UNIVERSITY OF EXETER

The Conference

Clostridium perfringens

1st – 4th December 2008

Torquay, Devon, UK

Programme and Abstracts

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Marie Curie Conferences and Workshops on Clostridia
Event 9
***Clostridium perfringens*: The Conference**

1st - 4th December 2008
The Grand Hotel, Torquay, Devon

Dear Delegates,

Clostridium perfringens has a unique place in the genus *Clostridium* as the etiological agent of a wide range of diseases of human and animals and as a prolific producer of a diverse range of toxins. The bacterium is probably most notorious as the etiological agent of gas gangrene, but nowadays diseases as diverse as food poisoning in humans, enterotoxaemias in domesticated livestock and necrotic enteritis in poultry are of greater economic importance. The molecular pathogenesis of many of these diseases is poorly understood, and for some, such as necrotic enteritis in poultry which are increasing in incidence, there is an urgent need to devise control measures. In parallel, an understanding of the modes of action of the toxins of *C. perfringens* is allowing their exploitation as novel therapies for diseases such as cancer.

This conference provides new researchers with an overview of our understanding of the pathogenic mechanisms of *C. perfringens* and highlights the research questions which will require addressing in the future.

Rick Titball

Clostridium perfringens: The Conference

PROGRAMME

Mon 1st December

Check in from 3pm
17:00 Registration
18.00 Welcome – Nigel Minton - Cavendish Room
18.30 Drinks reception
19.30 Private dinner in the Devonshire Room

Tue 2nd December

Devonshire Room

Genomics & genetics

09.00 Genetic tools - Julian Rood
09.40 Comparative genomic hybridization analysis suggests different epidemiology of chromosomal and plasmid-borne cpe-carrying *Clostridium perfringens* type A strains - Päivi Lahti
10.00 Distribution of putative spore germination genes in clostridium species – Yinghua Xiao
10.20 Generation of single-copy transposon insertions in *Clostridium perfringens* by electroporation of phage mu DNA transposition complexes - Anouk Lanckriet

10.40 Coffee

Enterotoxaemias of animals

11.00 Pathogenesis of disease - Francisco Uzal
11.40 Molecular basis of toxicity of Epsilon Toxin - Michel Popoff
12.20 *Clostridium perfringens* beta-toxin targets endothelial cells in necrotizing enteritis in piglets - Horst Posthaus
12.40 Toxin typing and antibiotic resistance profiles of *Clostridium perfringens* isolates associated with cases of sudden death and enterotoxaemia in veal calves in Belgium - Adeline Muylaert

13.00 Lunch

Human Disease

14.00 Gas gangrene - Rick Titball
14.40 Food poisoning - Bruce McClane
15.20 Epidemiology of food poisoning – Kathie Grant

16.00 Tea

16.20 Preliminary identification of claudin residues involved in *C.perfringens* enterotoxin binding and action - Susan Robertson
16.40 Recombinant *Clostridium acetobutylicum* expressing *C. perfringens* enterotoxin (*cpe*) for treatment of pancreatic cancer -Sandra Koenig

17.00 Close

19.30 Conference Dinner in the Devonshire Room

Wed 3rd December

Devonshire Room

09.00 Poster session and breakout discussion groups

10.00 Coffee

Necrotic enteritis of fowl

10.40 Pathogenesis of disease - Magne Kaldhusal
11.20 Molecular pathogenesis of disease - Filip van Immerseel
12.00 Intra-species growth-inhibition is an additional virulence trait of *Clostridium perfringens* in broiler necrotic enteritis- Leen Timbermont
12.20 Ecological and functional genomic studies for control of *Clostridium perfringens* infection in broiler chickens - Joshua Gong
12.40 Presence of the necrotic enteritis toxin B, *netB*, gene in *Clostridium perfringens* strains originating from healthy and diseased chickens - Lone Abildgaard

13.00 Lunch

Vaccines

14.00 Animal Vaccines - Keith Redhead
14.40 Necrotic enteritis vaccines – John Prescott
15.20 *Bacillus subtilis* spore vaccines: use of glutathione S transferase - Li Li

15.40 Tea

Diagnostics

16.00 New diagnostics methodologies - Joachim Frey

16.40 Close

19.15 Depart hotel for Kent's Cavern (coach provided)

19.30 Cave Tour

20.30 Carvery Dinner at Kent's Cavern

23.00 Depart Kent's Cavern for hotel (coach provided)

Thur 4th December

Breakfast and check out by 11am.

We would like to acknowledge the help of our generous sponsors (listed on the back cover), without whose help, this conference would have been impossible.

ORAL PRESENTATIONS

GENETIC ANALYSIS OF *CLOSTRIDIUM PERFRINGENS*

J.I. Rood, D. Lyras, J.K. Cheung, T.L. Bannam & M. M. Awad

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Clostridium perfringens is undoubtedly the pathogenic clostridium most amenable to genetic manipulation. In addition, unlike any other clostridium, there are three complete, annotated genome sequences available. For many years it has been possible to electroporate some virulent isolates at a frequency that is high enough to allow for the construction of chromosomal mutants by double crossover events. Unfortunately, many other strains are still not transformable. There is a well established series of shuttle vectors available for complementation studies, including shuttle plasmids that can be used to introduce genes into *C. perfringens* by RP4-dependent conjugative transfer from *Escherichia coli*. Finally, group II intron-based technology can also be used to construct defined chromosomal mutants in *C. perfringens*. We have used various combinations of these genetic tools to analyse the functional role of toxins in disease, to determine how these toxin genes are regulated and to carry out genetic analysis of the conjugation process in *C. perfringens.*

COMPARATIVE GENOMIC HYBRIDIZATION ANALYSIS SUGGESTS DIFFERENT EPIDEMIOLOGY OF CHROMOSOMAL AND PLASMID-BORNE *cpe*-CARRYING *CLOSTRIDIUM PERFRINGENS* TYPE A STRAINS

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[1]Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, 00014 Helsinki University, Finland

Enterotoxin-producing *C. perfringens* type A is a common cause of food poisonings. The cpe encoding the enterotoxin can be chromosomal (genotype IS1470-cpe) or plasmid-borne (genotypes IS*1470*-like-*cpe* or IS*1151*-*cpe*). The chromosomal cpe-carrying *C. perfringens* are a more common cause of food poisonings than plasmid-borne *cpe*-genotypes.

The chromosomal *cpe*-carrying *C. perfringens* type A strains are often present in retail foods and are generally more resistant to most food-processing conditions than plasmid-borne *cpe*-carrying strains. On the other hand, the plasmid-borne *cpe*-positive genotypes are more commonly found in human faeces than chromosomal *cpe*-positive genotypes and humans seem to be a reservoir for plasmid-borne *cpe*-carrying strains. Thus, it is possible that the epidemiology of *C. perfringens* type A food poisonings caused by plasmid-borne and chromosomal *cpe*-carrying strains is different.

A DNA microarray was designed for analysis of genetic relatedness between the different cpepositive genotypes of *C. perfringens* strains isolated from human and food samples. The DNA microarray contained two probes for all protein coding sequences in the three genomesequenced strains (*C. perfringens* type A strains 13, ATCC13124, and SM101).

The chromosomal and plasmid-borne *C. perfringens* genotypes were grouped into two distinct clusters, one consisting of the chromosomal cpe genotypes and the other consisting of plasmidborne *cpe* genotypes. Analysis of the variable gene pool suggests different epidemiology of *C. perfringens* food poisonings caused by chromosomal and plasmid-borne cpe-positive genotypes.

