ABSTRACTS

MARIE CURIE **ACTIONS**

CLOSTRIDIUM BOTULINUM
EPIDEMIOLOGY, DIAGNOSIS, GENETICS, CONTROL AND PREVENTION

16-19 JUNE 2008

DEPARTMENT OF FOOD AND ENVIRONMENTAL HYGIENE

FACULTY OF VETERINARY MEDICINE

UNIVERSITY OF HELSINKI

Clostridium botulinum –
Epidemiology, Diagnosis, Genetics, Control and Prevention

June 16-19, 2008
Helsinki, Finland

Dear Congress Delegate,

There is no doubt that *Clostridium botulinum* is still, nearly 200 years after its first reported emergence as a foodborne pathogen, a focus of increasing interest and intensive research. The targeted action of the botulinum neurotoxin has made this poisonous substance a central tool in the modern medical world. Development of novel genetic typing strategies has allowed establishment of more accurate phylogeny of the species *C. botulinum* and other neurotoxin-producing clostridia. The multitude of physiologies of the neurotoxigenic clostridia and their entrance mechanisms into the human and animal body, have brought the epidemiology of botulism into a new light and set new challenges for diagnostics.

Characteristic of the past couple of years has been the emergence of complete *C. botulinum* genomes, which has brought the study of the organism into a new blossoming. Understanding of the structure, composition, and location of the neurotoxin gene cluster has undergone renaissance. Genome-wide transcriptional analysis with DNA microarrays provides an efficient tool to screen for genetic mechanisms, and the newly developed gene mutation tools allow targeted study of gene functions.

An indication of the stature of *C. botulinum* in the European research field is this fully booked congress organised in Finland for the first time. Thanks to the EU Marie Curie Actions funding, the series of events *Clostridia* has attracted young scientists into the intriguing research field of clostridia. A continuation to this congress will be the practical workshop on the molecular epidemiology of *C. botulinum* to be organised later in 2008 at the University of Helsinki, a member of the League of European Research Universities, which has provided excellent facilities for *C. botulinum* research already over a decade.

We warmly acknowledge the kind support of the City of Helsinki, HK Ruokatalo Oyj, the Finnish Food Research Foundation, and University of Helsinki. On behalf of the organising committee we warmly welcome you to the congress and wish you a pleasant Midsummer time in Helsinki.

Miia Lindström Hannu Korkeala

Scientific Committee

Miia Lindström, University of Helsinki, Finland
Hannu Korkeala, University of Helsinki, Finland
Mike Peck, Institute of Food Research, UK
Nigel Minton, University of Nottingham, UK
Austin John, Health Canada, Canada

Organising Committee

Miia Lindström, University of Helsinki, Finland
Hannu Korkeala, University of Helsinki, Finland
Ying Chen, University of Helsinki, Finland
Elias Dahlsten, University of Helsinki, Finland
Annamari Heikinheimo, University of Helsinki, Finland
Sanna Hellström, University of Helsinki, Finland
Jacqueline Minton, University of Nottingham, UK
Mari Nevas, University of Helsinki, Finland
Sanna Piikkilä, University of Helsinki, Finland
Johanna Seppälä, University of Helsinki, Finland
Heimo Tasanen, University of Helsinki, Finland

PROGRAMME

Monday, June 16th 2008

Scientific programme
University Main Building, Modern side, Hall 5, Fabianinkatu 33 (City Centre)

18.00-18.45 Welcome lecture: A decade of *Clostridium botulinum* research in the University of Helsinki
Miia Lindström and Hannu Korkeala, Department of Food and Environmental Hygiene, University of Helsinki

Social event
University Main Building, Historical side, Teachers' Lounge, Unioninkatu 34 or guiding from Hall 5 (City Centre)

19:00-21:00 Reception hosted by Vice Rector Johanna Björkroth, University of Helsinki

Tuesday, June 17th 2008

Scientific programme
Info Centre Korona, Hall 2, Viikinkaari 11 (Viikki Campus)

Session I: *Epidemiology and prevention of* Clostridium botulinum *and botulism in humans and animals*

Session chair Mike Peck, Institute of Food Research, UK

09.30-10.15 Baked potatoes to beluga whales - botulism in Canada from 1985 to 2007
Catherine Paul, Bureau of Microbial Hazards, Health Canada, Ottawa, Canada

10.15-10.40 Phylogenetic analysis of *Clostridium botulinum* type A by multi-locus sequence typing
Eric Johnson, University of Wisconsin, Madison, USA

10.40-11.10 Break

11.10-12.05 Infant botulism: Global occurrence and molecular epidemiology in California
Stephen Arnon, California Department of Public Health, California, USA

12.05-12.30 Increasing incidence of botulism in cattle in Flanders
Bart Pardon, Ghent University, Merelbeke, Belgium

12.30-12.55 Animal botulinum toxicoses - German experiences
Helge Böhnel, Georg-August-University, Göttingen, Germany

13.00-14.00 Lunch

Session II: *Structure and function of botulinum neurotoxin*

Session chair Nigel Minton, University of Nottingham, UK

14.00-14.45 Botulinum neurotoxin complexes: Genetics, structure and function
Eric Johnson, University of Wisconsin, Madison, USA

14.45-15.10 Subtypes of the type B botulinum neurotoxin gene are widely distributed on extrachromosomal elements
Giovanna Franciosa, The Istituto Superiore di Sanità, Rome, Italy

15.10-15.40 Break

15.40-16.05 Effect of carbon dioxide on neurotoxin gene expression in non-proteolytic *Clostridium botulinum* types B and E
Ingrid Artin, University of Lund, Sweden

16.05-16.30 Expression of the neurotoxin cluster genes in *Clostridium botulinum* type E at optimum and low temperature
Ying Chen, University of Helsinki, Finland

Social event
City Centre

16:30-16:50 Bus departure from Viikki Info Centre Korona (Viikki campus) to the Hakaniemi Pier

17:00-18:45 Boat Tour in the archipelago of Helsinki, arrival at Market Square, South Harbour

19:00-20:00 Reception hosted by the City of Helsinki, Helsinki City Hall, Pohjoisesplanadi 11-13

Wednesday, June 18th 2008

Scientific programme
Info Centre Korona, Hall 2, Viikinkaari 11 (Viikki Campus)

Session III: *Diagnostics of* Clostridium botulinum *and botulism*

Session chair Dario De Medici, Istituto Superiore di Sanità, Italy

09.30-10.15 Application of different PCR based technologies for rapid screening of botulinum neurotoxins A, B, E, F producing *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*
Patrick Fach, AFSSA, French Food Safety Agency, Maisons-Alfort, France

10.15-10.40 Improved diagnosis of infant botulism by real time PCR of neurotoxin genes
Kathie Grant, Health Protection Agency, London, UK

10.40-11.05 Detection of *Clostridium botulinum* neurotoxin genes (bont) A, B, E and F with real-time-PCR
Ute Messelhäusser, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany

11.05-11.35 Break

11.35-12.00 Antibodies to *Clostridium botulinum* in blood in relation to the detection of *C. botulinum* in faeces of cows
Wieland Schroedl, University of Leipzig, Germany

12.00-12.25 MALDI-TOF MS fingerprinting as a powerful tool for the identification of *Clostridia* in the microbiological laboratory?
Anke Grosse-Herrenthey, University of Leipzig, Germany

Session IV: Food safety

Session chair Hannu Korkeala, University of Helsinki, Finland

12.30-13.15 Foodborne botulism, past present and future
Mike Peck, Institute of Food Research, Norwich, UK

13.15-14.45 Lunch

14.45-15.10 Growth of group I *Clostridium botulinum* at extreme temperatures
Katja Hinderink, University of Helsinki, Finland

15.10-15.35 Lag time variability in individual spores of non-proteolytic *Clostridium botulinum*
Mike Peck, Institute of Food Research, Norwich, UK

15.35-16.00 *Clostridium botulinum*, a pathogen for man and animal – a superior growth promoter for plants
Frank Gessler, miprolab, Göttingen, Germany

16.00-17.00 Poster session and refreshments

Social event
City Centre

19:00-01:00 Congress Dinner, Restaurant Olivia, Siltavuorenpenger 20 R (City Centre)
Music by The Queen an' Tools

Thursday, June 19th 2008

Scientific programme
Info Centre Korona, Hall 2, Viikinkaari 11 (Viikki Campus)

Session V: *Genetics and genomics of* Clostridium botulinum

Session chair Holger Brüggemann, Max Planck Institute for Infection Biology, Germany

10.00-10.30 Biological highlights from the genome of proteolytic *Clostridium botulinum*
Mike Peck, Institute of Food Research, UK

10.30-10.55 The glycosylated flagella of *Clostridium botulinum*
Catherine Paul, Bureau of Microbial Hazards, Health Canada, Ottawa, Canada

10.55-11.20 Structural and genetic characterisation of flagellar glycosylation in *Clostridium botulinum*
Susan Logan, National Research Council Institute for Biological Sciences, Ottawa, Ontario, Canada

11.20-11.50 Break

11.50-12.35 Gene tools and their application to *Clostridium botulinum*
Nigel Minton, University of Nottingham, UK

12.35-13.00 Cold shock increases the expression of *cspA* and *cspB* but not *cspC* in *Clostridium botulinum* ATCC3502
Henna Söderholm, University of Helsinki, Finland

13.00-13.10 Closing of the congress
Miia Lindström, University of Helsinki, Finland

13.10-14.20 Lunch

ORAL PRESENTATIONS

In alphabetical order

NOTES

EPIDEMIOLOGICAL FINDINGS FROM AFLP ANALYSIS OF 703 *CLOSTRIDIUM BOTULINUM* STRAINS ISOLATED FROM CALIFORNIA INFANT BOTULISM PATIENTS 1976-2001

S.S. Arnon[1], K.K. Hill[2], J.R. Barash[1], C. Helma[2], L. Ticknor[2], J.R. Payne[1], N. Dover[1] and H.A. Dabritz[1]
[1]Infant Botulism Treatment and Prevention Program, California Department of Public Health, Richmond, California 94804, USA.
[2]Los Alamos National Laboratory, Los Alamos, New Mexico 87544, USA.

