EUROPEAN COMMISSION

European Concerted Action QLK2-CT2001-01267

Genus *Clostridium*

Clostridia in medical, veterinary and food microbiology

Diagnosis and typing

Editor-in-chief
J. Mainil

Editorial board
C. Duchesnes, P.E. Granum, M.G. Menozzi, M. Peck,
S. Pelkonen, M. Popoff, E. Stackebrandt and R. Titball

2006 Directorate-General for Research
Key Action 2 – Control of infectious diseases EUR 21463 EN

Concerted Action QLK2-CT2001-01267

This book was produced as part of an EU Concerted Action project: « Pathology and Ecology of the Genus *Clostridium* in Humans, Animals and Foodstuffs: Identification, Epidemiology and Prophylaxis (Genus *Clostridium*)". This project is funded under the EU Quality of Life programme.

Proceedings and scientific booklets have been published after each of the following meetings:
1. Classification, molecular genetics and pathology of clostridia.
2. Protein toxins of the genus *Clostridium* and vaccination.
3. Diagnosis, epidemiology and antibiotic resistance of the genus *Clostridium*
4. Food microbiology and sporulation of the genus *Clostridium*

All the booklets are available on the project website (http://www.genusclostridium.net).

.

Cataloguing data can be found at the end of this publication.

Luxembourg: Office for Official Publications of the European Communities, 2006

ISBN. 92-79-00422.EPS

Printed in Luxembourg

PRINTED ON WHITE CHLORINE-FREE PAPER

List of contributors

Viveca BAVERUD
National Veterinary Institute – Bacteriology department – 751 89 Uppsala – Sweden
Viveca.baverud@sva.se; Phone : +46-18-67-40-00 ; Fax : +46-18-67-40-93

Eleftheria BOURTZI-HATZOPOULOU
Aristotle University of Thessaloniki – Faculty of Veterinary Medicine – Microbiology and infectious diseases – University Campus – 54006 Thessaloniki – Greece
bourtzi@vet.auth.gr; Phone: +30-2310-99-99-15 ; Fax: +30-2310-99-99-61

Jean-Philippe CARLIER
Institut Pasteur Paris - Centre National de Référence des Anaérobies - Toxines et Pathogénie Bactérienne - Rue du Dr Roux, 28 - 75724 Paris cedex 15 - France
jcarlier@pasteur.fr; Phone : +33-1-45-68-83-10 ; Fax : +33-1-40-61-31-23

Michel DELMEE
Université catholique de Louvain – Microbiology Unit – Avenue Hippocrate 54.90 – 1200 Bruxelles – Belgium
Delmee@mblg.ucl.ac.be; Phone: +32-2-764-94-41; Fax: +32-2-764-94-40

Piet DEPREZ
University of Ghent – Faculty of Veterinary Medicine – Internal Medicine Large Animals – Salisburylaan, 133 – 9820 Merelbeke – Belgium
Piet.deprez@ugent.ac.be; Phone: +32-9-264-75-80; Fax: +32-9-264-77-96

Christiane DUCHESNES
Université de Liège - Faculté de Médecine Vétérinaire - Bactériologie - Bât B43a - Sart Tilman - 4000 Liège - Belgium
cduchesnes@ulg.ac.be; Phone: +32-4-366-40-52 ; Fax : +32-4-366-42-61

Björn ENGSTROM
National Veterinary Institute – Poultry department – 751 89 Uppsala – Sweden
Bjorn.engstrom@sva.se; Phone : +46-18-67-40-00 ; Fax : +46-18-67-40-94

Per Einar GRANUM
The Norwegian Reference Laboratory - The Norwegian School of Veterinary Science - Food Hygiene - PO Box 8146 Dep - Ullevalsveien, 72 - 0033 Oslo - Norway
per.e.granum@veths.no; Phone: +47-22-59-70-52; Fax: +47-22-96-48-50

Magne KALDHUSDAL
National Veterinary Institute – Pathology department - PO Box 8156 Dep – Ullevalsveien, 68 - 0033 Oslo - Norway
per.e.granum@veths.no; Phone: +47-23-21-64-38; Fax: +47-23-21-63-03

Jacques MAINIL
Université de Liège - Faculté de Médecine Vétérinaire - Bactériologie - Bât B43a - Sart Tilman - 4000 Liège - Belgium
jg.mainil@ulg.ac.be; Phone: +32-4-366-40-50 ; Fax : +32-4-366-42-61

Didier MARLIER
Université de Liège – Faculté de Médecine Vétérinaire – Pathologie aviaire – Bât B42 – Sart Tilman – 4000 Liège – Belgium
dmarlier@ulg.ac.be; Phone: +32-4-366-40-11 ; Fax : +32-4-366-40-12

Maria Grazia MENOZZI
University of Parma - Microbiology - Pathology and Laboratory Medicine – Via Gramsci, 14 – 43100 Parma - Italy
mariagrazia.menozzi@unipr.it; Phone: +39-0521-98-88-85; Fax: +39-0521-99-36-20

Anastasios MINAS
Technological institute of Larisa – Microbiology – 51 223 – Greece
mhnas@ram.gr; Phone: +30-2410-611-069 ; Fax: +30-2410-611-070

Michael PECK
Institute of Food Research - Food Safety Science Division - Norwich Research Park - Colney - NR4 7UA Norwich - England - United Kingdom
mike.peck@bbsrc.ac.uk; Phone: +44-1603-25-52-51; Fax: +44-1603-50-77-23

Karl PEDERSEN
Danish Institute for Food and Veterinary Research – Department of Poultry, Fish and Fur Animals – Hangovej, 2 – 8200 Aarhus – Denmark
kpe@dfvf.dk; Phone: +45-72-34-68-93; Fax: +45-72-34-69-01

Sinikka PELKONEN
National Veterinary and Food Research Institute - Kuopio Regional Laboratory – PO Box 92 Neulaniementie, 4 - 70701 Kuopio - Finland
sinikka.pelkonen@eela.fi; Phone: +358-17-20-14-50; Fax: +358-17-20-14-58

Evanthia PETRIDOU
Aristotle University of Thessaloniki – Faculty of Veterinary Medicine – Microbiology and infectious diseases – University Campus – 54006 Thessaloniki – Greece
epetri@vet.auth.gr; Phone: +30-2310-99-98-54 ; Fax: +30-2310-99-99-61

Michel POPOFF
Institut Pasteur Paris - Centre National de Référence des Anaérobies - Toxines et Pathogénie Bactérienne - Rue du Dr Roux, 28 - 75724 Paris cedex 15 - France
mpopoff@pasteur.fr; Phone : +33-1-45-68-83-10 ; Fax : +33-1-40-61-31-23

Christine RHODE
DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - Molecular Systematics and Ecology - Mascheroder Weg, 1b - 38124 Braunschweig - Germany
chr@dsmz.de; Phone: +49-531-261-62-20; Fax: +49-531-261-64-18

Glenn SONGER
University of Arizona – Veterinary Science and Microbiology – 1117 east Lowell Street – 85721 Tucson – Arizona – USA
gsonger@u.arizona.edu; Phone: +1-520-621-2962; Fax: +1-520-621-6366

Erko STACKEBRANDT
DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - Molecular Systematics and Ecology - Mascheroder Weg, 1b - 38124 Braunschweig - Germany
erko@dsmz.de; Phone: +49-531-261-63-52; Fax: +49-531-261-64-18

David TAYLOR
University of Glasgow – Veterinary School – Bearsden Road – Bearsden G61 1QH – Glasgow – United Kingdom
d.taylor@vet.gla.ac.uk; Phone: +44-141-330-5396; Fax: +44-141-330-5602

Richard TITBALL
Defence Science and Technology Laboratory - CBD Porton Down – Porton Down - Salisbury - Wiltshire - SP4 0JQ - England - United Kingdom
rtitball@dstl.gov.uk; Phone: +44-1980-61-33-01; Fax: +44-1980-61-43-07

Preface

The purpose of the Concerted Action of the 5th Framework programme QLK2-CT2001-01267 'Pathology and Ecology of the Genus *Clostridium* in Humans, Animals, and Foodstuffs. Identification, Epidemiology and Prophylaxis' was to exchange across Europe scientific knowledge and technical know-how about the genus *Clostridium*. This Concerted Action brought together leading laboratories in the field with collaboration between human, veterinary and food microbiology laboratories.

Four workshops were initially planned with different themes. The first, entitled "Molecular genetics, classification, pathology and ecology of the genus *Clostridium*" was organised in Liège (Belgium) in January 2002; the second, entitled "Protein toxins of the genus *Clostridium* and vaccination", in Salisbury (England, UK) in November 2002; the third, entitled "Diagnosis, epidemiology and antibiotic resistance of the genus *Clostridium*" in Parma (Italy) in October 2003 and the fourth and final workshop, entitled "Food microbiology and sporulation of the genus *Clostridium*" in Oslo (Norway) in June 2004. In addition to the proceedings, a scientific booklet relating to the general theme of the workshop was edited after each meeting. These booklets are available in hard copy and on the website of the Concerted Action : **http://www.genusclostridium.net**.

These workshops, proceedings and scientific booklets have assisted and continue to assist in the exchange of scientific knowledge regarding clostridia, not only across Europe but also, it is hoped, on a worldwide scale.

Exchange of technical know-how will be achieved through the publication of this book 'Clostridia in Medical, Veterinary and Food Microbiology : Diagnosis and Typing'. The purpose of the book is to propose as much an overview as a summary of technical approaches in the following : clinical and necropsy diagnosis of clostridial diseases, collection of appropriate specimens for routine diagnostic laboratories, classical and molecular identification and toxintyping of clostridia, confirmation of problems of food spoilage by different clostridial species and specific food poisonings caused by *C. botulinum* and *C. perfringens* in humans. This book should be of considerable relevance to technical, medical and scientific staff members of laboratories working in the fields of clostridial diseases of animals and humans and of clostridial food microbiology.

Dr Isabel Mínguez Tudela
Directorate-General for Research
Biotechnology, Agriculture and Food Research
European Commission

Introduction

Molecular tools have induced new insight into the classification of the bacteria and their complexity is well exemplified by the taxonomy of the genus *Clostridium*. Since the description of *Clostridium butyricum*, the type species, in 1880 by Prazmowski[1], many bacterial species have been included in the genus *Clostridium*, while others have been reclassified in other genera. The genus *Clostridium* comprises nowadays up to 200 species of Gram-Positive spore-forming anaerobic rods, which are distributed worldwide. Fortunately most of the species are harmless non-pathogenic bacteria living in the environment, on plants, on the skin and on the mucosa of animals and humans (especially in the intestinal tract). Some of these species contaminate and grow on foods of vegetable and animal origins, causing food spoilage.

For several reasons, including their specific growth requirements, the diagnosis of infections and food contamination caused by clostridia still presents much difficulty at the clinical, bacteriological and molecular levels. Between 40 and 50 species are associated with clinical conditions in domestic animals and in humans. About 30 of them are considered to be minor pathogens. Fifteen species are major pathogens. Two of the major pathogens are also responsible for specific food poisoning in humans. The major pathogenic species produce several toxins that are responsible for the development of lesions and clinical signs. Those toxins are amongst the most potent in the microbial world and are still a basis for identification of several major pathogenic species.

The main purpose of this book is to present an overview of the parameters of the diagnosis of *Clostridium* in animals, humans and foodstuffs. In each chapter, an extended bibliography is given, to allow the reader to find additional and more detailed information on specific items.

Part I summarises necessary data for the clinical and necropsy diagnosis of the infections caused by clostridia.

Part II focuses on the growth conditions of the clostridial major pathogenic species, on their morphological and genetic identification and on the identification by various means of their virulence factors (toxintyping).

Part III presents information, scientific and legal, dealing with the specific problems of food microbiology: sampling, growth conditions, identification and typing procedures.

Finally an appendix gives additional relevant information concerning reference laboratories.

[1] Prazmowski A. (1880) Untersuchung uber die Entwickelungsgeschichte und Fermentwirking einiger Bacterien-Arten. Inaug. Hugo Voigt, Leipzig.

Table of contents

PART 1:
Clinical conditions, sampling and transport

1. Tetanus and botulism in humans

M.W. Peck and C. Duchesnes

1.1. Introduction

Tetanus and botulism are two severe neurological diseases. Tetanus is characterised by a spastic paralysis, while botulism causes a flaccid paralysis. Both diseases are due to neurotoxins produced by clostridia. In tetanus, *Clostridium tetani* is responsible for formation of the toxin. Seven different types of botulinum toxins (A through to G) are produced by six different clostridia (*C. botulinum* Group I, C. *botulinum* Group II, C. *botulinum* Group III, *C. argentinense, C. baratii* and *C. butyricum).*

1.2. Tetanus

1.2.1. Aetiology and epidemiology

Spores of *C. tetani* are found worldwide in the uppermost layers of the soil and in the digestive system of humans and of various animals. They are more frequently found in highly manipulated soils and are therefore more widespread in densely populated cities and in cultivated regions. The bacterium grows best at 37°C and is therefore rarely found in polar regions or at altitude. The spore is very resistant to antiseptics and heating. Killing of spores requires boiling for at least 4 hours or autoclaving for 12 minutes at 121°C (Bartlett, 2000b).

Tetanus in humans is divided into localised, generalised and neonatal forms. It is realistic to estimate that about 10^6 cases of tetanus occur per year worldwide, although most are not officially reported (Bleck, 2000a). Tetanus is of a great concern for to the WHO, and the WHO is developing a programme of vaccination, targeting the regions of Africa and Asia where neonatal tetanus is still common. The WHO states that the number of cases of neonatal tetanus was 500,000 in 2002 (http://www.who.int/vaccines-surveillance/ and http://www.who.int/vaccines/). Tetanus occurs mainly in the developing countries because of poor sanitary conditions at birth, and a lack of maternal immunisation (Vandelaer *et al*., 2003).

In developed countries, the number of tetanus cases is significantly lower because of widespread vaccine protection. The most susceptible persons are the elderly (Hyslop and Leach, 1998; Bleck, 2000a), as a result of a decrease in immunity with age. Injection intravenous drug users have recently become an important risk group because of a lack of asepsis and contamination of the drugs.

1.2.2. Pathogenesis

C. tetani is a non-invasive bacterium. Therefore, spores or bacteria must penetrate into the human (or other animal) through a disruption of the body's natural barrier. Besides obvious wounds, cases have been reported after puncture, laceration,

surgery, burn, medial otitis, dental infections, pregnancy and abortion. It should be noted that 20% of the cases occur without any recognised portal of entry. Neonatal tetanus is mostly due to poor sanitary conditions at birth and to the traditional septic practice of umbilical stump sealing with earth, principally in Africa.

In the tissues, spores germinate in tissues and, in anaerobic conditions, the bacterium will produce the neurotoxin (TeNT). Anaerobic conditions develop in deep wounds, in tissue necrosis and following simultaneous infection with other bacteria.

The toxin binds to specific receptors of the motorneuron at the neuromuscular junction. Tetanus toxin reaches the central nervous system after retrograde transport through the local peripheral motor nerve ends.

Because the amount of toxin produced is low (and the toxin is very highly potent), tetanus does not induce immunisation and, therefore, vaccination is required even after the disease (Bleck, 2000a).

1.2.3. Clinical signs

1.2.3.1. Generalised form

The incubation time between wound and the appearance of symptoms is highly variable (2 to 56 days), but is commonly reported to be less less than 14 days. A shorter incubation time corresponds to a more severe disease (Bleck, 2000a).

The first symptoms are restlessness, irritability, muscular pain, excessive sweating, dysphagia with hydrophobia and drooling and difficulty in opening the mouth. The first clinical signs are the spasms of the jaw muscle, leading to the well-known sign of lockjaw (trismus) and giving the typical aspect of the face (risus sardonicus). This is reported in 50 to 75% of cases (Bartlett, 2000b). Harmful spasms will progress to the muscles of the neck, back and abdomen before reaching the extremities. Opisthotonos results from spasms of the spinal muscles. Flexion and adduction of the arms with clenched fists and extension of the legs with plantar flexion of the toes is a typical attitude. These spastic waves are so powerful that bone fractures have been reported.

The autonomic nervous system is usually involved with an excessive adrenergic activity leading to tachyarrhythmia, fluctuating arterial blood pressure, laryngeal spasm, profound sweating and urinary retention (Hyslop and Leach, 1998). Consciousness, awareness and perception are not affected, but pain is very severe. Complications are due to the direct effect of the toxin: hypoxia, atelectasia, pneumonia due to spasms of the respiratory muscles, laryngeal spasm and fractures due to simultaneous spasms of agonists and antagonists. Secondary lesions, such as bedsores, thrombophlebitis and pulmonary embolism are due to the effects of prolonged confinement to bed, and also stress ulcers due to the intensity of the pain.

1.2.3.2. Localised and cephalic forms

This form is usually observed in partially immunised patients (Hyslop and Leach, 1998). Various levels of severity are recorded. Cephalic wound or medial otitis are the main portals of entry. In these particular cases, because the local concentration of toxin is high, a significant amount of it remains bound to the nerve

terminals at the neuromuscular junction in the infected site. This interferes with acetylcholine release by means indistinguishable from botulinal toxins, and first leads to paralysis of the muscles closest to the site, before spasticity occurs (Hyslop and Leach, 1998). A wound at a limb will usually first induce weakness, followed by localised spasms. There is considerable variation in severity in these localised forms but usually the prognosis for survival is excellent (Bartlett, 2000b).

1.2.3.3. Neonatal tetanus

This form is separated from the others mainly for epidemiological purposes, although the symptoms are the same as those of the generalised form. However, it affects newborns (in Africa) within the first ten days of life and is due to the contamination of the umbilical stump. It is fatal in more than 90% of the cases because of a lack of intensive care facilities. Neonatal tetanus is the most common form of tetanus across the globe.

1.2.4. Diagnosis

Diagnosis is usually made based on clinical observations. Organisms are rarely recovered from material removed from wounds (Bartlett, 2000b). According to Bleck (2000a), attempts to culture *C. tetani* from wounds are not useful in diagnosis since a)- cultures are frequently negative; b-) the organism might not contain the toxin-producing plasmid; c)- a positive culture may be present without disease in immunised patients.

In many countries, a single national laboratory is responsible for investigating cases of tetanus and for isolating and characterising implicated clostridia. Toxin detection, isolation and identification of the organisms require specialised techniques. Detection of the tetanus neurotoxin relies upon the use of a mouse bioassay. Identification of *C. tetani* organisms requires detection of the production of neurotoxins in culture supernatant, using the mouse bioassay.

This is a field in which new methods continue to be developed. For example, *in vitro* methods that detect biologically active toxin are being developed. Organisms which contain toxin gene fragments can be detected by PCR. For example, in response to an increase in cases of tetanus associated with heroin abuse in the UK in 2004, the Health Protection Agency has developed a more rapid laboratory test to examine samples of heroin for *C. tetani*. In trials so far, the new test has enabled detection of *C. tetani* in heroin samples within hours after enrichment culture. This is substantially quicker than with conventional laboratory methods (Akbulut *et al.*, 2004a).

1.3. Botulism

1.3.1. Aetiology and epidemiology

Botulinum toxin is produced by *C. botulinum* Group I (proteolytic *C. botulinum*), C. *botulinum* Group II (non-proteolytic *C. botulinum*), C. *botulinum* Group III and *C. argentinense,* and also by neurotoxigenic strains of *C. baratii* and

C. butyricum (Lund and Peck, 2000). *C. botulinum* is the term used for a complex of four species that are physiologically and phylogenetically distinct from each other, and they should be considered distinct species. Individual strains are capable of producing one or two of the seven antigenically distinct toxins types (A through to G). *C. botulinum* Group IV has been reclassified as a new species, *C. argentinense* (Suen *et al.*, 1988). Some strains of *C. baratii* and *C. butyricum* also produce botulinum toxins (Hall *et al*., 1985; Aureli *et al*., 1986). Of the seven toxin types, only four (types A, B, E and, F) produce human disease. As little as 30 ng of botulinum neurotoxin can be fatal (Peck, 2004).

The six species that produce botulinum neurotoxin are found worldwide in fresh water, salt water, soil and in lake and marine sediments. They are also found in the digestive system of animals and can therefore contaminate meat products in the slaughterhouse.

Botulism is very rare in Europe, although some EU countries have up to 100 cases of foodborne botulism each year (Peck, 2004). In the USA, since 1973, the median number of yearly reported cases is 71 for infant botulism, 24 for foodborne botulism and 3 for wound botulism. The case fatality rate has decreased progressively, with the development of intensive care facilities, from 60 to approximately 10 %. The progress of the disease is dependent on the age of the patient and promptness of treatment (Bleck, 2000b).

1.3.2. Pathogenesis

Various forms of botulism are distinguished and take account of the route of contamination (whether an infection or intoxication). These forms are: foodborne, wound, infant botulism and others.

Foodborne botulism is the well-known form, due to the ingestion of food containing preformed toxin. Food is contaminated by bacteria (in slaughterhouse) or more likely by spores (dust, unclean water), which can germinate if the conditions are appropriate. The vegetative forms will produce the toxin. Poor heating, inadequate refrigeration, and a neutral pH of the food may all contribute to the production of toxin. Sometimes, food will smell because of proteolysis due to the bacteria. But for non-proteolytic *C. botulinum,* there may be no odour and therefore the consumer may not be aware of the hazard. The toxin is absorbed through the intestinal wall and is carried by the blood stream before reaching peripheral cholinergic synapses, where it blocks acetylcholine release.

Infant botulism occurs in children up to one year of age, where the gut is contaminated by ingested spores. An underdeveloped intestinal flora will allow vegetative growth and formation of toxin. Soil and honey are the most common sources of spores. Honey consumption is therefore discouraged before the age of one year.

Wound botulism is, like tetanus, due to contamination of wounds by spores. As with tetanus, wounds presenting anaerobic conditions allow spore germination and production of toxin. Cases of botulism in intravenous drug users are increasing in many EU countries.

Other reported cases in adults have their origin in gut colonisation by *C. botulinum.* Achlorydia and altered commensal flora (e.g. as a result of antibiotic treatment or chemotherapy) or enteric lesions (surgery, inflammatory diseases) allow settlement of *C. botulinum* and *in situ* production of toxin (Bleck, 2000b).

1.3.3. Clinical signs

The incubation time for botulism is variable (3 hours to 14 days), but is commonly reported to be between 12 and 36 hours for foodborne botulism. A shorter incubation time corresponds to a more severe disease. The severity of the disease also depends on the type of toxin.

The first symptoms include blurred vision, dysphagia with dry mouth, weakness, nausea and apathy. The first clinical signs are associated with the eyes and facial muscles, which are more sensitive to any form of neuromuscular blockage than are skeletal muscles. Subsequent signs are: diplopia, inability to focus, dysphonia, dysarthria, eyelid ptosis, medial rectus paresis and abnormal pupillary reflex. Muscular weakness, and then paralysis, progresses from head to the neck and then to other parts of the body. Paralysis of the respiratory muscles is usually the cause of death (Bartlett, 2000a).

The cholinergic autonomic nervous system is usually involved, leading to dry mouth with decreased production of saliva, ileus or diarrhoea, urinary retention and alteration of heart rate. Specific features of botulism include: the lack of effect on consciousness, awareness and perception, fever is absent, paralysis is bilateral and/or symmetrical and there is no hypotension. Blurred vision is the only sensory deficit (Bleck, 2000b). Complications are mainly due to the failure of the respiratory muscles.

1.3.4. Diagnosis

Laboratory confirmation of a clinical diagnosis of botulism requires the detection of neurotoxin in a clinical specimen (serum, faeces, gastric contents and or food). Organisms may also be isolated from faeces, food and wound tissue. Isolation of *C. botulinum* organisms from a faecal specimen alone, without detection of toxin in a clinical specimen from that patient, does not confirm a clinical diagnosis of botulism. This is because the organisms are spore formers and are widespread in the environment. Moreover, neither does the failure to detect toxin and recover *C. botulinum* exclude the diagnosis.

In infants, however, the recommendation is to test stools for culture and toxin; two negative specimens obtained during the acute phase of disease will generally rule out this diagnosis (Bartlett, 2000a).

As with tetanus, in many countries, a single national laboratory is responsible for detecting botulism neurotoxins and for isolating and characterising implicated clostridia. Toxin detection, isolation and identification of the organisms require specialised techniques.

Detection of botulinum neurotoxins relies upon the use of a mouse bioassay and upon neutralisation of botulism symptoms with specific antisera. Isolation of *C.*

botulinum organisms requires enrichment, sometimes for up to 14 days. The organisms are very diverse, and there is no simple biochemical test(s) that will identify organisms as *C. botulinum*. Identification of *C. botulinum* organisms requires detection of the production of neurotoxins in culture supernatant, using the mouse bioassay. In addition, cases of botulism in humans have been caused by organisms that have been unequivocally identified as *C. baratii* or *C. butyricum*, but which produced botulinum neurotoxin. New and more rapid methods continue to be developed. *In vitro* methods that detect biologically active toxin are being developed (Lund and Peck, 2000). Organisms that contain toxin gene fragments can be detected by PCR (Akbulut, 2004a, b; Lindstrom *et al*., 2001). Further information is given in Chapter 17.

1.4. Bibliography

Akbulut D., Grant K.A. and McLauchlin J. (2004a). Rapid sensitive detection of clostridial toxin genes in clinical and environmental samples from injecting drug users. In: *Food microbiology and sporulation of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Peck M. and Granum P.E., Eds), pp 88-90, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Akbulut D., Grant K.A. and McLauchlin J. (2004b). Application of real-time PCR assay to detect fragments of the *Clostridium botulinum* types A, B and E neurotoxin genes for public health microbiology. In: *Food microbiology and sporulation of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Peck M. and Granum P.E., Eds), p 105, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Aranda E., Rodríguez M.M., Asensio M.A. and Córdoba J.J. (1997). Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe. *Lett. Appl. Microbiol.* **25**, 186-190

Aureli P., Fenicia L., Pasolini B., Gianfranceschi M., McCroskey L.M. and Hatheway C.L. (1986). Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. *J. Infect. Dis.* **154**, 207-211.

Bartlett J.G. (2000a) Botulism. In: *Cecil's Textbook of medicine.* 21st ed. (Goldman L. and Bennett J.C., Eds), pp 1673-1674, WB Saunders Co., Philadelphia, USA.

Bartlett J.G. (2000b) Tetanus. In: *Cecil's Textbook of medicine.* 21st ed. (Goldman L. and Bennett J.C., Eds), pp 1675-1676, WB Saunders Co., Philadelphia, USA.

Bleck T.P. (2000a) Tetanus. In: *Mandell, Douglas and Bennett's principles and practice of infectious diseases.* 5th ed. (Mandell G.L., Bennett J.E. and Dolin R., Eds), pp 2537-2543, Churchill Livingstone, Philadelphia, USA.

Bleck T.P. (2000b) Botulism. In: *Mandell, Douglas and Bennett's principles and practice of infectious diseases.* 5th ed. (Mandell G.L., Bennett J.E. and Dolin R., Eds), pp 2543-2548, Churchill Livingstone, Philadelphia, USA.

Bourée P. (2003) Immunity and immunization in the elderly. *Pathol. Biol*. (Paris), **51**, 581-585.

Centers for Disease Control and Prevention: Botulism in the United States, 1899-1996 (1998) Handbook for epidemiologists, clinicians, and laboratory workers. Centers for Disease Control and Prevention, Atlanta, USA.

FDA (2001) U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Bacteriological Analytical Manual Online, January 2001. http://www.cfsan.fda.gov/~ebam/bam-toc.html

Hall J.D., McCroskey L.M., Pincomb B.J. and Hatheway C.L. (1985) Isolation of an organism resembling *Clostridium baratii* which produces type F botulinal toxin from an infant with botulism. *J. Clin. Microbiol.* **21**, 654-655.

Hatheway C.L. (1998) Botulism. In: *Infectious diseases*. 2nd ed. (Gorbach S.L., Bartlett J.G. and Blacklow N.R, Eds), pp 1462-1463, WB Saunders Co., Philadelphia, USA.

Hielm S., Hyytiä E., Ridell J. and Korkeala H. (1996). Detection of *C. botulinum* in fish and environmental samples using polymerase chain reaction. *Int. J. Food Microbiol*. **31**, 357-365

Hyslop N.E. and Leach T.S. (1998) Tetanus. In: *Infectious diseases.* 2nd ed. (Gorbach S.L., Bartlett J.G. and Blacklow N.R., Eds), pp 1462-1463, WB Saunders Co., Philadelphia, USA.

Lalli G., Bohnert S., Deinhardt K, Verastegui C. and Schiavo G. (2003) The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol.* **11**, 431-437.

Lindström M., Keto R., Markkula A., Nevas M., Hielm S. and Korkeala H. (2001) Multiplex PCR Assay for Detection and Identification of *Clostridium botulinum* Types A, B, E, and F in Food and Faecal Material. *Appl. Environ. Microbiol.* **677**, 5694-5699

Lund B.M. and Peck M.W. (2000) *Clostridium botulinum*. In: *The Microbiological Safety and Quality of Food* (Lund B.M., Baird-Parker A.C. and Gould G.W., Eds), pp 1057-1109, Aspen, Gaithersburg, USA.

Peck M.W. (2004) *Clostridium botulinum* and foodborne botulism. In: *Food microbiology and sporulation of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Peck M. and Granum P.E., Eds), pp 27-37, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Roberts D., Hooper W. and Greenwood M. (1995) Practical Food Microbiology. PHLS, London, UK.

Sebald M. and Petit J.C. (1994) Laboratory methods, anaerobic bacteria and their identification. Institut Pasteur, Paris, France. (In French and English).

Speck M.L. (1976) Compendium of methods for the microbiological examination of foods, American Public Health Association, Washington, D. C.

Suen J.C., Hatheway C.L., Steigerwalt A.G. and Brenner D.J. (1988) *Clostridium argentinense* sp. Nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin group G and some nontoxigenic

strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. *Int. J. Syst. Bacteriol*. **38**, 375-381.

Sutter V.L., Citron D.M., Edelstein M.A.C. and Finegold S.M. (1985) Wadsworth Anaerobic Bacteriology Manual. 4th Edition. Star, Belmont, USA.

Vandelaer J., Birmingham M., Gasse F., Kurian M., Shaw C and, Garnier S. (2003) Tetanus in developing countries: an update on the maternal and neonatal tetanus elimination initiative. *Vaccine* **21**, 3442-3445.

2. Tetanus and botulism in animals

P.R. Deprez

2.1. Introduction

At first glance, tetanus and botulism are completely different diseases, since the main clinical symptoms are opposite, being paralysis in botulism and tetany in tetanus. There are, however, several common aspects to these two diseases, such as their worldwide distribution, their broad range of target species, their toxins and their high mortality rate in animals. Both syndromes have been known for centuries, but only modern bacteriology and biotechnology have been able to unravel the epidemiology and pathogenesis of these two diseases. Diagnosis, however, remains largely based on clinical symptoms.

2.2. Tetanus

2.2.1. Aetiology and affected species

Tetanus is caused by toxins produced by *Clostridium tetani*. This spore-forming anaerobe has a worldwide distribution and can be demonstrated in more than 50% of soil samples, especially in those from cultivated soils. The spread of spores by stools from healthy individuals may be an explanation for this higher occurrence in cultivated soil. Spores are not confined to the outdoor environment; they have been recovered from household dust and even in operating theatres. Lambs and horses seem to be the animals most vulnerable to developing tetanus. Adult ruminants and carnivores, especially scavengers, such as hyenas, seem to be less susceptible, probably due to the acquisition of a certain level of active immunity during their life (Smith, 2002 a). Lambs and horses, especially, suffer the generalised clinical form of tetanus, while carnivores more often develop a localised clinical form. Birds are much less susceptible than mammals.

2.2.2. Pathogenesis

The usual portal of entry of spores and bacilli is a deep and narrow wound (such as a nail wound at hoof level in horses) or a larger wound with necrotic tissues (after unclean castration of piglets and lambs) or through difficult and injurious farrowing in cows and bitches.

Spores germinate, *C. tetani* multiply and tetanus toxin is produced as soon as local oxygen tension is sufficiently low, as is common in such wounds. Nevertheless, the development of appropriate conditions may take so long that the original injury may have healed by the time the toxin has been produced.