DISTRIBUTION OF PUTATIVE SPORE GERMINATION GENES IN CLOSTRIDIUM SPECIES

Y. Xiao[1], A. Wagendorp[2], C. Francke[3], T. Abee[1], M. Wells-Bennik[2]

[1] Food microbiology laboratory, Wageningen University, Wageningen 6700 EV, Netherlands.[2] NIZO food research, Ede 6710 BA, Netherlands. [3] CMBI NCMLS, Radboud University Nijmegen Medical Centre, Nijmegen 6500 HB, Netherlands

Survival and persistence of Bacilli and Clostridia in the environment largely depends on their ability to produce spores which can germinate under favorable conditions. The process of spore germination is best understood in Bacilli. When a dormant spore senses an environment that can support the survival of a vegetative cell, germination of spores can be induced. Germination in response to nutrients is believed to be mediated by receptors that reside in the inner spore membrane, which are encoded by tricistronic so-called *ger* operons. Whereas *ger* family members are found in Bacillus and most Clostridia, the numbers of *ger* operons in Bacillus species tend to be higher than those found in Clostridia. Upon triggering of germination through the Ger receptor, full germination requires the removal of the spore-cortex. Hydrolysis of the cortex in the genus *Bacillus* is likely performed by germination-specific cortex-lytic enzymes. Several of these cortex lytic enzymes from bacilli and clostridium have been characterized. In the present study, the occurrence of known and putative Bacillus germination-related genes in their anaerobic relatives was analyzed by a homology search of 34 sequenced *Bacillus* genomes and 24 *Clostridium* genomes. The presence of the various genes involved in germination in various species will be discussed, with a particular focus on the Clostridia.

GENERATION OF SINGLE-COPY TRANSPOSON INSERTIONS IN CLOSTRIDIUM PERFRINGENS BY ELECTROPORATION OF PHAGE MU DNA TRANSPOSITION COMPLEXES

A. Lanckriet[1]*, L. Timbermont[1], L. J. Happonen[2], m. I. Pajunen[2], F. Pasmans[1], F. Haesebrouck[1], R. Ducatelle[1], H. Savilahti[2,3] and F. van Immerseel[1]

[1]Department of Pathology, Bacteriology and Avian Diseases, Research Group Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. [2]Research Program in Cellular Biotechnology, Institute of Biotechnology, Viikki Biocenter, PO Box 56, Viikinkaari 9, FIN-00014 University of Helsinki, Finland. [3]Division of Genetics and Physiology, Department of Biology, Vesilinnantie 5, FIN-20014 University of Turku, Finland.

Transposon mutagenesis is a widely used tool for the identification of genes involved in the virulence of bacteria. Until now, transposon mutagenesis in Clostridium perfringens has been restricted to the use of Tn916-based methods in laboratory reference strains. The system primarily yields multiple transposon insertions within a single genome, thus compromising its use in the identification of virulence genes. The current study describes a new protocol for transposon mutagenesis in Clostridium perfringens which is based on the bacteriophage Mu transposition system. The protocol was successfully used to generate single-insertion mutants both for a laboratory strain and a field isolate. Sequencing data revealed relatively even distribution of integrations although rRNA gene regions appeared to be slightly favoured. The integration is characterized by a duplication of the target site. Our method can be a valuable tool in large-scale screenings to identify virulence genes of C. perfringens.

PATHOGENESIS OF ENTEROTOXEMIAS IN ANIMALS

F.A. Uzal

California Animal Health and Food Safety Laboratory, University of California, Davis. 105 W Central Ave, San Bernardino, CA 92408, USA

Enterotoxemias are caused by bacterial toxins produced in the intestine, from where they are absorbed into the circulation and then act on other organs. Most enterotoxemias are produced by *Clostridium perfringens*, and there is a tendency to call all diseases produced by this microorganism "enterotoxemias". This is not exactly correct, since some diseases produced by *C. perfringens* are characterized by the production of toxins that act locally in the gut and are not absorbed into the circulation. Also, the use of the term enterotoxemia when referring to all diseases produced by *C. perfringens* is misleading, because each toxinotype of this microorganism produces a different disease with unique pathogenesis and clinical and pathological changes. *C. perfringens* type A disease has been confirmed in chickens and sheep, but the role of this microorganism in disease production of other species remains controversial. This disease in sheep is most likely mediated by alpha toxin, but in chickens other toxins (e.g. NetB) have been recently implicated in disease production. Type B disease occurs mostly in sheep and is characterized by necrotizing enteritis due to the action of beta toxin, although occasionally the animals may suffer neurological alterations as a consequence of the action of epsilon toxin. Type C disease occurs principally in neonates of most species and is mediated by the action of beta toxin. This age-predisposition is believed to be due to the inhibitory effect that the colostrum has on intestinal proteases, because beta toxin is very sensitive to the action of trypsin. This disease is also characterized by necrotizing enteritis with or without systemic involvement. Type D disease occurs mostly in sheep and goats. This disease in sheep is characterized by neurological clinical signs and lesions. In goats, although neurological signs and lesions are seen in the acute form of the disease, colitis is observed in the sub-acute and chronic forms. The neurological and enteric lesions of type D disease are produced by epsilon toxin. Type E disease has traditionally been considered a disease of rabbits, although it has been occasionally diagnosed in sheep and cattle. Iota toxin is believed to be responsible for the disease in rabbits. The role of enterotoxin and beta2 toxin in animal enterotoxemias is poorly understood and there is not firm evidence that these toxins produce diseases in animals.

MOLECULAR BASIS OF TOXICITY OF EPSILON TOXIN

M. R. Popoff

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Epsilon toxin is produced by *Clostridium perfringens* type B and D and is responsible for fatal enterotoxemia in animals, mainly in sheep. Overproliferation of toxigenic C. *perfringens* in the intestine produces large amounts of epsilon toxin, which enters the blood stream through the intestinal mucosa, and then diffuses in all organs, preferentially accumulating into the brain and kidneys. Epsilon toxin induces perivascular edema in the brain, and alters neuronal cells leading to the release of glutamate. Epsilon toxin also promotes extensive edema in the lungs, elevated blood pressure and extensive kidney damages known as "pulpy kidney disease". Epsilon toxin is one of the most potent bacterial toxins except the clostridial neurotoxins. Only few culured cell lines such as canine kidney cells (MDCK) or mouse kidney cells (mpkCCD) are sensitive to epsilon toxin. The toxin is secreted as a non-active precursor, which is activated by proteolytic removing of N- and C-terminal peptides. Epsilon toxin is structurally related to aerolysin, which is a pore forming toxin. Epsilon toxin binds to a specific receptor on target cells, heptamerizes and forms pores through the plasma membrane. But, epsilon toxin recognizes a distinct receptor than that of aerolysin, which has been identified as GPI-anchored proteins, and is much more potent to induce cell death by a yet poorly understood mechanism. Epsilon toxin causes slightly anion selective pores leading to a rapid loss of intracellular K^+ and to a Na^+ entry, which are accompanied by a rapid ATP depletion. Epsilon toxin induces a permeabilization of mitochondrial membranes leading to the release of cytochrome c as well as a mitochondrial-nuclear translocation of apoptosis-inducing factor, which is a caspase-independent cell death factor. However, cell death results from a necrotic process (absence of DNA fragmentation, entry of propidium iodide into the nuclei, no caspase activation) and not from apoptosis. Epsilon toxin is also highly potent to induce a rapid alteration of cell monolayer permeability without modifying the intercellular junctions, which is probably the basis for the extensive edema produced *in vivo*.

