system has not been clear. Recently, we reported that *agr* system plays important roles on toxin gene expression and VirS has part of the responsibility to sense the signal produced by *agr* region. Furthermore our latest data suggest that cell-cell signaling between other bacteria also has important role on toxin gene expression. These mechanisms seem to play important roles in infectious condition or in the intestine, and we have been analyzing the inter-species cell-cell signaling and also the relationship between intra-and interspecies signaling in detail.

P80

CHARACTERISATION OF A PUTATIVE QUORUM SENSING SYSTEM IN *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile is the major cause of health-care associated infections. However the factors that participate in *C. difficile* infection and the processes that regulate their expression remains poorly understood.
In *Staphylococcus aureus*, quorum sensing (QS) plays a central role in the regulation of virulence factors. The QS system of *S. aureus* is encoded by the accessory gene regulator (agr) locus and comprises of four genes. The pre-peptide (AgrD) is processed by AgrB and the mature auto-inducing peptide (AIP) is released into the medium. Free AIP binds to the histidine-sensor kinase AgrC, causing it to autophosphorylate. In turn, AgrC phosphorylates the response regulator AgrA, triggering an intracellular signal-transduction cascade which results in altered expression of several target genes.
Homologues of genes involved in QS have been identified in the genome sequence of *C. difficile* 630, a virulent multidrug resistant strain. These homologues have also been identified in *C. difficile* R20291, a ribotype 027 epidemic strain, which has been characterised to produce increased quantities of Toxin A and Toxin B. *C. difficile* R20291 has two *agr* loci, whereas *C. difficile* 630 only has one *agr* locus. The first *agr* locus present in both strains contains *agrB/D* homologues, although there are no apparent *agrA*/*agrC* homologues. However, the second *agr* locus in *C. difficile* R20291 contains homologues of all four *agr* genes. Insertional inactivation of the QS homologues were made in the two *C. difficile* strains and the effects on virulence assessed. Cytotoxicity assays indicated that there is a substantial reduction in toxin B production in *C. difficile* R20291 strains deficient in the second *agr* system compared to the wild-type strain. These results suggest that the second *agr* locus of *C. difficile* R20291 plays a regulatory role in toxin production.

P81

A NOVEL GENETIC SWITCH CONTROLS PHASE VARIABLE EXPRESSION OF CwpV, A *Clostridium difficile* CELL WALL PROTEIN

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Clostridium difficile is a nosocomial pathogen that can cause severe gastrointestinal infections. *C. difficile* encodes a family of cell wall proteins, some of which are implicated in pathogenesis. Here we have characterised CwpV, the largest member of this family. CwpV is surface expressed and post-translationally processed in a manner analogous to the major S-layer protein SlpA. Expression of *cwpV* is phase variable, with approximately 5 % of cells in a population expressing the protein under standard laboratory growth conditions. Upstream of *cwpV,* inverted repeats flank a 195 bp sequence which undergoes DNA inversion. Use of a *gusA* transcriptional reporter demonstrated that phase variation is mediated by DNA inversion; in one orientation *cwpV* is expressed while in the opposite orientation the gene is silent. The inversion region contains neither the promoter nor any of the open reading frame, therefore this system differs from previously described phase variation mechanisms. The *cwpV* promoter is located upstream of the inversion region and we propose a model of phase variation based on intrinsic terminator formation in the OFF transcript. A *C. difficile* site-specific recombinase able to catalyse the inversion has been identified. We have shown that CwpV binds to fibronectin, and we therefore propose that CwpV is a phase variable *C. difficile* adhesin. Further investigation into the role of CwpV during infection is underway.

P82

GENERATION OF A TETRACYCLINE SENSITIVE *CLOSTRIDIUM DIFFICILE* 630 STRAIN.

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The recent genome sequencing and improved techniques for genetic manipulation of *Clostridium difficile* are rapidly enhancing our understanding of this pathogen. Currently the only published genome is that of strain 630. This strain is resistant to multiple antibiotics therefore there are less suitable genetic markers that can be used to manipulate the strain. Recently we have been investigating the behaviour of an endogenous conjugative transposon Tn*5397*. Tn*5397* encodes resistance to tetracycline and is capable of transfer to and from *Bacillus subtilis* and also between *C. difficile* strains. In *C. difficile* it always enters the genome at the same *attB* site where it is completely stable over 30 days of continuous sub-culture on antibiotic free media. In order to see if a second copy of Tn*5397* would use an alternative *attB* site, if the first was occupied, a recombinant Tn*5397* was constructed in *B. subtilis* in which the *tet*(M) gene was replaced by *cat*P conferring resistance to chloramphenicol. After filter-mating into *C. difficile* 630 a single transconjugant was obtained. The incoming recombinant Tn*5397* had replaced the wildtype Tn*5397* and was inserted at the original *attB*. The transconjugant, designated *C. difficile* 630ΔTc, was resistant to chloramphenicol and sensitive to tetracycline. This strain provides researchers with an optional tetracycline sensitive strain.

P83

THE PROTEASE CspB IS ESSENTIAL FOR INITIATION OF CORTEX HYDROLYSIS AND DPA RELEASE DURING GERMINATION OF SPORES OF CLOSTRIDIUM PERFRINGENS FOOD POISONING ISOLATES

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The genome of the *Clostridium perfringens* food poisoning isolate SM101 encodes a subtilisin-like protease, CspB, upstream of the *sleC* gene encoding the enzyme essential for degradation of the peptidoglycan cortex during spore germination. SleC is an inactive pro-SleC in dormant spores that is converted to active SleC during spore germination, and Csp proteases convert pro-SleC to the active enzyme *in vitro*. In current work, the germination and viability of spores of a *cspB* deletion mutant of strain SM101, as well as *cspB* expression have been studied. The *cspB* gene was expressed only during sporulation, and only in the mother cell compartment. *cspB* spores were unable to germinate significantly with either a rich nutrient medium, KCl, or a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA), and the viability of these spores was ~10^4-fold lower than that of wild-type spores, although *cspB* and wild-type spores had similar viability on plates containing lysozyme. Germination of *cspB* spores was blocked prior to DPA release and cortex hydrolysis, and germination and viability defects in these spores were complemented by an ectopic *cspB*. These results suggest that Csp proteases are essential to generate active SleC and allow cortex hydrolysis early in *C. perfringens* spore germination. However, Csp proteases likely play another role in spore germination, since *cspB* spores did not release DPA upon exposure to germinants, while *sleC* spores have been shown previously to release DPA, albeit slowly, upon exposure to germinants.

P84

IRON ACQUISITION AFFECTS VIRULENCE OF Clostridium perfringens

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Iron is a fundamental element required for the growth and survival of almost all living organisms, but within the mammalian host most free iron is sequestered by host proteins and is not readily available to invading organisms. Bacterial pathogens have adopted numerous mechanisms for acquiring iron from host proteins during an infection, including direct acquisition of ferric iron from haem-associated proteins or from iron-scavenging siderophores. Ferric iron is then transported into the cytosol, where it can be utilised by the bacterial pathogen. Under anaerobic conditions bacteria can also transport ferrous iron using the transmembrane complex FeoAB, but little is known about iron transport systems in anaerobic bacteria such as the pathogenic clostridia. The overall aim of this project was to characterise iron acquisition systems in *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). The relative expression of these systems was determined using quantitative real-time RT-PCR assays that were specific for one gene from each system. Each of these genes was expressed, with *feoAB* generating the most abundant iron-uptake related transcripts. To further 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, under the conditions tested. Similarly, only the *feoB* mutant had a markedly reduced total iron content. Finally, preliminary data obtained using a myonecrosis virulence model indicated that mice injected with the *feoB* mutant had reduced virulence when compared to the wild-type strain. These studies suggest that FeoAB is the major system required for the uptake of iron into the cell and may play an important role in the pathogenesis of *C. perfringens* infections.

P85

ROLE OF FLAGELLA IN ASPECTS OF *CLOSTRIDIUM DIFFICILE* VIRULENCE

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Clostridium difficile is a major cause of antibiotic associated diarrhea (CDAD) worldwide and particularly affects the elderly and immunocompromised. While the pathogenesis of *C. difficile* colitis is primarily mediated by toxin A and toxin B, other accessory virulence factors are undoubtedly involved. In this study, we have initiated an investigation of the role of flagella in virulence. We have focussed on two strains, namely, *C. difficile*, 630Δerm and R20291. The latter is a so-called 'hypervirulent' ribotype 027 strain. The two strains exhibit a number of key phenotypic differences, most notably: (i) R20291 is less motile than 630Δerm in motility assays; (ii) electron microscopy (EM) showed that R20291 appears to possess a single flagellum, whereas *C. difficile* 630Δerm is peritrichously flagellated, and; (iii) strain R20291 adheres more strongly (3-fold) to Caco-2 cells than *C. difficile* 630Δerm. To date, we have generated several flagellar mutants (*fliC*, *flgE*, *fliD* and *motB*) in strain 630Δerm and a *flgE* mutant in R20291. All mutants where shown to be non-motile and, using EM, to lack visible flagella. To our surprise, mutants appeared to adhere more strongly than the wild strain to Caco-2 cells. Close contact of settled non-flagellated bacteria with the cells may lead to increased binding of an array of exposed surface components to cells. Currently, complementation of the phenotypes of flagellar mutants is being assessed, together with an evaluation of the colonization ability of the mutants compared to the wildtype strain using an axenic mouse model.