The toxin reaches the local peripheral motorneuron trunk at the neuromuscular junction and is transported along the axon into the central nervous system. In highly susceptible species (horses), the tetanus toxin is distributed via the bloodstream to the motorneuron trunks in the whole body.

In some cases in herbivores, the spores may be ingested and the gut appears to be the site of toxin production. The toxin subsequently crosses the intestinal wall and is transported by the bloodstream to the motorneuron ends.

2.2.3. Clinical signs

The incubation period of tetanus may vary between 1 and 4 weeks and depends on factors, such as the localisation of the portal of entry, local tissue conditions, the number of bacteria that have entered the body and the level of specific antibodies in the host. The first signs can be subtle; signs of colic in horses and bloat and constipation in ruminants have been reported. Lameness or stiffness in one limb is observed in some animals and this may reflect the localised form of tetanus as described in humans. In most cases, initial symptoms evolve within 24 hours into the typical form of tetanus, with generalised stiffness to spasticity and an extended head posture. Symptoms are dominated by hypertonia of the antigravity muscles, resulting in hyperextension of all limbs in animals, while in humans the arms are typically flexed with clenched fists. The posture of animals with tetanus is often described as a "sawhorse stance".

At the head, ears and lips may be retracted (risus sardonicus) and the jaw firmly fixed (lockjaw). Protrusion and rapid movement of the third eyelid is present, especially in horses, when the head is raised or when a menacing gesture is made. Difficulties in swallowing are often present, resulting in accumulation of saliva in the mouth and the presence of frothy saliva at the lip commissures. These difficulties in swallowing may lead to aspiration pneumonia. In a further stage, animals may become recumbent and, even with support in a sling, be unable to remain standing. The muscular spasms are tonic, but can become more pronounced and convulsive after auditory, visual or tactile stimulation. Increased muscular tonus and attempts to rise usually induce hypothermia in affected animals and profuse sweating in horses. When the respiratory muscles become affected, hypoxia may develop. Evolution of the disease may be fast, over the course of just a few days. However, especially in less affected animals, progression may be observed for up to 2 weeks after initial symptoms, due to the slow transport of the toxin in the axons. Tetanus can lead to death for several reasons, such as hypoxia, heart failure, autonomic dysfunction or secondary pathologies, including aspiration pneumonia and exhaustion (Smith, 2002a; Stöber, 2002a).

2.2.4. Diagnosis

2.2.4.1. Clinical diagnosis

Diagnosis of tetanus is almost exclusively based on typical clinical symptoms. In adult animals, a history of a wound 1 to 2 weeks before the onset of symptoms and the absence of active immunisation, may further support the presumptive diagnosis. In neonates, the absence of maternal immunisation may support the inclusion of tetanus in the differential diagnosis of tetanic conditions. Exclusion of other conditions that may resemble tetanus, such as (in horses)

hypocalcemic tetany, strychnine poisoning, meningitis, rhabdomyolysis or laminitis, can help in establishing a diagnosis (Smith, 2002a; Bartlett, 2000a).

2.2.4.2. Pathological lesions

On post-mortem examination, no characteristic lesions are present, apart from secondary lesions, such as those of aspiration pneumonia or, occasionally, bone fractures as a result of muscular spasms. Special attention should be paid to the presence of wounds (Stöber, 2002a).

2.2.4.3. Laboratory diagnosis

Bacteriological examination of wounds is occasionally performed, but recovery of the organism is infrequent. Swabs from wounds in anaerobic transport media may be submitted to a laboratory. Direct smears from wound fluid or tissue may show Gram-Positive rods, but polybacterial presence is common in contaminated wounds. Currently, there is no routine diagnostic method available for the identification of the toxin, except for the mouse bioassay (Bartlett, 2000a).

2.3. Botulism

2.3.1. Aetiology and affected species

Botulism is classically described as a disease caused by toxins produced by *Clostridium botulinum*. Since several species and groups of clostridia seem to be involved, it is more accurate to describe botulism as being a result of toxins from botulinum neurotoxin (BoNT)-producing clostridia (Johnson and Bradshaw, 2001).

When suitable conditions are present, spores of BoNT-producing clostridia develop into vegetative forms and may produce one of seven antigenically distinct neurotoxins (A, B, C1, D, E, F or G). Suitable growth conditions can be defined, in general, as anaerobic conditions with a protein source, sufficient temperature and moisture. For animals, these conditions can, for example, be found in decomposing cadavers, in insufficiently acidified ensiled feed or in decomposing vegetation in ponds. Botulinum neurotoxins have some species specificity: in horses botulism caused by types A, B and C is most common; from cattle, types B, C or D are mostly recovered; fur animals, such as ferrets and mink, are notoriously susceptible to botulism type C and, to a lesser degree, to types A and B; birds are sensitive to type A, very sensitive to types C and E but may harbour type D-producing strains in their gut without showing symptoms; fish seem especially vulnerable to type E (Bartlett, 2000b; Smith, 2002b).

2.3.2. Pathogenesis

The route of contamination in animals is primarily the ingestion of preformed toxins in food, water or carrion.

In some cases, as in shaker foal syndrome, spores of *C. botulinum* are ingested and are present in the gut. After germination of the spores, toxin is produced directly in the gut. (This is called toxicoinfection.)

The botulinum toxin subsequently crosses the stomach and intestinal wall and reaches the neuromuscular junctions via the bloodstream.

2.3.3. Clinical signs

2.3.3.1. Incubation period

In monogastric animals, the incubation period of foodborne botulism is usually short (an average of 18 to 36 hours), with a wide variation from a few hours to 8 days, depending on the amount of ingested toxin. In ruminants, the incubation period is usually somewhat longer and, after ingestion of smaller amounts of toxin, symptoms may be absent for over a week. This implies that, even after withdrawal of suspected feed, new cases may still appear after seven to ten days. For wound botulism and shaker foal syndrome, the incubation period is longer and an indicative period of 4 to 14 days is suggested in the literature (Smith, 2002b).

2.3.3.2. Clinical signs in farm animals (horses, cattle and pigs)

Depending on the amount of ingested toxin, initial symptoms may be subtle or overt in large domestic animals. Animals may be found dead or may die within a few hours, showing symptoms of lateral recumbency, paddling movements and progressively worsening dyspnoea.

In less acute cases, mild gastrointestinal symptoms, such as firm faeces and decreased rumen contractions in cattle, or mild abdominal discomfort in horses, can be the initial signs. These are quickly followed by decreased muscle tone and/or dysphagia. Decreased muscle tone in cattle may mimic the symptoms of milk fever, i.e. weakness and incoordination, evolving into recumbency. In horses, a shuffling gait with toe-dragging and muscle tremors are the initial signs, eventually leading to recumbency and increasing difficulty in rising. Lifting the head into the physiological position may become increasingly difficult for horses infected with botulism and this low head carriage can lead to both oedema of the head and difficulty in breathing.

Physical activity may have a negative effect on clinical evolution: it is believed that such activity results in further depletion of acetylcholine reserves and worsening of symptoms. Recumbent animals may remain sternally or become laterally recumbent in more advanced stages. For large animals, lateral recumbency is a poor prognostic sign and is often accompanied by symptoms of respiratory failure (due to paralysis of respiratory muscles) and by paddling movements in the terminal stages. Prolonged recumbency without respiratory failure remains prognostically poor because of complications associated with recumbency in heavy animals.

Dysphagia is present in most, but not all, cases of botulism in horses and cattle. Decreased masseter tone, decreased tongue strength and pharyngeal paresis or paralysis (bulbar paresis) may contribute to dysphagia each in variable degree. Dysphagia can express itself as an abnormally long time to consume a small amount of food, as a loss of saliva and/or food from the mouth, or as chewing for hours on a cud of hay or straw without swallowing. Testing tongue strength (tongue stress test)

by manually pulling the tongue from the mouth, testing masseter tonus by moving the mandible laterally and timing the uptake of a small amount of food are simple tests that should be done during the initial evaluation of animals suspected of having botulism. These tests may not be pathognomonic, but are at least indicative for botulism.

Due to the effects of toxin at the cholinergic nerve endings in the gastro-intestinal tract, constipation and signs of colic may be present early in the course of the disease. Moderate mydriasis and a poor pupillary response to light are additional clinical symptoms of botulism in large animals. The course of the disease depends largely on the initial evolution; mildly affected animals may show early signs of recovery after 7 to 14 days (Galey, 2001; Smith, 2002b; Stöber, 2002b).

Symptoms of shaker foal syndrome are comparable to botulism in adult horses, but evolution is usually slower. The foal will initially lie down more often than normal and will exhibit muscle tremors when forced to stand up. After suckling, the foal will lose milk from the mouth and tongue strength will be reduced. Mild pupillary dilation, ptosis, constipation and ileus may be present. As the disease progresses, the foal may become more and more lethargic and weak, develop secondary pathologies, such as aspiration pneumonia, and, when respiratory failure ensues, death may follow (Wilkins and Palmer, 2003).

Recently, a clinical syndrome in cattle, characterised by increased animal losses, reduced performance, a high incidence of lame, ataxic and paretic cattle and various digestive problems, has been suggested as the consequence of intestinal proliferation of toxigenic *Clostridium botulinum* ("visceral" botulism) (Böhnel *et al.*, 2001, Holzhauer, 2004).

In horses, there is increasing evidence that toxicoinfection with *Clostridium botulinum* type C may be linked to Grass Sickness. Neurotoxin has been detected in the gastro-intestinal contents of a significantly higher number of affected horses than in control horses. Moreover, antibodies against *Clostridium botulinum* type C and BoNT type C are lower in affected horses (Hunter *et al.*, 1999; Hunter and Poxton, 2001).

Pigs seem relatively resistant to botulism, at least our domestic and commercially raised pigs. Muscle paralysis, vomiting, feed and drink refusal and mydriasis are reported as symptoms.

2.3.3.3. Clinical signs in carnivores

There are very few reports on botulism in pets. Cats seem resistant to botulism, while a few case reports exist on botulism in dogs, mainly involving type C toxin. Symptoms of progressive flaccid paralysis dominate, starting with skeletal muscles and involving head musculature at a later stage (Borst *et al.*, 1986).

Other small mammals that eat meat and meat products, such as mink, ferrets and foxes, seem more prone to botulism. Toxin type C and, to a lesser degree, types A and B, have been reported. Large scale outbreaks in industrially raised fur animals are common and symptoms include the same clinical signs as those in large domestic

animals, i.e. some animals are found dead and others present varying degrees of flaccid paralysis and dyspnoea (Phaneuf *et al.*, 1972).

2.3.3.4. Clinical signs in birds

Flaccid paralysis is the main symptom of botulism in birds. First, the ability to fly and walk disappears and then the birds become unable to lift their neck. This inability to erect the neck is a typical symptom described as "limber neck". Paralysis of the inner eyelid is another characteristic symptom at this stage of the disease. Without treatment, birds will die because of drowning (in the case of waterfowl), emaciation or respiratory failure. A toxicoinfectious form of botulism has been described in broilers, which causes the same symptoms, eventually followed by diarrhoea with excess urates (Wobeser, 1997).

2.3.3.5. Clinical signs in fish

Regarding botulism in fish, mostly reported in farmed trout or salmon, the first observed symptom will be increased mortality. Individual fish may initially show agitation, followed by progressive paralysis of the fins, the tail fin being the last to become paralysed. Fish will, therefore, lose the ability to swim in a coordinated way and tend to sink to the bottom, tail fin first (Eklund *et al.*, 2004).

2.3.4. Diagnosis

2.3.4.1. Clinical diagnosis

Clinical symptoms of botulism are strongly indicative but not specific. Other causes of sudden death and nervous system disorders causing weakness and dysphagia must be included in differential diagnosis. These include milk fever or listeriosis in cows, herpes virus encephalomyelitis in horses, intoxication and autoimmune diseases (myasthenia gravis). Therefore, the diagnostic work-up will include elimination of other disease entities through clinical, biochemical and other diagnostic procedures and the positive identification of botulism as the primary cause of the symptoms (Smith, 2002b; Stöber, 2002b).

2.3.4.2. Pathological lesions

Necropsy findings are usually unremarkable, although in more protracted cases, secondary pathologies, such as aspiration pneumonia or lung congestion, may be present. These negative post-mortem results can be used as a diagnostic tool per exclusionem.

In cattle, congestion and haemorrhagic lesions can sometimes be observed in the proximal jejunum, and histological examination reveals local intestinal necrosis. These lesions are similar to those of clostridial enterotoxaemia in cattle and it is suggested that this may be the result of enterotoxins, such as C2, being locally produced by ingested clostridia (Martel, 2003).

2.3.4.3. Laboratory diagnosis

Diagnosis of botulism can be made by demonstrating 1) toxin in the patient (serum, gastro-intestinal contents, liver, wound), 2) *Clostridium botulinum* spores in the patient (gastro-intestinal contents, liver, wound), 3) toxin or spores in the feed or close environment of the patient or 4) an antibody response in a recovered patient (Smith, 2002b).

2.3.4.3.1. Sampling procedure and transport to the laboratory

Samples of serum (10 ml), ruminal or gastro-intestinal contents (200 g), faecal material and tissue samples (liver) for the detection of toxin should be taken as soon as possible after the onset of symptoms, or after death. The toxin is sensitive to heat (destruction after 20 minutes at 50 °C), but is stable for weeks when frozen. Transport on cold packs (4 to 8 °C) and in air- tight containers is preferred when it is possible for the sample to arrive at the laboratory within 24 hours.

Samples for bacteriology (tissue samples, ruminal or gastro-intestinal contents, faecal material, food, carcasses) should preferably be shipped on cold packs in air-tight and leak- proof containers, as freezing may reduce the recovery of bacteria on subsequent culture.

All containers should be clearly marked as possibly holding BoNT (Böhnel and Gessler, 2003).

2.3.4.3.2. Laboratory procedures

Toxin assay is available only in a small number of reference laboratories. Currently, the most sensitive test is the mouse bioassay. Serum or extracts of other submitted material is intraperitoneally injected into mice. The animals are then observed for 4 days for the development of clinical symptoms (wasp waist) or death. The injection of unneutralised samples and samples neutralised with specific antisera further confirms the presence and serotype of the toxin. ELISA techniques are currently being developed to reduce the use of laboratory animals and possibly to enhance the sensitivity of the test, but they are not yet approved as a reference test (Galey, 2001; Moeller *et al.* 2003; Ferreira *et al.*, 2004, Gessler and Böhnel, 2004).

Bacteriological examination of clinical samples or suspected food is carried out by anaerobic culture on appropriate media (for example egg yolk agar plates) and subsequent inoculation of suspected colonies takes place on broth media (Glasby and Hatheway, 1985; Shone and Tranter, 1995). The supernatant of those media can then be tested for the presence of toxin. Classic bacteriology will be combined in the near future with PCR assays for the demonstration of toxinogenic clostridia in clinical and environmental samples (Szabo *et al.*, 1992; Galey, 2001, Lindström *et al.*, 2001).

2.3.4.3.3. Interpretation of laboratory results

Although demonstration of toxin in the patient is the most valuable diagnostic technique, it remains difficult to achieve. This is because the mouse

lethality test will only be positive when there is still a significant amount of free toxin present in serum, tissue or gastro-intestinal contents. This test will, therefore, probably only be positive when samples are taken early in the course of the disease and/or when the patient has ingested a large amount of toxin. Preferred samples in the diagnosis of botulism in cattle are gastro-intestinal contents or those taken from the liver. In foals with shaker foal syndrome, intestinal contents may yield up to 20% positive results in toxin assay (Smith, 2002b). In birds, toxin can be demonstrated at a fairly constant rate in serum, intestinal contents or tissue samples. The low diagnostic sensitivity of mouse bioassay implies that multiple samples have to be examined before discarding botulism as a possible cause of symptoms. Anecdotal evidence suggests that even an extensive search for toxin may remain negative when all other symptoms indicate botulism as the cause of the disease (Heider *et al*., 2001; Cobb *et al*., 2002). Recent work has shown that one explanation for frequent negative diagnostic results when using the mouse lethality test in cattle may be their higher sensitivity to botulinum toxin in comparison with mice (Moeller *et al*., 2003).

Demonstration of spores in gastro-intestinal contents or tissues of animals with symptoms indicative of botulism, supports the diagnosis. Spores are normally rarely detected in faecal samples from foals or horses, but in cases suspected of botulism on clinical grounds, spores can be detected in 34% of faeces from adult horses and in 70% of foal faeces (Smith, 2002b).

Demonstration of spores or toxin in feed of animals showing symptoms of botulism may also help in diagnosis. This demonstrates that the animals are at risk and may help to identify the source of the toxin in order to prevent further fatalities. The presence of toxin in feed, in particular, is regarded as diagnostic evidence; the presence of spores is more indicative than diagnostic (Kelch *et al.*, 2000).

Where a test is available, demonstration of neutralising antibodies may allow a retrospective diagnosis in recovered animals (Smith, 2002b; Holzhauer, 2004).

Diagnosis of botulism, certainly when few animals are affected, can be difficult for several reasons: the small amount of toxin needed to kill an animal, the rapid disappearance of toxin from the circulation and the usually focal distribution of toxin in feed. Diagnosis is based, therefore, on exclusion of other causes, rather than on positive identification. The cost of repeated attempts to identify the bacterium or toxin may further limit diagnostic possibilities in veterinary medicine.

2.4. Bibliography

Bartlett J.G. (2000 a). Tetanus. In: *Cecil Textbook of Medicine*, 21st ed. (Goldman L. and Bennett J., Eds.), pp 1675-1676, W.B. Saunders Company.

Bartlett J.G. (2000 b). Botulism. In: *Cecil Textbook of Medicine*, 21st ed. (Goldman L. and Bennett J., Eds.), pp 1673-1674, W.B. Saunders Company.

Böhnel H. and Gessler F. (2003). Botulismusdiagnose seit 1995. Ein Erfahrungsbericht. *Berliner und Münchener Tierärtzliches Wochenschrift* **116**, 269-273.

Böhnel H., Schwagerick, B. and Gessler F. (2001). Visceral botulism – a new form of bovine *Clostridium botulinum* toxication. *J. Vet. Med. Series A* **48**, 373-383.

Borst G.H.A., Lambers G.M. and Haagsma J. (1986). Type-C Botulism in dogs. *Tijdschrift voor Diergeneeskunde* **111**, 1104-1105.

Cobb S.P., Hogg R.A., Challoner D.J., Brett M.M., Livesey C.T., Sharpe R.T. and Jones T.O. (2002). Suspected botulism in dairy cows and its implications for the safety of human food. *Vet. Rec.* **150**, 5-8

Eklund M.W., Poysky F.T., Paranjpye R.N., Peterson M.E. and Pelroy G.A. (2004). Susceptibility of coho salmon, Oncorhynchus kisutch (Walbaum), to different toxins of *Clostridium botulinum*. *Aquaculture Res.* **35**, 594-600.

Ferreira J.L., Eliasberg S.J., Edmonds P. and Harrison M.A. (2004). Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinal toxin in food. *J. Food Protect.* **67**, 203-206.

Galey F.D. (2001). Botulism in the horse. *Vet. Clin. North Am.: Equine Practice* **17**, 579-588.

Gessler F. and Böhnel H. (2004). Nachweis von Botulinum-Neurotoxinen. Ein methodischer Über- und Ausblick. *Tierärtzliche Umschau* **59**, 5-9.

Glasby C. and Hatheway C.L. (1985). Isolation and enumeration of *Clostridium botulinum* by direct inoculation of infant fecal specimens on egg yolk agar and *Clostridium botulinum* isolation media. *J. Clin. Microbiol.* **21**, 264-266.

Heider L.C., McClure J.T. and Leger E.R. (2001). Presumptive diagnosis of *Clostridium botulinum* type D intoxication in a herd of feedlot cattle. *Can. Vet. J.* **42**, 210-212.

Holzhauer M. (2004). Botulismus bei Rindern in den Niederlanden. *Tierärtzliche Umschau* **59**, 29-31.

Hunter L.C., Miller J.K. and Poxton I.R. (1999). The association between *Clostridium botulinum* type C with equine Grass Sickness: a toxicoinfection. *Equine Vet. J.* **31**, 492-499.

Hunter L.C. and Poxton I.R. (2001). Systemic antibodies to *Clostridium botulinum* type C: do they protect horses from Grass Sickness (dysautonomia)? *Equine Vet. J.* **33**, 547-553.

Johnson E.A. and Bradshaw M. (2001). *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. *Toxicon* **39**, 1703-1722

Kelch W.J., Kerr L.A., Pringle J.K., Rohrbach B.W. and Whitlock R.H. (2000). Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. *J. Vet. Diagn. Invest.* **12**, 453-455.

Lindström M., Keto R., Markkula A., Nevas M., Hielm S. and Korkeala H. (2001). Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Appl. Environm. Microbiol.* **67**, 5694-5699.

Martel J.-L. (2003). Le botulisme des bovins: un danger rare, mais présent. *Le Point Vétérinaire* **238**, 24-28.

Moeller R.B., Puschner B., Walker R.L., Rocke T., Galey F.D., Cullor J.S. and Ardans A.A. (2003). Determination of the median toxic dose of type C botulinum toxin in lactating dairy cows. *J. Vet. Diagn. Invest.* **15**, 523-526.

Phaneuf J.B., Vinet G. and Fredette V. (1972). Botulism on mink ranches in Quebec. *Can. Vet. J.* **13**, 75-77.

Shone C.C. and Tranter H.S. (1995). Growth of clostridia and preparation of their neurotoxins. *Curr. Top. Microbiol. Immunol.* **195**, 143-160.

Smith M.O. (2002a). Tetanus (Lockjaw). In: *Large Animal Internal Medicine*, 3rd ed. (Smith B.P., Ed.), pp 995-998, Mosby.

Smith M.O. (2002b). Botulism (Shaker foals; Forage Poisoning). In: *Large Animal Internal Medicine*, 3rd ed. (Smith B.P., Ed.), pp 1003-1008, Mosby.

Stöber M. (2002a). Tetanus. In: *Innere Medizin und Chirurgie des Rindes,* 4th ed. (Dirksen G., Gründer H.-D. and Stöber M., Eds.), pp 1068-1071, Parey Buchverlag.

Stöber M. (2002b). Botulismus. In: *Innere Medizin und Chirurgie des Rindes,* 4th ed. (Dirksen G., Gründer H.-D. and Stöber M., Eds.), pp 1113-1118, Parey Buchverlag.

Szabo E.A., Pemberton J.M. and Desmarchelier P.M. (1992). Specific detection of *Clostridium botulinum* type B by using the polymerase chain reaction. *Appl. Environm. Microbiol.* **58**, 418-420.

Wilkins P.A. and Palmer J.E. (2003). Botulism in foals less than 6 months of age: 30 cases (1989-2002). *J. Vet. Int. Med.* **17**, 702-707.

Wobeser G. (1997). Avian botulism – Another perspective. *J. Wildlife Dis.* **33**, 181-182.

3. Soft tissue infections in animals

J.G. Mainil

3.1. Introduction

The first stage of clostridial soft tissue infection in animals is the deposit of spores in the tissues: wound, derm and subcutaneous tissue, internal organs. Following the development of anaerobiosis, often after tissue necrosis, the spores will germinate; clostridia will multiply and produce their toxins. These toxins will act locally, digesting and lysing the tissue (gangrene, necrosis and abscedation), sometimes with gas production, and generally, after resorption in the bloodstream and distribution throughout the whole body (erythrolysis, lethal effect).

Soft tissue infections in animals are various (Songer, 1997, 2002, 2004). Their diagnosis can be performed clinically, bacteriologically or even molecularly on clinical specimens.

3.2. Clinical and necropsy diagnosis

Clostridial soft tissue infections in animals can be subdivided into specific and non-specific pathologies. All specific diseases can be suspected, or even in some cases diagnosed, based on criteria such as the target host species, clinical signs and lesions at necropsy (Table 3.1):

- necrotic and haemorrhagic myositis caused by *Clostridium chauvoei* in ruminants (blackleg) or by *Clostridium septicum* in any animal species (false blackleg, by extension of gas gangrene or of non-traumatic origin);
- necrotic hepatitis in ruminants caused by *Clostridium novyi* type B (black disease) or *Clostridium haemolyticum* (bacillary haemoglobinuria);
- hepatitis, myocarditis and enteritis in laboratory animals and young mammals caused by *Clostridium piliforme* (Tyzzer's disease);
- (gas) gangrene in subcutaneous or submucosal tissues and/or internal organs caused by a variety of clostridia: *Clostridium septicum*, *C. novyi* type A, *C. sordellii*, *C. chauvoei*, and, more rarely, *C. perfringens* type A;
- necrotic dermatitis in poultry caused by *Clostridium septicum*;
- enteritis and hepatitis in birds caused by *Clostridium colinum* (Quail disease).

Non-specific pathologies of soft tissue caused by clostridia are osteomyelitis, mastitis, meningo-encephalitis, external otitis, subcutaneous and internal abscesses, caused by the species named above, or less frequently by minor pathogenic clostridia.

3.3. Bacteriological diagnosis

Bacteriological diagnosis is often necessary for confirmation, but results are reliable only if lesions have been appropriately sampled and those samples appropriately transported. In some cases, growth of bacteria will not be necessary, because a diagnosis can be performed based on careful examination of Gram and Ziehl-Neelsen-stained smears from tissue samples sent to the laboratory. It is also possible to perform immuno-fluorescence or immuno-cytochemical assays on smears using clostridial species-specific whole cell immune sera and, in some cases, PCR assays. When growth of clostridia is necessary, the laboratory procedure targets one or more clostridial species, depending on the pathology suspected.

3.3.1. Sampling and transport

Quite often the laboratory receives the whole animal body for necropsy and, subsequently, bacterial analysis of suspect lesions (Table 3.2). Sometimes the dead animal will be necropsied directly by the practitioner. Reliable bacterial analysis can be effectively performed only if the body is sampled soon after death, especially if the outdoor temperature is high. Otherwise multiplication of clostridia originating from the gut will overwhelm any pathogenic species, even in the internal organs. This is particularly true for infections with gangrene-producing clostridia, since putrefaction starts even before death.

Tissue samples should be large enough (at least 4 cm^3) to include lesion area(s) and be stored in a jar with a catalyst, if not immediately introduced into an anaerobic cabinet. Fluids should be transported in air-free containers. All samples should be refrigerated and brought in, rather than sent, to the laboratory. If sent by post, they should be wrapped in two layers of leak-proof plastic bags. In the few cases where a cotton swab is taken, the transport medium must be appropriate for anaerobes. However, toxin detection is not possible in the case of cotton swabs.

3.3.2. Gram and Ziehl-Neelsen-stained smears

Gram and Ziehl-Neelsen stainings can be performed on smears prepared directly from clinical samples at the site of lesions (Table 3.2). The former gives information on the morphology of the bacteria; the latter on the spores, if any. Also a stained smear can reveal the presence of multiple bacterial species in the case of samples taken quite late after death. But all species become Gram-Negative in old cultures and when the cells are sporulating. Moreover, *Clostridium piliforme* always gives a negative reaction at Gram staining, and Giemsa staining must be used in order to see the intracellular bundles of bacilli.

Table 3.1: Main criteria for clinical diagnosis of specific clostridial soft tissue infections (Mainil and Duchesnes, 2002; Mainil *et al.*, 2002; Songer, 2004)

Pathology	*Clostridium*	Host species	Clinical sign(s)	Lesion(s)
Blackleg	*chauvoei*	Cattle, sheep >2 years	Lameness and local oedema (hindlimbs) Sudden death	Oedema, haemorrhages and necrosis in the muscles (mainly of the hindlimbs), rancid butter odour
False blackleg	*septicum*	Ruminants + others	See gas gangrene	See gas gangrene of muscles
(Gas) gangrene	*septicum, novyi* A, *sordellii, chauvoei,* *perfringens* A[1]	Ruminants (*C. novyi* A, *C. septicum*, *C. sordellii*, *C. chauvoei*, *C. perfringens* A) Horses (*C. sordellii*) Pigs (*C. septicum*) Others (various)	Warm, then cold, tumefaction, oedema and/or gas accumulation, cutaneous or mucosal wounds, internal organ infection Sudden death	Tissue oedema, haemorrhages and necrosis, gas in the subcutaneous and submucosal tissues and in internal organs (liver, muscles), rapid putrefaction of the body
Black disease	*novyi* B	Small (large) ruminants	None specific Sudden death	Subcutaneous venous congestion, focal hepatic venous infarcts, fluke infection
Bacillary haemoglobinuria	*haemolyticum*	Large ruminants (>1 year)	Anaemia, haemoglobinuria, jaundice	Large hepatic venous infarct(s), anaemia, red urine in the bladder fluke infection
Tyzzer's disease	*piliforme*	Young mammals (foals), laboratory animals	Weakness, fever, jaundice, diarrhoea, weight loss	Necrotic foci on the liver, heart, intestine (caecum) Pseudomembraneous typhlitis[2]
Necrotic dermatitis	*septicum*	Chickens	Incoordination, red skin, subcutaneous swelling	Oedema, necrosis and gas in the subcutaneous tissue
Quail disease[3]	*colinum*	Game birds, young poultry	Sluggish, inactive, anorexic	Ulcerative enteritis, necrotising hepatitis and splenitis

[1] and several minor pathogens
[2] For rabbits see Chapter 10
[3] For Quail disease caused by *Clostridium colinum* see Chapter 11

3.3.3. Immunoassays

Though the morphology of clostridial rods is indicative of their identity, accurate identification can be obtained only through biotyping or toxintyping. Because this procedure is difficult and time-consuming, especially in ill-equipped laboratories, it would be advantageous to obtain a good indication of clostridial identity without growing the bacteria.

Identification is possible using specific whole cell polyclonal immune serums in immuno-fluorescent reactions on smears prepared from tissue samples (Table 3.2). Fluorescent-labelled antiserums to *C. chauvoei*, *C. haemolyticum*, *C. novyi*, *C. piliforme*, *C. septicum and C. sordellii* are available.

Detection of *C. chauvoei* and *C. haemolyticum* has also been reported with the peroxydase-antiperoxydase (PAP) technique on formalin-fixed paraffin-embedded tissue sections (Giraudo Conesa *et al.*, 1995; Uzal *et al.*, 1992).

3.3.4. PCR on clinical specimens

Different PCRs (Polymerase Chain Reactions) have been developed for identification of clostridia (see Chapter 16). Some have been tested on clinical samples to detect and differentiate myositis and gas gangrene-associated clostridia: *C. chauvoei*, *C. novyi*, *C. septicum, C. sordellii* (Uzal *et al.*, 2003; Sasaki *et al.*, 2001, 2000a and b; Kojima *et al.*, 2001; Kuhnert *et al.*, 1997) and *C. piliforme* in intestinal specimens and faeces (Furukawa *et al.*, 2002; Ikegami *et al.*, 1999). However, PCRs are not yet used in routine diagnostic laboratories on clinical samples.

3.3.5. Demonstration of toxin production *in vivo*

With the exception of *C. colinum* and *C. piliforme* so far, major pathogenic clostridia produce toxins during infection (Titball *et al.*, 2003). The following toxins can be detected (Table 3.2) in tissue samples, exsudates and serosal effusions: α toxin of *C. perfringens* type A; α and/or β toxins of *C. novyi* types A, B and those of *C. haemolyticum*. Tests for these are immuno-chemical or animal assays (lethality in mice, dermonecrosis in guinea pigs). It must be emphasised that neutralising immune sera are not available for the α and β toxins of *C. novyi* and *C. haemolyticum.* Other toxins are not routinely detected.

3.3.6. Growth media and conditions

Though clostridia are anaerobes by definition, anaerobic requirements can vary between species. Amongst the species listed in this chapter, three are very strict in their requirement for anaerobiosis (*C. chauvoei*, *C. haemolyticum* and *C. novyi*). *C. haemolyticum* and *C. novyi* die within minutes of exposure to air. It is not always possible to obtain their growth in anaerobic jars and use of an anaerobic cabinet is recommended when possible. On the other hand, a species like *C. perfringens* can grow even if traces of oxygen are still present; it is therefore very easy to grow in any basically equipped laboratory. In addition, several species require the presence of blood and some enrichment in specific components (*C. chauvoei*, for example).

Table 3.2: Sampling and routine direct diagnostic procedures (Titball *et al.*, 2003; Mainil *et al.*, 2004; Popoff, 2004).