CLOSTRIDIUM PERFRINGENS BETA-TOXIN TARGETS ENDOTHELIAL CELLS IN NECROTIZING ENTERITIS IN PIGLETS.

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Institute of Animal Pathology, Vetsuisse-Faculty, University of Bern, Switzerland

Beta-toxin (CPB) is known to be the major virulence factor of *C. perfringens* type C strains, which cause necrotizing enteritis in animals and humans. The exact mode of action, in particular the cellular targets of CPB in the intestine of naturally affected animals, are still not completely resolved. To identify cellular targets of CPB in necrotizing enteritis, we retrospectively evaluated lesions of 104 piglets which were naturally affected by *C. perfringens* type C enteritis. Histopathologically, lesions were classified into peracute to acute and subacute cases based on the presence and extend of an inflammatory reaction. Peracute to acute cases dominated in piglets up to 3 days of age and were characterized by acute and deep coagulation necrosis of the mucosa, associated with massive hemorrhage in underlying layers and multifocal vascular necrosis. Subacute lesions, which were predominantly found in 1-3 weeks old piglets, additionally showed a marked neutrophilic inflammatory reaction and reduced numbers of vessels in the lamina propria and submucosa, due to widespread vascular necrosis. 50 piglets with typical *C. perfringens* type C enteritis and 13 control animals were further evaluated by immunohistochemistry, using a monoclonal anti beta-toxin antibody. We consistently demonstrated binding of CPB to vascular endothelial cells in lesions of peracute to acute necrotizing enteritis. Subacute cases, demonstrated reduced or no endothelial CPB staining. From these results we conclude, that the pathogenesis of *C. perfringens* type C enteritis involves binding of CPB to endothelial cells in the small intestine during the early phase of the disease. This direct CPB-endothelial cell interaction preceeds vascular necrosis in the natural disease. Thus, by targeting endothelial cells, CPB could specifically induce vascular necrosis, hemorrhage, and hypoxic tissue necrosis.

TOXIN TYPING AND ANTIBIOTIC RESISTANCE PROFILES OF CLOSTRIDIUM PERFRINGENS ISOLATES ASSOCIATED WITH CASES OF SUDDEN DEATH AND ENTEROTOXAEMIA IN VEAL CALVES IN BELGIUM

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Sudden death in suckling beef calves is often associated with an overgrowth of *Clostridium perfringens* in the small intestine (>106 CFUs per ml of content) following a decrease of the intestinal motility. *C. perfringens* produce toxins causing a necro-haemorrhagic enteritis and the sudden death after reaching the brain via the blood stream, so the name of « enterotoxaemia ». The *C. perfringens* isolated from typical cases belong to the toxin type A and can also produce the consensus β2 toxin. The purpose of this work was to study cases of sudden deaths with lesions of haemorrhagic enteritis at necropsy in veal calves and to compare the results with those obtained in suckling beef calves. The 14 calves studied so far belonged to beef cattle breeds. Their intestines were transported in an anaerobic jar within 2-4 hours to the diagnostic lab and the contents were ten-fold-diluted. Dilutions 3, 4 and 6 were plated onto Schaedler agar and incubated for 24 hours in an anaerobic cabinet. Up to ten typical colonies were sub for toxin typing and antibiotic sensitivity testing. The 14 calves were grouped as follows according to the necropsy findings : typical lesions (8 calves), suspicious lesions (3 calves), and absence of lesions (3 calves). Three of the calves with typical lesions were bacteriologically confirmed as cases of perfringens enterotoxaemia (>106 CFUs per ml), but 5 were not (<105 CFUs per ml). One of the calves with suspicious lesions was also bacteriologically confirmed as a case of enterotoxaemia (>106 CFUs per ml). Sixty-five isolates from seven calves were tested for antibiotic sensitivity by the agar disc diffusion assay. All were resistant to erythromycin, lincomycin and tetracycline and 54 to penicillin G and tylosin, but none was resistant to florfenicol. More cases will be studied. The detection of the α-, β-, ε-, ι-, β2 consensus, β2 atypic and entero-toxin-encoding genes will be performed by PCR amplification and the different amplicons found will be confirmed by sequencing. Further research is also needed to identify the aetiology of sudden death of bacteriologically negative calves.

GAS GANGRENE

R. W. Titball

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Clostridium perfringens biotype A strains are the causative agents of gas-gangrene in humans and in animals. Because of the requirement for anoxic host tissues for growth of the bacteria , the disease occurs mainly in diabetics, the elderly and traumatic injury victims where the blood supply to tissues is affected. The virulence factor of *C. perfringens* which plays the major role in gas gangrene is a zinc-dependent, phosphatidylcholine-preferring phospholipase C (α-toxin). The precise role of α-toxin in disease is not fully clarified. In the murine model of disease destruction of muscle tissue and haematuria appear to be the major consequences of the production of α-toxin. Although haemolysis is very occasionally seen in cases of gas gangrene or septicaemia in humans, it appears that the role of α-toxin might be much more subtle and a consequence of the perturbation of host cell metabolism. In this way it appears that α-toxin plays a role in the avoidance of host defence mechanisms, promoting the *in vivo* growth of *C. perfringens*, and finally causing the death of the host.

CLOSTRIDIUM PERFRINGENS TYPE A FOOD POISONING

Bruce A. McClane[1]

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Clostridium perfringens type A food poisoning is the 2nd most common cause of bacterial foodborne disease. This disease usually involves self-resolving diarrhea and abdominal cramps but it can be fatal in compro-mised people. It begins when ingested bacteria sporulate in the small intestines, allowing production of *C. perfringens* enterotoxin (CPE). CPE then binds to enterocytes, via claudin receptors, to form a small complex. Several small complexes oligomerize, in a lipid raft-independent process, to form a CH-1 complex on the membrane surface. The CH-1 prepore then inserts into the bilayer, producing a pore permeable to small molecules, especially cations. This produces a calcium influx that triggers cell death via apoptosis or oncosis. Enterocyte death then leads to desquamation of the intestinal epithelium, which cause fluid and electrolyte transport changes that manifest as diarrhea. The C-terminal half of the 319 amino acid CPE protein contains a C-terminal binding domain resembling the binding region of some *Bacillus thuringiensis* Cry toxins. The N-terminal half of CPE contains both a putative latch domain for oligomerization and a possible transmembrane stem domain for insertion. The *cpe* gene can be present on the chromosome or on large conjugative plasmids. Most food poisoning cases involve chromosomal *cpe* isolates, whose spores and vegetative cells are usually exceptionally resistant to food environment stresses such as heat, low temperature and preservatives. Recent studies have identified a variant small acid soluble protein (named SASP-4) that appears to mediate, in part, the heat and chemical resistance phenotype of spores made by chromosomal *cpe* isolates. The SASP-4 variant made by most chromosomal cpe isolates exhibits stronger DNA binding than the SASP-4 made by other *C. perfringens* isolates. Chromosomal *cpe* isolates apparently belong to a distinct cluster of *C. perfringens* type A isolates, suggesting that acquisition of the *cpe* gene by already environmentally-resistant isolates has produced an exceptionally fit foodborne pathogen.