P86

ANALYSIS OF CLOSTRIDIUM DIFFICILE CLINICAL ISOLATES RESISTANT TO FLUOROQUINOLONES IN ITALY

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Since few data are available on fluoroquinolone-resistance among Italian *Clostridium difficile* strains isolated in Italy, in this study we analyzed 147 toxigenic *C. difficile* isolates collected by the Istituto Superiore di Sanità, from 1985 to 2008, for susceptibility to fluoroquinolones and for a possible association between fluoroquinolone and MLSB-resistance. All strains were typed by PCR-ribotyping and analysed for susceptibility to moxifloxacin (MX), levofloxacin (LE), gatifloxacin (GA), ciprofloxacin (CI), erythromycin (EM) and clindamycin (CM) by the E-test method. All resistant strains were characterized for substitutions in GyrA and GyrB by gene sequencing and for the presence of an ermB genes by PCR assay. For convenience we divided the strains in two groups: the first group included 70 isolates from 1985 to 2001, the second group 77 isolates from 2002 to 2008. In total 50 isolates (34%) were found resistant to fluoroquinolones, with MICs between 8 and ≥32 □g/ml for MX and GA, 24 and ≥32 □g/ml for CI and ≥32 □g/ml for LE. 10% (7/70) belonged to the first group, whereas 56% (43/77) to the second group. All strains had the substitution Thr82 to Ile in GyrA, except one isolate in the first group and one isolate in the second group that showed the substitution Asp426 to Asn in GyrB. 98% (49/50) of the C. difficile isolates resistant to fluoroquinolones were also resistant to EM and/or CM. Among the resistant strains isolated from 1985 to 2001, 57% (4/6) had an erm(B) gene compared to 9% (4/43) among those isolated from 2002 and 2008. In total, six different PCR-ribotypes (001, 020, 012, 018, 078 and 126) were found. *C. difficile* strains resistant to fluoroquinolones and isolated from 1985 and 2001 belonged to type 001 (two strains), 126 (two strains) and 012, 020, 078 (one strain for each type). Fifty six percent (24/43) of the strains isolated between 2002 and 2008 belonged to type 126, 40% (17/43) to type 018 and 2% (1/43) to type 020 and 001, respectively. The results indicate an increase of fluoroquinolone-resistance among Italian clinical isolates, associated to the spread of clones 126 and 018.

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ROLE OF FIBRONECTIN BINDING PROTEIN A IN *CLOSTRIDIUM DIFFICILE* INTESTINAL COLONIZATION

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The fibronectin binding protein A (Fbp68) of *Clostridium difficile* appears to play a role in adhesion and to be involved in the colonization process with other surface adhesins.
In order to assess the role of FbpA in the colonization process, a mutant of FbpA was constructed in *C. difficile* 630 ∆erm strain by using the Clostron technology. The mutant was *in vitro* and *in vivo* characterized and compared to the isogenic wild type strain.
Inactivation of the *fbp*A gene was confirmed by PCR and by direct sequencing of the junction points. Absence of the protein was confirmed by Western blotting. A modified ELISA test was used to verify the incapacity of the mutant to bind to immobilized fibronectin. Adhesion of the FbpA mutant to human colonic epithelial cell line Caco2 was examined. Surprisingly, FbpA mutant seemed to adhere more than the wild type parental strain. The same experiment was carried out on mucus secreting HT29-MTX cell line. *In vivo* colonization assay in mice is in progress in order to better analyse the role of FbpA in *C. difficile* pathogenesis.

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IDENTIFICATION OF GENES REQUIRED FOR SPORE GERMINATION IN CLOSTRIDIUM DIFFICILE.

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Clostridium difficile is the leading cause of hospital-acquired diarrhoea and a huge burden to health services. *C. difficile* spores are highly resistant to many forms of disinfection and thus are able to persist on hospital surfaces and disseminate infection. Spore germination is defined as the irreversible loss of spore-specific properties and this process is essential in allowing outgrowth of the spore, subsequent vegetative cell formation and hence the pathogenesis of the organism. In order for complete spore core hydration and subsequent spore outgrowth to occur, the peptidoglycan cortex of the spore must be lysed. Cortex lytic enzymes are well understood in *Bacillus* and have been recently studied in *C. perfringens*. The development of ClosTron technology has now allowed us to inactivate, in two strains of *C. difficile* (genome strain 630 and a ribotype 027 strain, R20291), homologues of those genes important for cortex lysis in other spore formers. Using a combination of several different assays to study the mutants in detail, we have identified a gene essential for *C. difficile* spore germination. Knowledge of this complex process is important in understanding disease and in the future may have direct applications in preventing infection.

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NOVEL CHEMICAL PROBES OF SURFACE-LAYER FORMATION IN CLOSTRIDIUM DIFFICILE

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Clostridium difficile is responsible for recent epidemics of gastroenteritis and currently causes over twice as many deaths per year as the other major hospital 'superbug', MRSA. Since the bacterium is resistant to conventional antibiotics there is an urgent need to develop novel therapies. *C. difficile* secretes a family of proteins that are attached to the cell wall by non-covalent forces, producing a proteinaceous coat (the surface layer or S-layer) that surrounds the entire cell. These S-layer proteins (SLPs) are immunogenic in humans and play a role in binding to host cells. Synthesis of the *C. difficile* S-layer involves site-specific proteolytic cleavage of the SlpA precursor by an as yet unidentified *C. difficile* protease. Identification of the protease that cleaves SlpA has proven very challenging due to the lack of established genetic tools in *C. difficile*. Here we report the development of novel chemical probes that can disrupt S-layer formation by inhibiting protease activity. These inhibitors are potentially powerful chemical tools for both protease identification and exploring the process of S-layer formation. Inhibition experiments combined with bioinformatics analysis demonstrate that the site of proteolytic cleavage in SlpA is highly conserved, and is processed by a cysteine protease. Novel irreversible synthetic protease inhibitors based on the known cleavage site of SlpA in strain 630 are shown to disrupt formation of the S-layer when bacteria are grown in the presence of these compounds. The protease can also be labelled by synthetic inhibitors carrying an affinity tag, and we are currently using these 'activity-based probes' (ABPs) to isolate and identify the protease(s) involved in cleavage of SlpA. Future work will focus on cloning the target protease(s), developing novel and selective protease-inhibitor antibiotics against drug-resistant *C. difficile*, and applying this approach to related systems in pathogenic bacteria.

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USING REAL-TIME PCR TO QUANTIFY ADHERENCE OF CLOSTRIDIUM DIFFICILE TO POLARIZED HUMAN INTESTINAL TISSUE CULTURE CELLS AND INTESTINAL BIOPSIES

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Clostridium difficile infection (CDI) occurs when an individual's normal colonic microflora is disrupted, most commonly by treatment with broad-spectrum antibiotics. Recently, an epidemic strain of *C. difficile* has emerged worldwide that is resistant to commonly used treatments, hyper-produces Toxin A (TcdA) and Toxin B (TcdB), and produces a more severe disease. The use of therapies that remove the toxins from the body have to date been unsuccessful in treating CDI, suggesting that there are other factors involved in *C. difficile* virulence. Adherence and colonization are important initial steps in the infection process of many microorganisms. These, however, are still relatively poorly understood steps in the *C. difficile* pathogenic strategy. Here, we describe a novel method to investigate *C. difficile* adherence to polarized Caco-2 cell monolayers. As opposed to methods employing either light microscopy or plate counting procedures, we have developed a real-time PCR (qPCR) assay using a genomic DNA standard curve and primers designed to amplify a *C. difficile*-specific housekeeping gene, triose-phosphate isomerase (tpi). The use of polarized tissue culture cells allows us to monitor monolayer integrity under anaerobic conditions. In addition, qPCR reduces the length of standard plating assays because we do not have to rely on growth of *C. difficile* to obtain our results. This method has provided much more reproducible and consistent results. Moreover, we have successfully modified this method to quantify adherence of *C. difficile* to hamster cecal biopsy specimens. This assay will become the basis for studying adherence mechanisms and testing therapies against *C. difficile* in future studies. Understanding other potential virulence determinants of *C. difficile*, such as adherence, is important for the development of new approaches to treat CDI.