Pathology	***Clostridium***	**Samples**	**Gram stain**	**Immunoassays**	***In vivo* toxin demonstration**
Myositis	*chauvoei*	Muscle lesions	Yes	IFA[1], PAP[2]	No
	Septicum			IFA, PAP	No
(Gas) gangrene	*septicum*	Effusion fluids, organs (muscles, liver, spleen…), wound swab	Yes	IFA	No
	novyi A			IFA	Yes
	sordellii			IFA	No
	chauvoei			IFA	No
	perfringens A			No	Yes
Hepatitis[3]	*novyi* B	Liver lesions	Yes	IFA	Yes
	haemolyticum		Yes	IFA, PAP	Yes
	colinum		Yes	No	No toxin identified
	piliforme	(also faeces)	No (Giemsa)[4]	IFA	No toxin identified
Dermatitis	*septicum*	Skin, subcutaneous tissue	Yes	No	No

[1] IFA = Immuno-Fluorescence Assay
[2] PAP = Peroxydase-Antiperoxydase technique
[3] For Quail disease caused by *Clostridium colinum*
[4] Intracellular bundles of bacilli by Giemsa stain

3.4. Interpretation criteria

In general, the results of bacteriological analysis must be interpreted with care. Some clostridia originating from the gut (e.g. *C. septicum*, *C. perfringens)* will invade the body very rapidly after death, even during the agonic period, and overgrow other pathogenic species present in the internal tissue. Conversely, several clostridia are extremely O_2 sensitive and will die rapidly when exposed to air.

There are no general rules; each case is unique. Interpretation criteria must therefore include clinical signs and lesions, the delay between death and sampling, the outdoor temperature, the quality of sampling, transport conditions and the purity and number of bacteria observed and grown.

3.5. Bibliography

Furukawa T., Furumoto K., Fujieda M. and Okada E. (2002). Detection by PCR of the Tyzzer's disease organism (*Clostridium piliforme*) in feces. *Exp. Anim.* **51**, 513-516.

Giraudo Conesa L.C., Vannelli S.A. and Uzal F.A. (1995). Detection of *Clostridium chauvoei* in formalin-fixed, paraffin-embedded tissues of sheep by the peroxidase-antiperoxidase (PAP) technique. *Vet. Res. Commun.* **19**, 451-456.

Ikegami T., Shirota K., Une Y., Nomura Y., Wada Y., Goto K., Takakura A., Itoh T. and Fujiwara K. (1999). Naturally occurring Tyzzer's disease in a calf. *Vet. Pathol.* **36**, 253-255.

Kojima A., Uchida I., Sekizaki T., Sasaki Y., Ogikubo Y. and Tamura Y. (2001). Rapid detection and identification of *Clostridium chauvoei* by PCR based on flagellin gene sequence. *Vet. Microbiol.* **78**, 363-371.

Kuhnert P., Krampe M., Capaul S.E., Frey J. and Nicolet J. (1997). Identification of *Clostridium chauvoei* in cultures and clinical material from blackleg using PCR. *Vet. Microbiol.* **57**, 291-298.

Mainil J. and Duchesnes C. (2002). *Clostridium* in health and disease. In: *Molecular genetics, classification, pathology and ecology of the genus* Clostridium. Proceedings of the CA QLK2-CT2001-01267 (Mainil J., Duchesnes C., Eds.), pp 36-40. Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Mainil J., Duchesnes C., Frey J. and Stackenbrandt E. (2002). *Classification, molecular genetics and pathology of clostridia.* Scientific booklet of the CA QLK2-CT2001-01267, Presses Fac. Méd. Vét. ULg, Liège, Belgium. (http://www.genusclostridium.net)

Mainil J., Duchesnes C., Pelkonen S., Dubreuil L. and Menozzi M.G. (2004). *Identification, typing and antibiotic resistance of the genus* Clostridium. Scientific booklet of the CA QLK2-CT2001-01267, pp 12-14, Presses Fac. Méd. Vét. ULg, Liège, Belgium. (http://www.genusclostridium.net).

Popoff M. (2004). Overview of *Clostridium* identification. In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp 15-21, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Sasaki Y., Yamamoto K., Kojima A., Tetsuka Y., Norimatsu M. and Tamura Y. (2000a). Rapid and direct detection of *Clostridium chauvoei* by PCR of the 16S-23S rDNA spacer region and partial 23S rDNA sequences. *J. Vet. Med. Sci.* **62**, 1275-1281.

Sasaki Y., Yamamoto K., Kojima A., Norimatsu M. and Tamura Y. (2000b). Rapid identification and differentiation of pathogenic clostridia in gas gangrene by polymerase chain reaction based on the 16S-23S rDNA spacer region. *Res. Vet. Sci.* **69**, 289-294.

Sasaki Y., Yamamoto K., Amimoto K., Kojima A., Ogikubo Y., Norimatsu M., Ogata H. and Tamura Y. (2001). Amplification of the 16S-23S rDNA spacer

region for rapid detection of *Clostridium chauvoei* and *Clostridium septicum*. *Res. Vet. Sci.* **71**, 227-229.

Songer J.G. (1997). Molecular and immunological methods for the diagnosis of clostridial diseases. In: *The clostridia: molecular biology and pathogenesis* (Rood J.I., McClane B.A., Songer J.G. and Titball R.W., Eds), pp 491-503, Academic Press, Inc., USA

Songer J.G. (2002). Gas gangrene and myonecrosis. In: *Molecular genetics, classification, pathology and ecology of the genus* Clostridium (Mainil J. and Duchesnes C., Eds.), pp 53-62, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Songer J.G. (2004). Histotoxic clostridia. In: *Pathogenesis of bacterial infections in animals* (Gyles C.L., Prescott J.F., Songer J.G. and Thoen C.O., Eds), pp 125-130, Blackwell Publ., USA.

Titball R., Mainil J., Duchesnes C. and Popoff M. (2003). *Protein toxins of the genus* Clostridium *and vaccination.* Scientific booklet of the CA QLK2-CT2001-01267. Presses Fac. Méd. Vét. ULg, Liège, Belgium. (http://www.genusclostridium.net)

Uzal F.A., Belak K., Rivera E., Robles C.A. and Feinstein R.E. (1992). Bacillary haemoglobinuria diagnosis by the peroxydase-antiperoxydase (PAP) technique. *Zentralbl. Veterinarmed B* **39**, 595-598.

Uzal F.A., Hugenholtz P., Blackall L.L., Petray S., Moss S., Assis R.A., Fernandez Miyakawa M. and Carloni G. (2003). PCR detection of *Clostridium chauvoei* in pure cultures and in formalin-fixed, paraffin-embedded tissues. *Vet. Microbiol.* **91**, 239-248.

4. Clostridial abomasitis

J.G. Songer

Table 4.1: Clostridia causing abomasitis in domestic ruminants

***Clostridium* species**	**Abomasitis in:**
perfringens type A	neonatal and older calves, young lambs
perfringens type C	neonatal calves, young lambs (uncommon), older lambs (pine)
septicum	neonatal and older calves, young lambs

4.1. *Clostridium perfringens*

Clostridium perfringens is common in the environment and in the intestinal tracts of humans and domestic and wild animals. Infecting organisms may be from an exogenous source, but are often endogenous. It is perhaps the most frequently-isolated pathogenic bacterium; it is undeniably the most important cause of clostridial disease in domestic animals. The widely known typing system allows division of the species into 5 toxin phenotypes or genotypes. Truly non-toxigenic strains of *C. perfringens* (not producing any of the 4 major toxins) are rare.

There is considerable syndromic overlap between abomasitis (regardless of species) and enteritis/enterotoxaemia. The latter frequently involves abomasal damage as well.

Information about distribution of *C. perfringens* genotypes is accumulating as more diagnostic and research laboratories adopt PCR-based methods for toxin gene detection. There is evident disparity in prevalence of the various genotypes by geographic region, although type A remains the most commonly-isolated type overall. In North America, type A predominates, although types C (mainly from neonatal enteritis cases), D (mainly from ovine or caprine enterotoxaemia) and E (from bovine neonatal hemorrhagic enteritis) are not uncommon. In various studies, nearly 97% of isolates from various sources in Belgium were of non-enterotoxigenic type A, including all of those from cattle and horses with enterotoxaemia. Type C was limited to swine, type D was isolated from small ruminants with enterotoxaemia, and types B and E were not found. Types C and E were detected in Poland, while in Austria, type A was most common in sheep and type D in goats. Enterotoxaemia in goats, reindeer, and blackbuck were caused by type E. Type C was found only in swine. In Northern Greece, type B is apparently most prevalent in lambs (nearly 50% of cases of lamb dysentery), although type C accounted for 20% and type D 28% of all isolates. Types A and E were not found.

4.1.1. *C. perfringens* type A (α toxin)

4.1.1.1. Abomasitis in lambs and kids

Lamb enterotoxaemia (yellow lamb disease) and a similar condition in kids occurs sporadically in wet springs in the north-western US. Depression, anaemia, icterus and haemoglobinuria are common, and affected lambs die after a 6–12 h clinical course. Lesions begin in the abomasum and progress through the small intestine, with haemorrhagic necrosis throughout. Elevated numbers of *C. perfringens* (up to 10^7 colony-forming units per gram) are found in affected mucosa and intestinal contents. It is often difficult to distinguish yellow lamb disease from lamb dysentery, although type B infections (see below) are rare-to-non- existent in the US geographical range of yellow lamb disease.

4.1.1.2. Abomasitis in calves

Type A-associated abomasitis is more common in beef and dairy calves than in lambs, including calves raised for veal, but is similar in many respects to the disease in lambs. Infection may originate in contaminated colostrum, and the method of administration has associated risk factors; icy colostrum in large volumes is associated with development of disease.

Bloating is common, and the clinical syndrome ranges from severe diarrhoea, with a brief clinical course, to sudden death without premonitory signs. Gross lesions consist typically of haemorrhagic gastroenteritis. The abomasum is often grossly dilated, haemorrhagic and ulcerated, with thickened, emphysematous walls and folds. Microscopic lesions include severe necrosis and haemorrhage, with micro-colonies of large rod-shaped bacteria observed in the mucosa and submucosa. Rapid autolysis may obscure enteric lesions. Veal calves may experience similar infections at times of significant dietary change, not uncommonly as late as 4 months of age.

4.1.2. *C. perfringens* type C (α and β toxins)

Newborn animals are typically most susceptible to type C infection, likely following rapid gut colonisation in the absence of well established normal intestinal flora. Sudden dietary changes can alter flora and incite type C infection in older animals. Type C, shed in small numbers by dams, may be selectively enriched in the neonatal gut.

4.1.2.1. Abomasitis in neonatal calves, lambs, and kids

Robust, vigorous calves, often less than 10 days old, display signs of severe abdominal pain, sometimes with central nervous system signs including tetany and opisthotonos. Death may be peracute, occasionally without other clinical signs, but typically follows a clinical course of hours to days. Gross lesions include haemorrhagic, necrotic abomasitis and enteritis, and are similar to, though usually more severe than, type A infections.

Type C abomasitis in lambs resembles lamb dysentery, although colonic involvement, if any, is usually limited to the distal portion. Attack rates are often as high as 20%, and case fatality rates of 100% are not unusual. In these animals, and others affected by type C, β toxin is the virulence factor of note, and immunity is mainly antitoxic.

4.1.2.2. Abomasitis in adult sheep (struck)

Adult sheep (typically young ewes) in specific locales (e.g., Wales and the Romney Marsh areas of England), can be affected by an often peracute form of clostridial abomasitis and enteritis. Rapid death, which gives the impression of lightning strike, has resulted in application of the picturesque name "struck". Damage to gastrointestinal mucosa, caused perhaps by poor quality or frozen feed, encourages multiplication of type C in abomasal and small intestinal walls, resulting in mucosal necrosis, usually without dysentery or diarrhoea. Accumulation of fluid in the peritoneum and thoracic cavity, as well as central nervous system signs (including peracute death), suggest a role for β toxaemia, and rare cases have no gross lesions in the gut.

4.1.3. *C. perfringens* type B (α, β and ε toxins)

The natural history of lamb dysentery is much the same as for other forms of clostridial abomasitis and enteritis affecting neonates. Type B organisms, originating with the dam, multiply in the neonatal gut, resulting in abomasitis, as well as extensive haemorrhage and ulceration of the small intestine. Death is often peracute, but may be preceded by inappetence, abdominal pain, haemorrhagic diarrhoea, recumbency and coma. Older lambs may develop a chronic condition called "pine," in which infection of the abomasum and intestine manifests as chronic abdominal pain without diarrhoea.

4.2. *Clostridium septicum*

Clostridium septicum is commonly found in soil, and has also been isolated from the faeces of domestic animals and humans. It is frequently a post-mortem invader from the gut.

4.2.1. Abomasitis in sheep and calves (braxy)

In lambs or older sheep, the organism interacts with damaged abomasal epithelium and produces braxy or bradsot. It is responsible for large numbers of deaths in sheep in Great Britain, Ireland, Norway, Iceland and the Faroe Islands and has been reported in Europe, Australia, the United States and elsewhere. The invasion mechanism has not been characterised experimentally, but ingestion of coarse or frozen feed is associated with development of disease in both sheep and dairy calves. Impaired mucosal function may allow entry of the organism, followed by local multiplication and dissemination of toxins throughout the body, producing local lesions and toxaemia. The clinical course is often fulminant, with sudden death

of affected animals. Those not dying peracutely may exhibit lassitude and abdominal pain, and may occasionally produce haemorrhagic stools. Gross lesions include haemorrhagic, necrotic abomasitis, with diffuse suppurative inflammation; abomasal and proximal small intestinal walls are oedematous, haemorrhagic, necrotic, and emphysematous. Accumulation of fluid in body cavities, and other signs of bacteraemia and toxaemia, are common. Disease in calves is much the same, but occurs most commonly in neonates fed large volumes of barely-thawed, pooled colostrum.

α toxin is the key factor in pathogenesis of abomasitis, and antitoxic immunity protects against infection.

4.3. Diagnosis

Diagnosis can be based upon clinical signs, gross and microscopic findings at necropsy, Gram- and fluorescent antibody staining of direct smears, bacteriologic culture, and, in the case of *C. perfringens*, typing of isolates.

4.3.1. Clinical and necropsy findings

Clinical signs have much in common from organism to organism and host to host. Animals with peracute disease may simply be found dead, without premonitory signs. Those with acute disease will usually show evidence of depression, inappetence, abdominal pain and diarrhoea with blood, sometimes progressing to central nervous system signs, including opisthotonos. Subacute or chronic cases are more likely to experience haemorrhagic diarrhoea, although chronicity is rare.

Necropsy findings are dominated by extensive haemorrhagic necrosis of the abomasal epithelium and underlying tissues, often with a marked suppurative component. Signs suggestive of toxaemia are common in *C. septicum*- and *C. perfringens* type C-induced disease, and there is good evidence for systemic effects of their α and β toxins, respectively. Bacteraemia is a recognised component of *C. septicum* enteric infections in domestic animals and humans, and may also occur, at least terminally, in *C. perfringens* type A or type C infections.

It is common for similar lesions to extend into the small intestine and not uncommon for the colon to be affected as well.

4.3.2. Sampling procedures

Tissues should be preserved in buffered formalin for histopathologic examination. A portion of affected abomasum should be collected fresh for direct examination and bacteriologic culture. Contents, especially in the small intestine, can be preserved in a separate, sterile container or *ca* 15 cm of intestine can be ligated at each end for transport to the laboratory.

Due to the proclivity of these organisms for post-mortem invasion, euthanasia and necropsy is much preferable to examination of cadavers, especially if the interval since death is > 4 h.

4.3.3. Transport

Fresh tissues should be transported to the laboratory as rapidly as possible, and may be shipped on wet ice. Freezing may reduce the titre of organisms and toxin present, but is an acceptable alternative if shipping must be delayed for more than 24 hours.

4.3.4. Laboratory aspects of diagnosis

4.3.4.1. Direct smears

Gram-staining of direct smears from affected tissues is often useful in providing an indication of clostridial activity in a case. Comparison of organisms visualised with organisms isolated by bacteriologic culture is useful in determining the effectiveness of the latter.

Cells of *C. perfringens* stain as Gram-Positive-to-Gram-variable, short, plump rods with blunt ends ("box cars"). Staining of *C. septicum* may be uneven, regardless of the age of the culture. Cells are straight-to-curved rods, occurring singly or in pairs, but may also be pleomorphic, especially in lesions. Spores are oval, subterminal, and swell the cell.

The fluorescent antibody test is a useful direct, rapid differential aid for diagnosis of abomasitis. Conjugates are specific for myonecrosis agents, including *C. chauvoei, C. novyi, C. septicum*, and *C. sordellii*, but unfortunately none are routinely available for detection of *C. perfringens*. Nonetheless, a positive fluorescence assay for *C. septicum* in tissues from a freshly-necropsied clinical case is strongly suggestive of aetiology.

4.3.4.2. Toxin detection

Culture supernatant fluids or eluates from gut contents (trypsin-treated or untreated, neat or mixed with antiserum) may be examined for *C. perfringens* toxins, either in mice (injected IV) for lethality or guinea pigs (injected ID) for dermonecrosis. Toxin detection strongly suggests the existence of disease, but lability of these proteins, especially β toxin, may cause false negatives.

However, demonstration of toxins by *in vivo* assay has become less common, due to expense, variability of results and humanitarian concerns. Immunoassays have been developed for detection of toxins produced by *C. perfringens*, and the most common of these are enzyme immunoassays. These commercially available assays are quite helpful in establishing a diagnosis.

Detection of *C. septicum* α toxin is not a routine part of the diagnostic protocol.

4.3.4.3. Bacterial quantitation

Bacterial quantitation may be useful in establishing a diagnosis, but relative numbers (relative to “normal” animals or animals affected by other syndromes), rather than actual counts, are usually sufficient. This is more likely to provide practical information in, for example, type A enteritis in piglets (where lesions are

minimal) than in cases (bovine or ovine abomasitis) dominated by severe clinical signs and lesions.

4.3.4.4. Isolation and identification

Methods for isolation of enteric clostridia are as diverse as the veterinary microbiologists themselves. However, many would inoculate a pre-reduced, non-selective, blood-based agar medium. Increasing the agar content to 2% and supplementing with phenyl-ethanol help prevent swarming. Incubation is at 37 °C in an oxygen-free atmosphere, usually with hydrogen, nitrogen, and carbon dioxide. Presumptive identification of *C. perfringens* on this medium can often be made from its colony morphology (circular-to-stellate and smooth) and from the presence of a distinctive double zone of haemolysis. The inner zone of complete β-haemolysis is not always present, in that about 2% of isolates do not produce θ toxin, but strains failing to produce the outer zone of incomplete haemolysis (an effect of α toxin) are rare-to-non-existent. Growth of *C. perfringens* on egg yolk agar is characterised by lecithinase and lipase activity.

Clostridium perfringens and *Clostridium septicum* are likely to be aetiologic agents if they predominate in specimens obtained soon after death or euthanasia. Colonies of *C. septicum* are circular, 1–5 mm in diameter, and have rhizoid-to-irregular margins. They are slightly raised, translucent, grey, glossy and β-haemolytic. Many strains cultivated on media without phenyl-ethanol produce an almost invisible film over the entire agar surface due to swarming.

PCR genotyping can be a useful diagnostic tool in *C. perfringens* infections. Genes for α, β, ε, ι, and β2 toxins, as well as enterotoxin, can be detected in a multiplex assay; the correlation between major toxin phenotype and genotype approaches 100%, although silent enterotoxin and β2 toxin (apparently) genes have been reported.

If isolates of genotype C are obtained from animals with typical lesions in non- vaccinated herds, diagnosis is unequivocal. Diagnosis of genotype A infections is more complex, but if clinical signs and lesions are compatible and, if other aetiologies are ruled out by concurrent microbiologic examination, a diagnosis of type A enteritis should be considered.

4.3.4.5. Interpretation criteria for lab results

The major stumbling block in diagnosis of clostridial abomasitis is answering the question of aetiologic agent versus post-mortem invader. This is especially an issue with *C. septicum*. Thus, diagnostic data should be interpreted soberly, allowing for the possibility of alternative aetiologies and isolation of clostridia as a red herring.

4.4. Bibliography

Ballard J., Sokolov Y., Yuan W.L., Kagan B.L. and Tweten R.K. (1993). Activation and mechanism of *Clostridium septicum* alpha toxin. *Mol. Microbiol.* **10**, 627-634.

Billington S.J., Wieckowski E., Sarker M., Bueschel D., Songer J.G. and McClane B.A. (1998). *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. *Infect. Immun.* **66**, 4531-4536.

Ellis T.M., Rowe J.B. and Lloyd J.M. (1983). Acute abomasitis due to *Clostridium septicum* infection in experimental sheep. *Aust. Vet. J.* **60**, 308-309.

Eustis S.L. and Bergeland M.E. (1981). Suppurative abomasitis associated with *Clostridium septicum* infection. *J. Am. Vet. Med. Assoc.* **178**, 732-734.

Gordon V.M., Benz R., Fujii K., Leppla S.H. and Tweten R.K. (1997). *Clostridium septicum* alpha-toxin is proteolytically activated by furin. *Infect. Immun*. **65**, 4130-4134.

Meer R.R. and Songer J.G. (1997). Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am. J. Vet. Res.* **58**, 702-705.

Rood J.I. and Cole S.T. (1991). Molecular genetics and pathogenesis of *Clostridium perfringens. Microbiol. Rev.* **55,** 621-648.

Songer JG. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev*. **9**, 216-234.

5. Bovine enteritis and enterotoxaemia

J.G. Songer

It is challenging to separate bovine enteritis and enterotoxaemia into distinct syndromes, although the latter term seems to be reserved for cases in which sudden death is the hallmark.

Table 5.1: *Clostridium* and intestinal diseases in cattle

Species	Clinical entity	Host
perfringens type A	Enteritis and enterotoxaemia	Neonates and young calves
perfringens type B	Enterotoxaemia with necrotic/haemorrhagic enteritis, sudden death	Neonates
perfringens type C	Enterotoxaemia with necrotic/haemorrhagic enteritis, sudden death	Neonates
perfringens type D	Enteritis, with or without necrotic/haemorrhagic enteritis, sudden death	Young calves
perfringens type E	Haemorrhagic enteritis and sudden death	Young calves
septicum	Haemorrhagic enteritis	Neonates and young calves
sordellii	Possible involvement in enteritis and sudden death	Any age
difficile	Possible involvement in mild enteritis	Neonates and young calves

5.1. *Clostridium perfringens*

Enteritis and enterotoxaemia can be produced in calves by all *C. perfringens* toxin types (Table 5.1). Nevertheless, incidence of each toxin type can vary widely geographically (even within Europe) and some regions can be free of some of them. Cattle breed sensibility variations may also exist.

Information about distribution of *C. perfringens* genotypes is accumulating as more diagnostic and research laboratories adopt PCR-based methods for toxin gene detection. There is evident disparity in prevalence of the various genotypes by geographic region, although type A remains the most commonly-isolated type overall. In North America, type A predominates, although types C (mainly from neonatal enteritis cases), D (mainly from ovine or caprine enterotoxaemia) and Erom

bovine neonatal hemorrhagic enteritis) are not uncommon. Nearly 97% of isolates from various sources in Belgium were non- enterotoxigenic type A, including all of those from cattle and horses with enterotoxaemia. Type C was limited to swine, type D was isolated from small ruminants with enterotoxaemia, and types B and E were not found. Types C and E were detected in Poland, while in Austria, type A was most common in sheep and type D in goats. Enterotoxaemia in goats, reindeer, and blackbuck were caused by type E. Type C was found only in swine. In Northern Greece, type B is apparently most prevalent in lambs (nearly 50% of cases of lamb dysentery), although type C accounted for 20% and type D 28% of all isolates. Types A and E were not found.

5.1.1. *C. perfringens* type A (α toxin)

Toxins produced by type A strains (e.g., α and θ toxins) are systemically lethal under experimental conditions, and it is tempting to speculate as to a systemic role for these toxins in intestinal infection. However, unlike disease caused by types B, C, and D, there is little or no direct evidence that toxaemia occurs in animals experiencing sudden death due to type A infection.

Type A-associated enteritis is more common in beef and dairy calves, including those raised for veal, than in lambs. Feeding of icy colostrum in large volumes is a risk factor, especially if contaminated. Many affected calves are tympanic, depressed and lack appetite. The clinical course is seldom more than 12 hours and gross lesions consist typically of haemorrhagic gastroenteritis. The abomasum is often dilated and has emphysematous walls and folds. Haemorrhagic lesions may extend from abomasum into caecum and proximal colon. Microscopic examination reveals severe necrosis and haemorrhage, with micro-colonies of Gram-Positive rod-shaped bacteria and emphysema in mucosa and submucosa. As in the disease in lambs, this syndrome seems to be centred in the small intestine, which yields heavy growth of *C. perfringens* upon bacteriologic culture.

Enterotoxaemia is more common in small ruminants than in cattle. Nevertheless, Belgian Blue calves seem particularly prone to the “enterotoxémie” form of the disease, and > 10,000 die annually in Belgium. Affected animals experience haemorrhagic enteritis, with a high case fatality rate, and sudden death is common. Lesions may extend into the colon. Microscopic examination of affected tissues reveals necrosis of the villous tips, enterocytes, and the vascular axis of villi.

Type A *C. perfringens* aetiology of this form of enterotoxaemia has not been confirmed by fulfilment of Koch’s postulates, although inoculation of calves with pure cultures inconsistently reproduces enteritis. Numbers of *C. perfringens* in affected tissues (10^7–10^8 colony-forming units per gram of mucosa) is significantly higher in typically affected calves than in controls, although there may be no useful difference in counts on individual animals. When lesions are localised, numbers are substantially higher in lesions than in adjacent normal tissue. Thus, it may be that *C. perfringens*, normally present in small numbers in the small intestine, multiplies to higher numbers when conditions allow. Alteration of flora by sudden dietary change may also be an inciting factor.

5.1.2. *C. perfringens* type B (α, β and ε toxins)

Type B is best-known as the cause of lamb dysentery, but it also infects calves. They develop inappetence, abdominal pain, and bloody diarrhoea, and become recumbent and comatose. This is a true enterotoxaemia, and both β and ε toxins are demonstrable in the gut and in effusions throughout the body. Case fatality rates approach 100% and sudden deaths are common. Gross lesions include extensive haemorrhage and ulceration of the small intestine, and devastating haemorrhagic necrosis is observed microscopically.

Older lambs may develop a chronic condition called "pine," in which infection of the abomasum and intestine manifests as chronic abdominal pain without diarrhoea. This is apparently quite rare in calves.

5.1.3. *C. perfringens* type C (α, β toxins)

Newborn animals are typically most susceptible to infections by *C. perfringens* type C, perhaps due to ready colonisation of the gut in the absence of established normal intestinal flora. Alteration of flora by sudden dietary change may also be an inciting factor.

Infection of calves by type C may be sporadic, affecting only occasional calves in vaccinated herds. However, morbidity rates can reach 50%, with case fatality rates of 100% in non-vaccinated herds. This is perhaps most common in very young calves, where lack of normal flora provides unoccupied niches for multiplication of type C and production of β toxin. These animals often experience peracute disease, with diarrhoea and dysentery. Calves infected near the end of the neonatal period or later may have a longer clinical course and lower mortality rate, but seldom recover to normal production. Intestinal lesions are extensive and severe, but death may be due ultimately to β toxaemia. Tetany and opisthotonos are not uncommon. As in type B disease, hallmark signs are haemorrhagic necrosis affecting mucosa, submucosa, and muscularis mucosa of jejunum and ileum, and emphysema is also commonly observed in these tissues. The proximal colon may be affected in some cases.

5.1.4. *C. perfringens* type D (α, ε toxins)

Enterotoxaemia in suckling calves often follows dietary change. If a calf is denied access to the dam and then overfeeds when access is re-established, undigested nutrients entering the small intestine can stimulate rapid growth of type D. ε toxin has permease-like activity, facilitating its own absorption, and producing toxaemia, often with minimal enteritic lesions. Effects of ε toxin on the CNS and other tissues cause sudden death, preceded in some cases by clinical signs such as opisthotonos and convulsions. Focal encephalomalacia, which is apparently a chronic manifestation of enterotoxaemia in sheep, is uncommon in calves. Disease occurs rarely in adult cattle.

5.1.5. *C. perfringens* type E (α, ι toxins)

Dogma has held that type E enterotoxaemia in calves is rare. However, strains of type E comprise 3–5% of isolates from domestic animals with enteritis, and perhaps as many as 50% of isolates from calves experiencing haemorrhagic enteritis and sudden death in North America. Lesions are very similar to those produced by types A, B, and C in calves.

Strains of type E produce ι toxin, the A component of which is ADP-ribosylates actin. Little is known about pathogenesis, but ι toxin is assumed to play an important role. The enterotoxin gene, *cpe*, is detected routinely in type E isolates, but is silent.

5.1.6. Enterotoxigenic *C. perfringens*

Enterotoxigenic strains of *C. perfringens* have been detected in many species of animals, including cattle, but there is no direct evidence of a role for enterotoxin in bovine enteric disease.

5.2. *Clostridium sordellii* and *Clostridium difficile*

5.2.1. *Clostridium sordellii*

Clostridium sordellii is a common intestinal organism in cattle, and is not uncommonly a cause of myositis and sudden death. Large numbers of urease-negative strains have been found (in the company of large numbers of *C. perfringens* type A) in the intestines of 20% of Belgian Blue calves experiencing "sudden death syndrome". Curiously, these strains were non-toxigenic (lacking major toxin genes). Enteritis has been experimentally produced with toxigenic strains of the organism.

Two toxins, one haemolytic and one lethal, are antigenically and pathophysiologically similar to *C. difficile* toxins A and B, respectively. Like those toxins, *Clostridium sordellii* lethal toxin (TcsL) glucosylates Rac and Ral. But this toxin differs from other large clostridial toxins in its glucosylation of Ras.

It is assumed that these toxins play a role in pathogenesis.

5.2.2. *Clostridium difficile*

Clostridium difficile has been isolated from calves with diarrhoea, and inoculation with either purified toxins or spores results in mild diarrhoea. However, there is insufficient information at present to suggest that infection by this organism is an important cause of neonatal enteritis in calves.

5.3. Diagnosis

Diagnosis can be based upon clinical signs, gross and microscopic findings at necropsy, Gram- and fluorescent antibody staining of direct smears, bacteriologic culture, and, in the case of *C. perfringens*, typing of isolates.

5.3.1. Clinical and necropsy findings

5.3.1.1. Infection by *Clostridium perfringens* types A, B, C, and E

Affected animals may experience peracute death, or may have a brief clinical course with abdominal pain, hemorrhagic diarrhoea, and central nervous system signs. At necropsy, lesions may be seen in the abomasum, but the most dramatic are haemorrhagic necrosis, emphysema, and oedema in the small intestine. These may extend into the proximal colon.

5.3.1.2. Infection by *Clostridium perfringens* type D

Type D enterotoxaemia may or may not be accompanied by haemorrhagic enteritis. It is not unusual for calves to experience sudden death, or central nervous system signs such as opisthotonos, and have no remarkable changes in the small intestine. In others, the clinical picture is more like that in goats, with chronic fibrinous or haemorrhagic diarrhoea. In either case, if lesions are present at necropsy, they resemble those in infections by types A, B, C, and E.

5.3.1.3. Infection by *Clostridium sordellii* and *C. difficile*

Experimental infection with *C. sordellii* results in passage of soft faeces containing blood and mucus within a week after inoculation. Mild inflammatory changes have been reported. Culture filtrate produces similar clinical and pathological changes, with mild congestion and inflammation in jejunal and ileal mucosa and dilation of crypts and capillaries in colonic mucosa.

Gross lesions are apparently absent in *C. difficile* infection in calves. Animals infected experimentally or exposed to toxins A or B experience diarrhoea, but with only very mild inflammatory change in the small intestine and colon.

5.3.2. Sampling procedures

Tissues should be preserved in buffered formalin for histopathologic examination. A portion of affected intestine should be collected fresh for direct examination and bacteriologic culture. Contents, especially in the small intestine, can be preserved in a separate, sterile container or *ca* 15 cm of intestine can be ligated at each end for transport to the laboratory.