EPIDEMIOLOGY OF C. PERFRINGENS TYPE A HUMAN DIARRHOEAL DISEASE

Kathie Grant

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Clostridium perfringens type A is a common but under reported cause of gastroenteritis. It is responsible for several different forms of disease including food borne outbreaks, sporadic illness, as well as, antibiotic associated diarrhoea. Food borne illness generally presents 8-24 hours after the consumption of food containing high numbers of *C. perfringens* vegetative cells and disease symptoms include profuse diarrhoea, acute abdominal pain and nausea. Non- food borne diarrhoeal illness is often more severe and longer lasting than classical food poisoning, with symptoms of diarrhoea and acute abdominal pain often accompanied by the presence of blood and mucous in the faeces. Non-foodborne *C. perfringens* gastroenteritis is predominantly associated with the elderly population, with antibiotic associated *C. perfringens* diarrhoea occurring in hospitals or residential care settings and sporadic incidents occurring in the community. The clinical symptoms of all forms of *C. perfringens* diarrhoeal disease are due the effects of enterotoxin produced in the human small intestine when the organism sporulates. Most *C. perfringens* type A food poisoning strains carry their *cpe* gene on the chromosome and have spores that are resistant to extremes of temperature, whereas non-food poisoning strains carry their *cpe* genes on large plasmids and have spores that are more sensitive to temperature. Recently food poisoning strains with plasmid encoded *cpe* genes have been reported raising questions about when these strains enter the food chain. This presentation will focus on the epidemiology of human *C. perfringens* diarrhoeal illness in the UK, illustrating how the use of molecular methods is improving our understanding of this important gastrointestinal pathogen.

PRELIMINARY IDENTIFICATION OF CLAUDIN RESIDUES INVOLVED IN C. PERFRINGENS ENTEROTOXIN BINDING AND ACTION

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

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Clostridium perfringens enterotoxin (CPE) is responsible for the diarrhea and abdominal cramping associated with several common human gastrointestinal diseases. The action of this toxin begins with its binding to receptors to form a small (~90 kDa) complex. That complex shuttles into a larger CPE complex of ~450 kDa, forming a pore leading to cell death by apoptosis or oncosis. CPE can also remove occludin from the tight junctions (TJs) via a ~600 kDa complex. Previous studies with fibroblast transfectants and naturally-sensitive Caco-2 identified certain claudins (e.g., claudin -4) as CPE receptors. Claudins are a family of more than 20 different proteins that play an important role in maintaining the structure and function of epithelial and endothelial TJ's. Rat fibroblasts transfected with claudin-4 in which the second extracellular loop was substituted with that from claudin -2 (a non-CPE receptor) did not show any morphological damage when treated with the enterotoxin. Conversely Rat fibroblasts transfected with claudin -2 where the second extracellular loop was that from claudin -4 were able to bind to CPE and thus morphological damage was observed. To further unravel the interactions between CPE and claudins we produced claudin -4 receptor fragments; using those fragments in receptor inhibition studies we were able to block the actions of CPE on Caco-2 cells. This "protection" effect was not observed when using soluble fragments of claudin -2 and/or -1. Additionally site directed mutagenesis was employed to alter a specific amino acid in these receptor proteins to reverse their ability to serve as CPE receptors. Preliminary studies with these site directed mutants has revealed that switching this residue in a receptor claudin (claudin -4) with the residue present at the same position in a non-receptor claudin (claudin -1) abolishes their ability to protect Caco-2 cells from native CPE. The results of these inhibition studies suggest that CPE is interacting with a key residue on the second extracellular loop of claudin -4 and that by altering this residue (replacing it with that of a non-receptor claudin) claudin 4 no longer acts as a suitable CPE receptor.

RECOMBINANT *CLOSTRIDIUM ACETOBUTYLICUM* EXPRESSING *C. PERFRINGENS* ENTEROTOXIN (CPE) FOR TREATMENT OF PANCREATIC CANCER

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Pancreatic cancer carries the most dismal prognosis of all solid tumours. Due to the late clinical presentation, most patients only undergo palliative treatment. Genetically modified clostridia open a new possibility of anti-tumour treatment with enormous potential. Clostridial spores germinate only in the hypoxic regions of solid tumours and can deliver reactive agents directly to their targets. CPE is one of the 15 toxins known from *Clostridium perfringens*, is produced and released during the sporulation phase, and causes food borne diarrhea. The toxin was shown to target claudin receptors, which are 1000fold overexpressed in many pancreatic carcinoma cell lines. The binding of CPE to these receptors results in the formation of pores that ultimately cause cell death. C*lostridium acetobutylicum* DSM 792 was transformed with a vector carrying the gene for *Clostridium perfringens* enterotoxin, fused with a signal peptide sequence, and controlled by the *bdhA* promotor promoter. The modified strain produced and secreted 500 ng/ml of the toxin into the surrounding medium. This production is independent of sporulation and starts in the early exponential growth phase. The levels of production were sufficient to cause cell death in cytotoxicity tests with a pancreatic carcinoma cell line, but proved to be too low for therapy in an *in vivo* mouse model. Current work focuses on improved expression.

THE PATHOGENESIS OF NECROTIC ENTERITIS IN POULTRY

M.I. Kaldhusdal

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The pathogenesis of necrotic enteritis is incompletely understood. The presence in the intestine of *C. perfringens* alone is not sufficient to induce lesions. The presence of at least one of numerous influencing factors in the environment, feed or host appears to be necessary. These factors may work through the fulfilment of the following two requirements for induction of necrotic enteritis that have been proposed; (a) the presence of some factor causing damage to the intestinal mucosa, and (b) the presence of higher than normal numbers of intestinal *C. perfringens*. If these two requirements are fulfilled, lesions may develop often starting at the tips of the villi. Bacterial cells adhere to damaged epithelium and denuded lamina propria where they proliferate and induce coagulation necrosis. Attraction and lysis of heterophil granulocytes as well as further tissue necrosis and bacterial proliferation proceed rapidly.

For more than 20 years, *C. perfringens* alpha toxin has been assumed to be the most important factor involved in this process. But recent experimental evidence indicates that this toxin is not an essential virulence factor. Further, the NetB toxin of *C. perfringens* has been discovered recently. This toxin has been shown to be associated with virulence in a challenge experiment. Preliminary data suggest that NetB is prevalent in strains isolated from field outbreaks of necrotic enteritis, but it remains unclear if NetB is an essential virulence factor.

The pathogenesis of *C. perfringens*-associated liver disease is poorly understood. It has been proposed that *C. perfringens* or its toxins may reach the liver via the portal blood or via the bile tree. Cholangiohepatitis, which is the most common form of *C. perfringens*-associated liver disease, has been reproduced experimentally by inoculation of *C. perfringens* into the hepatoenteric bile duct or by ligation of both bile ducts. These results suggest that *C. perfringens* organisms and/or toxins may reach the liver and lead to bile stasis and inflammation of the bile tree.

MOLECULAR PATHOGENESIS OF NECROTIC ENTERITIS IN BROILER CHICKENS

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For many years it was believed that necrotic enteritis was caused by *Clostridium perfringens* alpha toxin, inducing intestinal epithelial cell lysis, followed by mucosal damage to the underlying tissue. Recent research is creating a paradigm shift in our understanding of the molecular pathogenesis. Histological studies have shown that initial damage occurs at the basement membrane and the lateral domain of the enterocytes, spreading throughout the lamina propria, with epithelial damage only occurring later in the process. Although alpha toxin can be used as antigen to induce protective responses, recent data show that this toxin plays no direct part in the pathogenesis of the induced gut lesions. A novel toxin, NetB, has recently been shown to be essential for lesion induction by outbreak strains. This pore-forming toxin is present in most, but not all, strains isolated from disease outbreaks. It is not present in *C. perfringens* strains from other sources. It is clear that a deeper understanding of molecular pathogenesis will facilitate development of effective disease-control measures, such as improved vaccines.