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CHARACTERISATION OF THE PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE* IN THE GOLDEN SYRIAN HAMSTER

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The Golden Syrian Hamster is widely regarded currently an important model of *Clostridium difficile* disease, as oral infection of animals pre-treated with antibiotics reproduces many of the symptoms observed in man. Several strains of *C. difficile*, have recently been characterised in detail using this model and a number of factors identified that affect progression of the infection and severity of disease outcome. In each case, the profile of infection has been monitored using a combination of qualitative and quantitative observations. These include symptoms such as onset of diarrhoea and loss of body temperature (by telemetry), colonisation of various regions of the gut by bacteriological counts of both vegetative cells and spores) and histological changes by light and electron microscopy. As part of these studies, images have been generated that suggest that several unique structures are expressed *in vivo* that enhance the capacity of the organism to attach and survive in the host. These include flagella-like structures, which appear expressed early in the infection process and pili-like structures that are expressed in the crypt regions of the gut, which maybe involved in attachment to the mucosal surface. Scrutiny of the available genomic sequence has identified a putative type IV pilus biosynthesis locus (including pilA-D). Antibody raised to one of the putative pilA proteins (CD3507) recognises this structure in vivo, suggesting a role for type IV pili in the infection process.

P92

TN*5397* HAS TWO PREFERRED INSERTION SITES IN *C. DIFFICILE* R20291: INSERTION INTO BOTH RESULTS IN AN ALTERATION OF CELLULAR MORPHOLOGY.

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Tn*5397* is a conjugative transposon originally isolated from *Clostridium difficile* 630. The element confers resistance to tetracycline and is capable of transfer to and from *Bacillus subtilis* and between *C. difficile* strains. The element has also been found in isolates of *Enterococcus faecalis* from animals. Work in our laboratory has shown that in *C. difficile* Tn*5397* has a highly preferred *attB* site into which it will always insert. In *B. subtilis*, where this site is absent, it will insert without any obvious site preference. However if the *C. difficile attB* site is cloned into the *B. subtilis* chromosome it will always use that site. In this work we wanted to determine how the element would behave in the epidemic ribotype 027 strain, R20291. In this strain a second preferred insertion site was identified and Tn*5397* will use each of these two sites with similar frequency. In R20291 both *attB* sites for Tn*5397* are within open reading frames that have the potential to encode proteins homologous to Fic from *Escherichia coli.* Although these genes have not been well investigated in bacteria, the available evidence indicates that they have a role in maintaining cell shape and in cell division. The strain containing Tn*5397* insertions in 2 *fic* homologues no longer forms filamentous cells. This phenotype could be complemented by cloning the wild-type *fic* into a plasmid vector. The implications of this observation for the virulence and physiology of *C. difficile* are discussed.

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FLAGELLAR GLYCOSYLATION IN CLOSTRIDIUM. DIFFICILE

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Glycosylation of both flagellin and S–layer structural proteins has been extensively reported for a wide range of bacteria including members of the Clostridial genus. Surface proteins such as flagellin and S-layer protein are thought to mediate adherence to host tissues and play a critical role in the process of bacterial colonization. Intact flagellin proteins have been purified from strains of *C. difficile* and analysed using QTOF and linear ion trap instruments. Top-down studies showed the flagellin proteins to have a mass greater than that predicted from the corresponding gene sequence. Additionally, diversity between the observed masses of glycan modifications was seen between strains. The sites of glycan modification on flagellins have been determined using electron transfer dissociation. Bioinformatic analysis of *C. difficile* genomes revealed diversity with respect to glycan biosynthetic gene content within the flagellar biosynthetic locus likely reflected by the observed flagellar glycan diversity. Insertional inactivation of either *fliC* or a glycosyltransferase gene from this locus using the Clostron mutagenesis system in strain *C. difficile* 630 resulted in an inability to produce flagella filaments at the cell surface and only minor amounts of unmodified flagellin protein in the glycosyltransferase mutant strain. Comparison of these two strains with *C. difficile* 630 was examined in a tissue culture model and Syrian hamster model of infection.

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IDENTIFICATION OF NATURAL TARGET CELLS OF CLOSTRIDIUM PERFRINGENS BETA-TOXIN.

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Clostridium perfringens type C is well known as a cause of necrotizing enteritis in animals and humans. The major virulence factor of pathogenic type C strains is beta-toxin, a member of the beta-pore-forming toxin family. The identification of natural target cells as well as the characterization of their dose dependent responses to pore formation by β-toxin are however lacking. We recently revealed endothelial binding of beta-toxin during the acute stage of necrotizing enteritis using immunohistological studies in spontaneously affected pigs and humans. To further evaluate whether endothelial cells are specifically targeted by beta-toxin, we established an *in vitro* system using primary porcine endothelial and control cell cultures. Beta-toxin derived from wild type *C. perfringens* type C strains and two different recombinant expression systems induced marked, dose dependent cytopathic effects and reduced viability of endothelial but not control cells. Pre-incubation with neutralizing monoclonal anti-beta-toxin antibodies abolished the cytopathic effect in a dose dependent manner. Immunofluorescent stainings showed rapid cellular retraction coinciding with a disruption of the actin cytoskeleton and localization of beta-toxin at the cell borders. Moreover, western blot analyses demonstrated multimeric beta-toxin at cell membranes.
In conclusion, our results provide evidence that *C. perfringens* beta-toxin induces cytoskeletal changes and lysis in porcine endothelial cells. Together with the *in situ* localization of beta-toxin at endothelial cells in lesions of necrotizing enteritis, we conclude that porcine endothelial cells represent natural targets of *C. perfringens* beta-toxin. Thus, disruption of the intestinal vasculature might play a major role in the pathogenesis of *C. perfringens* type C enteritis.

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ACTIVATION AND INHIBITION OF CLOSTRIDIUM DIFFICILE SPORE GERMINATION

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Spore formation by Clostridium difficile is a significant obstacle to preventing the spread of 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 and this process requires bile salts. Primary bile salts produced by the liver are composed mainly of cholate and chenodeoxycholate conjugated with either taurine or glycine We have shown that some cholate derivatives can act with glycine to induce the germination of C. difficile spores. Chenodeoxycholate was unable to stimulate the germination of C. difficile spores but was able to inhibit the growth of vegetative bacteria. In fact, we have shown that chenodeoxycholate can also competitively inhibit spore germination induced by cholate or taurocholate. These results suggest that chenodeoxycholate could be used as a therapy to prevent C. difficile spore germination and to prevent growth of spores germinated spores. To address this hypothesis, we have fed chenodeoxycholate to hamsters and detected an increase in chenodeoxycholate levels in fecal extracts. Experiments are ongoing to determine the effect of increased amounts of intestinal chenodeoxycholate on C. difficile pathogenesis.

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ANTIBIOTIC RESISTANCE: CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* CLINICAL ISOLATES

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Clostridium difficile is a well-known cause of diarrhea associated to antibiotic use. In this study, we analysed 204 toxigenic European *C. difficile* clinical isolates for resistance to metronidazole (MZ), vancomycin (VA), erythromycin (EM), clindamycin (CM), tetracycline (TC), moxifloxacin (MX) and rifampin (RIF). The E-test method was used to determine the MIC levels, whereas mechanisms of resistance were characterised by PCR and/or nucleotide sequencing. Twenty one isolates (10%) were multi-resistant, showing resistance to at least 3 different classes of antibiotics. Resistance to EM and/or CM was observed in 96 strains (47%), 7 strains (3.4%) were resistant (R) to TC, 70 (34%) to MX and 22 (11%) to RIF. Interestingly, all isolates R to MX were also R to EM or CM, and all strains R to RIF were R to MX, with the exception of only one strain. No isolate was R to MZ and VA. ermB gene was responsible for the resistance to EM/CM in 43 of the 87 examined strains (49%), whereas 51% resulted negative for ermB and also for other erm classes (AP,C,E,F,Q), except for one strain which showed an ermQ gene. Among the ermB-positive strains, different genetic organizations of the ErmB determinant were observed. All strains R to TC showed a tetM gene located on a Tn5397 transposon, whereas a Tn916-like element was observed in 21 of the 26 strains with an intermediate resistance (>6 -<16 µg/ml). Resistant and intermediate strains were also analysed for the presence of other tet genes (A,P,Q,W,O). The co-presence of a tetM and a tetW gene was observed in 4 resistant strains, whereas one intermediate isolate showed the co-presence of a tetM and a tetO gene. Fifty eight (93%) of the 62 isolates analysed for mutations in gyrA or gyrB showed the substitutions Thr82 to Ile in GyrA, whereas 4 strains showed a substitution in position 126 of GyrB. All the 22 isolates resistant to RIF showed one or two substitutions in RpoB. The majority of them (18/22) showed the substitutions His502 to Asn/Arg505 to Lys. The results indicate an increased propensity of *C. difficile* clinical isolates to acquire resistance to different classes of antibiotics and underline the importance of a constant monitoring of antibiotic susceptibility in this bacterium.