Due to the proclivity of these organisms for post-mortem invasion, euthanasia and necropsy is much preferable to examination of cadavers, especially if the interval since death is > 4 h.

5.3.3. Transport

Fresh tissues should be transported to the laboratory as rapidly as possible and may be shipped on wet ice. Freezing may reduce the titre of organisms and toxin present, but is an acceptable alternative if shipping must be delayed for more than 24 h.

5.3.4. Laboratory aspects of diagnosis

5.3.4.1. Direct smears

Gram-staining of direct smears from affected tissues is often useful in providing an indication of clostridial involvement. Comparison of organisms visualised with organisms isolated by bacteriologic culture is useful in determining the effectiveness of the latter.

Cells of *C. perfringens* stain as Gram-Positive-to-Gram-variable, short, plump rods with blunt ends ("box cars"). Cells are straight-to-curved rods, occurring singly or in pairs, but may also be pleomorphic, especially in lesions. Spores are oval, subterminal, and swell the cell.

5.3.4.2. Toxin detection

Culture supernatant fluids or eluates from gut contents (trypsin-treated or untreated, neat or mixed with antiserum) may be examined for *C. perfringens* toxins, either in mice (intravenously injected) for lethality or guinea pigs (intradermically injected) for dermonecrosis. Toxin detection strongly suggests the existence of disease, but lability of these proteins, especially β toxin, may cause false negatives.

However, demonstration of toxins by *in vivo* assay has become less common, due to expense, variability of results, and humanitarian concerns. Enzyme-immunoassays have been developed for detection of toxins produced by *C. perfringens*.

Detection of *C. difficile* toxins is also possible by immunoassays and detection of toxins in rectal swab samples or colonic contents is suggestive of toxin action in the gut. Tests for detection of *C. sordellii* toxins are not in routine diagnostic use.

5.3.4.3. Bacterial quantitation

Bacterial quantitation in the small intestine may be useful in establishing a diagnosis, but numbers (relative to “normal” animals or animals affected by other syndromes), rather than actual counts, are usually sufficient. This is more likely to provide practical information in, for example, type A enteritis in piglets (where lesions are minimal) than in calf disease, which is dominated by severe clinical signs and lesions.

5.3.4.4. Isolation and identification

Methods for isolation of enteric clostridia are as diverse as the veterinary microbiologists themselves. However, many would inoculate a pre-reduced, non-selective, blood-based agar medium. Increasing the agar content to 2% and supplementing with phenyl-ethanol both help prevent swarming. Incubation is at 37°C in an oxygen-free atmosphere, usually with hydrogen, nitrogen, and carbon dioxide. Presumptive identification of *C. perfringens* on this medium can often be made from its colony morphology (circular-to-stellate and smooth) and from the presence of a distinctive double zone of haemolysis.

The inner zone of complete β-haemolysis is not always present, in that about 2% of isolates do not produce θ toxin, but strains failing to produce the outer zone of incomplete haemolysis (an effect of α toxin) are rare-to-non-existent. Growth of *C. perfringens* on egg yolk agar is characterised by lecithinase and lipase activity. Isolation of *C. difficile* is less likely to be meaningful, in that it is often found in the absence of disease.

5.3.4.5. Interpretation criteria for lab results

The major stumbling block in diagnosis of clostridial abomasitis is answering the question of aetiologic agent versus post-mortem invader. Thus, diagnostic data should be interpreted soberly, allowing for the possibility of alternative aetiologies and isolation of clostridia as a red herring.

Diagnosis of infection by *C. sordellii* or *C. difficile* in cattle is equivocal at best. Isolation of *C. sordellii* raises the *possibility* of infection, given the experimental infection of calves with this organism. However, it is important to note that isolates from the bovine intestine are often incapable of producing the large clostridial toxins, and might not be expected to be virulent.

Clostridium difficile-associated disease has not been produced experimentally in calves. Thus, detection of the organism has relatively little meaning. Toxin detection is the gold standard for diagnosis and, since inoculation with purified toxins produces an effect in the calf gut, one might expect toxin positivity to indicate toxin action in the gut. This will require further study by both diagnosticians and researchers..

5.4. Bibliography

Al-Mashat R.R. and Taylor D.J. (1983). Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of *Clostridium sordellii*. *Vet. Rec.* **112**, 141–146.

Al-Mashat R.R. and Taylor D.J. (1983). Bacteria in enteric lesions of cattle. *Vet. Rec.* **112**, 5–10.

Billington S.J., Wieckowski E., Sarker M., Bueschel D., Songer J.G. and McClane B.A. (1998). *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. *Infect. Immun.* **66**, 4531–4536.

Daube G., China B., Simon P., Hvala K. and Mainil, J. (1994). Typing of *Clostridium perfringens* by in vitro amplification of toxin genes. *J. Appl. Bacteriol.* **77**, 650–655.

Garmory H.S., Chanter N., French N.P., Bueschel D., Songer J.G. and Titball R.W. (2000). Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol. Infect.* **124**, 61–67.

Gibert M., Jolivet-Renaud C. and Popoff M.R. (1997). Beta2 toxin, a novel toxin produced by *Clostridium perfingens*. *Gene* **203**, 65–73.

Manteca C., Daube G., Pirson V., Limbourg B., Kaeckenbeeck A. and Mainil J.G. (2001). Bacterial intestinal flora associated with enterotoxaemia in Belgian Blue calves. *Vet. Microbiol.* **81**, 21–32.

Meer R.R. and Songer J.G. (1997). Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am. J. Vet. Res.* **58**, 702–705.

Rood J.I. and Cole S.T. (1991). Molecular genetics and pathogenesis of *Clostridium perfringens. Microbiol. Rev.* **55,** 621–648.

Songer J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev.* **9**, 216–234.

6. Enteritis and enterotoxaemia in small ruminants

E. Bourtzi-Hatzopoulou, E. Petridou and A. Minas

6.1. Introduction

Clostridial enterotoxaemia is an acute, highly fatal infectious disease in animals. The clostridial group of diseases is caused by various toxin types of *C. perfringens.* In parts of Europe, especially in some Mediterranean countries, enterotoxaemia is the most common clostridial disease affecting sheep. Types B, C and D are of major importance to sheep and goats, while the role of type A enterotoxaemia in small ruminants is not clear. In small ruminants, *C. perfringens* types B, C and D cause lamb dysentery, struck and pulpy kidney disease respectively, while type E is not implicated.

6.2. Type A enterotoxaemia

Type A, which only produces the α toxin, is distributed worldwide and is well adapted to survive in the soil, as well as in the alimentary tract of animals and humans (Aiello, 1998). It is commonly found as part of the normal intestinal microflora of lambs and kids (Songer, 1996). Regarding the involvement of type A in small ruminant enterotoxaemia, some researchers believe that it is occasionally pathogenic, while its role in disease production is considered doubtful by others (Niilo, 1980). Recently, a survey investigating lamb dysentery in Greece revealed that *C. perfringens* type A was not isolated to diseased lambs, but was also present in the intestinal tract of healthy animals (Gkiourtzidis *et al*., 2001). It is well known that this toxigenic type is responsible for haemolytic disease in enterotoxaemic lambs, "yellow lamb disease", which has been described in the USA (Songer, 1998), but is unknown in most parts of the world.

6.3. Lamb dysentery

C. perfringens type B causes dysentery in lambs and kids. The disease is a severe and often fatal haemorrhagic enteritis, affecting these animals during the first 3–4 days of their life, although it may also affect older lambs and kids up to 2–3 weeks old. Because of the age of the affected animals, the assumption is that the micro-organism is usually acquired at birth from contaminated udder teats or from the hands of the farm labourer who looks after the animals (Martin and Aitken, 1999). The mixture of ingested virulent bacteria and large amounts of milk in the stomach favours multiplication of bacteria, which adhere to the small intestinal mucosa and produce large quantities of β toxin. The high susceptibility of this age group may be explained by the absence of microbial competition, low proteolytic activity in the neonatal intestine and low levels of pancreatic secretion (Biberstein and Hirsh, 1999). It is well known that β toxin is highly sensitive to the presence of proteolytic enzymes (trypsin) and this susceptibility partly explains why the disease

targets newborns. In the absence of trypsin (colostrum contains antitrypsin substances), β toxin retains its potency and leads to the onset of the disease (Radostits *et al*., 2000).

Outbreaks occur mainly towards the end of the lambing and kidding period. Sudden death without symptoms is the most frequent appearance of the disease in 1–4 day old, strong, well-fed lambs and kids. In acute cases, cessation of feeding and severe abdominal pain is accompanied by bloody diarrhoea, with recumbency, coma and death (Swift *et al.*, 1988). Incidence may be as high as 30%. Kids are more susceptible than lambs and their mortality can reach 100%. Death usually occurs 2–12 hours after the onset of symptoms.

In post-mortem examination, severe haemorrhagic enteritis affecting parts of the ileum, with blood-tinged fluid in the peritoneal cavity and pericardial sac are the major findings. Ulceration of congested intestinal mucosa may also be present. The stomach contains clotted milk, while petechiae can be found in the stomach mucosa (Timoney *et al.*, 1988).

In older lambs and kids, aged 2–3 weeks, the disease is characterised by listlessness, unwillingness to suckle and abdominal pain followed by foetid diarrhoea. The most prominent lesions are those of extensive haemorrhagic enteritis. The intestinal mucosa is congested and ulceration and necrosis may be present in the intestinal walls. The intestinal lumen and peritoneal cavity often contain haemorrhagic fluid. These animals survive for a longer period, 2–3 days (Swift *et al.*, 1988).

C. perfringens type C causes haemorrhagic enteritis in newborn lambs and kids worldwide. Beta toxin is also the major pathogenic factor here. Under favourable conditions, clostridia proliferate in the intestinal tract, leading to production of large quantities of toxin. Outbreaks are sporadic, affecting mostly strong, well-nourished lambs and kids younger than 3 weeks of age. Symptoms and lesions resemble those seen in type B lamb dysentery (Biberstein and Hirsh, 1999). Death occurs within hours of onset of the disease. Sudden death may occur without the observance of bloody diarrhoea or other symptoms. At necropsy, generalised haemorrhagic enteritis is present, but lesions are commonly confined to the jejunum and ileum. Distinct necrotic ulcers are not a feature. In severe cases, free blood may be present in the lumen of the intestine. Mesenteric lymph nodes are oedematous and sometimes haemorrhagic (Martin and Aitken, 1999

6.4. Struck

The disease in adult sheep, caused by *C. perfringens* type C, is known as "struck" because of the rapid and sudden death of the affected animals. Struck is well known in defined regions in different countries and usually occurs in adult sheep and goats at pasture, especially during the spring. Bacteria are ingested from the soil or may be present in small numbers in the intestinal tract of normal animals. Predisposing factors, such as a sudden dietary change, influence rapid multiplication of the micro-organism in the intestinal tract and lead to production of large quantities

of β toxin. Beta toxin plays a major role in the pathogenesis of the disease. As with other enterotoxaemiae, animals in good condition are most likely to be affected. The peracute form of struck manifests as sudden death, although some animals may be found in terminal convulsions. If found alive and suffering from the acute form, sheep are dull, reluctant to be moved and lame. Losses are usually sporadic (Radostits *et al.*, 2000).

Post-mortem findings are often absent. Haemorrhagic enteritis affects the jejunum and ileum, but few ulcers are present. Most often, patchy hyperaemia in the small intestine, and pericardial and thoracic effusion are present. Accumulation of fluid in the peritoneal cavity, along with congestion of peritoneal vessels and local haemorrhages, may be found (Kimberling, 1988).

6.5. Pulpy kidney disease

Type D enterotoxaemia (pulpy kidney disease) represents the most common appearance of enterotoxaemia in sheep and goats and has a worldwide distribution. Affected animals are usually in very good condition and well-nourished. Pulpy kidney disease occurs in all breeds and in either sex, but is usually encountered in 4–10 week old lambs and/or in lambs older than 6 months. Adults may be affected sporadically, but losses can be high. In most areas of Greece, pulpy kidney disease is the most commonly diagnosed type of clostridial disease (Papadopoulos, 2000).

The vast majority of cases of enterotoxaemiae appear when animals change their nutritional pattern. Pulpy kidney disease may occur during any season, but the highest incidence comes during the months following weaning, when fattening takes place. In some countries, most cases of the disease are diagnosed during the summer when sheep are grazing in harvested wheat fields. During this period, animals consume wheat straw and a large amount of wheat grain, which favours the multiplication of clostridia and produces high levels of ε protoxin. This process requires activation by trypsin. The high concentration of ε toxin slows intestinal motility, increases the permeability of the intestinal wall to toxins and aids the passage of toxin into the blood. Once in the blood, the toxin produces generalised toxaemia and leads to development of clinical signs (Quinn *et al.*, 2002).

In most cases, the disease is peracute and the animals are found dead. Some individuals, in subacute cases, may exhibit neurological signs, such as ataxia, blindness or convulsions just prior to death. Hyperglycaemia, together with glucosuria, is a typical finding of the disease. Gross lesions are variable but, in peracute cases, may be absent (Timoney *et al.*, 1988). These animals often have the rumen and abomasum filled with concentrated feed. The only post-mortem finding may be scattered hyperaemic areas in the intestines, especially in the ileum. The pericardial sac is often distended, with pale yellow or straw-coloured fluid. In addition, pulmonary oedema is common. Both pericardial and endocardial haemorrhage are present. ε toxin contributes to rapid autolysis of the kidneys, which is often the only reliable indication of the disease in animals necropsied within a few hours of death. Diagnosis can be established by histology of the brain, where focal

symmetrical encephalomalacia and symmetrical haemorrhagic lesions in the basal ganglia and midbrain may be present (Buxton *et al.*, 1978).

Pulpy kidney disease is more frequent and severe in sheep than in goats because of their differing nutritional habits. The onset of neurological signs, followed by sudden death, is more common in sheep. Goats are commonly affected with haemorrhagic enterocolitis. The chronic form of pulpy kidney disease occurs more often in goats, and these animals are more likely to show signs of diarrhoea before death (Uzal and Kelly, 1996).

6.6. Diagnosis

In most veterinary laboratories, routine diagnosis of lamb dysentery and enterotoxaemia in small ruminants is based on evaluation and interpretation of the following: clinical signs, gross and microscopic lesions, microbiological examination of appropriate specimens and the detection of toxin in gut content and/or in the bloodstream.

6.6.1. Clinical and necropsy findings

The criteria for clinical and necropsy diagnosis of the disease have already been described. In most cases, farmers are alarmed by the sudden death of newborn or adult animals. In order to avoid post-mortem invasion, liver, spleen and small intestine specimens (duodenum) are taken rapidly from those recently dead or euthanised animals exhibiting clinical symptoms of the disease. 10–20 ml of gut content is collected in a universal container (to which 1–2 drops of chloroform may be added), and is submitted to a laboratory where an ELISA or mouse neutralisation test can be performed (Matthews, 1999).

6.6.2. Laboratory aspects of diagnosis

The results of bacteriological culture must be interpreted with caution because *C. perfringens* strains are present in the gut of apparently healthy animals and can penetrate tissues of cadavers within a few hours of death (Quinn *et al*, 1994). Enumeration of *C. perfringens* from small intestine mucosa or content (the presence of an average of 10–12 bacteria per optical immersion field is a significant finding), can be an indicator of the presence of disease (Gkiourtzidis *et al*., 2001). Enumeration is achieved by smears with direct microscopy and/or culture in selective media, or by serial ten-fold dilutions of gut content from recently dead animals. Detection of one of the major toxins of *C. perfringens* can be a useful diagnostic finding, but does not necessary confirm the existence of disease, since toxins can be found in small quantities in the gut of apparently healthy adult animals.

The traditional mouse or guinea pig toxin neutralisation test is sensitive for the detection of α, β and ε toxins, but β may be destroyed if there is a delay with small intestinal contents reaching the laboratory. Failure to demonstrate the presence of toxin in the intestinal centrifuged supernatant (ileal) does not necessarily exclude a diagnosis of clostridial enterotoxaemia. Until recently, the mouse or guinea pig test

was widely used to demonstrate and identify major toxins in the small intestine, in the bloodstream and/or in the supernatant of *C. perfringens* culture. This method still remains the most valuable, but has now become less common on grounds of animal welfare, variability of results and high cost (Songer, 1996).

ELISAs can be used as an alternative to *in vivo* assays for demonstrating toxin in the same fluids. The sensitivity and specificity of ELISA for detection of *C. perfringens* toxins almost match those of mouse or guinea pig neutralisation methods. Failure to demonstrate toxins by ELISA may be as significant as by *in vivo* assay because of the lability of these toxins in the presence of proteases (Biberstein and Hirsh, 1999).

Polymerase chain reaction (PCR) can be employed to detect gene coding for the four major toxins (α, β, ε and ι toxins), for the recently described β2 toxin (Gkiourtzidis *et al*., 2001), and for enterotoxin.

6.7. Bibliography

Aiello S.E. (1998). Clostridial diseases. In: *Veterinary Manual*, 8th ed., Merck Publishing Group, Rahway, NJ, USA, p 447

Biberstein E.L. and Hirsh D.C. (1999). The clostridia. In: *Veterinary Microbiology*. (Hirsh D.C. and Zee Y.C., Eds), pp 233-245, Blackwell Science, Philadelphia, USA.

Buxton D., Linklater K. and Dyson D. (1978) Pulpy kidney disease and its diagnosis by histological examination. *Vet. Rec*. **102**, 241

Gkiourtzidis K., Frey J., Bourtzi-Hatzopoulou E., Iliadis N. and Sarris K. (2001) PCR detection and prevalence of α-, β-, β2-, ε-, ι- and enterotoxin genes in *Clostridium perfringens* isolated from lambs with clostridial dysentery. *Vet. Microbiol*. **82,** 39-43

Kimberling C.V. (1988). Jensen and Swift's diseases of sheep, 3rd ed., Lea and Febiger, Philadelphia, USA

Martin W.B. and Aitken I.D. (1999). Diseases of sheep. 3rd ed., pp 133-136, Blackwell Science, Philadelphia, USA.

Matthews J. (1999). Colic. In: *Diseases of the goat*. 2nd ed., p 226, Blackwell Science, England.

Niilo L. (1980) *Clostridium perfringens* in animal disease: a review of current knowledge. *Can. Vet. J.* **21**, 141-148

Papadopoulos O. (2000) *Infectious Diseases of Animals*, Aristotle University Press Services, Thessaloniki, Greece

Quinn P.J., Markey B.K., Carter M.E., Donnelly W.J. and Leonard F.C. (2002) In: *Veterinary Microbiology and Microbial Disease*, pp 91-94, Iowa Statte University Press, Ames, USA.

Quinn P.J., Carter M.E., Markey B.K. and Carter G.R. (1994). *Clostridium* species. In: *Clinical Veterinary Microbiology,* pp 191-208, Wolfe Publishing, London, UK.

Radostits O.M., Gay C.C., Blood D.C. and Hinchcliff K.W. (2000) Enterotoxemia caused by *Clostridium perfringens* types B, C and E. In: *Veterinary Medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses.* 9th ed., pp 770, WB Saunders, London, UK.

Songer J.G. (1996) Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev.* **9**, 216-234.

Songer J.G. (1998) Clostridial diseases of small ruminants. *Vet. Res.* **29**, 219-232

Swift B., Kimberling C. and Jensen R. (1988) Diseases of nursing lambs In: *Diseases of sheep* 3rd ed., p73, Lea and Febiger, Philadelphia, USA.

Timoney J.F., Gillespie J.H., Scott F.W. and Barlough J.E. (1988) Hagan and Bruner's microbiology and infectious diseases of domestic animal, 8th ed., Comstock Publishing Associates, Ithaca, N.Y., USA.

Uzal F.A., Kelly W.R. (1996) Enterotoxaemia in goats, *Vet. Res. Commun.* **20**, 481-492.

7. Enteritis and enterotoxaemia in pigs

D.J. Taylor

7.1. Introduction

Clostridia can cause enteric disease in pigs which is recognisable from clinical or pathological observations by the veterinarian and the farmer. Clostridial enteritis and enterotoxaemia occur principally in the sucking pig as recognisable syndromes, but these organisms can cause disease in older pigs, where the clinical signs are less easily identifiable. In addition to the specific syndromes described below, the existence of clostridia in the gut flora throughout life means that the organisms can respond rapidly to any change in enteric environment, colonise an enteric lesion and contribute to the clinical signs and pathology. Conditions in which clostridia colonise lesions are difficult to diagnose from clinical signs or pathology alone as they depend on the initiating disease and the presence of the organisms is usually confirmed by laboratory means such as histopathology and bacterial isolation. Finally, there is a group of conditions in the older weaned pig and in adults where sudden death occurs, associated with the presence of inflamed intestines. The frequent presence of clostridia in the intestinal contents of these animals may be related to the cause, but the sudden onset and rapid post-mortem change make their involvement subject to speculation. Experimental reproduction of this type of syndrome is difficult and, until it can be done repeatedly, the role of clostridia in these syndromes remains a matter of debate.

7.2. Defined syndromes in the sucking pig

Syndromes caused by *Clostridum perfringens* type A, type C and/or type B and *C. difficile* can be distinguished in the sucking pig. In all cases the identification of the syndrome on the ground is based on a combination of time of occurrence in the life of the pig, clinical signs and pathology. Confirmation is by demonstration of the agent and its toxins and is not considered here. Of these syndromes, *C. perfringens* type C is the most easily diagnosed on the ground. The presence of β2 toxin genes in *C. perfringens* strains involved in diarrhoea cannot yet be associated with any specific syndrome.

7.2.1. *Clostridium perfringens* type C enterotoxaemia

Clostridium perfringens type C produces a fatal necrotic and haemorrhagic enteritis in piglets under 7 days of age and may cause chronic infection in older piglets (Mackinnon, 1989). It occurs in most pig-keeping countries. Outbreaks often follow the introduction of infected breeding stock and disease persists in herds for up

to 2 months, but, where new stock is constantly introduced, outbreaks may continue for up to 15 months. Farrowing houses or areas may become heavily contaminated. Transmission of vegetative organisms occurs through faeces. Other animal species may be infected and may theoretically represent a source of infection for pigs.

Typically, three or four litters or part of a litter in a herd may be affected by severe disease but up to 50 litters have been reported to be affected in some outbreaks. Herds may be infected with the organism but typical disease may be absent. In some cases this results from early treatment with antimicrobials as treatment or prevention, but most commonly it results from the increasing practice of including *C. perfringens* type C toxoid in vaccines given to sows to prevent piglet diarrhoea (Bergeland, 1972; Ripley and Gush, 1983). Where protective antibody is present in the colostrum at adequate levels, no disease will be seen. Where levels are inadequate or intake is insufficient, the clinical signs may develop slightly later and be mild and difficult to recognise. When individual pigs have not been protected and develop the disease, its occurrence in these isolated animals may not be noticed.

7.2.1.1. Clinical signs

Affected pigs are normal at birth and may sicken on the first, or usually, the second day of life (36 hours after birth) and usually die within 12-24 hours of the onset of clinical signs. High mortality rates and the passage of reddish faeces are always suggestive of the presence of *C. perfringens* type C enterotoxaemia. A dramatic, profuse diarrhoea occurs and rapidly becomes claret-coloured. The hindquarters may be soiled with bloody faeces, but in some cases, the heat of creep lamps dries the faeces and the presence of diarrhoea may be overlooked. Affected piglets become weak, collapse and die. Piglets about to die may have greenish discolouration of the abdomen, subnormal temperatures or severe blood-stained diarrhoea. Some may be found dead without clinical signs being observed. Where acute disease is present, all members of the litter usually die.

In more chronic cases, shreds of necrotic material may appear in the reddish-brown watery faeces and chronically-affected animals become very thin and pale before death. Depression of growth rate may be a feature in chronically-infected piglets which do not die and they may remain stunted for some days after clinical recovery.

7.2.1.2. Pathology

Piglets which have died suddenly from *C. perfringens* type C enterotoxaemia are often in good condition and there may be no obvious presence of faeces on the perineum. Insertion of a probe or gentle pressure may allow faeces to be extruded. There are usually diarrhoeic and reddish in colour. There may be greenish discolouration of the abdomen, but the most obvious post-mortem finding is haemorrhagic enteritis in the jejunum.. The jejunum of affected piglets is swollen with an angry purplish-red colour and if death has been sudden, is usually full of pasty, blood-stained contents. In more chronic cases, the serosa of the intestine may

be pale and unevenly swollen. A diptheritic layer is present on the mucosa and the contents are watery with diphtheritic material.

7.2.1.3. Laboratory

Samples for further investigations should include unopened affected small intestine for bacteriology and fixed bowel wall for histopathology (Arbuckle, 1972).

Laboratory examination is necessary to confirm the identity of the organism present, as *C. perfringens* type B may also be found in this syndrome and it is not possible to distinguish clinically or pathologically between the two.

7.2.2. *Clostridium perfringens* type A enteritis and enterotoxaemia

C. perfringens type A causes, in piglets, a syndrome resembling that caused by *C. perfringens* type C but much less severe (Estrada Correa and Taylor, 1986; Nabuurs *et al*., 1983; Songer and Glock, 1998; Taylor and Olubunmi, 1982). The disease appears to be present in many countries and to be fairly common where it is recognised. As the organism is ubiquitous, being present in soil, intestinal contents and faeces, the disease could occur at any time, but is most commonly seen affecting some of the piglets in several litters on the same farm.

7.2.2.1. Clinical signs

C. perfringens type A may cause sudden death within 48 hours of infection or birth in non-immune piglets but more commonly causes diarrhoea. The main clinical signs are a pasty diarrhoea, loss of condition, lack of fever and low mortality beginning at 36-48 hours after birth. Affected diarrhoeic sucking piglets appear dull with sunken flanks and evidence of loss of condition. The perineal area is heavily pasted with yellowish faeces. The faeces present on the pen floor frequently contain some mucus but are often creamy or pasty containing flecks of fresh blood or of a pinkish tinge. Marked depression of growth occurs. A transient watery diarrhoea may be seen on some occasions with no mortality. Affected piglets rarely die, but those approaching death have low rectal temperatures and discoloured abdomens.

7.2.2.2. Pathology

The small intestine of dead piglets is often congested and its contents creamy or watery, but not blood-filled. Necrotic areas may be seen on the mucosal surface of the jejunal and ileal mucosa with a dissecting microscope and villous atrophy is obvious. The post-mortem findings do not prove that this is a case of *C. perfringens* type A enteritis. They do, however, distinguish the condition from that caused by Types C and B, as affected animals do not have the intensely reddened small intestine. Few changes, apart from congestion and darkening of the carcase are seen outside the gut. Similar findings may be made in chronically infected piglets. *C. perfringens* may contribute, by these changes, to syndromes initiated by other agents. *C. perfringens* type A may be isolated in profuse culture from the small intestines of animals with enteritis caused by a number of agents such as transmissible

gastroenteritis (T.G.E.), virus, rotavirus, coccidial and cryptosporidial infections. The lesions seen are those of the most important contributor to the syndrome present, but *C. perfringens* type A may contribute villous atrophy and the presence of diphtheritic material to the final lesions.

7.2.2.3. Laboratory

Samples of small intestine should be taken for laboratory diagnosis.

Successful therapy with a penicillin suggests that a *Clostridium* is involved, but confirmation requires the demonstration of *C. perfringens* type A by direct culture or immunofluorescence and the elimination of other causes of similar piglet disease such as Type C disease and coccidial infections.

7.2.3. *Clostridium difficile* typhlocolitis

C. difficile has occasionally been isolated from the intestines of individual pigs with chronic or haemorrhagic diarrhoea, but large-scale outbreaks in piglets in Canada, the USA and France confirm that the organism can cause disease. These outbreaks have enabled the disease to be distinguished clinically and pathologically following post-mortem examination (Songer *et al*., 2000; Waters *et al*., 1998; Waters *et al*., 2003). It is not clear to what extent the condition follows routine treatment of neonatal pigs with antimicrobials, but their use could contribute to the development of this disease.

7.2.3.1. Clinical Signs

The onset of this disease is anywhere between 1 and 7 days after birth. Affected piglets develop dyspnoea, emaciation, mild abdominal distension and scrotal oedema. The presence of abdominal distension and scrotal oedema are unusual and may suggest *C. difficile* disease. Diarrhoea may develop and affected animals may appear dehydrated with sunken eyes and have perineal faecal staining. It can cause preweaning losses of up to 90%, but mortality is usually less than 50%. High mortality rates would also be suspicious, particularly if routine treatments with antimicrobials had been given early in life.

7.2.3.2. Pathology

Piglets which have died from *C. difficile* infection may be dehydrated and have perineal faecal staining. There may be distension of the abdomen. The findings include ascites, with more than 50 ml of fluid being present in the peritoneal cavity, oedema of the mesocolon, hydrothorax and urate deposits in the kidneys. There may be scrotal oedema. Affected piglets have full stomachs. The mesocolic oedema is accompanied by large intestinal lesions including necrosis of the mucosa. There is local exudation of mucus, fibrin and neutrophils into the lumen of the colon and caecum and deep ulcers may give rise to transmural necrosis.

The post-mortem findings provide reinforcement of the provisional diagnosis as ascites, mesocolic oedema and colitis without major small intestinal involvement are unusual in piglets of this age group.

7.2.3.3. Laboratory

Final confirmation of the condition as *C. difficile* infection comes from laboratory demonstration of the organism, its toxin or toxin genes in the large intestine.

The organism can be demonstrated in direct smears of the colonic wall, by isolation on difficile agar and its preformed toxins can be demonstrated in colonic contents by Reverse Passive Agglutination Tests or ELISA and the presence of the B toxin-encoding gene can be detected by PCR. The samples required for this condition are portions of the large intestine rather than the small intestine as in the other conditions.

7.3. Defined syndromes in the weaned pig

The identification of clostridial enteritis and enterotoxaemia in weaned pigs is difficult, but one condition may be suspected on clinical grounds. The occurrence of sudden death associated with volvulus and haemorrhagic enteritis has been mentioned above, but the syndrome associated with *C. perfringens* type A is described here. It is a diarrhoea and loss of condition occurring from weaning onwards for a variable length of time and at different times in animals of the same group. This diarrhoea forms part of the 'colitis' complex of diarrhoeas and enteritides of weaned pigs and its development can be affected by the composition of the ration (Jestin *et al*., 1985), the presence of antimicrobial therapy or growth promoters in the feed and the use of products such as zinc oxide which affect the microbial flora. The recent ban on the use of high concentrations of copper and the forthcoming ban on the use of antimicrobial growth promoters in the European Union are likely to increase the amount of clostridial enteritis and enterotoxaemia in weaned pigs.

7.3.1. *C. perfringens* type A enterotoxaemia in weaned pigs

C. *perfringens* type A is a potential cause of and contributor to the 'colitis' complex of diarrhoeas. These are diarrhoeas of weaned pigs of any age from weaning to slaughter (but particularly from weaning to 40kg), the cause of which is not immediately apparent and which may result in loss of performance. Experimental infection of weaned pigs with enterotoxigenic strains of *C. perfringens* of porcine origin has resulted in a mild, featureless diarrhoea resembling that from which the organisms were isolated. The condition is rarely identified on the ground, largely because the majority of diarrhoeas of this type can be attributed to other agents such as *Brachyspira pilosicoli* or *Salmonella*. The role of *C. perfringens* type A in these diarrhoeas was first identified by Jestin *et al*. (1985) and confirmed by Estrada Correa and Taylor (1988).

The condition is present within individual farms, and transfer from farm to farm may occur with breeding stock. It is more common under unhygienic conditions. There may be an association with feed type and especially with the absence of antimicrobial growth promoters (Taylor and Estrada Correa, 1988) such as avilamycin. The syndrome of colitis has been identified in a number of countries including the UK, France, Belgium, Denmark, The Netherlands, Canada, the U.S. and Eire.

7.3.1.1. Clinical signs

Uncomplicated *C. perfringens* type A disease can be suspected from the clinical signs of greyish diarrhoea or soft motions, and loss of condition in weaned pigs from weaning until about 40 kg in weight. The syndrome is most common in the 8-10 week age group, but can occur later. In weaned pigs immunity is common but non-sporulating strains can induce transient nervous signs and minor faecal changes, sometimes accompanied by loss of condition in experimental infection. Sporulating strains cause a greyish, sometimes mucoid diarrhoea lasting 3-7 days or more after the onset of clinical signs. The faeces may froth and bubble immediately after being taken from the rectum. Inappetance and reduction in weight-gain occur. Loss of condition and faecal staining of the perineum and coat are common. Affected pigs appear hollow-flanked, dirty and restless. Growth rates may be depressed and feed conversion may be affected. Affected pigs are afebrile and do not die of uncomplicated infections.