INTRA-SPECIES GROWTH-INHIBITION IS AN ADDITIONAL VIRULENCE TRAIT OF CLOSTRIDIUM PERFRINGENS IN BROILER NECROTIC ENTERITIS

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Necrotic enteritis in broiler chickens, caused by *Clostridium perfringens*, emerged in many EU countries following the ban on growth-promoting antibiotics from animal feed. Clinically healthy chickens carry several different *Clostridium perfringens* clones in their intestine. In flocks suffering from necrotic enteritis, however, mostly only one single clone is isolated from all diseased animals. Selective proliferation of these clinical outbreak strains in the gut and spread within the flock seems likely, but a mechanistic explanation has not yet been provided. Necrotic enteritis associated strains could suppress the growth of normal microbiota strains. Therefore, 26 *Clostridium perfringens* strains isolated from healthy broilers and 24 clinical outbreak isolates were evaluated for their ability to induce intra-species growth-inhibition in an in vitro setup. A significantly higher proportion of the *Clostridium perfringens* clinical outbreak strains inhibited the growth of other *Clostridium perfringens* strains compared to *Clostridium perfringens* strains isolated from the gut of healthy chickens: 83% and 42%, respectively. It is proposed that in addition to toxin production, intra-species inhibition is a virulence trait that may allow certain *Clostridium perfringens* strains to cause necrotic enteritis in broilers.

ECOLOGICAL AND FUNCTIONAL GENOMIC STUDIES FOR CONTROL OF CLOSTRIDIUM PERFRINGENS INFECTION IN BROILER CHICKENS

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We studied microbial ecology and host response to *Clostridium perfringens* (CP) infection with an experimental infection chicken model. Chickens were fed antibiotic-medicated (bacitracin, 55 mg/kg) or non-medicated diets, and were challenged with CP (107 CFU/ml) through the diets at 18 days of age. Digesta and tissue samples were collected daily before and after the challenge, and were examined for cell proliferation of CP, gene expression of alpha-toxin, changes in the composition of ileal bacterial microbiota, and gene expression profiles in the spleens, in addition to recording animal performance. The cell proliferation of CP was highly correlated to alpha-toxin production during the development of necrotic enteritis (NE). The average CP counts in the ileal digesta of 5 log10 CFU/g was shown to be a threshold for developing NE disease. While major bacterial groups, such as *Enterococcus* genus and *Enterobacteriaceae* family, exhibited no change in their populations, the abundance of lactobacilli in the ileum was significantly reduced. In particular, the change of L. avarius correlated negatively with the CP counts. The analysis of gene expression profiles indicated that many immune-associated genes were significantly up-regulated in CP-infected chickens. These genes encode members of the Toll-like receptor pathway, antibody response, T cell markers, and inflammatory cytokines. The expression of a subset of functionally relevant genes was validated through quantitative RT-PCR assays. Both medicated and non-medicated chickens demonstrated similar annotation profiles with cell activity and regulation being the most dominant biological processes across time. We also had a separate effort to identify novel *C. perfringens* virulence factors in NE pathogenesis through the analysis of the genome sequence of an isolate known to cause NE disease in broilers. Genome comparison with other non-poultry strains revealed a number of putative genes unique to this isolate, including a putative hemolysin, a capsule biosynthesis locus, and several antibiotic resistance genes. We are developing a microarray based on this strain, which will be used to further identify and characterize genes related to NE disease. *Key words*: chickens, *Clostridium perfringens*, microbiota, immune response, pathogenesis

PRESENCE OF THE NECROTIC ENTERITIS TOXIN B, NETB, GENE IN CLOSTRIDIUM PERFRINGENS STRAINS ORIGINATING FROM HEALTHY AND DISEASED CHICKENS.

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Necrotic enteritis is a severe gastrointestinal disease in broiler chickens caused by virulence factors produced by C. perfringens. Alpha-toxin has for long been regarded as the main virulence factor, but recently other virulence factors have been suggested as well. Among these, a novel cytotoxic protein, NetB, was identified and presented as a crucial virulence factor mainly based on two observations: 1) Loss of cytotoxicity was associated with a mutation in the netB gene, whereas the wild-type and complemented mutants caused NE. 2) Presence of the netB gene in most strains isolated from chickens suffering from NE and absence of netB-specific product in strains isolated from various healthy animals, including mammals (Keyburn et al., 2008, PLOS Pathogens 4, e26).

We have screened 45 strains isolated from broilers with known disease status regarding NE and found no correlation between the health status of the chickens and the presence of the netB gene in the isolated strains. Therefore, even though NetB may well be important for the development of NE, other players still seem to be involved as well.

CLOSTRIDIUM PERFRINGENS VETERINARY VACCINES

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The *Clostridium perfringens* toxin types are responsible for a wider range of diseases in more types of animal than any other clostridial species. These diseases range from gangrene in poultry to necrotic enterotoxaemia in horses. The most commonly encountered are forms of enteritis and enterotoxaemia in poultry, sheep and pigs. The form of disease generated in the different species is believed to mainly depend on the major toxin or toxins that the pathogen produces. Due to the speed with which death usually follows the onset of clinical signs in many of these diseases immunoprophylaxis is the most important control measure. Vaccines against several *Cl. perfringens* diseases have existed for more than 50 years. Many of these vaccines are used to immunise pregnant animals to provide passive protection to their progeny via maternally derived antibodies. Such vaccines are often multivalent and may comprise inactivated cells, toxoids or both. There have been contradictory views as to whether antibacterial or antitoxic immunity is most important. It is now generally believed that antibody responses to the major toxins are the main protective mechanism. In fact, the Ph. Eur. monograph potency tests for *Cl. perfringens* vaccines are based on this presumption. However, due to economic considerations, veterinary toxoid vaccines rarely contain purified individual toxoids and usually comprise chemically toxoided whole supernatants. It is therefore difficult to differentiate between the protective contributions of the different major toxins and between them and the other minor toxins. However, with the application of recombinant technology and the ability to express genetically toxoided versions of the major toxins in vectors unrelated to clostridia their protective potential can now be assessed.

NECROTIC ENTERITIS VACCINES

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The recent years have seen many advances in understanding of *Clostridium perfringens* as an enteric pathogen of humans and animals, including recognition of novel toxins such as beta2 toxin (*cpb2)*, enterotoxin (*cpe)*, and necrotic enteritis toxin B-like toxin (*netB*), some of which are encoded on different conjugative plasmids. Given its importance to the broiler industry, there has been surprisingly little study of immunity to necrotic enteritis (NE), perhaps because it has been generally assumed to relate to antibody to alpha-toxin, but largely because the disease has been so well controlled by prophylactic use of antibiotics. Recently, however, Keyburn *et al*. (2006) showed that an alpha-toxin mutant retained virulence in a chicken NE model. Further work by this group (Keyburn *et al*., 2008) identified a novel toxin, NetB, that appears to be critical for the production of NE. Kulkarni et al. (2006) identified secreted proteins in an NE-producing isolate to which serum from birds immune to NE reacted strongly. Kulkarni *et al*. (2007) found that Immunization of birds with one of each of five purified proteins showed that immunized birds were to some extent protected against experimental NE, with particularly strong protection by alpha-toxin and good but lower protection by a hypothetical protein of unknown function and a truncated pyruvate:ferrodoxin oxidoreducatase (PFOR) protein. It seems that interference in enzymes involved in acquisition of nutrients provides a set-back to *C. perfringens* as a pathogen. Passive immunization of broilers by immunization of laying hens using secreted proteins is currently in commercial use, with reasonable success. The basis of this is unclear, but has been attributed to alpha-toxoid.