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BACTERIAL FLORA AND PROTOZOAL FAUNA OF COWS FROM FARMS WITH DIFFERENT BOTULISM BACKGROUNDS

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Botulism is caused by the neurotoxins of the 7 toxovars (A – G) of the spore-forming bacteria *C. botulinum*. Besides the well known acute form of botulism by the uptake of preformed toxin and subsequent flaccid paralysis and the uptake of bacteria via external wounds with production of toxin in the damaged tissue, a third form of botulism has been reported which is based on the uptake of bacteria via the alimentary tract, a successful colonisation of the intestines and the production of toxins within the infected organism. In cows, the chronic/visceral botulism causes unspecific symptoms like a decreasing milk yield, indigestion, apathy etc. Although, the impact of the autochthonous fauna and flora on the risk of developing chronic botulism is unquestioned, it is still cryptic which parameters matter most. To elucidate this problem, we collected samples of rumen juice and faeces of cows from farms with cases of acute (2 farms, 118 samples per substrate) and chronic botulism (3 farms, 100 samples) and from 2 farms without any botulism background (60 samples) and studied the bacterial flora of the rumen (3 farms) and the faeces (7 farms) by FISH (rumen fluid) and serial dilution and plate-count-method (faecal samples) and the protozoal fauna of the rumen (7 farms) as well by the use of a counting chamber and light-optical microscopy. Furthermore, we used an enrichment technique followed by an ELISA (in-house method) to look for *Cl. botulinum* A – D in all of the samples. It was possible to detect the following BoNTs: BoNT A in faeces (6 animals, 1 farm) and in rumen juice (13 animals, 2 farms), BoNT B in rumen juice (2 animals, 1 farm), BoNT C in faeces (2 animals, 2 farms) and in rumen juice (7 animals, 2 farms) and BoNT D in rumen juice (1 animal, 1 farm). All samples from the farms without botulism background were negative. There were significant differences in protozoal (*Dasytricha* spp., *Epidinium* spp., *Ophryoscolex* spp.) and bacterial parameters (rumen: EUB, EURY, Clit, DSS, Lab, Gam42a; faeces: *Enterococcus* spp., *Bacteroides* spp., total aerobic germ count, *Candida* spp.) between BoNT-positive and BoNT-negative cows.

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HUMAN INTESTINAL EPITHELIAL RESPONSE(S) TO *CLOSTRIDIUM DIFFICILE*

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C. difficile is a major nosocomial pathogen and cause of *C. difficile* infection (CDI). *C. difficile* possess two structurally similar toxins TcdA (308kDa) and TcdB (270kDa), which are the main virulence factors. Upon administration of antimicrobial agents the normal intestinal microbiota is altered and *C. difficile* otherwise silent intestinal bacteria are able to overgrow and cause disease. In this study (R20291, 630, A^+B^+) and (M68, CF5, A^+B^-) strains were investigated to examine their effects on intestinal epithelium. Disruption of intestinal epithelial cells (IECs) tight junctions (TJ) were investigated using occludin immunofluorescence assay, measurement of transepithelial electrical resistance (TEER), and FITC-dextran influx. Disruption of IECs triggers an innate immune response. IECs innate immune and antimicrobial response to infection with *C. difficile* was assessed employing reverse transcription polymerase chain reaction (RT-PCR) and confirming the results with Enzyme-linked Immunosorbent Assay (ELISA). Effect of infection on epithelial cell viability was assessed using ethidium homodimer uptake assay. It was observed that CF5 (asymptomatic, AB+) strain had the least effect on barrier integrity, cell death and proinflammatory cytokine production. Contrarily M68 (outbreak, A^-B^+) strain may have a greater effect on barrier function and immune response. Comparing effect of different strains on cell viability R20291 caused a greater cell death.

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TOXIN PRODUCTION AND SPORULATION OF HYPERVIRULENT *Clostridium difficile* STRAINS

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Toxigenic *Clostridium difficile* (CD) strains produce two toxins (TcdA and TcdB) during the stationary phase of growth, and cause antibiotic-associated diarrhea. CD isolates of the specific molecular type BI/NAP1/027 have been associated with severe disease outcomes and hospital outbreaks worldwide that have been postulated to be due to these “hypervirulent” (HV) strains producing more toxins, and at all growth phases.
Quantitative methods were used to determine growth rate, sporulation efficiency, toxin gene expression and toxin protein production of four HV and four genetically distinct non-HV CD human clinical isolates. Toxin production was assessed over 48 hours of growth using an enzyme-linked immunosorbent assay. Toxin gene (*tcdA* and *tcdB*), and toxin gene regulator (*tcdR* and *tcdC*) expression was measured using quantitative real-time PCR. Sporulation was quantified by heat-shock and selective bacterial plating. In all CD strains tested, toxins were undetectable during the exponential phase of growth. During the stationary phase of growth, HV CD strains produced, on average, 5-fold more toxin than 3/4 non-HV strains but less toxin than one non-HV strain. During exponential growth, *tcdA*, *tcdB* and *tcdR* showed low, similar baseline expression in both HV and non-HV strains, but were highly expressed in stationary phase in high toxin producing strains, consistent with high toxin production at the protein level. Interestingly, *tcdC* expression did not significantly diminish in stationary phase. HV CD strains also produced significantly more spores than all other strains; and for some HV strains, early, as well as rapid spore accumulation was observed.
We conclude from these data that the choice of comparator non-HV strains is critical in determining the significance of toxin production differences between HV and non-HV CD strains. A synergistic combination of increased toxin production, greater sporulation, and other factors including colonization ability as well as antimicrobial resistance is likely responsible for the severe and widespread disease associated with HV CD strains.

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FIBRONECTIN-BINDING PROTEINS OF CLOSTRIDIUM PERFRINGENS

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Genomic analysis of *C. perfringens* strain 13 revealed that this bacterium contains two genes (CPE0737 and CPE1847) that encode putative fibronectin (Fn)-binding proteins, named, FbpA and FbpB, respectively. These genes which were found to be constitutively expressed in *C. perfringens* were cloned and His-tagged, recombinant FbpA (rFbpA) and recombinant FbpB (rFbpB) were purified by Ni^{2+}-Sepharose column chromatography from transformed *E. coli*. Soluble Fn bound to immobilized rFbps. Fn binding to *C. perfringens* cells was inhibited by the presence of rFbps. To determine the Fn domain recognized by rFbps, we performed a plate binding assay using N-terminal 70 kDa peptide, III_1-C peptide, and 110 kDa peptide containing III_{2-10} of Fn. Both rFbps bound to the III_1-C peptide of Fn but not to the others. However, the III_1-C epitope of Fn is known to be cryptic in soluble Fn. Then, rFbp-binding proteins from Fn were purified by rFbp-affinity chromatography. The yield of purified proteins was approximately 1% of the applied Fn on a protein basis.

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CLOSTRIDIUM DIFFICILE SURVEY IN ITALIAN PIGGERIES USING DIFFERENT DIAGNOSTIC METHODS

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In order to investigate the role of *Clostridium difficile* (CD) in swine enteritis outbreaks, 79 faecal samples, 30 intestinal contents and 12 rectal swabs were collected in 31 different farms from pigs with an history of diarrhoea. Samples were stratified by growing phase (suckling, post-weaning, growing, fattening). Each sample was cultured in a selective medium for CD and the isolates were identified by a commercial biochemical panel kit and by means of a species-specific PCR. Each isolate was tested by multiplex PCR to reveal the presence of *tcd*A and *tcd*B genes encoding for toxin A and toxin B respectively. The samples were screened for CD toxins A and B by using a commercial ELISA. 26 intestinal contents and 73 faecal samples were tested by Real-Time PCR to enumerate CD Colony Forming Units (CFU) per g of sample. CD was recovered from 27 samples and the highest prevalence was detected in suckling pigs (43.5%). Thirteen strains tested positive for both *tcd*A and *tcd*B genes, one strain was *tcd*A-/*tcd*B-, whereas 13 resulted *tcd*A-/*tcd*B+. 28 samples resulted positive by Real-Time PCR and the highest CD amounts (10^2-10^3 UFC) were detected in samples that tested positive for toxins as well as at cultural examination. Toxin A and/or B were detected in 21 of the 27 samples positive for CD. This study highlights the involvement of CD in outbreaks of enteric disease in swine in Italian farms, irrespective of age, even though the highest prevalence was recorded in suckling pigs. Furthermore the enumeration of this enteric pathogen by Real-Time PCR coupled with ELISA toxin test provides a rapid and accurate tool for the diagnosis of clostridiosis caused by CD.