In 1976, coincident with the recognition of infant botulism an as intestinal toxemia and novel form of human botulism, the now California Department of Public Health (CDPH) began archiving an isolate of *Clostridium botulinum* from every California patient with infant botulism. The patient address at onset of illness was recorded for all patients, thereby identifying a geographic location for each isolate. In 2006 CDPH and Los Alamos National Laboratory began analyzing the California infant botulism strain collection by the Amplified Fragment-Length Polymorphism (AFLP) technique using the restriction enzymes *Eco*R1 and *Mse*I, as described by Hill et al., *J Bacteriol* 2007;189:818-832. To date, a total of 703 strains isolated from California patients during the 25-year period 1976-2001 have been analyzed, and a dendrogram has been constructed from these results.

Analysis of the clades in the dendrogram yielded a variety of epidemiological and microbiological insights. The isolates from the largest type A (N=312) and type B (N=108) clades were located throughout California. In contrast, a smaller clade (N=14; 1978-1991) of type B1 isolates localized to the eastern side of San Francisco Bay and the Sacramento River Delta area. A large clade of type A1 isolates (N=54; 1977-2001) localized almost entirely to San Francisco Bay Area counties and the Sacramento River counties in the Central Valley. Five smaller clades of type A isolates representing different parts of California that were bordered on either side by clades that contained type B isolates were identified as type A isolates that contained a “silent B” gene [i.e., were serotype A(B)]. Bivalent (Ba, Bf) isolates grouped into three different clades. In two clades (one A, one B), isolates from patients living in different geographical locations were found to be linked to the feeding of honey.

AFLP analysis of the CDPH collection of *C. botulinum* infant isolates has provided novel information about their geographic distribution, genetic relatedness and epidemiological connectedness that will become better understood when analysis of the entire collection is completed.

NOTES

EFFECT OF CARBON DIOXIDE ON NEUROTOXIN GENE EXPRESSION IN NON-PROTEOLYTIC CLOSTRIDIUM BOTULINUM TYPES B AND E

I. Artin[1,2,3], A.T. Carter[2], E. Holst[3], M. Lövenklev[1], D.R. Mason[2], M.W. Peck[2], and P. Rådström[1]
[1]Applied Microbiology, Lund Institute of Technology, Lund University, SE-221 00 Lund, Sweden, [2]Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK, [3]Division of Medical Microbiology, Department of Laboratory Medicine, Lund University, SE-223 62 Lund, Sweden.

When developing new food products and manufacturing solutions, it is important to consider not only the occurrence and quantity of pathogens in the food chain, but how the processing and subsequent storage and handling will affect them. This is especially relevant for food designed to have a long shelf-life. Understanding the influence of environmental factors, food preservatives, and type of packaging on microbial growth and expression of virulence factors is very important.
Quantitative reverse transcription PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to monitor neurotoxin gene (*cnt*) expression and neurotoxin formation in non-proteolytic *C. botulinum* types B and E. Relative gene expression varied with growth phase in both types, reaching a maximum in late exponential and early stationary phase.
We also found that while growth was slowed, relative *cnt* expression was increased with an elevated CO_2 concentration, with five-fold greater expression of *cntB* mRNA and accumulation of type B toxin at 70% CO_2 than at 10%. For type E the increase in expression at 70% CO_2 was two-fold greater, when compared with 10% CO_2.
In conclusion, these findings, shown with both qRT-PCR and ELISA, sheds a new cautionary light on the potential risks of botulism associated with non-proteolytic *C. botulinum* and the use of modified atmosphere packaging.

NOTES

BAKED POTATOES TO BELUGA WHALES - BOTULISM IN CANADA FROM 1985 TO 2007

JW Austin (presented by CJ Paul)
[1]Bureau of Microbial Hazards, Health Canada, Ottawa, ON, CAN

In Canada, since 1985, approximately 4.4 outbreaks of foodborne botulism occur annually, with an average of 2.5 cases/outbreak. The fatality rate of botulism in Canada has decreased from greater than 45% in the 1960's to less than 3%, due mainly to the availability of antitoxin to type E neurotoxin. Most botulism outbreaks in Canada have occurred in northern and west coast native communities. The foods involved were mainly raw meats from marine mammals, fermented meats such as muktuk (meat, blubber and skin of the beluga whale), raw fish or fermented salmon eggs. Type E was implicated in almost every case involving traditional foods. Commercial products are rarely implicated in outbreaks. Commercial pate caused two cases of type B botulism, while a cooked boneless pork product caused a single case of type A botulism. Two cases of type A botulism from commercial carrot juice were reported in October 2006. Garlic-in-oil, bottled mushrooms and a baked potato have been responsible for outbreaks involving food-service establishments. A cluster of three cases of colonization botulism, all in individuals with Crohn’s Disease, occurred from November 2006 to February 2007.

NOTES

ANIMAL BOTULINUM TOXICOSES: THE GERMAN EXPERIENCES

H. Böhnel[1], F. Gessler[2]
[1]Institute for Tropical Animal Health, Georg-August-University, Göttingen
[2]Miprolab GmbH, Göttingen, Germany

Clostridium botulinum is known as an anaerobe bacterium, producing spores and highly toxic metabolites (BoNT). Since about 100 years the disease "botulism" is known. Every textbook features

- Intoxication, soil borne disease
- Sudden onset, muscular paralysis, final death due to suffocation
- Different susceptibility of different animal species including man

In recent years there is evidence for an increasing number of cases of infection in man and animals. Almost nothing is known on chronic resorption/action of minute amounts on BoNT.

Following thousands of treated samples in our diagnostic laboratory we report several features of *C. botulinum* infection in animals and man

- Visceral botulism in cattle and pigs
- Grass sickness in horses
- Sudden infant botulism
- BoNT and bovine mastitis
- BoNT and right abomasal displacement
- BoNT and tonsils
- BoNT and diabetes
- BoNT and decubitus

Vaccination of animals is a promising means of prevention. The catastrophic situation to obtain vaccines as an example in Germany will be explained.

NOTES

EXPRESSION OF THE NEUROTOXIN CLUSTER GENES IN *CLOSTRIDIUM BOTULINUM* TYPE E AT LOW TEMPERATURE

Y. Chen, H. Korkeala, and M. Lindström.
Centre of Excellence in Microbial Food Safety Research, Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland

Being able to grow and produce neurotoxin at refrigeration temperatures, *Clostridium botulinum* type E possesses a significant safety risk for the modern food industry where the chill chain is the main bacterial growth-inhibiting factor. The expression of all six type E neurotoxin complex genes was monitored for the first time at either 10 or 30°C using quantitative real-time reverse transcription PCR (qRT-PCR). Different expression patterns of the toxin complex genes were observed at the two temperatures. The neurotoxin gene cluster had early and prolonged expression at 10°C when compared to that at 30°C. The expression levels had only little or no decline after 8 days of incubation at 10°C in contrast to those at 30°C. The maximal expression levels at 10°C were similar to (*ntnh* and *p47*), 37-65% lower than (*bont*/E, *orfx2*, and *orfx3*), or 90% lower than (*orfx1*) at 30°C. Evidence of different transcriptional mechanisms at 10 and 30°C was obtained. At 30°C, *bont*/E, *ntnh*, and *p47*, and *orfx1*, *orfx2*, and *orfx3* seemed to be expressed as two tricistronic operons whereas at 10°C mono- and bicistronic transcription seemed to prevail. The results suggest that the botulinum neurotoxin gene cluster has stable activity for extended time periods at low temperature. Novel strategies and additional control factors are therefore demanded to ensure the safety of refrigerated foods.

NOTES

APPLICATION OF DIFFERENT PCR BASED TECHNOLOGIES FOR RAPID SCREENING OF BOTULINUM NEUROTOXINS A, B, E, F PRODUCING *CLOSTRIDIUM BOTULINUM*, *CLOSTRIDIUM BARATII* AND *CLOSTRIDIUM BUTYRICUM*

P. Fach[1], P. Micheau[1], S. Perelle[1], P. Chablain[2] and M.R. Popoff[3]
[1]AFSSA, French Food Safety Agency, Research Laboratory on Food Quality and Food Processes, Maisons-Alfort, France. [2]GeneSystems SA, 35170 Bruz, France. [3]Institut Pasteur, Paris, France.