More recently, genes for some of these proteins have been cloned and expressed in an attenuated *Salmonella* vaccine vector and used by Kulkarni *et al*. (2008) to immunize broilers. Work is on-going in our laboratory using better expression systems and codon optimization to improve protection by selected epitope-mapped antigens. Further work is required before an effective vaccine (including appropriate delivery system) is produced for NE, and to define the optimum antigen(s).

BACILLUS SUBTILIS SPORE VACCINES: USE OF GLUTATHIONE S-TRANSFERASE

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Glutathione S-transferase (GST), is an essential detoxification enzyme in parasitic helminthes and is a major vaccine and drug target against Schistosomiasis and other helminthic diseases. GST, obtained from *Schistosoma japonicum*, is commonly used as an expression system for the expression and purification of recombinant proteins, which are fused to GST, in *Escherichia coli*.

In this work, we are using the 26 kDa GST from the *Sh. japonica* (SjGST) as a fusion partner with which to express the 52 kDa tetanus toxin fragment C (TTFC) of *Clostridium tetani*. It has shown that SjGST fused to TTFC fusion protein can be expressed using two routes, displayed on the spore surface as well as in the germinating spore. CotC and CotB have been used to express GST-TTFC on the spore surface and compared to expression of TTFC alone facilitate higher levels of expression, presumably the chimera is more stable. Likewise, GST-TTFC can be expressed at higher levels in the vegetative cell when fused to GST.

Our immediate plan now is to evaluate GST and GST-TTFC specific humoral immune responses and determine whether spores could be used as a mucosal vaccine against *Schistosoma japonicum* and possibly as a better spore based vaccine to tetanus.

DIAGNOSTIC METHODOLOGIES FOR *CLOSTRIDIUM PERFRINGENS*

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Clostridium perfringens causes several, mostly enteric diseases ranging from diarrhoea, to necrotising enteritis and enterocolitis in humans and animals. *C. perfringens*, in particular type A, is normally found as an inhabitant of the intestine of healthy human and most animal species. While the pathogenesis of many of the diseases caused by *C. perfringens* are still not clarified and seems to be multi-factorial, it is clear that the various toxins produced by *C. perfringens*, and in particular the main toxins are determinative factors for the sereneness of the disease and to a certain extent also for the host-specificity of the pathogen. Hence these factors must be included in the diagnosis. Routine bacteriological diagnosis is based on the isolation and identification of the pathogen to the species level. For medical and epidemiological purposes, but also for preventive measures such as vaccination, the identification of the toxins or toxin genes as well as the determination of the antibiotic susceptibility profile is crucial. The current designation of the *C. perfringens* types A – E needs to be revised, since it does not take into consideration two important toxins, the enterotoxin Cpe and poultry-specific necrotizing toxin NetB, for the designation of major toxin-types of *C. perfringens*. Several powerful PCR and multiplex real-time PCR methods have been described that give reliable profiles for the most relevant toxin genes in *C. perfringens* isolates. In contrast, very little is known on the nature of antibiotic resistance in *C. perfringens*. Recently we have developed a disposable microarray for the detection of antibiotic resistance genes in Gram-positive bacteria. Using this micro-chip, we have detected antibiotic resistance genes in *C. perfringens* and correlated the resistance gene profiles to the antibiotic resistance phenotypes. Analyzing a large number of *C. perfringens* isolated from diseased animals, single and multiple resistant strains carrying the aminoglycoside resistance genes *aph(3')-III* and *ant(6)-Ia*, the tetracycline resistance gene *tetP*, the streptothricin resistance gene *sat4*, the MLS_B resistance gene *erm*(B) and the chloramphenicol acetyltransferase gene *catP* have been detected in up to 35% of the isolates.

POSTER PRESENTATIONS

LIPID RAFTS ARE NOT REQUIRED FOR THE ACTION OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

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The action of bacterial pore-forming toxins typically involves membrane rafts for binding, oligomerization, and/or cytotoxicity. *Clostridium perfringens* enterotoxin (CPE) is a pore-forming toxin with a unique, multi-step mechanism of action that involves the formation of complexes containing tight junction proteins that include claudins and, sometimes, occludin. Using sucrose density gradient centrifugation, this study evaluated whether the CPE complexes reside in membrane rafts and what role raft microdomains play in complex formation and CPE-induced cytotoxicity. Western blot analysis revealed that the Small CPE Complex (SC) and the CPE Hexamer-1 Complex (CH-1), which is sufficient for CPE-induced cytotoxicity, both localize outside of rafts. The CPE Hexamer-2 Complex (CH-2) was also mainly found in non-raft fractions, although a small pool of raft-associated CH-2 was detected that is sensitive to cholesterol depletion with methyl-β-cyclodextrin (MβCD). Pre-treatment of Caco-2 cells with MβCD had no appreciable effect on CPE-induced cytotoxicity. Claudin-4 was localized to Triton X-100 soluble gradient fractions of control or CPE-treated Caco-2 cells, indicating a raft-independent association for this CPE receptor. In contrast, occludin was present in raft fractions of control Caco-2 cells. Treatment with either MβCD or CPE caused most occludin molecules to shift out of lipid rafts, possibly due (at least in part) to the association of occludin with the CH-2 complex. Collectively, these results suggest that CPE is a unique pore-forming toxin for which membrane rafts are not required for binding, oligomerization/pore formation, or cytotoxicity.

STRUCTURE-FUNCTION ANALYSIS OF THE HOLIN-LIKE TCDE FROM CLOSTRIDIUM DIFFICILE

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The pathogenicity locus of *C. difficile* exhibits an ORF (*tcdE*) located between the *tcdA* and *tcdB* genes encoding the 19 kDa protein TcdE. TcdE shows significant sequence homology to bacteriophage-encoded holins which are lytic proteins causing cell death of bacterial host cells. This finding led to the hypothesis that TcdE participate in the delivery of toxins A and B to the extracellular environment of *C. difficile*. Since expression of TcdE in *E. coli* leads to bacterial cell lysis, we used this property to study the influence of expressed (full length and deleted) TcdE on the growth profile of *E. coli.* Additionally, site-directed mutagenesis was performed to investigate a putative dual start motif which might account for TcdE protein of different length and function, as it is known for holins. The expression of deletion mutants lacking either the N- or C- terminus or both in *E. coli* resulted in inhibition of bacterial growth whereas a fusion construct of only the N- and C- termini (lacking the intermediate part) had no effect. For specific detection in Western blot analysis a polyclonal anti-TcdE antibody was generated. The expression pattern of wild type TcdE reflects a 19 kDa and a 16 kDa protein. Mutagenesis analysis confirmed a dual start motif enabling the expression of proteins with different length and function. The mutation within the ribosome binding site shifted the ratio of full length to truncated protein from 1:10 to 1:1. After induction of TcdE expression, recovery of the optical density was observed. This reversal in growth inhibition was due to base insertions into *tcdE* DNA which can be construed as a bacterial regulation against TcdE-induced toxicity. The altered expression pattern of the mutant TcdE was accompanied by a delayed recovery of bacterial growth reflecting a stronger selection force exerted by the 16 kDa protein, and thus revealing a more potent impact of the truncated TcdE. This conclusion is corroborated by the observation that a mutant construct expressing the full length TcdE exclusively, mediates a bacteriostatic but not a lytic effect.