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EPSILON-TOXIN-GFP IS A USEFUL TOOL TO DETECT MYELINATED STRUCTURES

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Epsilon-toxin, produced by Clostridium perfringens type B and D, is the main agent responsible for enterotoxaemia in livestock. Neurological and renal disorders are characteristic of the onset of toxin poisoning. Recombinant epsilon-toxin-green fluorescence protein (epsilon-toxin-GFP) and epsilon-prototoxin-GFP have already been characterized as useful tools to track their distribution in intravenously injected mice (Soler-Jover et al., J. Histochem. Cytochem., 2004). Using a mouse model of acute intoxication, we observed that epsilon-toxin-GFP, not only caused oedema but also crossed the blood-brain barrier and accumulated into the brain tissue. In some brain areas, epsilon-toxin-GFP bound to glial cells. Cytotoxicity assays with glial primary cultures, demonstrated the cytotoxic effect of epsilon-toxin upon both astrocytes and microglial cells (Soler-Jover et al.,Toxicon., 2007).
In the present work, epsilon-toxin-GFP fusion protein was used to incubate brain cryosections. Fluorescence microscopy analysis showed specific binding of epsilon-toxin to myelinic structures. Myelinated peripheral nerve fibres were also stained by epsilon-toxin. Binding to myelin was not only restricted to rodents, but was also found in humans, sheep and cattle. Moreover, in the brains of both sheep and cattle, besides the binding to myelin, the toxin strongly stained the vascular endothelium (Dorca-Arévalo, et al., Vet. Microbiol, 2008). To further characterize the binding we did different aproaches using proteases, detergents and a chemical deglycosylation (beta-elimination). Our results point to the involvement of O-glycosilated proteins in the binding.
Although the binding of epsilon-toxin to myelin does not directly explain its neurotoxic effect, this feature opens up a new line of enquiry into its mechanism of toxicity and establishes the usefulness of this toxin for the study of the mammalian nervous system.

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RECENT STUDIES ON *CLOSTRIDIUM BOTULINUM* IN NORTHERN IRELAND

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Botulism in cattle was rare in Northern Ireland until 1999, but since then some 764 cases from suspect outbreaks have been submitted to the Veterinary Sciences Division (VSD) of AFBI for diagnostic investigation. Botulinum neurotoxin has been detected in samples from 117 of these cases using the mouse bioassay, largely from the testing of intestinal samples rather than serum from suspect cases. The actual number of cases is likely to be higher. Increased numbers of suspect botulism cases have also been observed in the Republic of Ireland, Great Britain and some parts of continental Europe in recent years. Previous work at VSD has suggested a link between poultry litter and botulism in cattle, and further evidence in the current outbreak also supports this litter association. A project focussing on the diagnosis and epidemiology of botulism in cattle has been initiated at VSD. The initial objectives of this study are to determine the levels of botulinum toxin and the potential for the production of toxin by culture of the *C. botulinum* organism. within poultry litter samples as a potential exposure source for cattle, and also within the intestines of non-suspect botulism bovine post mortem cases to further validate the diagnosis of toxin detection in bovine gut samples. This work will also attempt to develop new methods for testing for the presence of the botulinum toxin by monoclonal antibody and PCR based assays as alternatives to the mouse bioassay, the use of which is raising welfare concerns. Preliminary results for this project will be presented.

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BINDING OF EPSILON-TOXIN FROM CLOSTRIDIUM PERFRINGENS TO RENAL SYSTEM

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Epsilon toxin, produced by *Clostridium perfringens* types B and D, causes fatal enterotoxemia, also known as pulpy kidney disease, in livestock. Recombinant epsilon-toxin-green fluorescence protein (epsilon-toxin-GFP) and epsilon-prototoxin-GFP were successfully expressed in *Escherichia coli*.
Epsilon-toxin-GFP and epsilon-prototoxin-GFP bound to endothelia in various organs of injected mice, especially the brain. However, fluorescence mainly accumulated in kidneys and mice injected with epsilon-toxin-GFP showed severe kidney alterations, including hemorrhagic medullae and selective degeneration of distal tubules (Soler-Jover et al., J. Histochem. Cytochem., 2004).
Here we show by direct fluorescence analysis on kidney and urothelium cryosections specific binding to distal and collecting renal tubule cells and also to the epithelium of the bladder in a range of species. To further characterize the binding we did different approaches using proteases, detergents and a chemical deglycosylation (beta-elimination). Our results point to the involvement of O-glycosilated proteins in the binding of epsilon-toxin in renal tissue.
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EPIDEMIOLOGICAL SURVEY OF OVINE AND CAPRINE ENTEROTOXAEMIA IN THE SOUTH OF ITALY

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Enterotoxaemia of sheep and goats is due to sudden and tumultuous intestinal replication of bacteria of the genus Clostridia. This leads to production of a large repertoire of toxins which cause the disease. An epidemiological survey was carried out in the South of Italy (Puglia, Basilicata, Calabria and Campania regions) to assess the clostridial species and the toxins involved in this pathology. Two hundred and forty animals, died after hyper-acute or acute disease with systemic signs and/or localized lesions of acute enteritis at necropsy, were included in the study population. Bacteriological analyses were performed on the small intestine content, on the liver and kidneys of all the animals. C. perfringens was detected in all the animals and the isolates were analysed by polymerase chain reaction (PCR), in order to determine the prevalence of the cpa, cpb, cpb2, etx, iap and cpe genes. The most prevalent toxin-type of C. perfringens was type A (84%), while C. perfringens type B, C, and E were not found. C. perfringens type D was isolated in 16% of the cases. About 24% of the isolates was cpb2 positive. The prevalence of cpb2 across the different C. perfringens types markedly varied. The β2-toxin gene cpb2 was detected in 4 out of 21 (19%) type A isolates, in 1 of 2 type D isolates, and in 1 of 2 type DE (cpe-carrying type D) isolates. This large survey on C. perfringens strains isolated from disease outbreaks revealed that both C. perfringens toxin-type A and toxin-type D, with or without the beta2 toxin, are the main toxin-types involved in enterotoxaemia outbreaks in Italy, in agreement with what observed in other European countries.

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OCCURANCE OF CLOSTRIDIUM BOTULINUM IN CATTLE SAMPLES IN GERMANY – AN INCREASING PROBLEM AND PTENTIAL RISK FACTOR FOR FOOD SAFETY?

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Clostridium (C.) botulinum (neurotoxintypes A-G)produces the most toxic food poisonous substance of bacterial origin. *C. botulinum* types C and D are frequently the causative agent of cattle botulism. *C. botulinum* is widespread in the environment and can be found in animals with acute or chronic botulism or to a much lower extend even in the intestines of healthy animals. Our aim was to determine the occurrence of *C. botulinum* in various samples of cattle herds which had problems with acute or chronic botulism. We tested viscera (liver, kidney and gut samples) from 20 cattle from 6 different herds, which died or were euthanized during an acute outbreak of botulism. 142 Faecal samples from 6 herds, where the presence of *C. botulinum* was assumed to be involved with the unsatisfying medical condition, and 115 faecal samples from 4 herds, where no specific health problems were reported, were also included in this study. The presence of *C. botulinum* was diagnosed with an ELISA, detecting soluble antigens of *C. botulinum* produced during an enrichment procedure. A positive result concerning viscera was confirmed by PCR and a detection of *C. botulinum* and its toxins in slide smears with a peroxidase conjugated polyclonal antibody Visceral samples: In 13 from 20 animals *C. botulinum* could be detected in various visceral samples. These animals belonged to 6 different flocks. In 6 liver samples from one flock no positive *C. botulinum* result could be obtained. Faecal samples: From 142 faecal samples from animals of 6 herds with considerable health problems 7 contained *C. botulinum*. From four of these herds viscera were obtained and in viscera of tree herds *C. botulinum* was detected. In contrast no *C. botulinum* could be detected in faecal samples of herds where no specific health problems were reported. Böhnel et al. [1] found considerable incidence of *C. botulinum* in tonsils from slaughtered cattle. In consideration of these, our results show that under specific conditions (agony) *C. botulinum* can translocate from the gut in to eatable viscera, and therefore be a potential risk factor for food safety

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ANTIBIOTIC INDUCTION OF *C. difficile* TRANSMISSION AND DISEASE IN MICE

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Clostridium difficile persists in hospitals by exploiting an infection cycle that is dependent on humans shedding highly resistant and infectious spores. Here we show that human virulent *C. difficile* can asymptomatically colonize the intestines of immunocompetent mice, establishing a carrier state that persists for many months. *C. difficile* carrier mice consistently shed low levels of spores but, surprisingly, do not transmit infection to cohabiting mice. However, antibiotic treatment of carriers triggers a highly contagious supershedder state, characterized by a dramatic reduction in the intestinal microbiota species diversity, *C. difficile* overgrowth and high-level spore excretion. Stopping antibiotic treatment normally leads to a recovery of the intestinal microbiota species diversity and suppresses *C. difficle* levels, although some mice persist in the supershedding state for extended periods. Spore-mediated transmission to immunocompetent mice treated with antibiotics results in self-limiting mucosal inflammation of the large intestine. In contrast, transmission to mice that are compromised in innate immune responses (Myd88$^{-/-}$) leads to a severe intestinal disease that is often fatal. Thus, mice can be used to investigate distinct stages of the *C. difficile* infection cycle and will serve as a valuable surrogate to study the spore-mediated transmission and interactions between *C. difficile* and the host/microbiota, and should guide infection control measures.