7.3.1.2. Pathology

The pathology of this condition is only clear when affected animals are killed. Because they rarely die, post-mortem examination is rarely carried out. Affected pigs may be hairy, in poor condition and covered with faeces. In classic 'colitis' only the large intestine is affected, but in this condition the posterior small intestine may be flaccid and contain mucous and bubbles of gas and the large intestines are flaccid and their contents are uniform, fluid, and filled with tiny bubbles and sometimes have an oily sheen. In early cases the mucosa appears to be normal even on histological examination, as it also does with electron microscopy scanning. Local loss of microvilli from colonic epithelial cells may occur. In some cases there may be more obvious colonic lesions and inflammation, with some epithelial cell shedding, may occur as the disease progresses.

7.3.1.3. Laboratory

Samples of ileum and its contents and large intestine and its contents are required for confirmation of the presence of enterotoxin and sporulating *C. perfringens* type A (Moller and Ahrens, 1996) as well as to rule out the presence of other agents. Samples of the wall of both regions should be taken for histological examination.

The presence of enterotoxin in faecal filtrates can be confirmed using commercial Reversed Passive Latex Agglutination tests, counter immunoelectrophoresis, ELISA and Vero cells toxicity. Sporulating strains may be isolated by dilution of faeces in ethanol for 1 hour or by heating at 80^{O}C for 10 minutes and then culturing anaerobically. The genes for the toxins concerned can be detected by PCR, but only confirm the involvement of the organism. Other causes of the 'colitis' syndrome such as the presence of *B. pilosicoli, Salmonella enterica, Yersinia enterocolitica* and *Eimeria sp* should be sought to confirm that *C. perfringens* type A is the cause of a particular outbreak.

7.4. Bibliography

Arbuckle J.B.R. (1972). The attachment of *Clostridium welchii* (*Cl. perfringens)* type C to intestinal villi of pigs. *J. Pathol.* **106**, 65-72.

Bergeland M.E. (1972). Pathogenesis and immunity of *C. perfringens* type C enteritis in swine. *J. Ann.Vet. Med. Assoc*. **160**, 658-671.

Estrada Correa A. and Taylor D.J. (1986). Enterotoxigenic *C. perfringens* type A as a cause of diarrhoea in pigs. Proceedings of the International Pig Veterinary Society **9**, 171.

Estrada Correa A. and Taylor D.J. (1988). Enterotoxigenic *C. perfringens* type A as a cause of diarrhoea in weaned pigs. Proceedings of the International Pig Veterinary Society **10**, 138.

Jestin A., Popoff M.R. and Mahe S. (1985). Epizootiological investigations of a diarrhoeic syndrome in fattening pigs. *Am. J. Vet. Res.* **46**, 2149-2151.

Mackinnon J.D. (1989). Enterotoxaemia caused by *C.perfringens* type C. *Pig Vet. J*. **22**, 119-125.

Moller K. and Ahrens P. (1996). Comparison of toxin neutralisation, ELISA and PCR tests for typing of *Clostridium perfringens* and detection of the enterotoxin gene by PCR. *Anaerobe* **2**, 103-110.

Nabuurs M.J.A., Haagsma J., Molen E.J. van der. and Heijden P.J. van der (1983). Diarrhoea in 1 to 3 week-old piglets associated with *C. perfringens* type A. *Ann. Rech. Vét*. **14**, 408-411.

Ripley P.H. and Gush A.F. (1983). Immunisation schedule for the prevention of infectious necrotic enteritis caused by *C. perfringens* type C in piglets. *Vet. Rec*. **112**, 201-202.

Songer J.G. and Glock R.D. (1998). Enteric infection of swine with *C. perfringens* Types A and C. *Swine Health and Production* **6**, 223 – 225.

Songer J.G., Post K.W., Larson D.J., Jost B.H. and Glock R.D. (2000). Infection of neonatal swine with *Clostridium difficile*. *Swine Health and Production* **8,** 185-189

Taylor D.J. and Estrada Correa A. (1988). Avoparcin in the prevention of enterotoxigenic *C. perfringens* type A infections and diarrhoea in pigs. Proceedings of the International Pig Veterinary Society **10**,139.

Taylor D.J. and Olubunmi P.A. (1982). Enteric disease in sucking and weaned pigs initiated by and associated with *C. perfringens* type A. Proceedings of the International Pig Veterinary Society **7**, 66.

Waters E.H., Orr J.P., Clark E.G. and Schaufele C.M. (1998). Typhlocolitis caused by *Clostridium difficile* in sucking piglets. *J. Vet. Diagn. Invest*. **10**, 104-108.

Waters M., Savoie A., Garmory H.S., Bueschel D., Popoff M.R., Songer J.G., Titball R.W., McClane B.A. and Sarker M.R. (2003). Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J. Clin. Microbiol*. **41**, 3584 – 3591

8. Enteritis and enterotoxaemia in humans (including food poisoning)

M. Delmée

This chapter will focus on 2 clostridial species implicated in enteritis and enterotoxaemia in humans: *Clostridium difficile* and *Clostridium perfringens*.

Clostridium difficile is the main aetiological agent of pseudomembranous colitis. Most of the time, this pathology is linked to antimicrobial therapy that disturbs the normal intestinal flora, allows *C. difficile* to develop when present, and causes disease. There is a wide range of symptoms from simple self-limiting diarrhoea to severe colitis.

Clostridium perfringens is often implicated in food poisoning, as are other micro-organisms, such as *Staphylococcus aureus* and *Bacillus cereus*. In these cases, enterotoxins produced in large amounts by bacterial growth in food cause intestinal disease when ingested.

8.1. Pseudomembranous colitis and antibiotic-associated diarrhoea caused by *C. difficile*

8.1.1. Clinical signs

C. difficile-associated disease (CDAD) should be suspected in every case of diarrhoea occurring after antimicrobial therapy. In some rare cases, colitis is induced by chemotherapy.

Diarrhoea may appear during treatment or after it has been discontinued. In the most severe cases, typical pseudomembranes can been seen at the surface of colonic mucosa by rectoscopy or colonoscopy. However, due to the difficulty of performing this type of examination and the lack of sensitivity, diagnosis is mostly obtained by laboratory tests on faecal specimens.

8.1.2. Laboratory diagnosis

Laboratory diagnosis of CDAD is based both on isolation of the pathogen in stool specimens by culture and on toxin detection. The latter can be performed either by detection of a cytopathic effect of a stool filtrate on cell lines or by direct enzyme immunoassay. Cytotoxin detection is advocated by many authors, especially in the USA, as the gold standard for diagnosis, but both culture and toxin detection are necessary to achieve an optimal result (Delmée, 2001).

Other indirect tests have been proposed in the past, such as gas liquid chromatography (GLC) performed on stool specimens, latex agglutination and CT-scans. However, these approaches do not reach sufficient sensitivity and specificity to be acceptable, unless used in addition to the other tests.

For optimal bacteriological diagnosis, only liquid stools should be accepted, except in the case of epidemiological investigation. Due to a rapid loss of cytotoxin activity, only freshly taken specimens should be processed and they should be stored at 4 °C, or less in cases where tests cannot be performed rapidly. Complete inactivation of cytotoxin has been demonstrated in about 20% of samples sent through the post (Brazier, 1993). On the other hand, cultures of *C. difficile* remain unaffected by ambient storage, due to sporulation. Repeated samples within 7 days of initial request seem to give little useful information (Renshaw *et al*., 1996).

8.1.2.1. Culture

Just after the discovery of the pathogenic role of *C. difficile*, George *et al*. proposed a selective agar plate called CCFA (cycloserine cefoxitin fructose agar) for its isolation from stool specimens (George *et al*., 1979). This medium is still in use in most laboratories.

Colonies of *C. difficile* are easily recognised on culture plates, due to their typical morphology (ground glass appearance) when observed with binoculars. GLC of an agar plot around a suspected colony is the best and easiest method for confirming identification (see also Chapter 13). Such a device, however, is not available in many laboratories. Individual biochemical tests or anaerobic panels may also be used, as well as a somatic antigen latex kit. Cross-reactions, however, have been documented with this latter reagent.

Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect toxin A or both toxins A and B. They may be used on culture or directly on faeces (see below), but, of course, will only recognise toxigenic isolates. Production of proline-aminopeptidase by disc test has recently been proposed as a means of rapid identification of *C. difficile*, when used in conjunction with the typical morphology of colonies (Fedorko and Williams, 1997).

8.1.2.2. Toxin detection

8.1.2.2.1. Cytotoxicity assays

This method consists of inoculating a filtrate of stool suspension into a cell culture and observing a cytopathic effect as a consequence of disruption of the cell cytoskeleton; this results in cell rounding in many cell lines. The effect is mainly due to toxin B, which is a thousand times more cytotoxic than toxin A.

Almost all cell lines commonly used in clinical microbiology laboratories can be used to detect faecal cytotoxin. Vero, HEp2, fibroblast, CHO and HeLa cells are the most common. Vero cell lines are considered by many to be the most sensitive. Confirmation of specificity is obtained by repeating the test with the addition of a specific antiserum directed against *C. difficile* or against *C. sordellii* toxins, which share the same antigens.

The cytotoxicity assay method has many advantages. It is sensitive and specific and it can also detect other clostridial toxins. On the other hand, it is

relatively slow in comparison with ELISA. In addition, a specific cytopathic effect (not neutralised by antiserum) can be observed in about two percent of cases, rendering impossible any interpretation. Having to maintain cell lines, which is both time consuming and expensive, especially if only a small number of specimens are processed, is another drawback.

8.1.2.2.2. Immunological assays

Many ELISAs have become commercialised over the last 10 years. Establishing a complete list is difficult, since not all are available in every country. Most use monoclonal anti-toxin A antibodies, although a few of them are designed to detect both toxins A and B. The reason why kits detecting both toxins have been developed is mainly because some isolates from clinical cases have been shown to produce only toxin B (Alfa *et al*., 2000). In our experience, such strains are very rarely observed. However, in England, they account for 3% of the strains referred to the reference laboratory for typing (Brazier, 1998) and outbreaks with A-negative, B-positive strains have also been described in Canada (al-Barrak *et al*., 1999).

Finally, in one of the latest kits, the detection of toxin A is coupled with the detection of a glutamate dehydrogenase (GDH), a *C. difficile*-specific enzyme found in toxigenic as well as non-toxigenic isolates. Although the sensitivity of toxin A detection is within the same range as other kits (79.4%), the addition of the GDH test allows us to obtain an excellent negative predictive value of infection by *C. difficile* (Landry *et al*., 2001; Barbut *et al*., 2000). A negative result for both tests at the same time means that the presence of a toxigenic strain of *C. difficile* can be excluded, with a reliability factor of 99.6 % (Barbut *et al*., 2000). This latest kit may be used as a rapid screening test to decide which stools need to be processed further.

There are numerous publications regarding the performance of different kits. When compared to faecal cytotoxin detection on cell lines, different ELISAs show a slightly lower sensitivity. In our experiments, serial two-fold dilutions of a positive faecal sample could be detected at a dilution of 1/64 with the cytotoxicity test, whereas this was the case only at ½ dilution for most ELISAs (unpublished results). The same observations have been made in clinical studies (Barbut *et al*., 1993; Barbut *et al*., 1997; Bentley *et al*., 1998; Fedorko *et al*., 1999; Jacobs *et al*., 1996; Vanpoucke *et al*., 2001).

Although less sensitive, ELISAs can be used as a screening test and may be useful in laboratories without tissue-culture facilities. Some kits are presented as individual panels, others as 96-well micro-plates. One of the main advantages of these kits is their rapidity, since results can be obtained within 20 minutes with some of them.

ELISA tests should always be combined with culture and, when negative with a positive culture, should be repeated by testing the strain isolated on the plate.

In several studies, toxigenic culture has been demonstrated as the most sensitive and specific technique for the diagnosis of CDAD (Barbut *et al*., 1993; Delmée *et al.*, 2005; Fedorko *et al*., 1999; Lozniewski *et al*., 2001; Staneck *et al*., 1996). In this approach, culture of faecal specimens on CCFA is followed by *in vitro*

determination of the toxigenicity of positive colonies by cytotoxin test or by ELISA. Most comparisons of ELISA kits against toxigenic culture have demonstrated lower performance on the part of ELISAs.

8.1.2.2.3. Genetic assays

Compared to other infectious diseases, molecular methods for the diagnosis of CDAD have been far less studied. This is probably due to the relatively satisfactory results obtained with classical methods, as described above, as well as to the difficulty of applying such methods to faecal specimens. This is known often to interfere with amplification procedures.

Direct detection of *C. difficile* genes in faecal specimens has also been tested. Oligonucleotide probes designed to detect toxin B have been used by Green (Green *et al*., 1994), with sensitivity and specificity in the same range as ELISAs. PCRs using sets of primers designed to detect toxin A or B have also been tested on stools by several authors (Kato *et al*., 1993; Gumerlock *et al*., 1993; Arzese *et al*., 1995; Wolfhagen *et al*., 1994). Procedures usually comprise a preliminary step to avoid inhibitory substances. So far, they have been tested on a small series of specimens and the results have not shown any significant improvement in comparison with classical methods.

Table 8.1: Proposed scheme for bacteriological diagnosis of CDAD

initial tests on stools			
culture	**faecal toxin**	**additional test**	**conclusion**
-	-		no CDAD
+	+		CDAD
+	-	strain *in vitro* toxigenicity	
		+	probable CDAD
		-	carriage of non-toxigenic *C. difficile*
-	+	repeated culture on CCFA + taurocholate	probable CDAD

8.2. Food poisoning due to *Clostridium perfringens*

8.2.1. Clinical signs

Acute liquid diarrhoea occurs rapidly (within 6 to 24 hours, most often 8 to 12 hours) after ingestion of contaminated food. It is often preceded by abdominal cramps. Fever or vomiting are more rare. Usually symptoms do not last for more than 24 hours. They are mainly caused by *C. perfringens* belonging to type A and producing the enterotoxin.

Necrotising enterocolitis has been described only in very rare cases. This is caused by *C. perfringens* type C.

8.2.2. Laboratory diagnosis

Diagnosis can be obtained by culturing either the patient's stool or the suspected food. The difficulty lies in the fact that *C. perfringens* may be present in normal stools as well as in non-implicated foods. However the number of bacteria differs: the usual count in normal stools is 10^3 to 10^4 cells per g, but from 0 to 10^3 in food.

The Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) currently accept (Olsen *et al*., 2000) several laboratory criteria for confirming the occurrence of a *C. perfringens* type A food poisoning outbreak: (a) demonstrating the presence of 10^5 *C. perfringens* organisms/g of stool from two or more persons (b) demonstrating the presence of 10^5 *C. perfringens* organisms/g of epidemiologically implicated food, or (c) demonstrating the presence of *cpe* gene in the faeces of several people involved in a food poisoning outbreak.

8.2.2.1. Culturing procedures for sporulation and enterotoxin production

In order to determine the enterotoxigenicity of *C. perfringens* from food or faeces, it is necessary to induce sporulation of the organism. Isolates are first subcultured in fluid thioglycollate broth and subsequently in one of the numerous proposed sporulation media (Peck *et al.*, 2004). The two recommended here are the modified AEA medium of Taniguti (Taniguti, 1969) and the modified (Harmon and Kautter, 1986a) medium of Duncan and Strong (Duncan and Strong, 1969; Labbe and Rey, 1979).

The resulting culture is checked for spores by using a phase-contrast microscope or by examining stained smears. Fewer than 5 spores per microscopic field is not considered adequate sporulation. A portion of the sporulated culture is then centrifuged for 15 mins at 10,000 x g and the cell-free culture supernatant tested for enterotoxin.

8.2.2.2. Rapid methods for detecting *C. perfringens* enterotoxin

A number of immunological assays have been reported for the rapid detection of enterotoxin. The most rapid methods available are the afore mentioned enzyme-linked immunosorbent assays (ELISAs) and Reverse Passive Latex Agglutination (RPLA) (Harmon and Kautter, 1986b). A rapid and inexpensive slide latex agglutination assay has also been reported (McClane and Snyder, 1987), which requires special test reagents and equipment not generally available.

A hydrophobic grid membrane filter-colony hybridisation method for the enumeration and isolation of *cpe* gene-carrying *C. perfringens* spores from faeces has been described (Heikinheimo *et al.*, 2004). First results seem promising and this may

be an aid in the investigation of faecal samples from patients suffering from food poisoning or other diseases caused by *cpe*-positive *C. perfringens*

8.3. Bibliography

al-Barrak A., Embil J., Dyck B., Olekson K., Nicoll D., Alfa M. and Kabani A. (1999) An outbreak of toxin A negative, toxin B positive *Clostridium difficile*-associated diarrhea in a Canadian tertiary-care hospital. *Can. Commun. Dis. Rep.* **25**, 65-69.

Alfa M.J., Kabani A., Lyerly D., Moncrief S., Neville L.M., Al-Barrak A., Harding G.K., Dyck B., Olekson K. and Embil J.M. (2000) Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **38**, 2706-2714.

Arzese A., Trani G., Riul L. and Botta G.A. (1995) Rapid polymerase chain reaction method for specific detection of toxigenic *Clostridium difficile*. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**, 716-719.

Barbut F., Kajzer C., Planas N. and Petit J.C. (1993) Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **31**, 963-967.

Barbut F., Lalande V., Daprey G., Cohen P., Marle N., Burghoffer B. and Petit J.C. (2000) Usefulness of simultaneous detection of toxin A and glutamate dehydrogenase for the diagnosis of *Clostridium difficile*-associated diseases. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**, 481-484.

Barbut F., Mace M., Lalande V., Tilleul P. and Petit J.C. (1997) Rapid detection of *Clostridium difficile* toxin A in stool specimens. *Clin. Microbiol. Infect.* **3**, 480-483.

Bentley A.H., Patel N.B., Sidorczuk M., Loy P., Fulcher J., Dexter P., Richards J., Borriello S.P., Zak K.W. and Thorn E.M. (1998) Multicentre evaluation of a commercial test for the rapid diagnosis of *Clostridium difficile*-mediated antibiotic-associated diarrhoea. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**, 788-790.

Brazier J.S. (1993) Role of the laboratory in investigations of *Clostridium difficile* diarrhea. *Clin. Infect. Dis.* **16** Suppl 4: S228-233.

Brazier J.S. (1998) The epidemiology and typing of *Clostridium difficile*. *J. Antimicrob. Chemother.* **41** Suppl C: 47-57.

Delmee M. (2001) Laboratory diagnosis of *Clostridium difficile* disease. *Clin. Microbiol. Infect.* **7**, 411-416.

Delmee M., Van Broeck J., Simon A., Janssens M. and Avesani V. (2005) Laboratory diagnosis of *Clostridium difficile*-associated diarrhoea: a plea for culture. *J. Med. Microbiol.* **54**, 187-191.

Duncan C. and Strong D. (1968) Improved medium sporulation of *Clostridium perfringens*. *Appl. Microbiol.* **16**, 82-89.

Fedorko D.P., Engler H.D., O'Shaughnessy E.M., Williams E.C., Reichelderfer C.J. and Smith W.I.Jr. (1999) Evaluation of two rapid assays for detection of *Clostridium difficile* toxin A in stool specimens. *J. Clin. Microbiol.* **37**, 3044-3047.

Fedorko D.P. and Williams E.C. (1997) Use of cycloserine-cefoxitin-fructose agar and L-proline-aminopeptidase (PRO Discs) in the rapid identification of *Clostridium difficile*. *J. Clin. Microbiol.* **35**, 1258-1259.

George W.L., Sutter V.L., Citron D. and Finegold S.M. (1979) Selective and differential medium for isolation of *Clostridium difficile*. *J. Clin. Microbiol.* **9**, 214-219.

Green G.A., Riot B. and Monteil H. (1994) Evaluation of an oligonucleotide probe and an immunological test for direct detection of toxigenic *Clostridium difficile* in stool samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **13**, 576-581.

Gumerlock P.H., Tang Y.J., Weiss J.B. and Silva J.Jr. (1993) Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. *J. Clin. Microbiol.* **31**, 507-511.

Harmon S.M. and Kautter D.A. (1986a) Improved media for sporulation and enterotoxin production by *Clostridium perfringens* enterotoxin. *J. Food Prot.* **49**, 706.

Harmon S.M. and Kautter D.A. (1986b) Evaluation of a reversed passive latex agglutination test kit for *Clostridium perfringens* enterotoxin. *J. Food Prot.* **49**, 523.

Heikinheimo A., Lindström M. and Korkeala H. (2004) Enumeration and isolation of *cpe*-positive *Clostridium perfringens* spores from feces. *J. Clin. Microbiol.* **42**, 3992-3997.

Jacobs J., Rudensky B., Dresner J., Berman A., Sonnenblick M., van Dijk Y. and Yinnon A.M. (1996) Comparison of four laboratory tests for diagnosis of *Clostridium difficile*-associated diarrhea. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**, 561-566.

Kato N., Ou C.Y., Kato H., Bartley S.L., Luo C.C., Killgore G.E. and Ueno K. (1993) Detection of toxigenic *Clostridium difficile* in stool specimens by the polymerase chain reaction. *J. Infect. Dis.* **167**, 455-458.

Labbe R.G. and Rey D.K. (1979) Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* type A. *Appl. Environ. Microbiol.* **37**, 1196-1200.

Landry M.L., Topal J., Ferguson D., Giudetti D. and Tang Y. (2001) Evaluation of biosite triage *Clostridium difficile* panel for rapid detection of *Clostridium difficile* in stool samples. *J. Clin. Microbiol.* **39**, 1855-1858.

Lozniewski A., Rabaud C., Dotto E., Weber M. and Mory F. (2001) Laboratory diagnosis of *Clostridium difficile*-associated diarrhea and colitis: usefulness of Premier Cytoclone A+B enzyme immunoassay for combined detection of stool toxins and toxigenic *C. difficile* strains. *J. Clin. Microbiol.* **39**, 1996-1998.

McClane B.A. and Snyder J.T. (1987) Development and preliminary evaluation of a slide latex agglutination assay for detection of *Clostridium perfringens* type A enterotoxin. *J. Immunol. Methods* **100**, 131-136.

Olsen S.J., MacKinnon L.C., Goulding J.S., Bean N.H. and Slutsker L. (2000) Surveillance for foodborne-disease outbreaks – United States, 1993-1997. *MMWR CDC Surveill. Summ.* **49**, 1-62.

Peck M.W., Granum P.E., Gould G.W. and Mainil J.G. (2004) *Foodborne clostridia and sporulation*. Scientific booklet of the CA QLK2-CT2001-01267, Presses Fac. Méd. Vét. ULg, Liège, Belgium (http://www.genusclostridium.net)

Renshaw A.A., Stelling J.M. and Doolittle M.H. (1996) The lack of value of repeated *Clostridium difficile* cytotoxicity assays. *Arch. Pathol. Lab. Med.* **120**, 49-52.

Staneck J.L., Weckbach L.S., Allen S.D., Siders J.A., Gilligan P.H., Coppitt G., Kraft J.A. and Willis D.H. (1996) Multicenter evaluation of four methods for *Clostridium difficile* detection: ImmunoCard C. difficile, cytotoxin assay, culture, and latex agglutination. *J. Clin. Microbiol.* **34**, 2718-2721.

Taniguti T. (1969) Sporulation media for *Clostridium perfringens*: a method with a new medium (AEA medium) for sporulation of *Clostridium perfringens* and some properties of formed spores. *J. Food Hyg. Soc. Jpn.* **9**, 219.

Vanpoucke H., De Baere T., Claeys G., Vaneechoutte M. and Verschraegen G. (2001) Evaluation of six commercial assays for the rapid detection of *Clostridium difficile* toxin and/or antigen in stool specimens. *Clin. Microbiol. Infect.* **7**, 55-64.

Voth D.E. and Ballard J.D. (2005) *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin. Microbiol. Rev.* **18**, 247-263.

Wolfhagen M.J., Fluit A.C., Torensma R., Poppelier M.J. and Verhoef J. (1994) Rapid detection of toxigenic *Clostridium difficile* in fecal samples by magnetic immuno PCR assay. *J. Clin. Microbiol.* **32**, 1629-1633.

9. Enterocolitis in horses

V. Båverud

Acute colitis in adult horses is a very severe disease with high mortality and often unknown aetiology (Cohen and Woods, 1999; Murray, 1992; Palmer, 1992). Some colitis cases result from infection with clostridia. Clostridial colitis in horses may be caused by toxigenic isolates of *Clostridium difficile* or *Clostridium perfringens* (Divers, 2002).

9.1. *Clostridium difficile* diarrhoea

C. difficile is a well known nosocomial pathogen in humans with antibiotic-associated diarrhoea. The bacterium can produce an enterotoxin, A and a cytotoxin, B. *C. difficile* has recently been reported as a pathogen in horses developing acute colitis when treated with antibiotics for problems other than gastrointestinal disorders (Båverud, 2002a, 2004; Båverud *et al*., 1997, 2003, 2004; Beier *et al*., 1994; Cosmetatos *et al.*, 1994; Gustafsson *et al.*, 1997, 2004; Madewell *et al.*, 1995; Magdesian *et al.*, 1997; Perrin *et al.*, 1993). Often, horses had been treated with antibiotics for some days when diarrhoea developed, and the majority of them had also been hospitalised (Båverud *et al.*, 1997, 2003; Gustafsson *et al.*, 1997).

C. difficile has also been isolated from diarrhoeic horses not treated with antibiotics (Båverud *et al.*, 2003; Beier *et al.*, 1994; Cosmetatos *et al.*, 1994; Magdesian *et al.*, 1997). This is also the case in other horse studies where there is no information as to whether or not the animals have been treated (Donaldson and Palmer, 1999; Weese *et al.*, 2000). Furthermore, *C. difficile* has been associated with acute colitis in mares when their foals have been treated orally with erythromycin and rifampicin for *Rhodococcus equi* pneumonia (Båverud *et al.*, 1998). In faecal samples from healthy adult horses, *C. difficile* is rarely found (isolation rate 0–1%) (Al Saif and Brazier, 1996; Båverud *et al.*, 1997, 2003; Jones *et al.*, 1987; Weese *et al.*, 2001). Healthy young foals less than 2 weeks old and non-diarrhoeic foals treated with antibiotics are often carriers of *C. difficile* (Båverud *et al.*, 2003). Other authors have reported on *C. difficile* being isolated from foals with diarrhoea (Jones *et al.*, 1987, 1988a, 1988b; Magdesian *et al.*, 1999, 2002; Weese *et al.*, 2001).

In an experimental infection after oral inoculation with *C. difficile* in mature horses, penicillin treatment was demonstrated to be a risk factor for establishment of *C. difficile* in the intestine (Gustafsson *et al*., 2004). Infection control measures to limit susceptibility of hospitalised horses and to decrease exposure to *C. difficile* may play a role in the control of *C. difficile*-associated disease. To prevent *C. difficile* diarrhoea in hospitalised horses judicious use of antimicrobials is important and also to minimise different stress factors at the clinic. Isolation of infected horses, cleaning and surface disinfection and routine handwashing by all staff are recommended (Båverud, 2004).

9.1.1. Clinical signs

C. difficile diarrhoea should be diagnosed if an adult horse develops diarrhoea during or after antibiotic treatment, if it is or has recently been hospitalised and if toxin A and/or B is identified in faeces. The clinical signs of a horse with antibiotic-associated diarrhoea caused by *C. difficile* cannot be differentiated from those of a horse suffering from diarrhoea caused by *C. perfringens* (Divers, 2002).

9.1.2. Laboratory procedures

9.1.2.1. Sampling and transport

A faecal sample (20–30 g) should be directly submitted to the laboratory for investigation (Weese *et al.*, 2000). If there is a delay in transport and/or in laboratory investigation, samples should be stored at −70°C (Jones, 2000). Multiple faecal samples should be taken on several consecutive days, as cytotoxin B is demonstrated one day later than a positive culture and also intermittently (Gustafsson *et al.*, 1997). *C. difficile* shows poor survival when faeces are exposed to air (Weese *et al.*, 2000). However, faeces may contain spores of *C. difficile* (Båverud *et al.*, 2003).

9.1.2.2. Culture

Faecal samples are cultured for *C. difficile* on a selective agar medium containing cycloserine (250 mg/L), cefoxitin (8 or 16 mg/L) and fructose (CCFA) (George *et al.*, 1979; Levett, 1985; Båverud, 2002b). Addition of bile salts, such as taurocholate, to a medium enhances germination of spores (Buggy *et al.*, 1983; Wilson *et al.*, 1982). Selective agar plates are available commercially. Isolates may be tested for antimicrobial susceptibility (Båverud *et al.,* 2003, 2004; Jang *et al.,* 1997; Weese *et al.,* 2001) and subtyped using molecular methods (Jang *et al.,* 1997; Madewell *et al.,* 1995; Magdesian *et al.,* 2002; reviewed by Brazier 1998; 2001).

9.1.2.3. Detection of toxin A and/or B

Cytotoxin B of *C. difficile* in faecal specimens may be investigated by tissue culture, e.g. on human diploid fibroblast cells or MRC-5 cells (Båverud *et al.*, 1997). Other cell lines may also be used (Delmée, 2001). Positive toxin samples should be confirmed by neutralisation with *C. difficile* antitoxin B (Båverud *et al.*, 1997).

Toxin A and/or B may be tested in faecal samples by using enzyme immunoassays. Commercial products are available for rapid detection of *C. difficile* toxins (O'Connor *et al.*, 2001; Turgeon *et al.*, 2003). Tests have been performed on faecal samples from horses, but not yet validated (Donaldson and Palmer, 1999; Weese *et al.*, 2001; Gustafsson *et al.*, 2004).

Isolates may be tested for the possession of toxin A and B genes by PCR (Båverud *et al.*, 2003; Madewell *et al.*, 1995). This test shows if the genes are present. However, it gives no information as to whether or not the toxins are expressed (Båverud *et al.*, 2003).

9.2. *Clostridium perfringens* diarrhoea

C. perfringens type A has been suggested as a pathogen in diarrhoeic horses (Ochoa and Kern, 1980; Wierup, 1977).

Wierup found that high intestinal counts of *C. perfringens* were associated with diarrhoea in mature horses (Wierup, 1977). Haemorrhagic enterocolitis was experimentally induced by enterotoxigenic type A *C. perfringens,* (Ochoa and Kern, 1980). Recently, *C. perfringens* enterotoxin was demonstrated in 16%–19% of faecal samples from mature horses with diarrhoea (Donaldson and Palmer, 1999; Weese *et al.*, 2001).

In foals, *C. perfringens* type A, B, C and D have been sporadically associated with enterocolitis (East *et al.*, 1998; Netherwood *et al.*, 1998; Stubbens, 1990; Traub-Dargatz and Jones, 1993).

The gene coding for the new β_2 toxin was recently found in some strains of *C. perfringens.* The β_2 toxin gene of *C. perfringens* was found in horses with typhlocolitis, whereas β_2 toxigenic isolates were not found in control horses (Herholz *et al.*, 1999).

9.2.1. Clinical signs

As *C. perfringens* may be isolated from healthy mature horses and neonatal foals (Tillotson *et al.*, 2002; Traub-Dargatz and Jones, 1993; Wierup, 1977; Wierup and DiPietro, 1981), the diagnosis of *C. perfringens* as a cause of diarrhoea in horses is complicated. According to Divers (2002), other causes should be ruled out. Divers suggests that, there should be large numbers ($>10^5$ colony forming units/ml faeces) of *C. perfringens* and demonstration of enterotoxin and sporulation. The β_2 toxin gene may be of further interest for study.

9.2.2. Laboratory procedures

9.2.2.1. Sampling and transport

For *C. perfringens* faecal sample (20–30 g) should be directly submitted to the laboratory for investigation. If there is a delay in transport and/or in laboratory investigation, freezing of samples at −70°C may be an alternative.

9.2.2.2. Culture and typing

Culture of *C. perfringens* is performed on blood agar plates for anaerobic culture. For typing (types A–E) of *C. perfringens* isolates, a multiplex PCR demonstrates the genes of the major toxins (Engström *et al.*, 2003).