These data indicate an essential role of the hydrophobic transmembrane domains of TcdE in inhibition of bacterial growth. The dual start motif regulates the ratio of full length (19 kDa) to truncated (16 kDa) TcdE. Truncated TcdE is proposed to be the active bacteriolytic protein whereas full length TcdE probably acts as its antagonist.

THE INITIATION OF ENDOSPORE FORMATION IN CLOSTRIDIUM ACETOBUTYLICUM

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Clostridium acetobutylicum forms heat-resistant endospores and produces solvents following a period of exponential growth in laboratory batch culture. Like the bacilli, the clostridia contain the master transition state regulator, Spo0A. In *Bacillus subtilis*, this phosphorylation-activated transcription factor orchestrates gene expression during the transition from the exponential to the stationary phase of growth. Previous work has shown that Spo0A also controls endospore formation and, in addition, solventogenesis in clostridia. However, Spo0F and in most cases Spo0B, which are key components of the phosphorelay responsible for activating Spo0A in the bacilli, do not appear to be present in the clostridia. The main objective of this investigation is to test whether, in the absence of a recognizable phosphorelay, Spo0A is directly phosphorylated by one or more sensor histidine kinases in these organisms.

Some 35 histidine kinases have been annotated in the *C. acetobutylicum* genome. Five of them (CAC0323, CAC0437, CAC0903, CAC2730 & CAC3319) are orphan kinases lacking an adjacent cognate response regulator. By analogy to other organisms, it is these orphan kinases that are most likely to play a role in the phosphorylation of Spo0A.

Recently developed “ClosTron” technology, which uses a mobile re-targeted group II intron to generate knockout mutations, is both reliable and efficient in *C. acetobutylicum*. We have employed it to inactivate the genes encoding these five orphan kinases. The mutant phenotypes have been carefully analysed and the results indicate that two, or possibly three, of these kinases are indeed involved in the initiation of sporulation in this organism.

BINDING OF EPSILON-TOXIN FROM CLOSTRIDIUM PERFRINGENS TO KIDNEYS AND NERVOUS SYSTEM

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Epsilon-toxin, produced by *Clostridium perfringens* type D, is the main agent responsible for enterotoxaemia, in livestock. Neurological and renal disorders are a 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.,2004 J. Histochem. Cytochem.). 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., 2007 Toxicon). Here we attempt to identify specific acceptor moieties and cell targets for epsilon-toxin in the mouse nervous system. Epsilon-toxin-GFP fusion protein was used to incubate brain sections. Confocal microscopy analysis showed specific binding of epsilon-toxin to myelinic structures. Protease treatments revealed that the binding was mainly associated to a proteinic component of the myelin.
Myelinated peripheral nerve fibres were also stained by epsilon-toxin. Moreover, the binding to myelin was not only restricted to rodents, but was also found in humans, sheep and cattle. Curiously, in the brains of both sheep and cattle, the toxin strongly stained the vascular endothelium. 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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ORIGIN OF CLOSTRIDIUM PERFRINGENS ISOLATES DETERMINES THE ABILITY TO INDUCE NECROTIC ENTERITIS IN BROILERS

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Necrotic enteritis in broiler chickens, caused by *Clostridium perfringens*, emerged in many EU countries following the ban on growth-promoting antibiotics from animal feed. Despite the importance of the disease, the pathogenesis is still not completely understood. In the current study, it was tested whether the origin of a given *Clostridium perfringens* strain is correlated with its ability to cause necrotic lesions in the gut of broilers. In a first experiment, *Clostridium perfringens* strains isolated from healthy flocks and isolates from outbreaks of necrotic enteritis were evaluated for the ability to cause gut mucosal necrosis in an experimental infection model in broilers. High, intermediate and low alpha toxin producing strains were chosen from each isolation source. Only the isolates from field outbreaks induced necrotic gut lesions, independent of the amount of alpha toxin produced *in vitro*. In a second experiment it was shown that alpha toxin producing isolates from calf hemorrhagic enteritis cases were not able to induce necrotic enteritis in poultry. These results suggest the presence of host specific virulence factors in *Clostridium perfringens* strains, isolated from chickens with necrotic enteritis lesions.

CONTROL OF *CLOSTRIDIUM PERFRINGENS* INDUCED NECROTIC ENTERITIS IN BROILERS BY BUTYRIC ACID, MEDIUM CHAIN FATTY ACIDS AND ESSENTIAL OILS

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The efficacy of butyric acid, medium chain fatty acids and essential oils or a combination of these products, for the control of necrotic enteritis was investigated using a necrotic enteritis model in broiler chickens. In a first experiment, four groups of chickens were fed a diet supplemented with either butyric acid, butyric acid in combination with medium chain fatty acids, butyric acid in combination with medium chain fatty acids and botanicals, or with botanicals only. In all groups except for the group receiving only butyric acid, a significantly lower number of animals with necrotic lesions was found compared to the infected, untreated control group.

In a second experiment the same products were tested but at a higher concentration. Moreover, an extra group fed a diet supplemented only with medium chain fatty acids was included. Reduction in the number of animals with necrotic lesions was found in all the treated groups.

These results suggest that butyric acid, medium chain fatty acids and botanicals counteract the development of *C. perfringens* associated gut lesions in poultry.

PREVALENCE OF NETB GENE IN CLOSTRIDIUM PERFRINGENS FIELD STRAINS ISOLATED FROM CHICKEN

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Clostridium perfringens (CP) is well known as the aetiological agent of necrotic enteritis (NE) in chicken. For over 30 years α toxin was considered the key virulence factors in this type of pathology. Recently a new toxin related to the appearance of NE called NetB has been described. The aim of this work was to evaluate the presence of genes coding for α (*cpa*), β (*cpb*), ε (*cpetx*), ι (*cpi*), β2 (*cpb2*), enterotoxin (*cpe*) and NetB toxins in CP field strains collected from chickens affected or not by enteric diseases. Seventy-two CP field strains were toxin typed: 22 isolated from chickens affected by NE, 38 from chickens with intestinal lesions not ascribable to NE and 12 from healthy chickens. 66/72 (91,6%) strains were positive for cpa gene (toxintype A) and 6 (8,3%) for cpa and cpb2 genes (toxintype A+β2). 24/72 (33.3%) CP were *NetB* positive and 91.6% of these was isolated from chickens affected by intestinal diseases: 14 with NE and 8 with macroscopic lesions other than NE. The number of NetB positive strains was significantly higher (p=0,002) in chickens affected by NE (61%) than in birds with different intestinal disorders (23%). Our preliminary results seem to confirm the involvement of NetB toxin in the pathogenesis of NE ,even if, its role should be verified by means of the evaluation of the toxin expression.

CHARACTERIZATION OF ILEAL BACTERIAL MICROBIOTA OF BROILER CHICKENS IN RESPONSE TO CLOSTRIDIUM PERFRINGENS INFECTION.