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TANNINS AS TOOL FOR THE CONTROL OF INTESTINAL DISEASES PRODUCED BY CLOSTRIDIUM PERFRINGENS

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There is a need to identify alternatives to heavy chemical use that can reduce the risk of livestock disease in a sustainable and environmentally friendly manner. Vegetable tannins are water-soluble polyphenolic compounds of varying molecular weights that occur abundantly in nature. The diet of many free-ranging wild animals contains significant amounts of tannins. Also, commercial tannins are already being used in animal industry as food additives as they can improve animal performance, either in monogastric animals or in ruminants improving the supply of protein post-ruminally. For these reasons, it results attractive to evaluate if tannins have the potential to avoid intestinal diseases produced by *C. perfringens*. Twenty isolates obtained from healthy animals or with intestinal disease were tested. The growth of *C. perfringens* (type A, B, C, D and E) was reduced in a dose-dependent manner in the presence of chestnut tannins (*Castanea sativa*; 80% hydrolysable tannins), quebracho tannins (*Schinopsis* lorentzii, 75% condensed tannins), combinations of both or a commercial formula (supplied by Silvateam & Cecil S.A.). These tannin extracts showed bactericidal activity in all the isolates tested (<1 mg/ml). Although the minimal inhibitory concentration of both tannins varied between isolates, no statistically differences were observed between isolates from healthy or sick animals. The most toxigenic strains were particularly sensitive to tannins. Comparative analysis showed that the concentrations of quebracho tannin required to inhibit the growth of *C. perfringens* type A, B, D or E were between 7-85 times higher than chestnut tannin (0.6-1.2 mg/ml vs. 0.003-0.15 mg/ml). Antibacterial effect of quebracho tannin was increased up to 20 times with the addition of 25% of chestnut tannin and 85 times with 75% of chestnut tannin. Antibacterial activity of commercial product was up to ~50 times higer than quebracho tannin alone. Both tannins were able to reduce alpha toxin lecitinase activity and epsilon toxin citotoxicity in MDCK cells. The incidence of the *C. perfringens* type D disease was highly reduced in tannin treated animals. These results suggest that tannin-supplemented diet can prevent some clostridial diseases.

P109

PRELIMINARY CHARACTERIZATION OF LARGE PLASMIDS OF NECROTIC ENTERITIS ISOLATES of *CLOSTRIDIUM PERFRINGENS*

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Clostridium perfringens type A isolates cause necrotic enteritis (NE), a common and important clinical and subclinical infection of broiler chickens. NE is usually controlled by the routine use of preventive antibiotics, but this type of use of antibiotics in animal production is increasingly under adverse scrutiny. Information about plasmids of NE isolates of *C. perfringens* is limited, therefore characterization of large plasmids found in NE isolates may identify potential avenues for use of this knowledge in control of the infection. In the current study, pulsed-field gel electrophoresis (PFGE) of a small collection of isolates of known virulence recovered from chickens with NE (CP1 to CP4, CP6) showed the variable presence of up to four different plasmids, whose sizes range from 48 to 97 kb. Southern blotting of pulsed-field gels showed that the *netB*, *cpb2* (atypical) and *tpeL* genes are carried on different large plasmids in some of these isolates. The"*cpb2*-plasmid" was maintained in all 5 isolates even after subculturing 90 times, whereas the "*netB*-plasmid" was lost in three of four isolates during passage, leading to complete attenuation of virulence for chickens. Retention of the "*netB*-plasmid" in the passaged isolate CP4 correlated with its retention of virulence. PCR studies revealed that plasmids of NE isolates do not carry the *cpe* gene which encodes the *C. perfringens* enterotoxin. It is known that *C. perfringens* plasmids possess a conserved backbone structure. Plasmids from NE isolates appear to carry conjugative transposon Tn*916*-like genes which in other *C. perfringens* plasmids encode conjugative transfer. These findings show the presence of plasmids in NE isolates carrying known (*netB*, *tpeL*) or putative (*cpb2* atypical) virulence genes and indicate that: (i) the most virulent NE isolates (CP1, CP4) can carry multiple plasmids, which may be lost with passage; (ii) plasmids of the non-virulent NE isolate (CP6) carry the *cpb2* gene but not the *netB* gene; and (iii) the presence of "*netB*-plasmids" are important for virulence and may be capable of conjugative transfer.

P110

IMMUNIZATION OF BROILER CHICKENS AGAINST NECROTIC ENTERITIS USING PURIFIED IMMUNOGENIC PROTEINS

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Necrotic enteritis (NE) is an important disease of broiler chickens caused by *Clostridium perfringens*. Secreted proteins of virulent and avirulent *C. perfringens* were electrophoretically separated and reacted with serum of chickens immune to NE. Three secreted protein bands unique to the virulent *C. perfringens* that reacted strongly with immune serum were identified by Mass Spectrometry as the toxin *C. perfringens* large cytotoxin (TpeL), Endo-beta-N-acetylglucosaminidase (Naglu) and Phosphoglyceromutase (Pgm). The genes encoding Naglu and Pgm proteins were cloned and their histidine-tagged recombinant proteins purified from *Escherichia coli* and used for intramuscular immunization of chickens. Groups of immunized and non-immunized control birds were then challenged with either strain CP1 or strain CP4 of *C. perfringens*. Of the two immunogens, Pgm immunization produced significant protection of birds although protection reduced as challenge severity increased. However, birds immunized with Naglu were protected from challenge only with strain CP4. Birds immunized with these proteins had antigen-specific antibodies when tested in ELISA. I n conclusion, this study demonstrated the partial efficacy of additional secreted proteins in immunity of broiler chickens to NE, and showed that there may be differences in the protective ability of immunogens depending on the infecting *C. perfringens* strain.

P111

BACTERIOPHAGES OF THE FAMILY SIPHOVIRIDAE CONTAIN AMIDASE ENZYMES THAT LYSE *CLOSTRIDIUM PERFRINGENS*

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In chickens *Clostridium perfringens* (Cp) is the etiologic agent of necrotic enteritis and causes gas gangrene along with being the third leading cause of bacterial food-borne gastroenteritis in humans. While the disease in poultry can be controlled by antibiotics, there is increasing pressure to ban antimicrobial growth promoters (AGP) in animal feed. Bans of AGP's have yielded an increased incidence of Cp-associated necrotic enteritis in poultry which could potentially threaten human health. Consequently, screening of bacteriophages lytic for Cp was performed utilizing filtered samples obtained from poultry intestinal material, soil, sewage and poultry processing drainage in the southeastern USA. Bacteriophages had icosohedral heads with approximate 100 nm tails characteristic of the *Siphoviridae* family in the order *Caudovirales* and DNA genomes of approximately 40kb. Interestingly each one of the phages had host ranges restricted to specific Cp isolates. Two putative lytic enzyme genes from phiCP39O and phiCP26F were cloned, expressed in *E. coli* and the proteins purified to homogeneity. The actual Edman degradation/mass spectrometry and predicted amino acid sequences of the two recombinant proteins were homologous to amidases. The phage amidases were identical at the C-terminus (cell-wall binding domain), but only 55 per cent similar to each other at their N-terminal catalytic domain. Both recombinant lytic enzymes were able to lyse parental phage host strains of Cp as well as other Cp isolates assayed but not other clostridial species. Phage host-range specificity supports the use of phage lytic proteins rather than use of whole-phage preparations to control disease-causing bacteria.

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COMPARATIVE ANALYSIS OF CLOSTRIDIUM BOTULINUM TYPE C ISOLATES FROM BOTULISM OUTBREAKS IN SWEDISH BROILER FARMS

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Type C botulism, a paralytic disease caused by neurotoxin producing Clostridium botulinum, has in recent years become a serious problem in poultry flocks in Sweden. During 2008, botulism outbreaks were confirmed in 13 different broiler farms. This is the highest number since 2003 when the first outbreak was registered. The reasons behind this increase are not fully understood and speculations about a contaminated common source made it important to investigate if the same clone was responsible for the 2008-year outbreaks. C. botulinum type C was isolated from poultry caeca or liver from seven of the affected farms. The isolates were analyzed by pulsed field gel electrophoresis (PFGE). Cells had to be fixed in formaldehyde, and HEPES buffer was used for electrophoresis to prevent DNA degradation by endogenous DNase activity and Tris radicals. The PFGE patterns from SmaI and SalI digests were identical for all seven isolates. This finding might suggest that the bacteria are derived from a common source.

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CLOSTRIDUM PERFRINGENS AND ABOMASAL ULCERS IN CALVES: IS THE BACTERIUM CAUSATIVE?

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Abomasal ulceration and abomasitis are important problems in veal calves. High prevalence rates have been reported, indicating the economical importance of the disease. *Clostridium perfringens* is one of the most suspected bacterial agents to induce abomasal ulcers. If *C. perfringens* is causative, one should expect that (1) the bacterium can be isolated from the ulcers in high numbers in nearly pure cultures, (2) the bacterium can be isolated at different spots of the diseased tissue and even in the gut of the cases, in clonal form, as confirmed by genetic tools, (3) disease can be induced after inoculation in calves in order to fulfill the postulates of Koch. The first two points were tested in Belgian Blue calf, i.e. a case of sudden death caused by abomasal ulceration. Macroscopical lesions included abomasal nonperforating ulcers containing hemorrhagic material. Histologically, abomasal ulcers presented as demarcated areas of coagulative mucosal necrosis and haemorrhage, sometimes extending into the submucosa. Moderate numbers of neutrophils and macrophages were infiltrating these lesions, demarcating small numbers of rod-shaped bacteria adherent to the surface of the necrotic mucosa. Isolates collected from different ulcers in the abomasum and from the gut yielded a clonal population of C. perfringens type A, as confirmed by PFGE analysis. This clearly suggests, at least in this calf, an etiological role of *Clostridium perfringens* in the abomasal ulcer induction, although other additional factors may also be involved. Inoculating this particular clone, AU1, in a calf model and reproduction of abomasal ulcers will be essential to confirm a role of C. perfringens in the disease.