Botulinum neurotoxins (BoNT) are produced by phenotypically and genetically different Clostridium species including Clostridium botulinum, and some strains of Clostridium baratii and Clostridium butyricum. Four real-time PCRs working with the same melting temperatures, each being specific to the genes bont/A, bont/B, bont/E, and bont/F, and enables a toxin type-specific identification have been developed. Owing to the very low G+C content of *C. botulinum* and due to the high genetic diversity within the BoNT genes we used some degenerate and locked nucleic acid oligonucleotides. The specificity of the assay for *Clostridium botulinum* types A-G, *Clostridium butyricum* type E and *Clostridium baratii* type F was demonstrated using a panel of BoNT producing clostridia representing all seven toxin serotypes. In addition, exclusivity of the assay was demonstrated using non-botulinum toxin producing clostridia (21 strains) and various other bacterial strains. Using purified DNA, the toxin type-specific assays had a sensitivity of 100fg – 1000fg of total DNA in the PCR tube (25-250 genome equivalents). The limit of detection of the assays ranged from 10^3 to 10^4 cells/ml by using calibrated Clostridial suspension. After a 48h enrichment in anaerobic conditions, *C. botulinum* type A has been detected in a naturally contaminated sample of “foie gras” suspected in a *C. botulinum* outbreak. Further development of the methods on the GeneSystems® PCR based technology which relies on the multiplex real-time PCR amplification of several DNA targets within a unique GeneDisc would represent an improvement of the methods. Some preliminary results with a first GeneDisc are presented.

NOTES

SUBTYPES OF THE TYPE B BOTULINUM NEUROTOXIN GENE ARE WIDELY DISTRIBUTED ON EXTRACHROMOSOMAL ELEMENTS

G. Franciosa[1], A. Maugliani[1], C. Scalfaro[1], P. Aureli[1]
[1]Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, ITALY

Five distinct subtypes (B1, B2, B3, bivalent B and non-proteolytic B) have been described for BoNT/B, the neurotoxin accounting for most human botulism cases in Italy and Europe, and the second leading cause of botulism in the United States. Unexpectedly, the genes encoding BoNT/B1 and bivalent B subtypes have very recently been shown to be located on large plasmids in two peculiar strains of *C. botulinum*; whether plasmid encoded BoNT/B genes are rare or widespread among *C. botulinum* strains is unknown. We developed here a PCR-RFLP assay for the rapid identification of the BoNT/B gene subtypes in a panel of *C. botulinum* isolates. Nonradioactive probes and hybridization tools were then applied to analyse the chromosomal or extrachromosomal location of the BoNT/B genes.
All five subtypes of the BoNT/B gene were detected. BoNT/B2 was more prevalent among *C. botulinum* strains from Italy, whereas BoNT/B1 prevailed among strains from the US. BoNT/B3 and non-proteolytic B were identified in a low number of strains. Bivalent BoNT/B subytpe was atypically detected in several strains only harbouring the BoNT/B gene. Conversely, two rare *C. botulinum* type Ab (bivalent) strains were found to carry BoNT/B1 and B3 subtypes, respectively; since both strains contain a BoNT/A2 gene, they represent two novel A2/B1 and A2/B3 bivalent variants. The BoNT/B1 gene was chromosomally located in one of these two strains, whereas the BoNT/B3 gene was plasmid encoded in the other one. A gene coding for BoNT/B3 was located within the chromosome of another *C. botulinum* type B strain. In the remaining strains, the BoNT/B1 genes were predominantly carried by large plasmids; the BoNT/B2 genes were mainly located on the chromosome of strains; the non-proteolytic BoNT/B gene was identified in a single strain and was plasmid encoded; all bivalent BoNT/B genes atypically detected in the non-bivalent *C. botulinum* strains were located on plasmids. Our results clearly indicate that the presence of BoNT/B1 and bivalent B genes on plasmids is more widespread than previously known. Furthermore, this is the first demonstration that the genes encoding subtypes BoNT/B2, B3 and non-proteolytic B can also be carried by extrachromosomal elements. Finally, the genomic location of the genes encoding the same BoNT/B subtype can vary among different *C. botulinum* strains.

NOTES

CLOSTRIDIUM BOTULINUM, A PATHOGEN FOR MAN AND ANIMAL – A SUPERIOR GROWTH PROMOTER FOR PLANTS

Frank Gessler[1], Azuka N. Iwobi[2], Michael Schmid[2], Anton Hartmann[2], Helge Böhnel[3]
[1]miprolab GmbH, Göttingen, Germany, gessler@miprolab.com
[2]Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Department Microbe-Plant-Interactions, Neuherberg, Germany
[3] Institute for Applied Biotechnology in the Tropics at the University of Göttingen, Germany

Clostridium botulinum is well-known for its pathogenicity due its highly potent neurotoxins. Generally considered being a soil-bacterium, few aspects are known on the soil life of this species.
In a field experiment the topsoil of the experimental plots was mixed with compost spiked with increasing amounts of *C. botulinum* type D spores. A grass seed mix with clover was sown. We found that *C. botulinum* persisted over 3 years, the whole observation period. In spring of year 2 of the experiment the growth of clover was observed, but exclusively on the plots treated with *C. botulinum* spores. The yield correlated to the amount of spores introduced into soil at the beginning of the experiment. In the higher spore concentrations the total biomass increased by 2.6 times compared to the controls. Investigations of the root zone revealed an increasing spore gradient from the rhizosphere to the root surface of the clover.
In a subsequent growth chamber experiment the colonization of clover by toxigenic and non-pathogenic *C. botulinum* strains was investigated. Spores of the bacteria, in combination with *Rhizobia* were applied to the clover. The harvested plants were subjected to fluorescence *in situ* hybridization (FISH). Both toxin-producing and non-pathogenic *C. botulinum* strains were identified as endophytic colonizers of clover. Additionally, a plant growth promotion effect was observed in the growth chamber experiment as well.

NOTES

IMPROVED DIAGNOSIS OF INFANT BOTULISM BY REAL TIME PCR OF NEUROTOXIN GENES

K. A. Grant, I. Nwafor, C. Curson, O. Mpamugu, V. Mithani and J. Mclauchlin
HPA Centre for Infections, 61 Colindale Ave, London, NW9 5EQ, UK (kathie.grant@hap.org.uk)

Infant botulism is a rare disease caused by growth and toxin production of the bacterium *Clostridium botulinum* in the enteric tract of infants, usually less than 12 months of age. Up until 2007 there had only been 6 reported cases of infant botulism in the UK. Diagnosis of infant botulism is established by demonstrating botulinum neurotoxin and/or BoNT producing organisms in a faecal specimen. The mouse bioassay is the gold standard method for detecting BoNT in clinical and food samples. The assay is highly sensitive but usually takes several days to perform; is costly; involves the use of live animals and is usually run without controls. In the UK Reference Laboratory real time PCR assays for neurotoxin genes are used to assist in the rapid detection and identification of *C. botulinum* in cases of wound and food botulism. Here we describe the use of these assays to detect BoNT A,B,E and F genes, to assist in the laboratory diagnosis of two unrelated cases of infant botulism that occurred within two weeks of one another in the latter half of 2007. In October 2007, samples from two infants (aged 4 and 8 months) with suspect botulism at different hospitals in London were received by the Reference Laboratory. Following a series of conventional and PCR based tests the diagnosis of infant botulism was confirmed. The cases were sporadic and apparently unrelated, with one found to be due to type A and the second to type B.
These assays used in conjunction with the MBA improved the rapidity of diagnosis and enabled the real time monitoring of the organism in the infant gut. Food and environmental samples were rapidly screened by PCR for the presence of *C. botulinum* thus improving disease investigation. The use of real time PCR assays for the detection of BoNT genes has substantially improved the diagnostic process for infant botulism facilitating rapid and appropriate patient management.

NOTES

MALDI-TOF MS FINGERPRINTING AS A POWERFUL TOOL FOR THE IDENTIFICATION OF CLOSTRIDIA IN THE MICROBIOLOGICAL LABORATORY?

Anke Grosse-Herrenthey[1], Thomas Maier[3], Frank Gessler[4], Reiner Schaumann[2], Helge Böhnel[4], Markus Kostrzewa[3], Monika Krüger[1]
[1]Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, University of Leipzig, [2]Institute of Medical Microbiology, Faculty of Medicine, University of Leipzig, [3]Bruker Daltonik GmbH, Leipzig; [4]Institute of Agronomy and Animal Production in the Tropics, University of Göttingen, Germany

The identification and characterization of Clostridia, with well over 150 species in this genus, is not an easy task for a microbiological laboratory. In addition to this, traditional methods based on the metabolic properties of the bacteria require rigorously standardized media and growth conditions to assure the attainment of reproducible results. 16S rDNA sequencing is assumed to be the gold standard for bacterial classification but is nonetheless a time-consuming and costly technique.
Here, we describe the utilization of matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for whole cell fingerprinting in combination with a dedicated bioinformatic software tool to distinguish between various clostridial species.
A total of 64 clostridial strains from 31 pathogenic and apathogenic species served as a reference database. Starting with a single colony it was possible to correctly identify a Clostridium species within minutes. Even species which are difficult to differentiate by traditional methods, such as *C. chauvoei* and *C. septicum* could be identified correctly. It was not possible to distinguish between toxovars of *C. perfingens*, whereas the differentiation between the four metabolic groups of *C. botulinum* was carried out correctly.
To conclude, our findings indicate that, inasmuch as the technology employed is based on a high-quality reference database, MALDI-TOF may serve as an effective tool for the swift and reliable identification and classification of clostridia.