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We previously determined that the development of necrotic enteritis (NE) in broilers was highly correlated to cell proliferation of *Clostridium perfringens* (CP) and its production of alpha toxin. This study investigated the response of dominant ileal bacteria during NE development using bacterial profiling and quantitative PCR (QPCR) techniques. Chickens were on antibiotic-medicated (bacitracin, 55 mg/kg) or non-medicated diets, and were challenged with CP (107 CFU/ml) through the diets at 18 days of age. Ileal digesta was collected daily before and after the challenge for 5 days. The PCA (Principal Components Analysis) analysis of DGGE profiles of major ileal bacteria showed a significant difference between the bacterial profiles of 2 days post-infection (PI) chickens treated or untreated with bacitracin. QPCR assays subsequently verified our previous observation on the cell proliferation of CP and identified changes in the population of lactobacilli, although no changes in other dominant bacteria, such as *Enterococcus* genus and *Enterobacteriaceae* family, were detected. On days 3 and 4 PI, the abundance of *L. avarius* significantly decreased, while *L. salivarius* demonstrated a remarkable reduction only on day 2 PI in non-medicated birds compared with medicated ones. The changes in the population at both the group level of *Lactobacillus* and the species level of *L. avarius* correlated negatively with the CP counts in the ileum, suggesting that *L. avarius* was suppressed by CP infection. This observation warrants further studies on the mechanisms underlying the ecological change and for the development of novel probiotics to control CP infection. *Key words*: chickens, *Clostridium perfringens, Lactobacillus,* DGGE, QPCR

MICROARRAY ANALYSIS OF GENE EXPRESSION WITHIN THE SPLEEN OF BROILERS IN RESPONSE TO CLOSTRIDIUM PERFRINGENS INFECTION

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Clostridium perfringens (CP) causes necrotic enteritis (NE) in chickens. Understanding the host response to CP infection is essential for developing strategies to improve CP immunity for the control of NE. However, such required knowledge is limited. The objective/purpose of this study was to investigate molecular mechanisms of the response. Gene expression profiles in the spleens were examined with a 44K Agilent chicken genome microarray by comparing RNA from spleen tissues of antibiotic-medicated and non–medicated birds before and after CP infection. A total of 600 Ross broilers were reared in 12 pens with six pens on medicated (bacitracin at 55 ppm) and six pens on non-medicated Starter diets immediately after hatch. At 18 days of age, birds were challenged with CP and spleens were collected from 12 birds of each group on days 18 (before infection), 19, 20, and 22. Directly-labelled cDNA was prepared from splenic total RNA for microarray hybridizations. LOWESS-normalized microarray signal intensity was analyzed using a mixed model including treatment, time, array, dye, and all interactions among treatment and time to identify significantly differentially expressed genes between treatments and time points ($p < 0.01$). Expression profiles indicated that many immune-associated genes were significantly up-regulated in CP-infected chickens. These genes encode members of the Toll-like receptor pathway, antibody response, T cell markers, and inflammatory cytokines. The expression of a subset of functionally relevant genes was validated by quantitative RT-PCR assays. Functional annotation of significantly differentially expressed genes revealed similar annotation profiles between medicated and non-medicated chickens with cell activity and regulation being the most dominant biological processes across time. Further functional studies are required to identify targets for preventative measures as alternatives to antibiotics. *Key words*: *Clostridium perfringens*, necrotic enteritis, microarray, gene expression, antibiotics.

RETROSPECTIVE STUDY ON NECROTIZING ENTERITIS IN PIGLETS IN SWITZERLAND.

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The re-emergence of necrotizing enteritis in Swiss pig breeding farms raised concern that, besides *C. perfringens* type C strains, additional *C. perfringens* toxinotypes might be involved in this disease. Therefore, we retrospectively investigated the association of necrotizing enteritis with *C. perfringens* type C or different toxinotypes. We evaluated pathological lesions, routine diagnostic bacteriology results, and multiplex real-time PCR analyses from DNA extracts of archived intestinal samples of 199 piglets from our diagnostic case load. 96.5% of necrotizing enteritis cases and 100% of herds affected by necrotizing enteritis were positive for *C. perfringens* type C genotypes. Animals without necrotizing enteritis revealed a significantly lower detection rate of type C genotypes. Non affected piglets showed a high prevalence for beta-2-toxigenic *C. perfringens* type A strains. A parallel epidemiological study evaluating swab cultures of 800 live piglets from 21 different herds revealed *C. perfringens* type C isolates only in herds which were affected by necrotizing enteritis. These type C isolates were detected both in vaccinated (type C toxoid vaccine) and unvaccinated herds. In 100% of non-affected and non-vaccinated herds, beta2-toxigenic *C. perfringens* type A strains were detected. On the individual animal level, 99.8% of *C. perfringens* cultures from these herds were positive for beta2-toxigenic *C. perfringens* type A strains. Collectively, our data indicate that outbreaks of necrotizing enteritis in piglets in Switzerland cannot be attributed to newly emerging pathogenic toxinotypes, but are due to the spread of pathogenic *C. perfringens* type C strains. Beta2-toxigenic type A strains are highly prevalent, both on the individual animal and herd level, and thus cannot be attributed to any particular enteric disease in Swiss pig farms.

ANALYSIS OF THE PREVALENCE, RISK FACTORS AND MOLECULAR TYPING OF *CLOSTRIDIUM PERFRINGENS*, A CAUSATIVE AGENT OF ANTIBIOTIC ASSOCIATED DIARRHOEA.

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Clostridium perfringens has been reported as the cause of up to 15% of cases of antibiotic-associated diarrhoea (AAD). Diagnosis of *C. perfringens* AAD can be performed by detection of *C. perfringens* enterotoxin (CPE) in stool samples. CPE is encoded by the *cpe* gene, which generally has a plasmid location in those *C. perfringens* isolates causing AAD. The prevalence of *C. perfringens* AAD was determined using, initially a commercially available ELISA and then confirmed by culture techniques followed by PCR methods. 265 (n=265) stool specimens which were submitted for *Clostridium difficile* toxin testing were also tested for the presence of CPE. 7% (n=18) of the specimens were positive for CPE using ELISA. Culture and PCR techniques confirmed the majority of ELISA positive results. However, 6 anomalous results were obtained. Three ELISA positive samples did not grow when cultured on Neomycin agar and on repeat ELISA testing were found to be CPE negative. They were thus deemed false positive results. The other 3 ELISA positive samples were cultured and Gram stain on these cultures yielded 2 isolates that were Gram negative bacilli and 1 isolate that was a Gram positive coccus, thereby excluding the possibility that they were Clostridial species. They were therefore also deemed to be false positive results. Twelve ELISA positive samples were tested using a duplex PCR assay and the *cpe* gene was detected in 10 of these 12 samples. The risk factors associated with *C. perfringens* AAD such as increasing age, length of hospital stay and use of broad-spectrum antibiotics were evaluated for each patient. Statistical analysis of patient demographics revealed a strong relationship between gender and a moderate relationship between age and the probability of testing positive for CPE, with males >60 years being most at risk. It is plausible to suggest that if the aetiology of a patient's diarrhoea is undetermined after routine testing and symptoms persist, then testing for *C. perfringens* should be performed and detection of CPE needs to be established as part of routine testing in clinical microbiology laboratories.

Clostridium perfringens: The Conference

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