P114

CD34 NEUTRALISES THE CYTOTOXIC ACTIVITY OF C. DIFFICILE TcdB IN VITRO

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Conventional antibiotic therapy for antibiotic associated colitis has the limitation that the disease is mediated by toxins and their expression may be enhanced by sub lethal levels of antibiotic therapy. This form of treatment does not neutralize them. Individuals who had recovered from *C. difficile* infection were immunologically profiled by sequencing the dominant antibody genes. Representatives of these were expressed in *Escherichia coli* as histidine tagged scFvs and these were used to screen for the immune dominant antigens where recovery correlated with a humoral response. This identified Toxin B (TcdB), a high molecular weight toxin previously described in the cause of *C. difficile* pathology.
CD34 was an example of such a scFv which bound to TcdB in both ELISA and dot blot format. The heavy chain represented about 10% of the antibody sequences from one particular patient who had recovered from colitis due to *C.difficile*. Furthermore, we demonstrated that 3□g/ml CD34 had the ability to neutralise the cytotoxic activity of *C. difficile* TcdB from culture supernatants in a tissue culture assay. We discuss the potential use of CD34 as a targeted *C. difficile* therapy.

P115

DEVELOPMENT OF A NOVEL CLOSTRIDIUM DIFFICILE RECOMBINANT PROTEIN VACCINE TARGETING THE RECEPTOR BINDING DOMAINS OF TOXIN A AND B AND ADJUVANTED WITH TLR-5 AGONIST SALMONELLA TYPHIMURIUM FLAGELLIN

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Clostridium difficile is the leading cause of nosocomial infectious diarrhea. *C. difficile* produces two toxins (CDA and CDB), and systemic and mucosal anti-CDA antibodies prevent or limit *C. difficile*-associated diarrhea. Here we report the development of a novel recombinant *C. difficile* vaccine containing the receptor binding domains of toxin A (CDA-RBD) and B (CDB-RBD) fused with *Salmonella typhimurium* flagellin, which is a potent Toll-like receptor (TLR) 5 ligand. A number of studies have clearly demonstrated that flagellin is a potent adjuvant that promotes robust immune responses when it is given with a protein antigen. The fusion protein constructs (CDA-RBD-FliC and CDB-RBD-FliC) retains full TLR 5-stimulating activity in an in vitro cell-based NF-kB induction assay. To evaluate the immunoadjuvantative properties of flagellin, we intraperitoneally immunized mice on days 0 and 7 with adjuvanted or unadjuvanted CDA-RDB and CDB-RBD constructs using a prime-boost immunization protocol. Mice immunized with CDA-RBD and CDB-RBD developed prominent anti-CDA and anti-CDB IgG in serum, and anti-CDA and anti-CDB IgA responses in stool. Sera from immunized mice were able to neutralize *C. difficile* toxin A and B activity in an in vitro cell culture assay. Our results suggest that incorporation of flagellin in our toxin A/B RBD vaccine can provide a strong adjuvant effect, resulting in a potent and efficacious vaccine against *C. difficile,* an important cause of morbidity and mortality against which current preventative strategies are failing.

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SURFACE LAYER PROTEINS FROM CLOSTRIDIUM DIFFICILE INDUCE INFLAMMATORY AND REGULATORY CYTOKINES IN DENDRITIC CELLS.

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Clostridium difficile exerts its pathological action mainly by the activity of toxin A and toxin B. Less known is the role that S-layer proteins (SLPs), predominant surface components of the bacterium, may play in pathogenesis. Since SLPs are basic components of the bacterium and immunodominant antigens, a role in pathogenesis and specifically in the inflammatory process has been postulated.
We have investigated the ability of SLPs to modulate the function of human monocyte-derived dendritic cells (MDDC) and to induce inflammatory and regulatory cytokines influencing the natural and adaptive immune response. SLPs extracted from the clinical isolate C253 induced maturation of MDDC and secreted large amounts of IL-10 and IL-12p70. MDDC treated with C253-SLPs enhanced proliferation of allogeneic T cells and induced a mixed Th1/Th2 orientation of naïve CD4 T cells.
Overall, these data proved the immunomodulatory role of SLPs that may contribute to *C. difficile* pathogenicity by perturbing the fine balance of inflammatory and regulatory cytokines. Our findings are of interest also in the light of the possible use of SLPs in a multicomponent vaccine against *C. difficile* infections for high risk patients. We intend to further expand the knowledge on the ability of SLPs to affect the immune response of the host, in particular focusing our studies on the hypervirulent strain *C. difficile* 027.

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FREQUENCY OF EPIDEMIC BI/NAP1/027 *CLOSTRIDIUM DIFFICILE* IN THREE NORTH AMERICAN SURVEYS AND EVIDENCE OF REDUCED RESPONSE TO TREATMENT

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Hines VA Hospital obtained and typed 1374 *Clostridium difficile* (CD) isolates from patients with CD infection (CDI) in 3 North American (NA) studies during November 2005-February 2009. Restriction endonuclease analysis (REA) typing on 548 isolates voluntarily submitted from 45 locations in 25 states revealed that 54% (296/548) were REA group BI (PCR ribotype 027 and PFGE type NAP1). In 2 randomized clinical trials of tolevamer and fidaxomicin, the incidence of BI was 36% (159/444) and 36% (137/382), respectively. In the fidaxomicin (FDX) vs. vancomycin (VAN) trial, treatment response rate (cessation of diarrhea by end of treatment) for either VAN or FDX was 85.4% (117/137) for BI isolates vs. 95.5% (234/245) for non-BI isolates (P=.001). For VAN the BI response rate was 84.7% (61/72) vs. 94.4% (119/126) for non-BI (p=.037), and for FDX the BI response rate was 86.2% (56/65) vs. 96.6% (115/119) for non-BI (p=.012). FDX and VAN were not significantly different in response rate, and there was no significant difference in recurrence rate for BI, however, FDX significantly reduced the recurrence rate of non-BI CD isolates compared to VAN (7.8% vs. 25.5%) p<.001, as well as for all patients (13.3% vs. 24%) p<.005. CDI caused by BI isolates is common in NA hospitals and does not respond as well to treatment as CDI caused by non-BI isolates, in part explaining increased morbidity and mortality with BI/NAP1/027.

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CHARACTERISATION OF CLOSTRIDIUM DIFFICILE SPORES

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Clostridium difficile, an anaerobic, spore-forming bacterium is currently the commonest cause of hospital acquired infection in the UK. In Wales alone 3000 cases were reported in patients over 65 from July 2007 to June 2008. Factors that contribute to the pathogenicity of the organism include the ability to produce a resistant spore which enables the organism to survive for months on contaminated hospital surface and resist the attentions of commonly used biocides. In order to better understand the contribution of this important structure to the virulence of bacterium we initiated a study to characterise the properties of a collection of twenty diverse clinical isolates of C. *difficile* which included hyper-virulent 027,106 and 001 ribotypes. No relationship was observed between an isolate ribotype and its ability to produce spores when cultured in degassed BHI broth. We did find, as have others, that inclusion of 1% sodium taurocholate in the enumeration medium increased the spore count of some isolates. To further characterise the physical properties of these spores the relative hydrophobicity of each isolate was determined using a Hexadecane based microbial adhesion to hydrocarbon assay. The results suggest a relationship between hydrophobicity and PCR ribotype with hyper-virulent isolates such as 027 tending to be predominantly hydrophobic. To determine if hydrophobicity relates to discernable differences in spore structure we employed Transmission Electron Microscopy to compare the structure of hydrophobic DS1813 (77%) and hydrophilic DS1748 (14%) isolates. Our results suggest that these differences relate to the presence or absence of the exosporium- the outer layer of the spore. In subsequent studies we demonstrated that exposure of spores to sodium taurocholate or the chlorine based biocide HazTabs (1000ppm) affected both spore structure and hydrophobicity. In conclusion we found significant variations in the properties for the 20 C. *difficile* isolates examined in this study. Our results highlight the need to examine a representative collection of diverse isolates and caution against drawing conclusions based on a single isolate.