NOTES

GROWTH OF GROUP I *CLOSTRIDIUM BOTULINUM* AT EXTREME TEMPERATURES

K. Hinderink, M. Lindström, H. Korkeala
Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, 00014 Helsinki University, Finland

The growth at low and high temperature of 23 Group I *Clostridium botulinum* strains comprising toxin types A, AB, B, and F was studied. The minimum and maximum growth temperatures and maximum growth rates at 20°C, 37°C and 42°C of each strain were determined. A temperature-gradient incubator (GradiplateW10, BCDE Group, Helsinki, Finland) was used to study minimum and maximum growth temperatures. The maximum growth rates were calculated from optical density measurements of bacterial cultures incubated in an automated turbidity reader (Bioscreen C Microbiology Reader, Oy Growth Curves AB Ltd, Helsinki, Finland).
The minimum growth temperatures varied from 12.8°C to 16.5°C with an average of 14.5°C. The maximum growth temperatures showed even larger variation from 40.9°C to 48.0°C with an average of 45.4°C. At 20°C all strains grew substantially slower than at 37°C, the commonly accepted optimum growth temperature for Group I *C. botulinum*. However, when comparing maximum growth rates at 37°C and 42°C, it was found that eight type B strains grew significantly faster at the higher temperature. Seven of these strains additionally possessed a maximum growth temperature higher than average. Thus their optimum growth temperature appeared to be higher than 37°C. Taking all strains in consideration, a significant correlation between the maximum growth rates at 42°C and the maximum growth temperature was found. This correlation did not apply for the type A strains alone.
The largest strain variation was found within the toxin type B. This type is known to have a wide genetic diversity compared to the other Group I toxin types. In accordance, genetically very similar type B strains showed similar growth performance.
The observed variation in minimum and maximum growth temperatures and growth rates at different temperatures between strains of Group I *C. botulinum* and within the toxin types should be taken in account when selecting model strains for studies to evaluate the safety of food products.

NOTES

BOTULINUM NEUROTOXIN COMPLEXES: GENETICS, STRUCTURE AND FUNCTION

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Clostridium botulinum comprises a heterogeneous group of organisms, all of which have the distinctive characteristic of producing botulinum neurotoxin (BoNT). BoNTs can be immunologically distinguished using homologous antitoxins into seven serotypes, designated A-G. Previous studies have identified that the BoNT genes of all strains of *C. botulinum* and neurotoxigenic strains of *Clostridium butyricum* and *Clostridium baratii* all have a set of genes located upstream of the BoNT gene that are organized as neurotoxin clusters. Previous data indicated that there exist two primary types of neurotoxin clusters: (a) a hemagglutinin (HA) cluster and; (b) an orfX cluster with genes of unknown function, but we have found considerable diversity in the genes and their arrangement in clusters, indicating that considerable recombination and possibly lateral transfer of the toxin gene clusters has occurred. The toxin gene clusters encoding BoNT/A3, BoNT/A4 and certain bivalent gene clusters were found to be located on large plasmids. Genes and gene fragments of the HA and orfX clusters have been cloned and expressed in *E. coli*, the proteins purified, and antibodies prepared for immunological characterization of the clusters and for preliminary structural analysis. Oral feeding studies in mice have demonstrated that the BoNT/A toxin cluster is considerably more stable than purified BoNT/A and that foodstuffs and other factors affect its oral toxicity.

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PHYLOGENETIC ANALYSIS OF *CLOSTRIDIUM BOTULINUM* TYPE A BY MULTI-LOCUS SEQUENCE TYPING

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Clostridium botulinum comprises a heterogeneous group of neurotoxigenic clostridia for which the phylogeny and evolutionary relationships are poorly understood. *C. botulinum* type A has recently been shown to produce multiple neurotoxin subtypes, suggesting that it is not monophyletic as previously reported, but is comprised of distinct lineages. The elucidation of these evolutionary relationships necessitates the use of experimental methods that can distinguish *Clostridium* lineages, which are time and cost effective, and can be accurately and reproducibly employed in different laboratories. Multi-Locus Sequence Typing (MLST) has been successfully used as a reproducible and discriminating system in the study of eukaryotic and prokaryotic evolutionary biology, and for strain typing of various bacteria. In this study, MLST was applied to evaluate the evolutionary lineages in the serotype A group of *C. botulinum*. For MLST analysis, we initially evaluated fourteen housekeeping genes (*gapdh, tuf, sod, oppB, hsp60, dnaE, aroE, pta,* 23S *rDNA, aceK, rpoB,* 16S *rDNA, mdh,* and *recA*) for amplification and sequence analysis. In the first phase of the analysis, thirty *C. botulinum* type A strains producing subtype BoNTs A1 - A4 were examined. Results of this pilot study suggested that seven of the genes (*mdh, aceK, rpoB, aroE, hsp60, oppB,* and *recA)* could be used for elucidation of evolutionary lineages and strain typing. These seven housekeeping genes were successfully applied for the elucidation of lineages for 73 *C. botulinum* type A strains, which resulted in 24 distinct sequence types (STs). This strategy should be applicable to phylogenetic studies and typing of other *C. botulinum* serotypes and *Clostridium* species.

NOTES

STRUCTURAL AND GENETIC CHARACTERISATION OF FLAGELLAR GLYCOSYLATION IN *CLOSTRIDIUM BOTULINUM*

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Flagellins from *Clostridium botulinum* were shown to be posttranslationally modified with novel glycan moieties by top down MS analysis of purified flagellin protein from strains of unique toxin serotypes. Detailed analysis of flagellin from two strains of *C. botulinum* were shown to be modified in *O*-linkage by a novel glycan moiety of mass 417 Da. The structural characterisation of the carbohydrate moiety was completed utilising both MS and NMR and it was shown to be a novel legionaminic acid derivative. ETD-MS and ETD-cidMS was successfully utilised to map the 7 sites of *O*-linked glycosylation eliminating the need for chemical derivatisation of tryptic peptides prior to analysis. Marker ions for novel glycans as well as a unique C terminal flagellin peptide marker ion were identified in a top down analysis of the intact protein. These ions have the potential to be utilised for rapid detection and discrimination of *C.botulinum* cells and for botulinum neurotoxin (BoNT) contamination. This is the first report of glycosylation of Gram positive flagellar proteins with the “sialic acid like” nonulosonate sugar, legionaminic acid. Bioinformatic analysis of available *C. botulinum* genomes identified a flagellar glycosylation island containing homologs of genes recently identified in *Campylobacter coli* which have been shown to be responsible for the biosynthesis of legionaminic acid derivatives.

NOTES

DETECTION OF CLOSTRIDIUM BOTULINUM NEUROTOXIN GENES (BONT) A, B, E AND F WITH REAL-TIME-PCR

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Intoxications with *C. botulinum* toxins belong also in Germany to the rare occurring food-poisonings; the mortality, however, is very high. In the last years the risk for the consumer´s health is mainly due to not commercially produced food cans or salted bacon. Because of the difficult and time-consuming cultural detection of *C. botulinum*, PCR methods to screen for the toxin genes A, B, E and F, which are relevant in human medicine, have been used increasingly during the last years. These comprised mostly conventional PCR methods which are not so efficient to screen large numbers of samples. Therefore we designed for the *C. botulinum*-detection in our routine laboratory two probe-based real-time-PCR-assays, one triplex-PCR-assay for the detection of BONT A and B in combination with an Internal Amplification Control (IAC) and one duplex-system for the detection of BONT E and F, both based on the Taqman-technology. As IAC we used the pUC 19 plasmid. Both assays were tested for specifity and sensitivity with pure cultures first and then with artificially contaminated food samples (meat, fish and honey) after enrichment. The sensitivity with pure cultures was 10 cfu/ml for all four toxin genes. Food samples contaminated with 10 cfu/g, were reliably detected as positive with real-time-PCR after enrichment. Subsequently we checked 147 routine samples with our real-time-PCR-systems, the conventional PCR-system of Lindström et al., 2002 and the cultural method in parallel. We examinated mainly food samples (honey, carps, canned meat and vegetables), but also animal feeding stuff and environmental samples. Six samples were positive with the real-time-PCR and the convential PCR-system of Lindström et al., 2002 (five samples for BONT B and one sample for BONT E), but only five samples were positive with the cultural method. The results of our examinations indicates, that the two real-time-PCR-assays are suitable in a routine laboratory for a fast screening for the presence of *Clostridium botulinum* type A, B, E and F in food samples.