9.2.2.3. Detection of toxin

Enterotoxin may be demonstrated in faecal samples by using enzyme immunoassays (Donaldson *et al.,* 1999; Weese *et al.,* 2001). Presence of the enterotoxin gene and the β_2 toxin gene may be demonstrated through PCR of *C. perfringens* isolates (Engström *et al.*, 2003).

9.3. Bibliography

Al Saif N. and Brazier J.S. (1996) The distribution of *Clostridium difficile* in the environment of South Wales. *J. Med. Microbiol.* **45**, 133-137.

Båverud V. (2002a) *Clostridium difficile* infections in animals with special reference to the horse: a review. *Vet. Quarterly* **24**, 203-219.

Båverud V. (2002b) *Clostridium difficile* in horses. Doctoral thesis. ISSN 1401-6257, ISBN 91-576-6378-5. Swedish University of Agricultural Sciences

Båverud V. (2004) *Clostridium difficile* diarrhea: infection control in horses. *Vet. Clin. North Am. Equine Pract.* **20**, 615-630.

Båverud V., Gustafsson A., Franklin A., Lindholm A. and Gunnarsson A. (1997) *Clostridium difficile* associated with acute colitis in mature horses treated with antibiotics. *Equine Vet. J.* **29**, 279-284.

Båverud V., Franklin A., Gunnarsson A., Gustafsson A. and Hellander-Edman A. (1998) *Clostridium difficile* associated with acute colitis in mares when their foals are treated with erythromycin and rifampicin for *Rhodococcus equi* pneumonia. *Equine Vet. J.* **30**, 482-488.

Båverud V., Gustafsson A., Franklin A., Aspán A. and Gunnarsson A. (2003) *Clostridium difficile*: prevalence in horses and the environment, and antimicrobial susceptibility. *Equine Vet. J.* **35**, 465-471. *Erratum* in 2003; **35**,706.

Båverud V., Gunnarsson A., Karlsson M. and Franklin A. (2004) Antimicrobial susceptibility of equine and environmental isolates of *Clostridium difficile*. *Microb. Drug Resist.* **10**, 57-63.

Beier R., Amtsberg G. and Peters M. (1994) Bakteriologische Untersuchungen zum Vorkommen und zur Bedeutung von *Clostridium difficile* beim Pferd. *Pferdeheilkunde* **10**, 3-8.

Brazier J.S. (1998) The epidemiology and typing of *Clostridium difficile. J. Antimicrob. Chemother.* **41**, Suppl. C, 47-57.

Brazier J.S. (2001) Typing of *Clostridium difficile. Clin. Microbiol. Infect.* **7,** 428-431.

Buggy B.P., Wilson K.H. and Fekety R. (1983) Comparison of methods for recovery of *Clostridium difficile* from an environmental surface. *J. Clin. Microbiol.* **18**, 348-352.

Cohen N.D., Woods A.M. (1999) Characteristics and risk factors for failure of horses with acute diarrhoea to survive: 122 cases (1990-1996). *J. Am. Vet. Med. Assoc.* **214**, 382-390.

Cosmetatos I., Perrin J., Gallusser A., Nicolet J. and Straub R. (1994) Faecal isolation of *Clostridium difficile* and its toxins from horses with typhlo-colitis. 7th Intern. Conference on Equine Infectious Diseases, June 8-11 1994, Tokyo, Japan. Poster session 69.

Delmée M. (2001) Laboratory diagnosis of *Clostridium difficile* disease. *Clin. Microbiol. Infect.* **7,** 411-416.

Divers T.J. (2002) Postoperative complications-colitis; Clostridial diarrhea in adult horses; Acute diarrhea in adult horses – other causes; Clostridial

enterocolitis in foals. In: *Manual of Equine Gastroenterology*, 1st ed. (Mair T., Divers T., Ducharme N. and Saunders W.B., Eds), pp 230-232, 410-412, 422-425, 499-502.

Donaldson M.T. and Palmer J.E. (1999) Prevalence of *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A in faeces of horses with diarrhea and colic. *J. Am. Vet. Med. Ass.* **215**, 358-361.

East L.M., Savage C.J., Traub-Dargatz J.L., Dickinson C.E. and Ellis R.P. (1998) Enterocolitis associated with *Clostridium perfringens* infection in neonatal foals: 54 cases (1988-1997). *J. Am. Vet. Med. Ass.* **212**, 1751-1756.

Engström B.E., Fermér C., Lindberg A., Saarinen E., Båverud V. and Gunnarsson A. (2003) Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. *Vet. Microbiol.* **94**, 225-235.

George W.L., Sutter V.L., Citron D. and Finegold S.M. (1979) Selective and differential media for isolation of *Clostridium difficile. J. Clin. Microbiol.* **9**, 214-219.

Gustafsson A. (2004) Antibiotic associated diarrhea in horses, with special reference to *Clostridium difficile*. Doctoral thesis. ISSN 1401-6257, ISBN 91-576-6652-0. Swedish University of Agricultural Sciences.

Gustafsson A., Båverud V., Gunnarsson A., Rantzien M.H., Lindholm A. and Franklin A. (1997) The association of erythromycin ethylsuccinate with acute colitis induction in horses in Sweden. *Equine Vet. J.* **29**, 314-318.

Gustafsson A., Båverud V., Gunnarsson A., Pringle J. and Franklin A. (2004) Study of faecal shedding of *Clostridium difficile* in horses treated with penicillin. *Equine Vet. J.* **36**, 180-182.

Herholz C., Miserez R., Nicolet J., Frey J., Popoff M., Gibert M., Gerber H. and Straub R. (1999) Prevalence of β2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. *J. Clin. Microbiol.* **37**, 358-361.

Jang S.S., Hansen L.M., Breher J.E., Riley D.A., Magdesian K.G., Madigan J.E., Tang Y.J., Silva J. Jr. and Hirsh D.C. (1997) Antimicrobial susceptibilities of equine isolates of *Clostridium difficile* and molecular characterization of metronidazole-resistant strains. *Clin. Infect. Dis.* **25** (Suppl 2): S266-267.

Jones R.L. (2000) Clostridial enterocolitis. *Vet. Clin. North Am. Equine Pract.* **16**, 471-485.

Jones R.L., Adney W.S. and Shideler R.K. (1987) Isolation of *Clostridium difficile* and detection of cytotoxin in the feces of diarrheic foals in the absence of antimicrobial treatment. *J. Clin. Microbiol.* **25**, 1225-1227.

Jones R.L., Adney W.S., Alexander A.F., Shideler R.K. and Traub-Dargatz J.L. (1988a) Hemorrhagic necrotizing enterocolitis associated with *Clostridium difficile* infection in four foals. *J. Am. Vet. Med. Ass.* **193**, 76-79.

Jones R.L., Shideler R.K. and Cockerell G.L. (1988b) Association of *Clostridium difficile* with foal diarrhea. In: Proceedings 5th International Conference on Equine Infectious Diseases, pp 236-240, University Press of Kentucky, Lexington, Kentucky.

Levett P.N. (1985) Effect of antibiotic concentration in a selective medium on the isolation of *Clostridium difficile* from faecal specimens. *J. Clin. Pathol.* **38,** 233-234.

Madewell B.R., Tang Y.J., Jang S.S., Madigan J.E., Hirsh D.C., Gumerlock P.H. and Silva J.Jr. (1995) Apparent outbreaks of *Clostridium difficile*-associated diarrhea in horses in a veterinary medical teaching hospital. *J. Vet. Diagn. Invest.* **7**, 343-346.

Magdesian K.G., Madigan J.E., Hirsh D.C., Jang S.S., Tang Y.J., Carpenter T.E., Hansen L.M. and Silva J. Jr. (1997) *Clostridium difficile* and horses: a review. *Reviews Med. Microbiol.* **8** (Suppl 1), S46-S48.

Magdesian K.G., Hirsh D.C., Jang S.S. and Madigan J.E. (1999) Characterisation of *Clostridium difficile* isolates from an outbreak of enteritis in neonatal foals. In: Proceedings Eighth International Conference on Equine Infectious Diseases, Dubai, 23rd-26th March, 1998. 1st ed. (Wernery U., Wade J.F., Mumford J.A. and Kaaden O.-R., Eds.), pp 561-562, R and W Publications (Newmarket) Limited.

Magdesian K.G., Hirsh D.C., Jang S.S., Hansen L.M. and Madigan J.E. (2002) Characterization of *Clostridium difficile* isolates from foals with diarrhea: 28 cases (1993-1997). *J. Am. Vet. Med. Assoc.* **220**, 67-73.

Murray M.J. (1992) Acute colitis. In: *Current Therapy in Equine Medicine 3*, 1st ed. (Robinson N.E., Ed.), pp 244-250, W.B. Saunders Company, Philadelphia, USA

Netherwood T., Wood J.L., Mumford J.A. and Chanter N. (1998) Molecular analysis of the virulence determinants of *Clostridium perfringens* associated with foal diarrhoea. *Vet J.* **155,** 289-294.

Ochoa R. and Kern S.R. (1980) The effects of *Clostridium perfringens* type A enterotoxin in Shetland ponies - clinical, morphologic and clinicopathologic changes. *Vet. Pathol.* **17***,* 738-747.

O'Connor D., Hynes P., Cormican M., Collins E., Corbett-Feeney G. and Cassidy M. (2001) Evaluation of methods for detection of toxins in specimens of feces submitted for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol*. **39,** 2846-2849.

Palmer J.E. (1992) Diarrhea. In: *Veterinary Gastroenterology,* 2nd ed., (Anderson N.V., Ed.), pp 646-654, Lea and Febiger, Philadelphia, USA.

Perrin J., Cosmetatos I., Gallusser A., Lobsiger L., Straub R. and Nicolet J. (1993) *Clostridium difficile* associated with typhlocolitis in an adult horse. *J. Vet. Diagn. Invest.* **5**, 99-101.

Stubbings D.P. (1990) *Clostridium perfringens* enterotoxaemia in two young horses. *Vet. Rec.* **127**, 431.

Tillotson K., Traub-Dargatz J.L., Dickinson C.E., Ellis R.P., Morley P.S., Hyatt D.R., Magnuson R.J., Riddle W.T., Bolte D. and Salman M.D. (2002) Population-based study of fecal shedding of *Clostridium perfringens* in broodmares and foals. *J. Am. Vet. Med. Assoc.* **220**, 342-348.

Traub-Dargatz J.L. and Jones R.L. (1993) Clostridia-associated enterocolitis in adult horses and foals. *Vet. Clin. North Am. Equine Pract.* **9**, 411-421.

Turgeon D.K., Novicki T.J., Quick J., Carlson L., Miller P., Ulness B., Cent A., Ashley R., Larson A., Coyle M., Limaye A.P., Cookson B.T. and Fritsche T.R. (2003) Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J. Clin. Microbiol.* **41**, 667-670.

Weese J.S., Staempfli H.R. and Prescott J.F. (2000) Survival of *Clostridium difficile* and its toxins in equine faeces. *J.Vet. Diagn. Invest.* **12**, 332-336.

Weese J.S., Staempfli H.R. and Prescott J.F. (2001) A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. *Equine Vet. J.* **33,** 403-409.

Wierup M. (1977) Equine intestinal clostridiosis, an acute disease of horses associated with high intestinal counts of *Clostridium perfringens* type A. *Acta. Vet. Scand. (Supplementum)* **62**, 1-182.

Wierup M. and DiPietro J.A. (1981) Bacteriologic examination of equine fecal flora as a diagnostic tool for equine intestinal clostridiosis. *Am. J. Vet. Res.* **42**, 2167-2169.

Wilson K.H., Kennedy M.J. and Fekety F.R. (1982) Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. *J. Clin. Microbiol.* **15**, 443-446.

Acknowledgements

The author is grateful to Anders Franklin, Anders Gunnarsson and Agneta Gustafsson for their valuable comments on the manuscript.

10. Enteritis and enterotoxaemia in rabbits

D.G. Marlier

Clinical condition	Clostridial species
Tyzzer's disease	*Clostridium piliforme*
Clostridial enterotoxaemia	*Clostridium spiroforme*

10.1. Introduction

The aetiological diagnosis of digestive disorders in rabbits is always very exacting to perform. Such diagnosis is illusive without systematic secondary parasitical and bacteriological examinations. Basic investigations for Tyzzer's disease and *C. spiroforme* infections should always be included by veterinarians in the protocol for diagnosis.

When these diseases are suspect in rabbits, the best way for owners and practitioners to obtain an accurate diagnosis is to send a representative batch of animals directly to a specialized laboratory. This batch should include recently deceased animals and typically sick, untreated ones, which would be sacrificed for complementary examinations. A complete history of death rates and clinical signs must be provided. The data should include the type and the size (expressed in number of does) of specimens from the rabbitry and the feeding, breeding, hygiene and management practices of same.

10.2. *Clostridium piliforme* infections in European rabbits (Tyzzer's disease)

Tyzzer's disease, while best known in rodents, also occurs in rabbits. It is caused by *Clostridium piliforme*, an intracellular Gram-Negative and filamentous *Clostridium*.

10.2.1. Clinical signs

On a clinical basis, both acute and chronic forms are described in rabbits (Prescot, 1977). In broiler animals, from small as well as from intensive rabbitries, acute cases are more frequently observed in young animals around weaning age (5 to 8–9 weeks old) than those of breeding age.

Clinical signs reflect the acute digestive syndrome, which results in depression, diarrhoea and high mortality rates (up to 50%).

Chronic forms are usually seen in the breeding stock (does and bucks). Chronic weight losses associated with bad to very bad conditions are the only clinical signs.

10.2.2. Pathological lesions

At necropsy, gross lesions may be observed on the caecum, liver and myocardium. Moderate to extremely severe pseudo-membranous acute typhlitis with moderate to severe oedema of the caecal wall is the most frequent finding. Some acute ileitis and colitis may also be observed. Unlike in rodents, necrotic foci in the liver parenchyma are rarely seen in rabbits which gives rise to diagnosis difficulties (Prescot, 1977; Pawaiya *et al.*, 1999). Some necrotic foci and degenerative lesions may also be found in the myocardium (Lee *et al*., 1976; Pawaiya *et al.*, 1999).

In chronic forms stenosis of the ileum is sometimes observed (Okerman, 1994).

10.2.3. Laboratory

The confirmation of Tyzzer's disease is always difficult to perform. When gross lesions are present, tissues (caecal wall, liver and myocardium) fixed in neutral, buffered 10% formalin should be collected at necropsy and submitted to the laboratory. *C. piliforme* may be observed in histopathologic sections after Warthin-Starry silver-impregnation staining (Okerman, 1994; Pawaiya *et al.*, 1999), though results are often disappointing. For a more specific diagnosis, immunofluorescence tests such as those conducted for mice (Savage and Lewis, 1972) or rats (Fries and Svendsen, 1978) can be adapted to rabbit tissues.

Serological tests (indirect immunofluorescence test, ELISA) have been described (Fries, 1977a; 1977b; Waggie *et al*., 1987), but they are seldom used for diagnosis, as their results are controversial.

The isolation and propagation of *C. piliforme* are both time and labour consuming, since *C. piliforme* does not grow in artificial media. Culturing, in the yolk sac of chicken embryonated eggs (Fries, 1977c) and in primary chick embryo livers (Waggie *et al.*, 1987), has been described, but the use is limited to research methods.

Theoretically, PCR reactions such as those developed for cattle (Ikegami *et al.*, 1999, Furukawa *et al.*, 2002) should work in rabbits.

10.3. *Clostridium spiroforme* enterotoxaemia

In rabbits, enterotoxaemia has been identified by Lesbouyries and Berthelon since 1936. For a long time, its aetiology was incorrectly attributed to *C. perfringens* type E infection until Carman and Borriello (1982) demonstrated the aetiological role of *C. spiroforme*. This bacteria is an helically coiled *Clostridium* that produces a toxin that can be neutralised by anti-*Clostridium perfringens* type E sera (Carman and Borriello, 1984). This bacteria was reported to be present in 52 % of rabbits with digestive disorders and in 83 % of the respective rabbitries (Licois, 1989).

10.3.1. Clinical signs

The "spontaneous forms" of the disease mostly occur in young broiler rabbits (3–4 to 8-9 weeks old), but they may also affect older animals such as does

about to nest. Usually, there are few typical clinical signs: either the rabbits are in poor condition and show watery diarrhoea or are simply found dead.

"Antibiotics-induced *C. spiroforme* enterotoxaemia" also occurs. It follows the administration of clindamycin, lincomycin (Licois, 1980) or ampicillin (Peeters, 1989) to rabbits. After the administration of the antibiotics, feed intake quickly decreases, and mortality may begin within 2 days after the administration and continue until 3 weeks after discontinued use. Total mortality rate may be as high as 50 %. The same clinical signs, but with lower mortality rate (up to 20%), may also be observed after long-term use of other antibiotics such as tetracyclin and neomycin (Peeters, 1989).

10.3.2. Pathological lesions

At necropsy a watery caecal content, usually associated with a severe to extremely severe acute haemorrhagic typhlitis, is commonly observed. Lesions sometimes are spread out to the distal part of the ileum and proximal part of colon. There may also be some mucus in the colon. The bodily condition of the animal depends upon the delay between infection and death.

10.3.3. Laboratory

The final diagnosis is based on the observation of numerous, typically curled, U-shaped or spiralled *C. spiroforme* rods after Gram staining of the caecal contents (Borriello *et al.*, 1986). A more sensitive method based on centrifugation of caecal content at 20000 g (15 min.; 4°C) and observation of the supernatant-pellet interphase after Gram staining has been proposed (Holmes *et al.*, 1988).

Routinely, isolation of *C. spiroforme* is rarely performed. If required, it can be isolated from caecal content after high-speed centrifugation by the method of Holmes *et al.* (1988).

The presence of ι-like *C. spiroforme* toxin in caecal content of affected animals may be demonstrated by the method of Carman and Borriello (1983). It is almost never done routinely, and the diagnosis is questionable, as 90 % of the strains seem toxinogenic (Licois, 1989). As previously mentioned, the easiest way to diagnose *C. spiroforme* enterotoxaemia is to send recently deceased or typically sick animals to the lab. Otherwise the use of fresh caecal content is also appropriate

10.4. Bibliography

Borriello S., Davies H. and Carman R. (1986). Cellular morphology of *Clostridium spiroforme*. *Vet. Microbiol.* **11**, 191-195.

Carman R. and Borriello S. (1982). *Clostridium spiroforme* isolated from rabbits with diarrhoea. *Vet. Rec.* **111**, 461-462.

Carman R. and Borriello S. (1983). Laboratory diagnosis of *Clostridium spiroforme*-mediated diarrhoea (iota enterotoxaemia) of rabbits. *Vet. Rec.* **113**, 184-185.

Carman R. and Borriello S. (1984). Infectious nature of *Clostridium spiroforme* mediated rabbit enterotoxaemia. *Vet. Microbiol.* **9**, 497-502.

Fries A. (1977a). Detection of *Bacillus piliformis* (Tyzzer) antibodies in serum samples of mice and rabbits (fluorescent antibody technique). *Zeitschr. Fur Versuchstierkd.* **19**, 117-125.

Fries A. (1977b). Studies on Tyzzer's disease: application of immunofluorescence for detection of *Bacillus piliformis* and for demonstration and determination of antibodies to it in sera from mice and rabbits. *Lab. Anim.* **11**, 69-73.

Fries A. (1977c). Studies on Tyzzer's disease: isolation and propagation of *Bacillus piliformis*. *Lab. Anim.* **11**, 75-78.

Fries A. and Svendsen O. (1978). Studies on Tyzzer's disease in rats. *Lab. Anim.* **12**, 1-4.

Furukawa T., Furumoto K., Fujieda M. and Okada E. (2002). Detection by PCR of the Tyzzer's disease organism (*Clostridium piliforme*) in feces. *Exp. Anim.* **51**, 513-516.

Holmes H., Sonn R. and Patton N. (1988). Isolation of *Clostridium spiroforme* from rabbits. *Lab. Anim. Sci*. **38**, 167-168.

Ikegami T., Shirota K., Une Y., Nomura Y., Wada Y., Goto K., Takakura A., Itoh T. and Fujiwara K. (1999). Naturally occurring Tyzzer's disease in a calf. *Vet. Pathol.* **36**, 253–255.

Lee Y., Hirose H., Oghiso Y., Goto N., Takahashi R. and Fujiwara K. (1976). Myocardiopathy in rabbits experimentally infected with the Tyzzer's organism. *Jap. J. Exp. Med.* **46**, 371-382.

Lesbouyries G. and Berthelon M. (1936). Enterotoxémie du lapin. *Bull. Acad. Vet. France* **9**, 74-82.

Licois D. (1980). Action toxique de certains antibiotiques chez le lapin. *Rec. Méd. Vét*. 915-919.

Licois D. (1989) Affections digestives d'origine infectieuse et/ou parasitaire. In : *Pathologie du lapin de compagnie et des rongeurs domestiques* (Brugère-Picoux J., Ed.), pp 139-164, Ecole Nationale Vétérinaire d'Alfort, Maisons Alfort, France.

Okerman L. (1994). Diseases of the digestive system. In: *Diseases of Domestic Rabbits*. (Price C.J., Ed.), pp 71-91, Blackwell Scientific Publications, Oxford.

Pawaiya R., Mishra N., Bhagwan P. (1999). Pathology of *Bacillus piliformis* infection in rabbits. *Indian J. Anim. Sci*. **69**, 924-927.

Peeters J. (1989). Ademhalings en spijsverteringsstoornissen in de industriële slachtkonijnenhouderij. *Diergeneeskd. Memorandum* **3**, 99-108.

Prescott J. (1977). Tyzzer's disease in rabbits in Britain. *Vet. Rec*. **100**, 285-286.

Savage N. and Lewis D. (1972). Application of immunofluorescence to detection of Tyzzer's disease agent (*Bacillus piliformis*) in experimentally infected mice. *Am. J. Vet. Res.* **33**, 1007-1011.

Waggie K., Spencer T. and Ganaway T. (1987). An enzyme-linked immunosorbent assay for detection of anti-*Bacillus piliformis* serum antibody in rabbits. *Lab. Anim. Sci*. **37**, 176-179.

11. Enteritis and enterotoxaemia in birds

B. Engström, M. Kaldhusdal and K. Pedersen

Clinical condition	Clostridial species	Host species
Necrotic enteritis	*Clostridium perfringens*	Chickens, turkey poults, domestic ducks, ostrich chicks, captive capercaillies and wild ducks and geese
Ulcerative enteritis	*Clostridium colinum*	Quail, chickens, turkeys, pigeons and game birds, such as partridge, pheasants and grouse reared in captivity

11.1. Necrotic enteritis

Necrotic enteritis is caused by *Clostridium perfringens*, the *Clostridium* species associated with most health problems in poultry today. Clinical necrotic enteritis is most often seen in broilers and turkey poults, but may also be seen in young broiler breeders and layers kept on litter bedding. *C. perfringens* may also cause subclinical infection in the intestine and liver: subclinical necrotic enteritis and *C. perfringens*–related hepatitis. In young layer chickens (Fossum *et al*., 1988) a disease with gizzard lesions has been reported as a condition related to infection by *C. perfringens*. Similar lesions in the gizzard have been observed in broiler chickens and turkey poults, and *C. perfringens* has also been cultivated from such lesions in broilers.

11.1.1. Clinical signs

Acute outbreaks usually occur in broilers and turkeys of at least 2 weeks of age, but are less frequently observed in flocks above 7 weeks old. Mortality is variable, but about 1% per day during a week is quite common in a flock. Signs of the disease are general depression, anorexia and acute death. Diarrhoea is seldom seen in cases of acute outbreaks, but may be found in more protracted cases. Clinical signs are variable, and mild necrotic enteritis is probably more common than the clinical form of the disease. Although the clinical symptoms of mild and subclinical disease are less conspicuous, the effects on production performance may be significant.

Gizzard erosions are observed at roughly the same age as gut lesions in clinically healthy broiler chickens.

11.1.2. Pathological lesions

A typical lesion in acutely diseased chickens appears as severe extensive necrosis of the mucosa in the small intestine. Alpha toxin produced by *C. perfringens*

is assumed to cause necrosis of intestinal mucosa, starting at the tips of the villi (Long *et al*., 1974). Necrotic lesions may become widespread, with diphtheritic membranes and severe intestinal dysfunction. Alpha toxin may gain access to the bloodstream, leading to the development of toxaemia, which, together with intestinal lesions, contributes to clinical illness and, in some cases, death (Al-Sheikhly and Truscott, 1977; Fukata *et al*., 1988).

Subclinical necrotic enteritis (SNE) is characterised by focal ulcers and necroses in the mucosa of the anterior small intestine (Kaldhusdal and Hofshagen, 1992). SNE has been found in broilers from 1–2 weeks of age up to slaughter. Up to 80% of examined birds in affected flocks may have typical lesions in the small intestine.

C. perfringens-related hepatitis is characterised by cholangiohepatitis or focal necroses in the liver. These lesions are usually found during meat inspection at slaughter, but rarely in birds with gut lesions. In most cases, no signs of disease in the flock will have been detected during the rearing period. Livers with cholangiohepatitis are usually enlarged, firm, pale, and mottled with a lobulated pattern (Randall, 1991). The gall bladder wall may be inflamed or necrotic and chicken carcasses sometimes show jaundice and/or ascites. Livers with focal necroses are characterised by multiple nodules either on the cut surface or protruding beneath the liver capsule. The lesions are probably caused by infection with *C. perfringens* in the liver and bile ducts, leading to obstruction of the bile ducts (Sasaki *et al.*, 2000). Bacteria may ascend from the duodenum via the bile ducts to the liver or come to the liver via *vena portae*.

11.1.3. Sampling procedure and transport to the laboratory

Freezing of specimens should be avoided, however, they should be kept and transported as cool as possible. Whole birds should be wrapped in fluid-absorbing paper and packed in plastic bags to avoid leakage. All sampled material should be transported as fast as possible to the laboratory.

In cases of clinical disease, necropsy of dead birds may be performed in the field. Alternatively, or as a supplement, whole birds that have died with the suspected disease may be submitted to a laboratory for necropsy and further examination. Individuals should be selected that have died as recently as possible, or else moribund birds should be euthanised for the purpose. Several birds should preferably be submitted, since pathological lesions and bacteriological findings may vary.

If subclinical disease is suspected, live birds are sampled at random from the flock. Again, the birds may be examined in the field and/or submitted (alive) to a laboratory for examination.

Instead of submitting whole birds, organ specimens may be collected and submitted. However, due to a high risk of contamination and proliferation of contaminant flora, such material is not optimal. For (semiquantitative) bacteriology, specimens are collected with cotton-tipped applicators and sent to the laboratory in transport tubes containing a suitable medium (e.g. Amies' medium with charcoal).

Specimens may be collected from small intestinal lesions, gall bladders of affected livers or gizzard lesions.

11.1.4. Laboratory procedures

On a routine basis, semiquantitative examination for *C. perfringens* on blood agar plates is adequate support for a presumptive diagnosis based on pathology. However, positive identification of *C. perfringens* requires inoculation on differential media (Wages and Opengart 2003).

11.1.4.1. Birds that have died with suspected necrotic enteritis

Gross necropsy findings of the small intestine are usually diagnostic, but the potential role of coccidia should be investigated (intestinal smears and/or histology, or oocyst counts). Bacteriology is essential if gut lesions are considered difficult to distinguish from those of ulcerative enteritis.

11.1.4.2. Birds sampled alive from a flock with suspected subclinical necrotic enteritis

The birds must be examined immediately after euthanasia, because diagnostic gut lesions may disappear rapidly after death. The role of bacteriology is the same as described for dead birds. Further, intestinal contents from newly euthanised birds can be used for quantitative bacteriology. In broiler chickens, caecal or ileal contents of birds with necrotic enteritis usually contain at least 1 million *C. perfringens*/g (median counts often 100 million *C. perfringens*/g), whereas median counts of birds without such lesions have been reported to be 100.000 *C. perfringens*/g (Kaldhusdal and Hofshagen, 1992). There is a large individual variation in *C. perfringens* counts of birds with and without necrotic enteritis, and the counts are significantly influenced by the age of the birds.

11.2. Ulcerative enteritis (quail disease)

Ulcerative enteritis (quail disease), caused by *Clostridium colinum* occurs in many species of birds, including chickens, turkeys, pigeons and game birds, such as partridge, pheasants and grouse reared in captivity. Quail is, however, the most susceptible species. The disease does not affect waterfowl. In chickens, ulcerative enteritis often accompanies or follows coccidiosis, immunosuppressive viral infections (e.g. chicken infectious anaemia virus and infectious bursal disease virus), or physical stress. Ulcerative enteritis is not a commonly diagnosed disease in broilers or turkeys in Europe today.

11.2.1. Clinical signs

Individual signs vary with the duration of the disease, from acute death to watery droppings, listlessness, anorexia and emaciation. Chickens and turkeys experience outbreaks in the rearing period of 4–10 weeks and mortality varies from 2% up to 12%.

11.2.2. Pathological lesions

Lesions include at first, small, round, superficial ulcers with haemorrhagic borders, occurring in the mucosa of the small intestine, the caecum and upper large intestine. Later the ulcers become deeper and may perforate the intestinal wall, causing peritonitis. Liver lesions are variable and involve yellow mottling, large irregular yellow areas, and disseminated grey or yellow foci. The spleen may be enlarged, congested, and haemorrhagic (Wages, 2003).

Gross post-mortem lesions (presence of gut, liver and spleen lesions) are usually diagnostic.

11.2.3. Sampling procedure and transport to the laboratory

Sampling and submission procedures are as described for necrotic enteritis above.

11.2.4. Laboratory procedures

The possible significance of coccidia, histomoniasis and *C. perfringens* (necrotic enteritis) must be evaluated.

Further, necrotic liver tissue may be crushed between two glass slides, fixed by heat, and stained using the Gram method. If *C. colinum* is present, large Gram-Positive rods containing subterminal spores, and free spores can be seen. A fluorescent antibody test has been found to be highly specific for the diagnosis of ulcerative enteritis (Berkhoff and Kanitz, 1976).

C. colinum is strictly anaerobic and needs an enriched medium for growth (e.g. tryptose-phosphate agar with 0.2 % glucose, 0.5 % yeast extract adjusted to pH 7.2, to which 8 % horse plasma is subsequently added).

11.3. Bibliography

Al-Sheikhly F. and Truscott R.B. (1977) The pathology of necrotic enteritis of chickens following infusion of crude toxins of *Clostridium perfringens* into the duodenum. *Avian Dis.* **21**, 241-255.

Berkhoff G.A. and Kanitz C.L. (1976) Fluorescent antibody tests in diagnosis of ulcerative enteritis. *Avian Dis.* **20**, 525-533.

Fossum O., Sandstedt K. and Engström B.E. (1988) Gizzard erosions as a cause of mortality in white leghorn chickens. *Avian Pathol.* **17**, 519-525.

Fukata T., Hadate Y., Baba E., Uemura T. and Arakawa A. (1988). Influence of *Clostridium perfringens* and its toxins in germ-free chickens. *Res. Vet. Sci.* **44**, 68-70.

Kaldhusdal M. and Hofshagen M. (1992) Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological and bacteriological findings in a mild form of necrotic enteritis. *Poult. Sci.* **71**, 1145-1153.

Long J.R., Petit J.R. and Barnum D.A. (1974) Necrotic enteritis in broiler chickens. II. Pathology and proposed pathogenesis. *Can. J. Comp. Med. Vet. Sci.* **38**, 467-474.

Randall C.J. (1991) *A Colour Atlas of Diseases and Disorders of the Domestic Fowl and Turkey* (2nd edition), pp 134-135, Wolfe Publishing Ltd.

Sasaki J., Goryo M. and Okada K. (2000) Cholangiohepatitis in chickens induced by bile duct ligations and inoculation of *Clostridium perfringens*. *Avian Pathol.* **29**, 405-410.

Wages D.P. and Opengart K. (2003). Necrotic Enteritis. In: *Diseases of Poultry,* 11th ed. (Saif Y.M., Ed.), pp 781-785, Iowa State Press, Iowa, USA.