P119

CRYSTAL STRUCTURE OF A CATALYTICALLY ACTIVE ENDOPEPTIDASE DERIVATIVE OF *Clostridium botulinum* TOXIN A

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Botulinum neurotoxins (BoNTs) modulate cholinergic nerve terminals to result in neurotransmitter blockade. BoNTs consists of catalytic (LC), translocation (Hn) and cell-binding domains (Hc). The binding function of the Hc domain is essential for BoNTs to bind the neuronal cell membrane, therefore removal of the Hc domain results in a product that retains the endopeptidase activity of the LC but is non-toxic. Thus, a molecule consisting of LC and Hn domains of BoNTs, termed LHn, is a suitable molecule for engineering novel therapeutics. The structure of LHA at 2.6Å reported here provides an understanding of the structural implications and challenges of engineering therapeutic molecules that combine functional properties of LHn of BoNTs with specific ligand partners to target different cell types.

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ORAL VACCINATION AGAINST *CLOSTRIDIUM DIFFICILE* WITH CWP84 ENCAPSULATED WITHIN PECTIN BEADS

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Clostridium difficile is a bacterium that causes disease of the intestine, particularly after treatment with antibiotics. The bacterium produces two toxins (A and B) that are responsible for the pathology of the disease. *C. difficile* exerts its pathological effects at the intestinal surface; thus, a vaccine that stimulates mucosal immunity in the gut should be an appropriate line of defense against this pathogen. A number of bacterial virulence factors are implicated in the colonization process which is the first step of pathogenesis. Blocking the primary stages of infection namely the colonization of the mucosal surface, may be an effective strategy to prevent *C. difficile* infection. Cwp84 is a cystein protease which is highly immunogenic in patients with *C. difficile*-associated-disease, suggesting that Cwp84 could play an important role in the physiopathology of *C. difficile*. In particular, Cwp84 could contribute to the cleavage of the extracellular matrix host proteins to facilitate the degradation of host tissue integrity.
The aim of this study was to evaluate the surface protease Cwp84 encapsulated within pectin beads as an oral vaccine candidate in a hamster model. Pectin is non-toxic, not digested by gastric or intestinal enzymes and almost totally degraded by pectinolytic enzymes produced by the colonic microbiota. Pectin beads therefore appear as an adequate vehicle to specifically deliver Cwp84 to the colon.
Hamsters were divided in two groups. The first one was immunized with pectin beads encapsulating Cwp84 and a control group received similarly unloaded beads. After three immunizations by the intragastric route, hamsters received Clindamycine. Then, five days later, they were challenged with spores of a toxigenic strain of *C. difficile*. Post challenge survival was followed.
On one hand, two days after spores administration all the control hamsters have deceased. On the other hand, about 40% of hamsters administered with Cwp84-loaded beads survived 10 days after challenge, proving that oral vaccination provides partial protection.

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BACTERIOCIN E1073 PRODUCED BY ENTEROCOCCUS FAECIUM LWP1073 IS EFFECTIVE FOR TREATING COMMENSAL CLOSTRIDIUM PERFRINGENS INFECTION IN BROILERS

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Enterotoxin-producing *Clostridium perfringens* type A bacteria occupy a significant place in the etiological structure of food-borne infections in humans. One potential approach to minimize infections associated with food-borne pathogens is to control the carriage of *C. perfringens* in broilers. For this purpose we propose that a preparation of bacteriocin E1073 could be used (molecular mass of 3,256Da, pI = 8.2, 34 amino acids, class IIa, inhibits in vitro the growth of *C. perfringens*, *Salmonella* spp., *Campylobacter* spp.). For treating experimentally induced non-disease causing commensal *C. perfringens*-associated infection we used 14-day old commercial broilers. The infection was reproduced by challenging chickens with *C. perfringens* along with simultaneously providing them with a high roughage diet (50% rye flour + 50% fish meal). The birds were provided with drinking water added with bacteriocin E1073 in different concentrations for 3 days. The bacteriocin in doses of 32.8 and 16.9 mg/bird produced a significant therapeutic effect. The concentration of *C. perfringens* in control birds was 1.2 x 106 CFU per one gram of iliac homogenate versus *C. perfringens*-free samples from treated birds. Bacteriocin E1073 was also effective against mixed infections caused by *C. perfringens* and *C. jejuni*, and *C. perfringens* together with *C. jejuni* and *S. enteritidis*. These pathogens either were not identified or their concentrations were reduced by 103-106 CFU. Results demonstrated that bacteriocin E1073 is a promising therapeutic alternative to antibiotics for elimination of *C. perfringens* from pre-slaughter commercial broiler chickens.

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PROTEOMIC ANALYSIS OF THE *CLOSTRIDIUM DIFFICILE* SPORE COAT.

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The spore form of *Clostridium difficile* is a vehicle for widespread contamination and infection in environments such as care homes and hospital wards, where they can persist in the environment through long periods of dormancy. The precise mechanism whereby the coat protects against reactive chemicals is not known, but the coat proteins may perhaps react with and detoxify such compounds. It provides a mechanical integrity and excludes large toxic molecules while allowing small nutrient molecules to penetrate and interact with the germination receptors. Despite spores having such a vital role in contamination and disease transmission, little is known about the structure and biology of the spore coat or the molecular mechanisms that lead to sporulation in *C. difficile*. In the present study various protein extraction methods which included both physical (eg. sample homogenisation with beads) and chemical (eg. spore coat solubilisation buffers) procedures were used to investigate the release of proteins from these robust structures. 2-D PAGE and LC-MS-MS together with data from full genome analysis (currently in progress) is being used to provide a landscape of the spore coat subproteome. These data will be used be used to elucidate the pathways that regulate sporulation and provide insight into processes that can be used to restrict spore survival and dissemination.

P123

PURIFICATION AND PARTIAL CHARACTERIZATION OF A NOVEL ANTIMICROBIAL PEPTIDE FROM CLOSTRIDIUM PERFRINGENS STRAIN 56

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Necrotic enteritis in broiler chickens is associated with netB positive *Clostridium perfringens* type A. Single strain dominance is found in the gut of broiler chickens suffering from necrotic enteritis: within an outbreak, all affected animals carry the same clonal *C. perfringens* strain in the affected tissue. It is known that *C. perfringens* is capable of secreting factors inhibiting growth of other *C. perfringens* strains and this characteristic is more prevalent in outbreak strains compared to normal microbiota strains. This characteristic could lead to extensive and selective presence of a strain that contains the genetic make-up enabling to secrete toxins that cause gut lesions. A bacteriocin-like inhibitory substance (BLIS) from a necrotic enteritis associated *C. perfringens* strain, exhibiting antibacterial activity against other *C. perfringens* strains, was purified and characterized. Amino acid sequence analysis indicated that the BLIS is an 11.5kDa fragment of a 22kDa protein and that it constitutes the C-terminal part of this protein. The 22kDa protein is a cytoplasmic membrane protein without signal peptide. The antibacterial activity of the purified BLIS was abolished by proteolytic enzymes trypsin and proteinase K and by heat treatment (10min at 80°C). The purified BLIS showed inhibitory activity over a wide pH-range (4.0 to 10.0). Since the antibacterial activity against other *C. perfringens* strains of the purified BLIS had a narrower spectrum than the crude supernatant of the secreting strain, most probably different unknown bacteriocins are produced.

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ANTI-TUMOR ACTIVITY OF CLOSTRIDIUM DIFFICILE TOXIN B

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Toxin B (TcdB) is a key virulent factor of *Clostridium difficile* and induces intestinal inflammatory disease. Because of its potent cytotoxic and proinflammatory activities, we investigated the anti-tumor activity of TcdB. Using a mouse colorectal cancer model, we have found that CT26 cells were highly sensitive to the cytotoxicity of TcdB. Exposure of CT26 cells to TcdB induced their cytokines/chemokines production. Cells were undergoing apoptosis since effector caspases 3 and 7 were activated and phosphatidylserine was exposed on the cell surfaces. These apoptotic bodies were ready to be phagocytosed by dendritic cells. Co-incubation of the intoxicated CT26 cells with dendritic cells stimulated their activation and cytokine production. Immunization of mice with the TcdB-intoxicated CT26 cells elicited a potent anti-tumor immunity that protected mice from lethal challenge of the same tumor cells. The anti-tumor immunity was long-term since mice were survived from the rechallenge of a lethal dose of the tumor cells two months post immunization. More dramatically, vaccination of the TcdB-intoxicated cells generated a potent therapeutic immunity that completely rejected pre-established tumor. The anti-tumor immunity generated was tumor-specific since the immune mice were protected from challenge of the parental, but not irrelevant, tumors. Intratumor injection of TcdB induced the tumor regression. Further experiments demonstrated that the intact apoptotic bodies were important for the immunogenicity of the intoxicated tumor cells. Freeze/thaw cycles of the intoxicated CT26 cells resulted in a reduced immunogenicity of these cells. Blocking apoptosis induction of the TcdB-exposed CT26 cells with a pan-caspase inhibitor z-VAD also significantly decreased their potency for the induction of anti-tumor immunity. Taken together, our data demonstrated that *C. difficile* toxin TcdB had a potent anti-tumor activity by direct killing of cancer cells and enhancing immunogenicity of the dying tumors, thus provoking anti-tumor immunity. Our finding may have implications in designing tumor-targeted killing and antitumor vaccines.

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