NOTES

GENE TOOLS AND THEIR APPLICATION TO CLOSTRIDIUM BOTULINUM

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Despite the medical and industrial importance of the genus *Clostridium* our understanding of their basic biology lags behind that of their more illustrious counterpart, *Bacillus*. The advent of the genomics era has provided new insights, but full exploitation of the data becoming available is being hindered by a lack of mutational tools for functional genomic studies. Thus, in the preceding decades the number of clostridial mutants generated has been disappointingly low. On the one hand, the absence of effective transposon elements has stymied random mutant generation. On the other hand, the construction of directed mutants using classical methods of recombination-based, allelic exchange has met with only limited success. Indeed, in the majority of clostridial species mutants are largely based on integration of plasmids by a Campbell-like mechanism. Such single crossover mutants are unstable. As an alternative, recombination-independent strategies have been developed that are reliant on retargeted group II intron. One element in particular, the ClosTron, has been devised which provides the facility for the positive selection of mutants. ClosTron-mediate mutant generation is extremely rapid, highly efficient and reproducible. Moreover the mutants made are extremely stable. Its deployment considerably expands current options for functional genomic studies in *Clostridium botulinum*.

NOTES

INCREASING INCIDENCE OF BOTULISM IN CATTLE IN FLANDERS

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Since 2003 an increasing incidence of botulism herd outbreaks has been reported in several European countries (e.g. England, Wales and Ireland). Botulism in cattle is caused by neurotoxins produced by *Clostridium botulinum type B, C or D*. Type C and D are poultry associated and most common in Europe. Type B is associated with forage. In the period 2001-2008 31 herd outbreaks of botulism were diagnosed at the faculty of Veterinary Medicine of Ghent University. The number of outbreaks rose spectacularly in 2003 and remained at a higher incidence up to 2008. Outbreaks affected both dairy and beef herds and occurred at pasture and in stabled animals. There was no seasonal distribution. Diagnosis was confirmed in 42% of the outbreaks by the mouse-toxin test. In 21% no diagnostic efforts were made. All outbreaks were type D, with one outbreak not being further determined. There were no outbreaks of the zoonotic type B. In total 59 samples were send to the reference lab for analysis. The percentage positive samples for liver and rumen fluid were respectively 29 and 22%. No serum samples were positive. On 4 of the 6 most recent outbreaks, a poultry stable was present on the farm. Most other outbreaks were also poultry associated. Also the national animal health organisation (DGZ) reports an increase in possible outbreaks with 118 suspicions in 2007-2008. Preventive measures in Belgium are limited to hygienic procedures and prevention of accidental spreading of carcasses in bovine feed or pastures. Vaccination is currently not allowed in Belgium. Treatment with anti-toxin is extremely expensive, not effective in all cases and needs type-identification. In general the possible risks for public health are thought to be minimal. In Belgium the precautionary principle is applied and preventive measures for public health are taken. In case of botulism suspicion milk delivery is forbidden until 17 days after the onset of the last clinical case. After conformation UHT-treatment or sterilisation is obligatory until 17 days after the last clinical case. The increasing European incidence of botulism in cattle, current public health policy and the lack of financial support for affected farms are strong arguments to enhance vaccine registration in Europe.

NOTES

THE GLYCOSYLATED FLAGELLA OF *CLOSTRIDIUM BOTULINUM*

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Clostridium botulinum produces large amounts of the flagellin protein, FlaA, the structural subunit of the flagellar filament. FlaA is present in culture supernatant and is detectable using mass spectrometry or with flagellin antibodies, supporting the idea that this protein could be used as a surrogate biomarker for botulinum neurotoxin. While useful for detection, FlaA is also highly variable, due to variations in *flaA* gene sequence and unique posttranslational modifications. This variation can be used to differentiate between closely related strains, particularly those of *C. botulinum* Group I. We have developed methods for strain identification using this variability in flagellin including: a rapid colony PCR method for determining group and *flaA* allele; top down mass spectrometry for detection of posttranslational modification; high resolution strain matching by FlaA intact mass profile; and microarray profiling of the flagellar glycosylation island.

During investigations of FlaA posttranslational modification (PTM), we discovered that some strains modify FlaA with nonulosonate sugars that are structurally similar to glycan modifying the flagellins of *Campylobacter* and *Helicobacter.* We have characterised the novel PTM for type A strain FE9909ACS and are working towards a complete structural elucidation of this new nonulosonate sugar. The type F strain Langeland has a similar PTM to FE9909ACS, and with the recent completion of the Langeland genome sequence, a genetic origin for this new PTM can be postulated.

We have used methods developed for FlaA-based identification for the investigation of several recent cases of botulism, including adult colonization botulism, referred to the Botulism Reference Service for Canada.

NOTES

FOODBORNE BOTULISM, PAST PRESENT AND FUTURE

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Foodborne botulism is an intoxication caused by consumption of pre-formed botulinum neurotoxin. Botulinum neurotoxins are the most potent substances known, with as consumption of little as 30ng sufficient to cause illness and death. Foodborne botulism is principally caused by neurotoxins formed by two physiologically and genetically distinct organisms, proteolytic *Clostridium botulinum* and non-proteolytic *C. botulinum*. Proteolytic *C. botulinum* is a mesophile, and associated botulism cases have involved improper preservation of home-prepared foods, underprocessing and/or poor storage of commercial foods, while non-proteolytic *C. botulinum* is a psychrotroph, with botulism cases often involving dried or vacuum-packed fish.

Anecdotal evidence suggests that foodborne botulism may have occurred in ancient cultures, and that some dietary laws and taboos may have evolved to prevent the disease. The word "botulism" is derived from the Latin word "*botulus*" meaning sausage, and was given to a disease associated with consumption of blood sausage characterised by muscle paralysis, breathing difficulties and a high fatality rate in central Europe in the 18th and 19th century. In Belgium in 1897, van Ermengem first isolated a causative organism from home-made raw salted ham and the spleen of a man who later died of botulism. A great number of botulism outbreaks occurred in the early part of the twentieth century, and were associated with commercial and home canning processes, particularly in the USA.

Through the identification and implementation of appropriate effective control measures, the incidence of foodborne botulism is lower than a century ago. Today, outbreaks are often associated with home-prepared foods, where known control measures have not been implemented. Botulism outbreaks involving commercial processing are less common, but can be associated with significant medical and economic consequences. It is essential that all new processing technologies are introduced safely, and that they are not associated with an increase in the incidence of foodborne botulism. Minimally-heated refrigerated foods are a particular concern.

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BIOLOGICAL INSIGHTS FROM THE GENOME OF PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* STRAIN HALL A

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The complete genome sequence of the type A1 strain of proteolytic *C. botulinum* Hall A (ATCC 3502, NCTC 13319) was determined and annotated. It consisted of a chromosome (3.89Mb, 3650 coding sequences (CDSs)) and plasmid (16.3kb, 19 CDSs); %GC = 28.2%. The sequenced genome confirms the heterogeneity of the *Clostridium* genus, with only 568 *C. botulinum* CDSs (16%) shared with other sequenced clostridia (*C. acetobutylicum*, *C. perfringens*, *C. tetani* and *C. difficile*), while 1511 CDSs (41%) have orthologues in at least one of the sequenced clostridia, and 1571 CDSs (43%) are unique to *C. botulinum*, compared to the other sequenced clostridial genomes. In common with other sequenced clostridia (except for *C. difficile*), there is little evidence of recent horizontal gene acquisition. Consistent with its proteolytic phenotype, the genome harbours a large number of genes encoding secreted proteases and enzymes involved in uptake and metabolism of amino acids. The genome also reveals a previously unknown ability to degrade chitin.

The annotated genome sequence was used to produce a microarray of PCR products (100-500 bp). Not all CDSs were suitable for PCR amplification due to repeat sequences and the low G+C content. The microarray contains 3433 DNA species, corresponding to approximately 94% of all chromosomal CDSs, in addition to 19 probes from the plasmid. The array includes extra features that will facilitate the genotyping of new strains isolated from, for example, disease outbreaks (e.g. probes for all neurotoxin and neurotoxin-associated genes (haemagglutinin, NTNH, cntR etc)). Comparative genomic indexing studies showed that the two prophages of the sequenced Hall A strain were not found in other strains. Other strains of proteolytic *C. botulinum* and *C. sporogenes* were closely related (84-92% of Hall A CDS). There was synteny between the genomes. DNA from strains of other clostridia (non-proteolytic *C. botulinum* and *C. difficile*) hybridised more weakly confirming that they are members of evolutionarily distinct groups.

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LAG TIME VARIABILITY IN INDIVIDUAL SPORES OF NON-PROTEOLYTIC *CLOSTRIDIUM BOTULINUM*

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Spores formed by pathogens such as *Clostridium botulinum* are important in food safety as they are able to resist many of the processes, such as cooking, used to kill vegetative cells. When growth of *C. botulinum* occurs in a food it will often initiate from just a few spores contaminating the product. Here the distribution of times to growth in packs will reflect the heterogeneity of times to growth from individuals. Quantifying lag times from individual spores and the associated variability is therefore an important part of understanding the hazard associated with such organisms.