Wages D.P. (2003). Ulcerative Enteritis (Quail Disease). In: *Diseases of Poultry,* 11th ed. (Saif Y.M., Ed.), pp 776-780, Iowa State Press, Iowa, USA.

PART 2: Identification and characterization

12. Shipping and safety

C. Rohde

12.1. Introduction

The information given in this chapter refers to packaging and shipping regulations for cultures of infectious microorganisms assigned to Risk Group 2 or 3 (Risk Group 4 includes highly pathogenic viruses only, no bacteria). Among the clostridia, there are several pathogens and toxin producers allocated to Risk Group 2 (WHO, 1993; Europ. Parliament, 2000). *Clostridium botulinum*, *C. perfringens* and *C. tetani* are especially noted for their pathogenicity and are considered as a potential danger for public health. Regulations are therefore in place governing correct handling of such organisms, so permits from Health Authorities are required. Furthermore, the Dual-use aspect has to be considered: before shipping an infectious substance, it is essential to know the destination of the consignment and to ensure that the receiver is authorised to work with the material and has adequate facilities. Supply to private persons or unknown institutions is to be avoided. When shipping outside the country, the sender must be sure the microorganism does not fall under export restrictions such as the Biological and Toxin Weapons Convention (BTWC) or other national legislation. In the case of listed "select agents" like *C. botulinum* and *C. perfringens* export requirements must be adhered to, including possible export permits or end-user certificates. If anything is unclear, the national Export Offices, Departments of Commerce or Foreign Offices should be consulted (Export Controls-Brief Outlines; Smith *et al.*, 1999; Weller *et al.*, 1999; Europ. Parliament, 1994).

Abbreviations used

ADR	Accord européen relatif au transport international des marchandises dangereuses par route
DG	Dangerous Goods
DGR	Dangerous Goods Regulations (**IATA**)
IATA	International Air Transport Association (Montreal, Geneva)
ICAO	International Civil Aviation Organization (Montreal)
PI	Packing Instruction (**IATA**)
SD	Shipper's Declaration for Dangerous Goods (transport by air)
UPU	Universal Postal Union (Berne)
UN	United Nations (Geneva)
WHO	World Health Organization (Geneva)

12.2. The Risk Groups

The WHO Risk Group definitions offer a unique classification system for microorganisms (WHO, 1993) although global harmonisation of their assignments to the Risk Groups has not necessarily been achieved, due to different public health care

standards in the countries or regions, vaccination and hygiene status of the populations, climatic and possibly other considerations. Therefore, slight variations in different countries may occur. The Risk Group characterises the pathogenicity of an organism, the mode and relative ease of transmission, the degree of risk to both an individual and a community, and the reversibility of the disease through the availability of known and effective preventive agents and treatment. Risk Group 2 (moderate individual risk, limited community risk) includes microorganisms that can cause human or animal disease, but are unlikely to spread since there is usually effective prophylaxis or treatment available.

12.3. Definitions

12.3.1. Non-infectious biological substances

are substances containing viable microorganisms known not causing any disease in humans or animals. Organisms assigned to Risk Group 1 may be considered as non-infectious. They do not fall under any dangerous goods transport regulations. However, other restrictions may apply and certain packaging and shipping rules have to be followed. See "International Shipping Regulations" (author's note: plant pathogenic microorganisms do not fall under DG transport regulations, but they may of course fall under quarantine or other import restrictions (Europ. Parliament Council Directive, 2000).

12.3.2. Dangerous goods

are substances or articles capable of posing a risk to health, safety, property or environment while they are being transported by surface carriers or air (Orange book, 2004; IATA DGR, 2005). Dangerous goods (DG) are allocated to one of 9 classes and to a specific UN number.

12.3.3. Infectious substances

are substances known to contain, or reasonably expected to contain, pathogens. Pathogens are microorganisms (including bacteria, viruses, rickettsiae, parasites, fungi) and other agents such as prions which can cause disease in humans or animals (Orange book, 2004; IATA DGR, 2005). Organisms grouped with Risk Group 2, 3 or 4 are infectious substances and transport regulations for DG fully apply.

12.3.4. Genetically modified microorganisms

refer to organisms in which the genetic material has been purposely altered through genetic engineering in a way that does not occur naturally. For the purpose of international transport regulations, such organisms are divided into the following categories (Orange book, 2004; IATA DGR, 2005):

- genetically modified microorganisms meeting the definition of an infectious substance must be classified in DG Class 6, Division 6.2 and assigned to UN 2814, UN 2900 or UN 3373, as appropriate; this means the

fact of being an infectious substance has priority over the fact of being genetically modified.

Genetically modified organisms and microorganisms which do not meet the definition of infectious substances but which are capable of altering animals, plants or microbiological substances in a way which is not normally the result of natural reproduction. They must be assigned to UN 3245 (DG class 9).

- animals which contain, or are contaminated with, genetically modified microorganisms meeting the definition of an infectious substance must not be transported by air unless exempted by the States concerned;

12.4. The philosophy behind shipping rules

While the availability and world-wide exchange of microbial cultures including pathogens is essential for progress in research and application, for screening, reference and similar purposes, it is obvious that adequate safeguards have to be observed in such a way that biological material presents no hazard to the environment or to people who may handle it during transport. There is a need to ensure that shipping of possibly hazardous materials is managed with a sound knowledge of the relevant regulations. Biological substances are very unlikely to cause a problem during transit and can be transported efficiently provided they are properly packed, labelled, declared and the quantities per package respect proper limits. The universal principle of the triple packaging system (primary receptacle, secondary containment and outer packaging) as required by the UN Model Regulations (Orange book, 2004) and correct labelling should be strictly followed in order to avoid rejected shipments, civil lawsuits and penalties. Therefore, all shippers of infectious substances should be thoroughly conversant with the respective regulations, which are not just guidelines. It is important to know that these are legal requirements and compliance is not optional. When cultures of infectious substances are transported, these are classified as DG, Class 6, Division 6.2, assigned to UN 2814 (affecting humans) or UN 2900 (affecting animals only), but by far most of those microorganisms allocated to Risk Group 2 can be sent under the new dereglemented shipping requirements as set in force for diagnostic specimens, UN 3373: the UN Model Regulations, 13th revised edition, have moved away from using the Risk Group allocation for packaging and transport regulation. Two new Shipping Categories are in place instead:

Category A: an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease to humans or animals. The UN Model Regulations and IATA have published an indicative examples list of organisms of Category A, covering many examples of the Risk Groups 3 and 4.

Category B is defined as follows: An infectious substance which does not meet the criteria for inclusion in Category A. Infectious substances in Category B

must be assigned to UN 3373. The proper shipping name of UN 3373 is either "Diagnostic specimens", "Clinical specimens" or "Biological substance, Category B".

12.5. International organisations and the plethora of implementation rules

(see "Abbreviations used")

In view of the risk that accidents, involving any means of transport and any carrier, might lead to the dispersal of dangerous biological material, international organisations have arranged for discussions on this subject:

The UN Committee of Experts on the Transport of Dangerous Goods (www.unece.org) regularly updates the Model Regulations, known as the "Orange Book", which is the basis for international transportation regulations for DG specified items for all carriers (Orange book, 2004). It contains information on safe handling, classification and packaging with an up-dated list of all the existing UN numbers. The ICAO Council uses these recommendations as the basis for its Technical Instructions for the Safe Transport of Dangerous Goods by Air (Council of ICAO, 2005-2006). The Dangerous Goods Regulations (DGR) (IATA DGR, 2005) are published by IATA and are the legally binding basis for shippers and carriers of dangerous goods to be transported by air. However, IATA has additional requirements which are necessarily more restrictive than the ICAO Technical Instructions on account of several State or operator variations. The Universal Postal Union UPU publishes the International Postal Convention (UPU, 1996) and shippers should know that there are restrictions in place for sending infectious substances by postal mail: many countries prohibit the movement of biological substances and especially infectious substances through postal services. The WHO Risk Group definition scheme (WHO, 1993) is fundamental for shippers of cultures of infectious substances in order to classify their material correctly before offering for transport. For DG transport by road in Europe, it is important to be aware of the ADR requirements (ECE, 1997). Other modes of transport (rail: RID or maritime waterways: IMO) do not play such a predominant role as air and road transport.

12.6. International shipping regulations

12.6.1. Transport of Biological Materials by Postal Services

National Post Offices are internationally linked together in the Universal Postal Union (UPU**,** Berne, Switzerland). UPU constantly updates the Letter Post Compendium, one of the Universal Postal Convention Acts (UPU, 1996) (last revision at Bucharest in 2004). The Convention describes detailed regulations for the conditions of acceptance and marking of items containing Perishable Biological Substances and Infectious Substances (Articles RE 412 and RE 413, respectively) and for special packing requirements (Article RE 207).

12.6.1.1. Non-infectious biological substances

Exchange of perishable biological substances (non-infectious) shall be restricted to countries whose postal administrations have declared their willingness to admit such items, whether reciprocally or in one direction only. In addition, national import or export restrictions as well as quarantine regulations should be taken into account. Also, international mail containing biological material is usually subject to customs clearance. The green Customs Declaration label for postal mail is required. The type and number of materials (e.g. 4 bacterial cultures) and the value should be entered on this label. Declare "No commercial value" in case of free exchange between laboratories or any other supply free of charge. It is recommended to send biological material by First Class, Priority or Express Letter Mail. Please note that no biological material is permitted in postal parcels, but in letter mail only. If perishable biological substances (non-infectious) are not accepted by postal services, such material cannot be sent to or within that country by mail. Shipments have to be made by freight using a courier. The courier decides the mode and route of transport.

12.6.1.2. Infectious substances

Tracking must be possible where infectious substances can be shipped by postal mail (registered mail system). Observe all restrictions by UPU, IATA and the respective National Postal Authorities. For safety reasons, airlines ask post offices to ship infectious substances only if in total conformity with the DGR. Air mail containing infectious substances (if permitted!) must therefore be accompanied by a Shipper's Declaration for Dangerous Goods (SD) in duplicate, signed by an authorised and trained person (see “Shipper’s Responsibilities”) if sent under UN 2814 or UN 2900. The documents and all additional permits are placed in a plastic envelope affixed to the outer shipping container. DG declaration documents are not required when infectious substances classified as Category B, UN 3373, are shipped.

In fact, it is hardly possible to ship infectious substances by international postal mail because very few countries permit infectious substances in postal mail. The **UPU** Acts generally forbid DG in the mail and **IATA** DGR states under chapter 2.4., “Dangerous Goods in Air Mail: … except as permitted in 2.4.1: The dangerous goods listed in this subsection may be accepted in mail for air carriage subject to the provisions of the National Postal Authorities concerned (author’s note: these include the senders’, consignee’s and all transit countries!) and the parts of these Regulations which relate to such materials. (a) Infectious substances, provided a "Shipper's Declaration" accompanies the consignment…” (IATA DGR, 2005).

(Freeze-) dried non-infectious microbial cultures may not be considered to be perishable.

Such cultures may therefore be shipped by mail to any country as far as no general import restrictions exist. Infectious, non-perishable cultures, however, should be handled in any case as infectious perishable microbial cultures (the DGR fully apply).

12.6.2. Transport of biological materials by freight

12.6.2.1. Shipper's responsibilities

Not only trans-border shipping but also transports over long distances within countries are often carried out by air. The IATA general philosophy appears very reasonable when one considers possible risks during air transport caused by fluctuations in air pressure, temperature or vibrations. Air freight is carried out by airlines, which are greatly concerned in the transport of dangerous goods, so packaging is the most essential component of safe transport.

The shipper is responsible for correct identification, classification, packaging, marking and labelling of all DG (infectious substances) intended for transport. In the case of air transport, full compliance with the IATA DGR (IATA DGR, 2005) is required. Only the shipper has an exact knowledge of the contents of a consignment and carriers must rely on the shipper's ability to properly package infectious substances with all the documentation needed.

If applicable, the Shipper's Declaration for Dangerous Goods (SD) must be correctly completed and signed by a trained person:

Shippers of DG are required to attend one initial then subsequent training courses on DG transport: "Training must be provided or verified upon the employment of a person in a position involving the transport of dangerous goods by air. Recurrent training must take place within 24 months of previous training to ensure knowledge is current..." (IATA DGR, 2005).

This measure is implemented by the National Aviation Authorities, whose addresses can be found on the IATA web site (www.iata.org/dangerousgoods). Alternatively to the obligatory IATA training course, a shipper may commission an authorised agent for packaging and shipping of DG. Any person signing a IATA SD form must have the above mentioned training certification. In case of infectious substances Category A (UN 2814 or UN 2900) transport, the shipper must have made advance arrangements with the consignee. He must have received confirmation that the substance may be legally imported without delay in delivery and must also make advance arrangements with the operator to ensure expeditious carriage (IATA DGR, 2005). It is important to know that it is forbidden to carry infectious substances including those of UN 3373 or other DG in or as passengers' checked-in or carry-on luggage. Severe penalties are in place.

12.6.2.2. The IATA regulations

The UN has developed procedures for the safe transport of all dangerous goods (Orange book, 2004). ICAO has used these recommendations as the basis for developing the regulations for the safe transport of DG by air (biannually updated) (Council of ICAO, 2005-2006). The IATA DGR (IATA DGR, 2005) are annually updated and published as a book containing all the requirements of the ICAO Technical Instructions. By following the IATA DGR, all international laws for shipping DG on land and by air are met. The DGR are available in Chinese, English, French, German and Spanish language versions and represent a user-friendly manual

(it can be purchased from national companies, or as CD-ROM directly from IATA: http://www.IATA.org/cargo/dg).

12.6.2.3. Packaging and documentation

Cultures of infectious substances of Category A (UN 2814 or UN 2900) are to be packed according to IATA Packing Instruction PI 602 requiring a UN certified combination packaging that is to be used systematically. Any self-made "safe" packaging is not permitted. The UN packaging resembles a strong and certified (specification marking on packagings) version of the triple packaging containment principle for biological substances. There is a growing and cheaper market now for such ready-to-use packagings for infectious substances that carry all the necessary labels and markings on their outer high quality fibreboard box. The Shipper's Declaration for Dangerous Goods form is the essential document for transport of DG including highly pathogenic Category A substances, along with the road transport declaration for DG, the transport emergency card and the airway bill. All these signed documents are not required for shipment of infectious substances of UN 3373, Category B (mostly Risk Group 2 organisms). However, the requirements set by Packing Instruction PI 650 apply.

12.6.2.4. Shipment example case: steps in summary

The organism to be sent is a dangerous infectious substance, affecting humans (UN 2814) or animals (UN 2900) classified in Category A → to be shipped as a Class 6.2 dangerous goods by freight; national postal mail is rarely permitted, air mail is prohibited. Independently of the net weight, the UN Model Regulations apply for all modes of transport requiring a UN certified combination packaging system (Packing Instruction 602). The shipper is a trained person and is responsible for the consignment, including the documents. The IATA DGR have to be followed. Choose approved courier service and clarify ALL steps, including possible IATA limitations before offering the consignment to the courier (destination manageable? Fast delivery? Door-to-door or door-to-airport?). Make advance arrangements with the consignee (announce date and kind of shipment) and make clear the 24-h emergency contact telephone number.

12.6.3. Transport of diagnostic specimens and of cultures of microorganisms meeting the definition of Category B

The UN Model Regulations have modified the shipping regulations for infectious substances and for the first time created a new UN number, UN 3373, that is applicable for diagnostic specimens and for such infectious substances sent as cultures if the definition of Category B is met (see above). The responsible shipper has to properly classify the substance prior to transport: for these considerations, the definitions of the Risk Groups and the allocation of microorganism species to them is still very helpful. According to the legal requirements and including shipments of

Category B, it is mandatory that shippers of infectious substances are trained persons to ensure knowledge is up-to-date

12.7. Bibliography

Council of ICAO (International Civil Aviation Organization). Technical Instructions for the Safe Transport of Dangerous Goods by Air. Doc 9284-AN/905. Addendum to the 2005-2006 Ed.

Economic Commission for Europe (ECE) (1997). ADR Accord européen relatif au transport international des marchandises dangereuses par route. English version

European Parliament Council Directive (1994). 3381/94 setting up a Community regime for the control of exports of goods with dual use. OJ No. L 367, pp. 1-12 of 31.12.1994

European Parliament Council Directive 2000/29/EC (2000). Protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. OJ No. L.169, p. 1 of 10.07.2000

European Parliament Council Directive 2000/54/EC (2000). Protection of workers from risks related to exposure to biological agents at work. OJ No. L262, pp. 21-45 of 18.09.2000

Export Controls-Brief Outlines. Bundesausfuhramt (German Federal Export Office), Eschborn, Germany: regular updates published

IATA (International Air Transport Association) (2005). *Dangerous Goods Regulations*, 46th ed., Montreal, Geneva.

Orange Book (2004) Recommendations on the Transport of Dangerous Goods.13th ed., UN, New York, ISBN 92-1-139057-5

Smith D., Rohde C and Holmes B. (1999). Handling and distribution of micro-organisms and the law. *Microbiology Today* **26**, 14-16

Universal Postal Union (1996). Universal Postal Convention, Compendium of Information, Berne (International Bureau). Edition of January 1996.

Weller R. *et al.* (1999). Universities and the Biological and Toxin Weapons Convention. *ASM News* **65**, 403-409

World Health Organization (1993) Laboratory Biosafety Manual, 2nd ed. (revised; interim guidelines). World Health Organization, Geneva, ISBN 92- 4-154450-3

13. Bacteriological identification and antibiotic sensitivity testing

J.G. Mainil, M.G. Menozzi, S. Pelkonen, M. Delmée

Clostridia are Gram-Positive rod-shaped spore-forming anaerobic bacteria. Their *in vitro* growth, identification in the routine diagnostic laboratory and antibiotic sensitivity testing must therefore follow specific rules and use specific materials. In this chapter the general diagnostic methodology, the specific growth requirements, the different orientation and identification assays and the antibiotic sensitivity testing of clostridia are described. In some of the sections, special emphasis will be placed on the growth and identification of *Clostridium difficile*.

13.1. General methodology

Probably the most important prerequisite in the laboratory diagnosis of any clostridial species is a correct clinical sample sent following the strict requirements of anaerobes (Chapter 12). Although the clostridial species differ in their requirements for anaerobiosis (Table 13.1), with some being even aero-tolerant, all samples should be plated onto appropriate blood agar plates for anaerobic incubation and onto classical blood agar plates and selective agar plates for enterobacteria for comparative aerobic growth. During the incubation period of up to five days the plates should be examined daily for colony growth.

The whole procedure may sometimes take several days and is often not compatible with the field practise, in either human or veterinary medicine. But following the whole procedure is absolutely necessary to prevent the diagnostic laboratory from using identification and antibiotic sensitivity tests for anaerobes on non-strictly anaerobic bacteria. Precise clinical data from the doctor or the veterinarian are also of importance in the identification of the major and minor pathogen clostridia.

13.2. Specific growth requirements

Clostridia grow best on enriched media, but apart from being anaerobes, the majority of species have no particular growth requirements (with the exception of the intracellular *C. piliforme*, of course!).

13.2.1. Growth media

Clostridia grow on commercially available 5% sheep blood agar plates for anaerobes, such as CDC anaerobe blood agar and Schaedler blood agar. Other classical 5% sheep blood agar, such as Columbia, Brucella and Brain-Heart Infusion, must be supplemented with yeast extract, vitamin K1 and haemin to favour growth of anaerobes (Bisping and Amtsberg, 1988; Quinn *et al.*, 1994; Atlas and Park, 2000). Anaerobic storage or pre-incubation of the plates for a few hours in anaerobiosis

enhances the chance of isolation of clostridia. Colony swarming can be prevented by growing the bacteria on phenylethyl alcohol blood agar (PEA) or on 4% agar media. The species of the genus *Clostridium* can also grow on dozens of selective blood agars for use in medical or food microbiology (Bisping and Amtsberg, 1988; Quinn *et al.*, 1994; Atlas and Park, 2000; chapter 17), several of which are commercially available, with, for instance, aminoglycosides or colistin/nalidixic acid to inhibit coliforms and other Gram negative bacteria, or Columbia with cycloserin for isolation of *C. perfringens* (Manteca *et al.*, 2001), or the selective media for *C. difficile* (see section 13.2.4).

One of the problems is the variation in the colony morphology and in the zone(s) of haemolysis when the clostridia grow on different media, leading to confusion in the pre-identification criteria. The origin of the blood is also of importance. The zone(s) of haemolysis by bovine isolates of *C. perfringens* toxin type A are broader and clearer when grown on ox blood agar than on sheep blood agar (Manteca, Mainil and Daube, unpublished data).

Growth is also possible in liquid media. The broth medium must be supplemented with serum and reducing agents, such as thioglycollate or cooked meat broths. An alternative is to add liver pieces (from rabbits) to BHI broth. The broth with a piece of rabbit liver is most probably the best liquid medium (Kaeckenbeeck and Mainil, unpublished), but its preparation is time- and labour-consuming. In most liquid media the clostridia grow best in the reduced portion of the medium, i.e. at the bottom of the tube, close to the piece of meat or of liver. All liquid media must be heated and boiled for 5-10 minutes prior to inoculation, in order to expel the absorbed traces of O_2, followed by cooling at 37°C (Quinn *et al.*, 1994).

13.2.2. Atmosphere

Because they are anaerobes, the clostridia were separated early from the genus *Bacillus* (Mainil *et al.*, 2004). In addition, all clostridia prefer to grow in an atmosphere with 2 to 10% CO_2 (Quinn *et al.*, 1994). Nevertheless, variations exist in their anaerobic requirements (Table 13.1). Some species are very strict anaerobes and do not grow in presence of even traces of O_2. Conversely, other species are aerotolerant (*C. histolyticum*, *C. tertium*) and can still grow either in a mixture of 5% O_2, 10% CO_2, 85% N_2, or even in air, though less extensively than in absence of O_2. In contrast with *Bacillus* species, *Clostridium* species can sporulate only in absence of O_2 (Peck *et al.*, 2004).

13.2.3. Incubation

Colony growth normally takes 24h to 48h at a near-neutral pH and 37°C, but may be delayed up to 5 days for some species (*C. botulinum*, *C. colinum*) or in non-optimal conditions (Table 13.1). Incubation inside an anaerobic cabinet is the best alternative and is very flexible, allowing daily examination of the plates. But this equipment is of course very expensive. Incubation in anaerobic jars with envelope systems (GasPak®) generating anaerobiosis (H_2 + CO_2) is the second best alternative. However daily examination becomes costly since new packs must be used each time.

Moreover, contact with O_2, even shortly, can already reduce the viability of the most strictly anaerobic species (Table 13.1). The third alternative is the anaerobic jar with replacement of the air by N_2 and 5-10% CO_2. A catalyst must be present inside the jars to remove the remaining O_2 traces and to allow the growth of strict anaerobes. However, most often this system does not favour the growth of the most strictly anaerobic clostridia, while favouring the growth of more aerotolerant species, leading to diagnostic confusions and errors.

Liquid media have the advantage of being aerobically incubated. However, one must also be careful, since not all of them support the growth of the most strictly anaerobic species.

Whatever system is used, a strictly aerobic bacterial species, like *Pseudomonas aeruginosa*, and a strictly anaerobic bacterial species should be grown in parallel as controls for anaerobiosis.

13.2.4. *Clostridium difficile*

Just after the discovery of the pathogenic role of *C. difficile*, George *et al.* proposed a selective agar plate called CCFA (cycloserine cefoxitin fructose agar) (George *et al.*, 1979) for isolation from stool specimens of humans (chapter 8), horses (chapter 9) and pet animals (Borrelio and Honour, 1981; Quinn *et al.*, 1994). This medium containing yeast extract, haemin, vitamin K, and cysteine is still used in most laboratories. The selective agents are cycloserine at the concentration of 500 mg/L and cefoxitine at 16 mg/L. These concentrations have been reduced to 250 mg/L and 10 mg/L respectively in some studies (Levett, 1985). Cefotaxime may also be used instead of cefoxitine at the concentration of 4 mg/L. The original formulation included egg-yolk, which may be replaced by blood. This is commercially available from several companies. Stools are directly inoculated and incubated in an anaerobic atmosphere for 48h. Anaerobic pre-incubation of the plate may also improve the recovery rate (Peterson *et al.*, 1996). The use of an Anoxomat® system, which enables the obtention of an anaerobic atmosphere in the jar within one minute, allows the reading of the plates after only a 24 hour incubation period (Delmée, unpublished data). It is worth saying that, in the vast majority of cases, the use of a standard CCFA medium with a standard inoculation procedure is quite satisfactory for the diagnosis of most cases of *C. difficile*-associated diarrhoea. Culture is the most sensitive method but it is not very specific, due to the possibility of isolating non-toxigenic isolates. It is a slow method, but one that allows the testing of strains for toxigenicity. It is also the only method for epidemiological investigations.

13.3. Identification to the species

The shapes of the colony and of the haemolysis zone(s) are important orientation criteria. Gram and Ziehl-Neelsen (acid-fast) stained smears must be performed to study the morphology of the cells and of the spores. Any suspect colony must subsequently be subcultured anaerobically, aerobically and microaerobically to prove the requirement for anaerobiosis, before applying the identification tests (without forgetting that a few minor pathogen species are aerotolerant). Identification

is performed by study of the biochemical properties, by gas liquid chromatography and by molecular methods, including toxin typing (Bisping and Amtsberg, 1988; Alen and Baron, 1991; Barrow and Feltham, 1993; Quinn *et al.*, 1994; Hirsh and Biberstein, 2004; Popoff, 2004).

13.3.1. Colony morphology and haemolysis zone(s)

In strict anaerobic conditions, colonies appear within 24 to 48 hours. Some species are, however, fastidious growers and require longer incubation (Table 13.1). The three criteria to examine carefully are the colony morphology (surface, form), the presence/absence of colony swarming and the haemolysis pattern (number and width of haemolysis zones).

13.3.2. Orientation tests

The orientation tests are generally similar to those for non anaerobes : Gram and Ziehl-Neelsen stained smears, catalase, cytochrome peroxidase and motility tests. Production of lecithinase and lipase are also considered useful pre-identification tests.

Though clostridia are Gram-Positive bacteria, Gram stained smears display a mixture of Gram-Positive and Gram-Negative bacterial cells after *in vitro* growth. In Gram stained smears, most clostridia appear as straight or curved rods, but the shape of a few species can also vary from coccoid rods to long filamentous forms. The ends of the rods are rounded, tapped or blunt. The cells of many species occur singly or in pairs and several can also form chains of varying lengths and numbers of cells. In chains, the individual cells are of similar length (*C. chauvoei*) or can be of greatly different lengths (*C. septicum*). The chains formed by a few species can form tight coils or spiral-shaped (*C. spiroforme*) configurations. Specific arrangements are, however, more frequent and typical in smears from the lesions than in smears from the colonies. The shape and arrangements of the major pathogen clostridia in Gram stained smears from colonies (Table 13.1) are of course very important for pre-identification, but other minor or non pathogen clostridial species can resemble one or other of the major pathogen species.

In modified Ziehl-Neelsen (acid-fast) stained smears, spores are ovoid or spherical, centrally, subterminally or terminally localized, and cell-distending. Some species sporulate very easily and quickly, while others only rarely.

Clostridia give negative results at the catalase (in contrast with *Bacillus* species) and cytochrome peroxidase tests. Some species can, however, give a weakly positive result at the catalase test. On the other hand, most species are motile, but quickly loose their motility in the presence of O_2.

13.3.3. Identification tests

Most clostridia are saccharolytic or proteolytic, or both, but a few species are neither. The biochemical-based minisystems [e.g. API20A® (Analytab Products, Plainview, NY)] are best suited for identification of saccharolytic, fast growing clostridia. With these systems the identification can be obtained after 24 to 48hrs of

anaerobic incubation by reading the colour reactions manually. A code number is generated and interpreted from the code book or computerized database provided by the manufacturer [e.g. ATB Expression® (bioMérieux, Marcy l'Etoile, France)]. The rapid pre-existing enzyme-based minisystems (e.g. RapID ANA II® [Innovative Diagnostic Systems, Atlanta, GA], AN-Ident and API-ZYM [Analytab Products, Plainview, NY], ANI card® [Vitek systems, Hazelwood, MO], ATB 32A® [bioMérieux, France], Rapid Anaerobe Identification panel® [Baxter Healthcare Corp., MicroScan Division, West Sacramento, CA], BBL Crystal™ ANR ID System [Becton Dickinson Microbiology Systems, Cockeysville, MD]), utilise chromogenic or fluorogenic/chromogenic substrates for detection of bacterial glycosidases, aminopetidases and some other enzymes. They are best suited for rapid identification of slow growing clostridia. The inoculation of a heavy bacterial suspension, equal to McFarland standard n° 3 to 5, allows the reading of the results of the reactions after 4 hours of aerobic incubation. The identification can be reached either manually by using a code book or by a computer-assisted automated reader, such as ATB Expression® (bioMérieux, Marcy l'Etoile, France) and MicroScan (AutoScan-4) (Engelkirk *et al*., 1992; Summanen *et al*., 1993).

Nevertheless, none of the identification kits is 100% reliable, even for the identification of the major and minor pathogenic species (60-80%). So, final identification is completed by anti-whole cell antibody immunofluorescent assays (*C. chauvoei*, *C. septicum*, *C. sordellii*, *C. novyi*, *C. haemolyticum*; chapter 3), genetic tests (chapter 14), gas liquid chromatography (chapter 15) and/or toxin typing assays (chapter 16), if necessary (Allen and Baron, 1991; Quinn *et al.*, 1994).

13.3.4. *Clostridium difficile*

Colonies of *C. difficile* are easily recognised on culture plates, due to their typical ground glass appearance when observed under a magnifying glass (Table 13.1). A yellow-green or chartreuse fluorescence under ultraviolet illumination is another characteristic of the colonies, but this may vary with the medium used. The typical odour (horse manure) is also an aid to identification. Gas liquid chromatography (GLC) of an agar plot around a suspected colony is the best and easiest method for definite identification. *C. difficile* displays a typical GLC profile, with large amounts of butyric and iso-caproic acids (chapter 15). Such a device, however, is not available in many laboratories. Individual biochemical tests or anaerobic panels may also be used (rapid pre-existing enzyme-based minisystems are strongly recommended), as well as a somatic antigen latex kit. Cross-reactions, however, have been documented with this latter reagent.

Production of proline-aminopeptidase by a disc test has been recently proposed as a means of rapid identification of *C. difficile*, when used in conjunction with the typical morphology of the colonies (Delmée, 2001, 2004). A direct enzyme immunoassay (EIA) for toxin A (chapter 16) may also be used but then, of course, it will only recognise toxigenic isolates. Enzyme immuno-assays detecting glutamate-dehydrogenase (GDH) , an enzyme specific to *C. difficile,* can also help to identify this species, in faeces and after *in vitro* growth (Snell, 2004 ; Delmée, unpublished

data). In combination, these two EIAs for toxin A and for GDH can identify and differentiate non-toxigenic and toxigenic isolates of *C. difficile*, either after *in vitro* growth or directly in faeces (TRIAGE *C. difficile* [Biosite, San Diego, CA]).

13.4. Antibiotic sensitivity and resistance

13.4.1. Intrinsic and acquired resistance

All or most clostridia species are intrinsically resistant to aminoglycosides (like all anaerobes), sulfonamides (*C. perfringens* is one exception) and diaminopyrimidines. Acquired resistance to members of other families of antibiotics, especially to the macrolides, lincosamides, tetracyclines and phenicols, has progressively developed at different frequency rates in clostridia species of clinical importance. Several clostridia species can indeed harbour antibiotic resistance genetic determinants on transferable molecules (plasmids, transposons, conjugative transposons) and may serve as reservoir of antibiotic resistance genes for other clostridial species and even genders (Cato *et al.*, 1986; Dubreuil, 2004; Mainil *et al.*, 2004).