The lag period between a spore being exposed to conditions suitable for growth and the start of exponential growth will depend upon the time required for germination, emergence, elongation and first cell division. Each of these stages may be affected by processing regimes and the food storage conditions. Currently very little is known about the variability and duration of these stages and any relationships between them. We have used phase contrast microscopy and image analysis to follow individual spores of non-proteolytic *Clostridium botulinum* strain Eklund 17B from dormancy, through germination and emergence, to cell division. The results obtained were used to predict the time to toxin in a test food to illustrate the use of lag time distributions in risk assessment.

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ANTIBODIES TO *CLOSTRIDIUM BOTULINUM* IN BLOOD IN RELATION TO THE DETECTION OF *C. BOTULINUM* IN FAECES OF COWS

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Habitats of *Clostridium (C.) botulinum* are found in the environment but also in the gastrointestinal tract of animals and man. Specific antibodies are the main humoral defence factors of the adaptive host immune response. They are also interesting for the indirect diagnoses of diseases and for epidemiological investigations. The antibody classes IgG and IgM against *C. botulinum* type A, B, C and D were detected in blood samples from dairy cows with newly developed indirect enzyme-immunoassays. The main results are: (i) antibodies against *C. botulinum* type A to D were detectable in blood samples of dairy cows without specific vaccination or botulism history, and (ii) a negative relation between the measured values of antibodies and the positive detection of *C. botulinum* type A and D in faeces of the same animals by using a method of toxin enrichment and toxin detection by ELISAs. With these methods *C. botulinum* was detectable in several samples. Furthermore, some positive results were confirmed by immunocytochemistry (immunoperoxidase method) and PCR.
We conclude that naturally occurring or induced antibodies to *C. botulinum* are detectable in blood of dairy cows. They may have a protective potential to *C. botulinum* in the gastrointestinal tract. The results demonstrate the infection or contamination of cows with *C. botulinum*.

NOTES

COLD SHOCK INCREASES THE EXPRESSION OF *cspA* AND *cspB* BUT NOT *cspC* IN *CLOSTRIDIUM BOTULINUM* ATCC3502

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Changes in growth temperature cause multiple alterations in the physiology and metabolism of bacteria and there is a wide variation between the gene expression profiles of different bacteria at low temperatures. However, the meaning of different genes and regulation systems for bacterial cold tolerance is not yet fully understood. Genome-wide transcriptional analysis with DNA microarrays (Institute of Food Research, Norwich, UK) was employed to study the effect of low temperature on gene expression of group I *Clostridium botulinum* type A strain ATCC 3502 and to screen for interesting genes for further research. Based on statistical analysis, approximately 600 genes were either up- or down-regulated (FDR<0,01) 30 minutes after cold shock from 37°C to 15°C. After incubation at 15 °C for 2 and 5 hours changes were seen in the expression of approximately 1000 and 1300 genes, respectively. The first genes selected to be studied more closely included cold shock protein (Csp) coding genes. Csp-coding genes are known to be important for cold tolerance in most organisms having a role in both transcriptional and translational regulation. The genome of *C. botulinum* ATCC 3502 contains three Csp-coding genes, *cspA, cspB* and *cspC*. Real time quantitative RT-PCR studies confirmed the microarray results showing that the expression of *cspA* and *cspB* was significantly increased after cold shock. However, the expression of *cspC* was not influenced by temperature change but remained constant troughout the study. Therefore it seems obvious that *cspA* and *cspB* have an important role in the cold tolerance of *C. botulinum* ATCC3502 whereas *cspC* may be related to some other function. An ongoing deletion mutant analysis will give more information about the importance of Csp-coding genes.

POSTER PRESENTATIONS

In alphabetical order

NATIONAL SPECIALTIST AND REFERENCE SERVICES FOR HUMAN BOTULISM: THE UK EXPERIENCE

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The first cases of human botulism in the UK were detected in 1922, and although rare, this disease still has the potential to be a serious public health emergency. The epidemiology of human botulism has changed in the UK over the past 10 years resulting in an increase in incidence. Two unrelated cases of infant botulism occurred in 2007 and an unprecedented six incidents of food-borne botulism occurred since 1998. However, the most common presentation in the UK is wound botulism which was not detected before 2000. Over 120 cases of wound botulism have now been detected which were exclusive to illegal injecting drug users. The HPA Food Safety Microbiology Laboratory (FSML) is the UK national reference laboratory for *Clostridum botulinum* offering specialist and reference facilities to confirm clinical diagnoses of human botulism and to assist in the public health response to this disease. FSML provides services for the detection of botulinum neurotoxin and for the detection, isolation and identification of *C. botulinum*. The laboratory receives less than 250 samples per year including serum, faeces, wound tissue, other body fluids, food, environmental samples, illegal drugs and cultures,. A combination of mouse bioassay, real-time PCR assay and conventional microbiological culture provides rapid detection and confirmation of a clinical diagnosis. Services are fully accredited to the ISO standard 17025. Molecular fingerprinting of isolates is also available using DNA amplified fragment polymorphism analysis.
As a response to the changing pattern of human botulism in the UK, FSML has been active in the development of improved laboratory strategies resulting in a robust testing regime with better control of tests and detection of laboratory cross-contamination events. This provides a faster, less expensive and ethically more acceptable approach to conventional laboratory diagnostic approaches. This presentation will outline the UK approach to developing improved specialist and reference services for human botulism.

DEVELOPMENT OF A REVERSE-TRANSCRIPTASE REAL TIME-PCR FOR THE DETECTION OF NEUROTOXIN-PRODUCTION OF *CLOSTRIDIUM BOTULINUM* TYPE A, B, E AND F IN FOODSTUFF AS AN ALTERNATIVE TO THE MOUSE-BIOASSAY

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Clostridium botulinum is an obligatory anaerobic, gram-positive, endospore-forming straight rod, which is a member of the family Clostridiaceae (NCBI Taxonomy, 2006). The ubiquitous occurring elicitor is able to produce seven different types of neurotoxins (BoNT/A-G). The different *C. botulinum*-toxins belong to the most effective biological poisons and cause the clinical picture of botulism (Bulander and Eisgruber, 2007).The foodstuff-related botulism is a life-threatening nutritional intoxication (Bulander and Eisgruber, 2007). For the examination of a suspicious food-sample according to DIN 10102 and § 64 LFGB (German federal collection of methods) (L 06.00-26) a quantity of at least 54 mice is needed. Additional examinations of clinical samples from intoxicated humans raise the number of mice which need to be infected up to 216. By means of biochemical tests it is intended to characterize *C. botulinum* strains having different toxin-producing abilities. Based on these results a Reverse-Transcriptase Real Time-PCR shall be established and evaluated, in order to detect the *C. botulinum* neurotoxin production out of various samples.
The aim of this project is to implement an alternative method by means of a Reverse-Transcriptase Real time-PCR to the mouse-bioassay which is still considered as the “golden standard”.

THE FIRST FINDINGS ON THE EFFECT OF SEVERAL ENVIRONMENTAL FACTORS TO THE OCCURRENCE OF AVIAN BOTULISM OUTBREAKS IN A HUNGARIAN WETLAND HABITAT

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From 1959, botulism outbreaks among wild waterfowl, caused by the Gram-positive, obligate anaerobe spore-forming bacterium *Clostridium botulinum* type C were reported regularly from many wetland sites in Hungary. Some of these habitats, as Lake 'Kis-Balaton', are nature reserves, giving shelter to hundreds or thousands of birds.
High air- and water temperatures and shallow, stagnant, saline water with low dissolved oxygen were believed favourable for outbreaks. In consonance with these findings, numerous studies reported from outbreak sites with similar parameters. However, many wetlands with these characteristics are not known as sites of botulism, and outbreaks are often occur on deep, well-oxygenated lakes or in late winter or early spring. Water temperature, pH, salinity, redox potential and invertebrate biomass could influence the occurrence of outbreaks, but some factors were not consistently different between outbreak and non-outbreak sites.
In our study, regularly collected water-quality parameters (temperature, pH, conductivity, water-soluble oxygen, organic matter content (COD)) and weather data (daily air temperature and daily rainfall values) were compared between high (HR) and low (LR) avian botulism risk areas of the Lake 'Kis-Balaton', in HR and LR periods of time (5-5 years between June and September, with or without documented avian losses, respectively). Statistical analyses (one-way ANOVAs and paired-samples t-test) showed significant differences between HR and LR years in water temperature, pH, WSO and COD values in 3 of 5 (60%) HR and 3 of 5 (60%) LR areas. Significant difference was also observed between the air temperature values of HR and LR years, but the length of dry periods (without rainfall) were almost equal, and the number of dry days was higher in LR than in HR years. Although positive relationship was found between the incidence of avian botulism cases and some environmental conditions, the exact role of other examined factors in the facilitation of the outbreaks remained unclear.

AVIAN BOTULISM IN ITALY: APPLICATION OF A DUPLEX PCR ASSAY AS A USEFUL TOOL FOR THE ISOLATION OF NEUROTOXIGENIC STRAINS

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Avian botulism is a paralytic and often fatal disease of wild and farming birds occurring worldwide and caused by *Clostridium botulinum* type C and D. The disease is often diagnosed only on clinical and epidemiological features because of the difficulty of laboratory confirmation. Particularly, the need to isolate the toxigenic strains in order to produce an auto-vaccine, is a hard step depending on the frequent loose of the gene encoding the neurotoxin. Avian botulism has been documented also in Italy but, due to the lack of a specific surveillance system on animal botulism, the epidemiological and clinical data are not already systematically collected.

We reported a recent experience on repeated outbreaks of avian botulism occurred in the North–East of Italy. A duplex PCR for *bont/*C and *bont/*D genes, together with Internal Amplification Control (IAC), was optimised and successfully utilized for the laboratory confirmation of the outbreak and to facilitate the isolation of the neurotoxigenic strain used to produce the autovaccine. The IAC was included in the PCR protocol to avoid false negative results due to the lack of the reaction caused by inhibitory substances.

It is also discussed a project to organize in Italy a surveillance system, in collaboration with the Italian Ministry of Health, the National Reference Center for Botulism at the ISS, state veterinary diagnostic laboratories (IZS) and other stakeholders to collect all epidemiological, clinical and microbiological data connected with animal botulism outbreaks and particularly with avian botulism.

DRIED ROACH (“VOBLA”) AS A POSSIBLE SOURCE OF *CLOSTRIDIUM BOTULINUM* SPORES AND RECENT RECALLS RELATED TO THIS PRODUCT

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Vobla is a Russian word for Caspian roach or *Rutilus rutilus caspicus* which has been salt-dried for several weeks. Vobla originated in Russia, and is a common Russian "snack" that goes well with beer or vodka. It is generally eaten without sauces or side dishes. Vobla could be considered as raw fish, but it is neither raw nor cooked, but rather salt-cured. It is soaked in brine for two weeks and then is thoroughly air-dried for another two. Uneviscerated fish has been linked previously to outbreaks of botulism poisoning related to salted fish products.
Clostridium botulinum spores are more likely to be concentrated in the viscera than in any other parts of fish in such a product, because viscera could provide protective environment with low salt concentration for bacteria.
U.S. Food and Drug administration in December, 2007 recalled dried roach- vobla produced in Latvia because the possible contamination with *Clostridium botulinum* spores. The sale of this product was prohibited under New York State Department of Agriculture and Markets regulations, moreover Latvian food control authorities carrying out complete investigation and further information were asked from the US Food and Drug administration.

THE ROLE OF FLAGELLA IN CLOSTRIDIUM BOTULINUM HALL A

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Clostridium botulinum produces an extremely potent neurotoxin (CNT) which, in addition to being important from a virulence standpoint, has multiple pharmaceutical applications. *C. botulinum* CNTs inhibit the release of neurotransmitters at the myoneural junctions of nerve endings in the peripheral nervous system, resulting in severe flaccid paralysis from relatively small doses. The paralysis caused by the neurotoxin is the organism's predominant method of virulence and as such the neurotoxin has been the main focus of research. Although much is known about the action of the neurotoxin, comparatively little is known about the organism itself. This is predominantly a result of the relative lack of effective molecular tools in *Clostridium*. One area which has not been thoroughly studied yet in clostridia is the flagella complex. As well as having a major fundamental role in motility, bacterial flagella complexes are intimately linked to virulence. A recently developed method of disabling genes within clostridial species by targeted insertion with a group II intron has been utilised to inactivate *sigD* in *Clostridium sporogenes*. This gene encodes a sigma factor responsible for expression of late flagellar genes. This inactivation results in a non-motile phenotype. The same insertional mutagenesis procedure has been used to inactivate several genes in *C. botulinum* encoding flagella components. The phenotypic effects brought about by their inactivation in *C. botulinum* have been analysed and the role of the flagella in different cellular functions has been assessed.

This work has been supported by Ipsen BioPharm Ltd.

CLOSTRIDIUM BOTULINUM D TOXIN SANDWICH ELISA

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The incidence of botulism in cattle due to *Clostridium botulinum* toxin has increased significantly in Northern Ireland in recent years, with only 1 confirmed positive case detected in 1999 rising to 35 confirmed positive cases detected in 2003. Confirmation of clinical diagnosis has been dependent on the "Gold Standard" mouse lethality test and mainly type D toxin has been detected. The present study was undertaken to raise monoclonal antibodies (Mabs) to this toxin in order to investigate their potential application in a sandwich ELISA (sELISA) to replace the mouse lethality test. Mabs against *Clostridium botulinum* type D toxin were produced by fusing spleen cells from BALB/c mice immunised with type D toxoid, with mouse myeloma line SP2/0-Ag14. The hybridomas F319 7A10 and 5B5 were chosen for cloning on the basis of high antibody titre. The tissue culture fluids from the cloned hybridomas were caprylic acid purified and some of the purified immunoglobulin was biotinylated. Mab 7A10 was finally selected for capture and biotinylated 5B5 for detection, and this sELISA arrangement demonstrated a sensitivity of 1.9 ng per ml with type D toxin complex. The Mabs were found to be D specific when tested against type *C. botulinum* A, B, C, D, E, F and G toxins. Suspect and non-suspect *C. botulinum* bovine diagnostic cases, submitted to the Veterinary Sciences Division Belfast, were tested, both directly and after heat shock and pre-enrichment treatment, using the type D sELISA and the results compared with the mouse lethality test.

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 631 cases from suspect outbreaks have been submitted to VSD for diagnostic investigation. Botulinum neurotoxin has been detected in samples from 98 of these cases using the mouse bioassay. The actual number of cases is likely to be higher. This increase in botulism has also been observed in the Republic of Ireland, GB and some parts of Europe. 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. It has been postulated that this may be due to the reduction of antibiotics in poultry meal allowing the proliferation of *Clostridium botulinum* in carcasses in the litter as a part of the natural carbon re-cycling process. 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 *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. The 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 from this project will be presented.

CHARACTERISATION OF A PUTATIVE AGR SYSTEM IN CLOSTRIDIUM BOTULINUM AND CLOSTRIDIUM SPOROGENES

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One of the most notorious members of the genus *Clostridium* is *C. botulinum*, the causative agent of botulism. Aside from wound botulism in intravenous drug users, the major clinical concerns relate to intestinal toxaemia (common in the USA) and foodborne botulism - the major UK concern. To help prevent botulism, it would be useful to understand the regulatory mechanisms that control changes in growth rate, virulence factor production and spore formation/ germination. Such adaptive responses are reliant on the co-ordinate control of gene expression mediated by cell-to-cell communication systems, i.e., Quorum Sensing (QS).

The genome sequence of the *C. botulinum* Group I strain ATCC 3502 has recently been determined. *In silico* analysis has revealed the presence of two distinct loci capable of encoding proteins with homology to AgrB and AgrD of the *Staphylococcus aureus* agr quorum sensing system. We have begun the functional characterisation of these genes in order to determine whether they play a role in quorum sensing. The equivalent regions were shown to be present in *C. sporogenes*, and to be highly conserved. Regions of conservation are also apparent with similar loci in other clostridia and, to a lesser extent, in staphylococci.

Modulation of the expression of the identified agr genes is a prerequisite to determining their function. We have used antisense RNA expression to down regulate the *agrB* genes in *C. sporogenes* and have demonstrated that this inhibits sporulation. Two *agrD* mutants have also been generated in *C. botulinum* using our newly-developed ClosTron system, and these mutants also show reduced sporulation efficiency.

HUMAN BOTULISM IN ITALY, 1984-2007

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Botulism is a neuroparalytic illness caused by botulinum neurotoxins produced by *Clostridium botulinum*, and rarely by neurotoxigenic strains of *Clostridium baratii*, and *Clostridium butyricum*. To date 5 forms of natural human botulism are recognized: two toxic forms (foodborne botulism and iatrogenic botulism) and three infective forms (wound botulism, and intestinal toxemia botulism in infant and in adult). Recently also inhalational botulism, caused by voluntary and/or accidental release of toxin was classified.
In Italy botulism has been included since 1975 among the statutory notifiable diseases, and since 1990 to “immediate reporting”. All natural forms of botulism have been diagnosed in Italy.
The NRCB at the Istituto Superiore di Sanità performs the laboratory confirmation of suspected cases of botulism and a collection of clinical, microbiological and epidemiological data. An active surveillance on the infective forms of botulism is also conducted from 1984.
We report NRCB data of laboratory confirmed botulism cases from 1984 to 2007.
A total of 283 cases of foodborne botulism, from 177 incidents, were confirmed. In 76 incidents (42.9%) food vehicle was identified, while in 44 (24.9%) it was unknown. Moreover in 57 (32.2%) incidents, food vehicle was highly suspected.
Home canned foods were associate in 76.3 % of incidents in which food vehicle was identified. 71.1 % of them were canned vegetables. Type B toxin accounted for 77.4% of foodborne incidents.
The 31 cases of intestinal toxemia botulism (28 infant and 3 adult), were recognized in Italy with the highest number of cases in Europe. *C.botulinum* type B accounted for 61.3% of cases, and *C. butyricum* type E accounted for 6 cases (19.4%). Four cases of wound botulism were also reported, all caused by *C.botulinum* type B.

CHARACTERISATION OF THE NEURONAL UPTAKE OF BONT/C1 AND D

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Among the seven serotypes of botulinum neurotoxin (BoNT/A-G) only BoNT/A, B, E and F are pathogenic in humans. BoNT/C1 and D cause botulism in animals, BoNT/C1 preferentially in birds and BoNT/D in cattle. Although the latter types were considered untoxic in humans if taken up by the gastro-intestinal route they cause botulism if taken up parenterally. The differences in toxicity in animals and humans may be due to lack of transcytosis through the mucosal membrane of the gut. Due to the parenteral human pathogenicity of BoNT/C1 and D we want to characterize their neuronal uptake mechanism compared to BoNT/A and B, identify receptor binding sites in BoNT/C1 and D and characterise the interaction of receptors with their respective binding pocket.
The dual receptor model describing the primary binding to complex gangliosides and subsequent interaction with a protein receptor has been proven for BoNT/A, B and G. An analogous binding mechanism of BoNT/C1 and D to the receptors is determined employing synaptosomes derived from fowl and mice. Productive binding, i.e. binding that leads to inhibition of exocytosis is studied in isolated nerve-muscle preparations derived from mice. The ganglioside dependent binding of BoNT/C1 and D is demonstrated using nerve-muscle preparations of ganglioside-deficient mice. The putative binding pockets in BoNT/C1 and D molecules are predicted by molecular modelling and are analysed by site directed mutagenesis.
The results provide insights how the neurotoxins are navigated from the blood circulation into the neuronal target cell and may answer the question whether the occurrence of BoNT/C1 and D in water and animals is hazardous for humans.

TYPE C BOVINE BOTULISM OUTBREAK: THE FIRST CASE IN FINLAND

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Nine of 90 cattle on a dairy farm died after being fed non-acidified silage contaminated by animal carcasses. Clinical sings of the affected cattle included mainly ataxia, anorexia and apathy, but also flaccid paralysis of tongues was observed. All the affected cattle died or were euthanized. A necropsy investigation was conducted on two died animals and gastrointestinal, liver, blood and silage samples were analysed for *Clostridium botulinum*. Type C botulinum neurotoxin gene was detected in one heifer intestinal, ruminal, abomasal and liver samples by polymerase chain reaction (PCR) and the neurotoxin was detected by the mouse bioassay. *Clostridium botulinum* type C was isolated from liver samples. The isolated strain was identified with amplified fragment length polymorphism analysis (AFLP) as Group III *Clostridium botulinum*. This is the first type C bovine botulism outbreak diagnosed using PCR combined with subsequent isolation and identification of the disease strains by AFLP. This is also the first confirmed case of bovine botulism in Finland. The use of acidification process is recommended in silage production to inhibit *C. botulinum* spore germination and toxin production in silage and thus to prevent further botulism outbreaks. However, acidification process does not prevent botulism hazard if the toxin is preformed in silage.

THE INITIATION OF ENDOSPORE FORMATION IN CLOSTRIDIUM BOTULINUM

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The ability of pathogenic clostridia to form endospores is considered to be one of the most important virulence factors due to their persistence and resistance in the environment. It is therefore important that the processes that control and regulate sporulation are elucidated. A better understanding of the physiology of *Clostridium botulinum* (the principal target of food processing in the UK) would make it possible to meet the increasing requirement of achieving tighter food safety margins without compromising product safety.

The proteins involved in the initiation of sporulation (Stage 0) are best studied in the model Gram-positive bacterium *Bacillus subtilis*. Sporulation is controlled by the phosphorylation status of the master regulator Spo0A. The intracellular and extracellular signals that induce sporulation are conveyed to this transcription factor by a variety of proteins (sensor histidine kinases, response regulators, and a phosphotransferase) which together constitute a phosphorelay.

The recent completion of several clostridial genome sequencing projects has highlighted that clostridia do not possess a recognisable phosphorelay. They all contain a Spo0A homologue, and *C. tetani* does posses a protein with weak homology to the Spo0B response regulator phosphotransferase. However, none of the clostridia contain a homologue to response regulator Spo0F. Therefore, how is Spo0A phosphorylated in clostridia?

One hypothesis being tested is that Spo0A is directly phosphorylated by sensor histidine kinases which do not possess any cognate response regulator, so called ‘orphan’ sensor histidine kinases. Analysis of the genome sequence of *C. botulinum* highlighted the presence of five orphan sensor histidine kinases. Work is presented here on the inactivation and subsequent characterisation of these orphan sensor histidine kinases utilising the clostridial universal knockout technology recently developed at the University of Nottingham (ClosTron technology).

SPOILAGE OF VACUUM-PACKED RAW MEAT DUE TO *Clostridium estertheticum*

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Clostridium species were not associated with meat spoilage (except bone-taint), until 1989 when *Clostridium estertheticum* (Cle) was first described as the source of blown pack. The spoilage of vacuum-packed chilled-stored meat is mainly caused by two closely-related clostridia (*Cl. estertheticum* subsp *estertheticum* and *Cl. estertheticum* subsp *laramiense*), although other psychrophilic or psychotolerant clostridia occasionally cause similar spoilage. The problem was first reported in meat from southern Africa and the United States, but has since been reported in New Zealand, Ireland, the United Kingdom, Brazil and Chile. There is no evidence that they produce toxins. However, the smell of the spoiled meat is extremely offensive.

Characteristics of *Cl. estertheticum* spoilage

Pack A grossly distended pack is an obvious sign of spoilage, but sometimes infected packs just become loose without being blown. On opening both grossly-distended and loose packs have offensive odours, described as 'vomit-like', 'sulphurous' and 'hydrogen sulphide-like'. The meat retains its colour.

Gas and volatile compounds The ratio of gases is variable. The major gases are hydrogen, carbon dioxide, and nitrogen. Cle produces esters, sulphur-containing products, butanol and butanoic acid.

Microbiological profile Numbers and types present (except for Cle) often do not differ from those in unspoiled packs of the same age (predominantly lactic acid bacteria, with lower numbers of *Brochothrix thermosphacta* and Enterobacteriaceae, and sometimes yeasts and pseudomonads). However, on one occasion Cle was the only microbe detected.

Detection and isolation Cle is difficult to detect using cultural methods as it is psychrophilic, with a maximum temperature for growth of about 14^{o}C. A PCR-RFLP and a real time-PCR method have been developed in our laboratory, which can be used for testing meat and environmental samples. There is currently no selective isolation medium, although most strains are haemolytic, so heat or ethanol treatment to inactivate vegetative competitive flora, followed by plating on blood agar is sometimes successful. We are attempting to devise a selective culture medium, which will require incubation for about 9 days at 12^{o}C.

COMPARATIVE GENOMICS BETWEEN GROUP I (PROTEOLYTIC) AND GROUP II (NON-PROTEOLYTIC) *C. BOTULINUM* STRAINS

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The previously sequenced and published genome of Group I (proteolytic) *C. botulinum* strain Hall A has revealed interesting information about the lifestyle of this organism. The genome of a second *C. botulinum* strain, Eklund 17B, has been recently sequenced at the Sanger Institute using a combination of capillary and 454 sequencing technologies. This strain was selected for sequencing because it belongs to Group II (non-proteolytic) *C. botulinum* and produces type B neurotoxin, both characteristics are most frequently associated with foodborne botulism in Europe. In addition Eklund 17B is the most extensively studied and characterised strain of Group II *C. botulinum*.
The genome is now in two contigs and it is anticipated that the complete sequence will be available in the near future. Analysis of the Eklund 17B genome and comparative analysis with the genome of HallA strain will be presented.
Comparative genomics of two *C. botulinum* genomes should reveal important data and will undoubtedly lead to an improved understanding of the physiology of *C. botulinum*.

AN ASSAY FOR THE BOTULINUM TOXINS THAT REQUIRES FUNCTIONAL BINDING AND CATALYTIC DOMAINS WITHIN THE NEUROTOXIN

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Increasingly, botulinum neurotoxin serotypes are being further subdivided into subtypes which are not necessarily detected by antibodies raised against another subtype. This reduces the applicability of detection assays which are based on antibody capture.
A novel capture method has been employed to retrieve active toxin from contaminated media which uses synaptosomes. After capture, the synaptosome-bound toxin can be incubated in the presence of its substrate (Vesicle Associated Membrane Protein (VAMP) in the case of serotypes B and F, Synaptosomal Associated Protein of 25kDa (SNAP-25) for serotypes A and E). The cleaved substrate can then be detected using serotype-specific antibodies raised against the unique cleaved product of each toxin serotype.
Synaptosomes have a number of advantages over antibodies as a capture phase. All BoNT serotypes and subtypes are captured and very small samples volumes may be used for the detection of biologically active toxin in complex media such as serum.

INDEX

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ATTENDEES

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