For instance, resistance of human isolates of *C. difficile* and of human and ruminant isolates of *C. perfringens* to macrolides and lincosamides is mediated by different *erm* (Erythromycin Resistance Methylase) genes, possibly originating from *Enterococcus* or *Streptococcus*, some of which are located on a conjugative transposon or on a plasmid. Similarly, resistance to tetracyclines is most frequently mediated by the *tetA* gene amongst human, ruminant and poultry *C. perfringens* isolates, that can be located on a conjugative plasmid, and by the *tetM* gene amongst human *C. difficile* isolates, that can be located on a conjugative transposon which is similar to a conjugative transposon of *Enterococcus faecalis*. A third example is the resistance of *C. perfringens* and *C. difficile* to chloramphenicol, which is mediated by two different CAT (Chloramphenicol Acetyl Transferase) enzymes in *C. perfringens*, one of which is synthesised by transposon located genes (*catP*) and is closely related to the *catD* genes of *C. difficile*, located on a similar transposon. Different percentages of isolates of other species are also resistant to these three families of antibiotics, but the mechanisms for this are mostly unknown (Cato *et al.*, 1986; Dubreuil, 2004; Mainil *et al.*, 2004; Mastrantonio and Spigaglia, 2004)

Acquired resistance to other antibiotics, like β lactams (mainly penicillins, more rarely cephalosporins), glycopeptides, fluoroquinolones and metronidazole have also been described. In most species the percentage of resistant isolates remain low. Nevertheless, a high percentage of resistant isolates is found in some minor and major pathogen clostridia. These resistances develop as a consequence of mutations in chromosomal genes or after acquisition of new genes (such as those coding for the production of β lactamase active on penicillins in *C. butyricum*, *C. clostridioforme, C. innocuum* and *C. ramosum*) or are mediated by still unidentified mechanisms (Cato *et al.*, 1986; Dubreuil, 2004; Mainil *et al.*, 2004).

13.4.2. Antibiotic sensitivity testing

The determination of the sensitivity/resistance patterns of a clostridial isolate may be important clinically in human medicine, but much less in veterinary medicine. In human hospital medicine, clostridia are tested for antibiotic sensitivity in particular cases, such as the occurrence of several cases of infection or the persistence of an infection in a patient despite instauration of treatment(s) based on the intrinsic sensitivity of the species isolated. Another exception, in veterinary medicine, is probably *C. difficile*-associated diarrhoea in horses (chapter 9) and pet animals. The main problem is the availability of the results only after 3 to 5 days, so that a treatment must be empirically initiated by the clinician. Testing the antibiotic sensitivity patterns of clostridia is therefore important mainly for epidemiological purposes in both human and veterinary medicines, not only for local hospitals, but also because these patterns can vary according to geography following the epidemiology of the specific resistance genetic determinants.

The antibiotics to test are narrow and/or broad spectrum β lactams (penicillins, cephalosporins and carbapenems, with β lactamase inhibitors), glycopeptides (vancomycin, teicoplanin), macrolides (erythromycin), lincosamides (clindamycin), phenicols (chloramphenicol, florfenicol), tetracyclines, recent fluoroquinolones, and/or metronidazole (Prescott *et al.*, 2000). Macrolides can not be tested in anaerobic conditions with CO_2 concentrations between 5 and 10%. It must also be remembered that chloramphenicol (but not florfenicol) was banned years ago for use in farm animals, and that metronidazole has been banned more recently in the European Union. Bacitracin has been used for years in poultry to prevent enterotoxaemia caused by *C. perfringens*, but is today also banned in the European Union. Nitrofuranes are recommended against *C. colinum* infections in birds (Prescott *et al.*, 2000; chapter 11), but are also not allowed in farm animals in the European Union.

A variety of methods is available for susceptibility testing of anaerobic isolates, although problems are encountered with all of them. Agar plate methods include agar dilution, Wadsworth modified agar dilution, spiral gradient endpoint (SGE [Spiral System Instruments, Bethesda, MD]), PDM Epsilometer (E- test [AB Biodisk, Solna, Sweden]) and disk diffusion. Broth methods include broth dilution, broth micro-dilution and broth disk-elution. Not all of these procedures are currently endorsed (e.g. disk diffusion and broth disk-elution) by the NCCLS (National Committee for Clinical Laboratory Standard, 2004) whose guidelines are followed by the majority of the commercially available systems (Engelkirk *et al.*, 1992 ; Summanen *et al.*, 1993).

Agar dilution, broth macro- and micro-dilution are the techniques suggested to be used in research studies to determine the minimal inhibitory concentrations (MIC) of different antibiotics against a clostridial isolate in anaerobic conditions. The systems used in routine diagnostic laboratories are generally based on broth micro-dilution methods. There are commercial sources for frozen trays (MSI/Micro-Media System [Cleveland, OH], MicroTech Medical System [Aurora, CO] and Innovative Diagnostic Systems [Atlanta, GA]) or lyophilized trays [Radiometer

America/Sensititre, Westlake, OH]). The limited number of appropriate anti-anaerobic drugs in some of these trays is a disadvantage, but the laboratories can be supplied with custom-poured MIC panels by MicroTech Medical System and by Sensititre (Engelkirk *et al*., 1992 ; Summanen *et al*., 1993).

E-test is another agar-diffusion method used successfully for anaerobes. It is quite easy to perform and to read; it shows a high categorical agreement with the agar dilution reference method in research studies but it is costly. Therefore this method is particularly suited for studying individual isolates against few selected agents (Engelkirk *et al*., 1992 ; Summanen *et al*., 1993).

In human anaerobic bacteriology the MIC values are interpreted and the tested strains categorized (Susceptible, Intermediate, Resistant) according to the breakpoint concentrations recommended either by NCCLS (National Committee for Clinical Laboratory Standard, 2004), or by the EUCAST (European Committee on Antimicrobials Susceptibility Testing, 2003), or by national committee guidelines (e.g. Comité de l' Antibiogramme de la Société française de Microbiologie [2005], Deutsches Institute für Normung [2004]) in an attempt to achieve a good predictive value on the basis of *in vivo* correlations (Kahlmmeter *et al.*, 2003).

13.5. Bibliography

Atlas R.M. and Parks L.C. (2000). Handbook of microbiological media. CRC Press, Boca Raton, FA, USA.

Allen S.D. and Baron E.J. (1991). *Clostridium*. In: *Manual of clinical microbiology*, 5th edition (Balows A., Hausler W.J.Jr., Hermann K.L., Isenberg H.D., Shadomy H.J., Eds), pp. 505-521, ASM Press, Washington D.C., USA.

Barrow G.I. and Feltham R.K.A. (Eds) (1993). Cowan and Steel's manual for the identification of medical bacteria, 3rd edition, pp. 83-86, Cambridge University Press, Cambridge, UK.

Biberstein E.L. (1990). The Clostridia. In: *Review of veterinary microbiology* (Biberstein E.L. and Zee Y.C., Eds), pp. 295-310, Blackwell Scientific Publications, Cambridge, MD, USA.

Bisping W. and Amtsberg G. (Eds) (1988). Genus: *Clostridium*. In: *Colour atlas for the diagnosis of bacterial pathogens in animals*, pp. 79-100, Verlag Paul Parey Publishers, Hannover, Germany.

Borrelio P.S. and Honour P. (1981). Simplified procedure for the routine isolation of *Clostridium difficile* from faeces. *J. Clin. Pathol.* **34**, 1126-1127.

Carter G.R. (1986). Clostridia. In: *Essentials of veterinary bacteriology and mycology*, 3rd edition, pp.129-136, Lea and Febiger, Philadelphia, Pa, USA.

Cato E.P., George W.L. and Finegold S.M. (1986). Genus *Clostridium* Prazmowski 1880. In: *Bergey's manual of systematic bacteriology*, Volume 2, (Sneath P.H.A., Mair N.S., Sharpe M.E. and Holt J.G., Eds), pp. 1141-1200, Williams and Wilkins, Baltimore, MD, USA.

Comité de l'Antibiogramme de la Societé Française de Microbiologie (2005). Communiqué 2005 (Soussy C.J., Coordonnateur), Institut Pasteur, Paris, France. (http://www.sfm.asso.fr/nouv/general.php?pa=2)

Delmée M. (2001). Laboratory diagnosis of *Clostridium difficile* disease. *Clin. Microbiol. Infect.* **7**, 411-416.

Delmée M. (2004). Diagnosis, epidemiology and antibiotic resistance of *Clostridium difficile*. In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp 30-37, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Deutsches Institute für Normung (1998). Medical microbiology – Susceptibility testing of pathogens to antimicrobial agents – Part 4: Evaluation classes of the minimum inhibitory concentration breakpoints of antibacterial agents. DIN 58940-4, Bbl. 1, (http://www2.din.de/index.php?lang=en)

Dubreuil L. (2004). Clostridia: antibiotic susceptibility, present and near future. In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp. 49-55, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Engelkirk P.G., Duben-Engelkirk J. and Dowell V.R.Jr. (1992). Principles and Practice of Clinical Anaerobic Bacteriology (Hoffman S. and Kelley S.G., Eds), Star Publishing Company, Belmont, CA, USA.

European Committee on Antimicrobial Susceptibility Testing (2003). EUCAST definitions of antimicrobial susceptibility and resistance in relation to "clinical breakpoints" and "epidemiological cut-off values" (http://www.srga.org/eucastwt/MICTAB/index.html)

George W.L., Sutter V.L., Citron D. and Finegold S.M. (1979). Selective and differential medium for isolation of *Clostridium difficile*. *J. Clin. Microbiol.* **9**, 214-219.

Hirsh D.C. and Biberstein E.L. (2004). *Clostridium*. In: *Veterinary microbiology*, 2nd edition (Hirsh D.C., MacLachlan N.J. and Walker R.L., Eds), pp. 198-214, Blackwell Publishing, Oxford, UK.

Kahlmeter G., Brown D.F.J., Goldstein F.W., MacGowan A.P., Mouton J.W., Osterlund A., Rodloff A.C., Steinbakk M., Urbaslova P. and Vatopoulos A. (2003). European harmonization of MIC breakpoints for antimicrobal susceptibility testing of bacteria. *J. Antimicrob. Chemother.* **52**, 145-148 (http://www.srga.org/Eucastwt/bpsetting.htm)

Levett P.N. (1985). Effect of antibiotic concentration in a selective medium on the isolation of *Clostridium difficile* from faecal specimens. *J. Clin. Pathol.* **38**, 233-234.

Mainil J., Duchesnes C., Pelkonen S., Dubreuil L. and Menozzi M.G. (2004). *Identification, typing and antibiotic resistance of the genus* Clostridium. Scientific booklet of the CA QLK2-CT2001-01267, Presses Fac. Méd. Vét. ULg, Liège, Belgium. (http://www.genusclostridium.net)

Manteca C., Daube G., Pirson V., Limbourg B., Kaeckenbeeck A. and Mainil J. (2001). Bacterial intestinal flora associated with enterotoxaemia in Belgian Blue calves. *Vet. Microbiol.* **81**, 21-32.

Mastrantonio P. and Spigaglia P. (2004). Molecular studies on resistance determinants in *Clostridium difficile* clinical isolates In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp. 55-59, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

National Committee for Clinical Laboratory Standards (2004). Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved Standard, 6th edition, NCCLS, Document M11-A6. Wayne, PA: NCCLS. (http://www.clsi.org/template.cfm?Section=Area_Committee_on_Microbiology)

Peck M., Granum P.E., Gould G. and Mainil J. (2004). *Foodborne clostridia and sporulation*. Scientific booklet of the CA QLK2-CT2001-01267, Presses Fac. Méd. Vét. ULg, Liège, Belgium. (http://www.genusclostridium.net)

Peterson L.R., Kelly P.J. and Nordbrock H.A. (1996). Role of culture and toxin detection in laboratory testing for diagnosis of *Clostridium difficile*-associated diarrhea. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**, 330-336.

Popoff M. (2004). Historical aspects of clostridial toxin identification. In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp 15-21, Presses Fac. Méd. Vét. ULg, Liège, Belgium.

Prescott J.F., Baggot J.D. and Walker R.D. (2000). Antimicrobial therapy in veterinary medicine, 3rd edition, Iowa State University Press, Ames, IA, USA.

Quinn P.J., Carter M.E., Markey B. and Carter G.R. (1994). *Clostridium* species. In: *Clinical veterinairy microbiology*. Chapter 17, pp. 191-208, Wolfe Publishing, London, UK.

Snell H., Ramos M., Longo S., John M. and Hussain Z. (2004). Performance of the TechLab *Clostridium difficile* chek-60 enzyme immunoassay (EIA) in combination with the *Clostridium difficile* Tox A/B II EIA kit, the triage *Clostridium difficile* panel immunoassay, and a cytotoxin assay for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **42**, 4863-4865.

Summanen P., Baron E.J., Citron D.M., Strong C., Wexler H.M. and Finegold S.M. (1993). Wadsworth anaerobic bacteriology manual. (Hoffman S.and Baron E.J., Eds), Star Publishing Company, Belmont, CA, USA.

Table 13.1: General morphological and cultural criteria of major pathogenic clostridia (Carter, 1986; Bisping and Amtsberg, 1988; Biberstein and Zee, 1990; Allen and Baron, 1991; Quinn *et al.*, 1994; Hirsh and Biberstein, 2004).

Clostridium	**Morphology**	**Spores**	**Culture features**	**Colonies/Haemolysis**
argentinense Toxintype G	1.5 to 2.0 x 1.5 to 10.0 µm Motile Singly or in pairs Straight rods	Oval, subterminal, bulging, rare	Very strict anaerobe Up to five days	Circular to irregular, raised, transluscent, smooth, shiny Haemolysis zone
botulinum Group I Toxintypes A/B/F Proteolytic	0.5 to 1.5 x 3.0 to 20.0 µm Motile Singly or in pairs Straigth to slighly curved rods	Oval, subterminal, bulging	Very strict anaerobe Up to five days	Circular to irregular, flat to raised, transluscent to semi-opaque, gray Haemolysis zone
botulinum Group II Toxintypes B/E/F Nonproteolytic	0.8 to 1.5 x 2.0 to 16.0 µm Very motile Singly or in pairs Straight rods	Oval, subterminal, bulging (usually)	Very strict anaerobe Up to five days	Irregular, raised, translucent to opaque, gray-white Haemolysis zone
botulinum Groupe III Toxintypes C/D	0.5 to 2.5 x 3.0 to 22.0 µm Motile Singly or in pairs Straight rods	Oval, subterminal, bulging	Very strict anaerobe Up to five days	Circular to slightly irregular, flat to raised, transluscent, gray-white Haemolysis zone
chauvoei	0.6 to 1.5 x 3.0 to 8.0 µm Pleimorphic, motile Singly or in short chains Straight sides, rounded ends	Oval, subterminal, bulging	Very strict anaerobe Needs blood, cysteine Slow growth	Compact, spherical, smooth, greyish Slight haemolytic
colinum	1.0 x 3.0 to 4.0 µm	Oval, subterminal, bulging, rare	Very fastidious, primary growth in broth	Growth on blood agar only after several passages
difficile	0.5 to 2.0 x 3.0 to 17.0 µm Motile Singly or in short chains Slender rods	Oval, subterminal, slightly bulging	Fastidious, Special blood agar	Rhizoid (grounded glass), flat or low convex, opaque, gray-white
haemolyticum	0.5 to 1.5 x 3.0 to 6.0 µm Motile, mainly singly Straight sides, rounded ends	Oval, subterminal, bulging, abundant	Very strict anaerobe Slow growth	Lenticular, then woolly Haemolysis zone

Table 13.1 (continued). General morphological and cultural criteria of major pathogenic clostridia (Carter, 1986; Bisping and Amtsberg, 1988; Biberstein and Zee, 1990; Allen and Baron, 1991; Quinn *et al.*, 1994; Hirsh and Biberstein, 2004).

Clostridium	**Morphology**	**Spores**	**Culture features**	**Colonies/Haemolysis**
histolyticum	0.5 to 1.0 x 1.5 to 9.0 µm Singly or in short chains Straight rods	Oval, subterminal, slightly bulging	Can grow aerobically No spore formation during aerobic growth	Circular to irregular, flat to low convex, translucent to semi-opaque, gray-white Haemolysis zone
novyi	0.5 to 1.5 x 3.0 to 10.0 µm Motile, mainly singly Straight sides, rounded ends	Oval, subterminal, bulging, abundant	Very strict anaerobe Slow growth	Haemolysis zone gray-white Haemolysis zone
perfringens	1.0 to 1.5 x 4.0 to 8.0 µm Singly or in pairs, encapsulated Thick, straight sides	Oval, subterminal, bulging, rare	Aero-tolerant	Round, smooth, opaque glistening, ochre Haemolysis zone(s)
piliforme	0.5 x 8.0 to 10.0 µm Motile, intracellular	?	Only on cell- containing media	Not relevant
septicum	0.6 to 1.0 x 3.0 to 8.0 µm Motile, long chains including filamentous forms (10-30 µm) Straight or slightly curved	Oval, subterminal, bulging	Strict anaerobe Rapid growth	Swarming, cottony (veil-like or lawn-like) Haemolysis zone
sordellii	0.5 to 1.5 x 1.5 to 10.0 µm Motile, singly or in pairs Straight	Oval, central or subterminal, not or weakly bulging	Strict anaerobe	Round to irregular, translucent to greyish
spiroforme	0.3 to 0.5 x 2.0 to 10.0 µm Non motile Coiling, long chains	Round, terminal (subterminal), slightly bulging	Strict anaerobe	Circular, convex, shiny, Semi-opaque to opaque, gray-white to gray-brown
tetani	0.5 to 1.0 x 2.0 to 18.0 µm Motile, mainly singly Straight, slender	Round, terminal, bulging	Strict anaerobe Slow growth	Circular or rhizoid, flat, translucent, gray (swarming) Narrow haemolysis zone

14. Molecular Typing and Identification Methods

E. Stackebrandt

14.1. Introduction

The main aim of this chapter is to discuss the molecular characterisation of clostridia. However, some preliminary remarks on molecular identification in general are necessary to provide a background.

14.1.1. Classification, characterisation and identification of micro-organisms in general

Classification is the theory and process of ordering organisms into recognisable groups. Different classification schemes may exist in parallel. However, today the initial step of classification is mainly based upon characterisation of gene coding sequences for RNA of the small subunit of ribosomes (named 16S rRNA gene in the following pages), the sequences being the basis for unravelling evolutionary lines of descent.

In the pre-molecular era, classification systems were based, for example, on selected cellular and metabolic properties (such as presence or absence of spores and of photosynthetic apparatus) or the relationship to oxygen. Moreover, at the dawn of bacterial systematics, classification was even based on shape, colour or motility. It is obvious that characterisation and classification are inter-dependent, as the type of classification defines the range of properties to be determined.

Present day classification, following the polyphasic approach, is much more demanding than any other previous classification strategy. This is because a broad spectrum of properties needs to be determined at the genetic level (information carried directly on the genes, including non-coding regions) and at the epigenetic level (information carried on the products of the genes).

Bacterial systematics consists of a series of sequential steps. The first step is characterisation, i.e. obtaining data on the properties of an organism. These properties are then compared to those of described species, usually following a dichotomic key or focusing on salient characteristics. Results will show whether the isolate can be identified, i.e., affiliated to a described taxon. If identification is not possible, the isolate is likely to represent a novel taxon which may be described following more extensive characterisation. The extent to which characterisation needs to be performed depends upon the range of properties assigned to phylogenetic neighbouring taxa, to which the new taxon belongs. If sufficient properties have been determined allowing the organisms to be classified (i.e. placed into a systematic framework), a name is given to the appropriate rank of the classified organism. It is named as a new species either of an established genus or of a novel genus. It may be concluded that the purpose and efficiency of a taxonomy is tested by its use in identifying organisms (Stackebrandt *et al*., 1998).

A chapter on molecular identification methods should not give the impression that non-molecular approaches have lost their significance (even though the molecular approach may one day replace these more traditional methods). In fact, molecular identification constitutes only one, albeit large, segment of a broader spectrum of methods required unambiguously to affiliate isolates to taxa today. Nucleic acid based identification approaches also have obvious advantages over more traditional methods. These are: cost, time, reproducibility, reliable authentification, existence of cumulative databases and their universal application. Nucleic acid based identification approaches are especially advantageous when large strain numbers from the clinical environment need to be identified rapidly, some expressing the most potent toxins known.

14.1.2. Prerequisites for a suitable identification marker

Nucleic acid stretches suitable for the identification of members of a given phylogenetic level are molecules showing some of the same characteristics also demanded of suitable phylogenetic markers. The stretches should be present among all representatives of a given group of phylogenetically related organisms in order to enable their study. Since every individual sequence position can only carry information on a rather narrow range of evolutionary time, an increasing number of independently evolving positions or regions raises the number of phylogenetic levels that can be detected.

Depending on the method applied, a nucleic acid stretch should contain at least one region of conserved structure to allow one PCR primer. The conserved region would also have a second primer targeting a taxon-specific variable stretch, to amplify a specific DNA fragment of a wide range of taxa. Between these conserved regions, the nucleotide composition should contain one or more regions of high sequence divergence (e.g., for sequence analyses of intergenic space [ITS] regions and intrageneric stretches). In addition, the rrn operons (coding for rRNA) should contain sequence idiosyncrasies (to allow fluorescent *in situ* hybridisation [FISH]), together with discriminative sequence analyses and/or electrophoretic separation of DNA fragment of different lengths.

The sequence should contain one, preferably more, restriction sites to allow generation of DNA patterns by Amplified rRNA Digest Restriction Analyses (ARDRA), a 16S rRNA gene-based RFLP method.

The availability of the rapidly increasing database of sequenced genes and of completely sequenced genomes will facilitate the comparative search for nucleic acids suitable for identification. Likewise, the following will facilitate the development of identification protocols: the curated database of certain housekeeping genes (MLST, Multi-Locus Sequence Typing), search programs for tandem repeats (MLVA, Multi-Locus Variable-number tandem repeat Analysis) and probe design (ARB software).

Table 14.1 lists some of the genes presently being investigated in detail in order to unravel the phylogenetic structure of prokaryotes. Table 14.2 compares the

suitability of the gene coding for 16S rRNA and gene coding for proteins to serve as phylogenetic markers.

Table 14.1: Examples of genes used for phylogenetic studies

Widely used conservative and ubiquitous gene coding for	**Genes of restricted use and not ubiquitous gene coding for**
16S rRNA, 23S rRNA, alanyl-tRNA	Pyrophosphatase
Ribosomal proteins	Ferredoxin oxidoreductase
DNA gyrase B, DNA synthase, recA	Chitinase, serine proteinase, biotin synthase
HSP70 (DNAK), HSP60 (chaperonin)	Acetyl-CoA acytransferase, melonavate pathway

Table 14.2: Major differences between gene coding for 16S rRNA and proteins, as judged from the viewpoint of phylogenetic significance

Property	**16S rRNA gene**	**Protein-coding genes**
Intracellular amplification	mainly multiple gene copies	single copy
Universally distributed	+	some, mostly not
Availability of conservative PCR primers	+	Rarely
Evolution	orthologous	orthologous, but often paralogous
Degeneration of the code	-, not coding for proteins	+
Database	large (<50.000 sequences)	rare (few substantial)

14.2. Molecular characterisation of clostridia

14.2.1. The 16S rRNA genes

Determination of the 16S rRNA gene sequence is among the first steps applied in the identification process. Either purified nucleic acid preparations or crude extracts of bacterial cells can be used for *in vitro* amplification of *rrn* genes (= rDNA). In principle, a few bacterial cells are sufficient to perform the analysis. The worldwide application of rDNA gene sequencing makes description of a detailed protocol unnecessary. The reader is referred to the publication by Rainey and Stackebrandt (1992); PCR primer sequences have been indicated by Lane (1991) and updates are regularly included in recent publications on the phylogeny and systematics of prokaryotes. The almost complete gene is amplified using two primers located at the 5' terminus (position 27 forward) and the 3' terminus (position 1525 reverse). A series of internally binding primers (usually 14–20 bases in length) (Lane, 1991) allows rapid sequencing by means of the primer-directed chain termination method.

More than 98% of the *Clostridium* species type strains have been sequenced (Stackebrandt and Hippe, 2001), including pathogenic species (Stackebrandt *et al*.,

1999), the remaining species being unavailable from public collections. Major findings of phylogenetic studies have demonstrated that the genus *Clostridium* constitutes a broad assembly of distantly related major branches within the Firmicutes (Collins *et al*., 1994). Studies have also shown that the species *C. botulinum*, phenotypically circumscribed by the presence of neurotoxins, embraces at least five moderately related strain clusters, each being defined by the presence of a unique neurotoxin. Nearly full length 16S rRNA gene sequences have been subjected to RFLP analysis, digesting the amplificate with restriction enzymes (e.g. TaqI, RsaI), resulting in the discrimination of *C. perfringens* from other species (Cordoba *et al*., 2001) and in the classification of pathogenic strains (Gürtler *et al*., 1991).

14.2.1.1. Variable 16S rRNA gene regions

As the position of variable regions is known where the majority of species-specific nucleotide differences occur, only parts of the complete gene need to be sequenced. Within the 16S rRNA gene, variable regions are located between the following positions: (I) 60 and 106, (II) 146 and 219, (III) 440 and 494, (IV) 588 and 651, (V) 821 and 879, (VI) 997 and 1044, (VII) 1117 and 1154, (VIII) 1240 and 1296, as well as (IX) 1435 and 1466 (*E. coli* numbering). The degree of variation differs between members of the main lines of descent (phyla and orders). Amplification of rDNA stretches by polymerase chain reaction (PCR), flanking the variable regions, provides easy access to sequenceable material. The sequences of the members of *Clostridium* differ mainly in the variable regions located in region I. Long insertions with moderate sequence similarities have been detected in variable region I in *Clostridium paradoxum* (Rainey *et al*., 1996) and in members of the genus *Desulfotomaculum* (Stackebrandt *et al*., 1997), a neighbouring taxon of *Clostridium*. The multiple *rrn* operons may show strong micro-heterogeneity, requiring a cloning step for unambiguous sequence determination.

Regions displaying length heterogeneity are ideal candidates for identification protocols based on electrophoretic analysis of PCR products, especially when species-specific PCR primers are used (Van Dyke and McCarthy, 2002). Application of two species-specific PCR primers, followed by restriction analysis of one of the resulting fragments, has led to the discrimination of *C. estertheticum* from other organisms detected in vacuum-packed meat (Helps *et al*., 1999). Sensitivity can be increased by application of nested PCR, where amplification is achieved by applying two sets of PCR primers, the second one amplifying a subset of the fragments generated by the first set of primers (Klijn *et al*., 1995)

14.2.1.2. Oligonucleotide probes targeting *rrn* genes

The identification of highly variable regions with the gene coding for 16S rRNA has led to the development of oligonucleotide probes directed against these mainly species-specific regions. The phylogenetically broader the taxonomic group, the more difficult it is to design a single probe capable of detecting all taxon members. This is especially true for members of the genus *Clostridium,* which is

phylogenetically heterogeneous. A broad group would also detect members of other genera that cluster with certain *Clostridium* species (e.g. *Ruminococcus, Eubacterium, Sarcina*), while a species-cluster-specific probe would exclude detection of a major part of the *Clostridium* species.

Originally, radioactively and digoxigenine labelled probes (Rainey and Stackebrandt, 1992) were designed that hybridised with one of the following: membrane-bound rRNA, isolated DNA or DNA released from lysed filter-fixed colonies. Examples were *C. perfringens*-specific probes (Rönner *et al*., 1994a) and probes directed against *C. botulinum* (Rönner and Stackebrandt, 1994b). Later, fluorescently labelled probes were developed that were able to target RNA directly within the cells. Application of multiple probes carrying different fluorescent labels to the same 16S rRNA, increased sensitivity and allowed detection of non-cultured cells directly within the environment (Amann *et al*., 1995).

Little data is available for clostridial species, as detection of these organisms in environmental samples has not yet been of high research priority. Examples include the use of fluorescence *in situ* hybridisation (FISH) probes in the enumeration of human colon bacteria (Harmsen *et al*., 2000a) and intestinal flora in breast-fed and formula-fed newborn infants (Harmsen *et al*., 2000b). A combination of a method of PCR amplification of a 16S rRNA gene region, followed by probe hybridisation, increases the sensitivity of detection of *Clostridium tyrobutyricum,* a bacterium involved in the late blowing of certain cheeses (Klijn *et al*., 1995**).**

14.2.1.3. 23S rRNA genes and intercistronic spacer regions

Some data is available for the 23S rRNA gene, though this molecule is mainly used for phylogenetic studies. Partial 23S rRNA gene sequences have been used to detect *C. chauvoei* in clinical materials (Sasaki *et al*., 2000). Using primers directed against the 3' terminus of 16S rDNA and the 5' terminus of 23S rDNA, the intergenic spacer region is accessible for amplification followed by electrophoretic separation of the resulting fragments. One example is the molecular differentiation of some clostridia associated with “blown pack” spoilage (Broda *et al*., 2003), a species causing the clinical diagnosis in cows of blackleg or malignant oedema (Sasaki *et al*., 2001). A further example is the identification of intestinal clostridia, including several species pathogenic to humans and animals (Song *et al*., 2002).

14.2.2. Multi-Locus Sequence Typing (MLST)

This method has been introduced to base population genetics and epidemiology on a molecular level. Stretches of about 400–500 nucleotides of seven genes, coding for housekeeping proteins, are carefully analysed and differences in alleles recorded. Examples of genes used in MLST are DMSO reductase, aspartokinase glutamin synthase, thymidilate kinase, phosophomannomutase and anthranilate synthase. The selection of genes needs to be evaluated for each new taxon undergoing investigation, as genes common to a particular set of organisms may not be universally distributed. Prominent examples are strain-rich species such

as *Neisseria gonorrhoea* and *N. meningitidis, Yersinia pestis and Y. pseudotuberculosis,* as well as *Bacillus anthracis* and *B. cereus*. The evolutionary distance between strains is quantified in MLST as the number of differing loci. All strains identical to a particular strain at 5 or more loci are considered members of a clonal complex, or "ecotype". Each species consists of several ecotypes, which should be considered the starting point for independent evolution from within a species as defined today.

The topology of trees of housekeeping genes not involved in recombination is similar to that of trees representing the core of the genome, i.e. genes coding for rRNA. These organisms are considered clonal organisms. In cases where the topologies of these two sets of genes differ, recombination has occurred and these organisms are considered panmictic. Epidemic populations are panmictic, but contain islands of clonal structure, i.e., the population structure is not coherent.

No MLST data is as yet available for clostridia, but will very likely be generated because of the availability of strain-rich species with significant pathogenic potential.

14.2.3. Analysis of genomic DNA

The analyses of DNA patterns comprise a broad range of different techniques involving electrophoreses, including restriction fragment analyses and the specific and non-specific generation of PCR-mediated amplification products.

14.2.3.1. Restriction Fragment Length Polymorphism (RFLP)

This method should be considered a traditional approach. Originally it was used in two-dimensional physical mapping by separation of macro-restriction fragments using pulsed field gel electrophoresis (PFGE). This included a wide spectrum of different variations, for example: CHEF (Contour-clamped homogeneous electric field), FIGE (Field inversion gel electrophoresis), TAFE (Transverse alternating field electrophoresis) and PHOGE (Pulsed homogeneous orthogonal field gel electrophoresis). The advantage of high one-dimensional electrophoretic RFLP resolution was soon evaluated for taxonomic purposes and is used today for the discrimination of highly related strains. The quality of results depends on the generation of high molecular DNA and reproducible separation techniques, as well as on sophisticated software programs to recognise small differences in migration behaviour of fragments. Only a small amount of data is available for members of *Clostridium*, such as in the study by Cordoba *et al.* (2001). This study differentiated *C. perfringens* from other strains of other *Clostridium* species.

14.2.3.2. Amplified Fragment Length Polymorphism (AFLP)

AFLP is the method indexing variations in the whole genome, depending on the presence of restriction sites, and is one which is highly reproducible. The method consists of three steps. Firstly, total DNA is digested using 2 restriction enzymes and

the resulting restriction fragments subsequently ligated to halfsite-specific adaptors. Secondly, a subset of the fragments is selectively amplified, using 2 restriction-site/adaptor-specific primers. Use of fluorescently labelled primers (FAFLP), will refine this method. Thirdly, the PCR products (30–50 fragments of size 80–550 bp) are electrophoretically separated on polyacrylamide gels and fragments numerically evaluated by computer assistant analysis (Vos *et al*., 1995; Janssen *et al*., 1996). The AFLP method has been used to detect *C. novyi* and *C. perfringens* in drug users (McLauchlin *et al*., 2002) and to investigate *C. perfringens* food poisoning in seven outbreaks in the UK. A comparison of PFGE and AFLP has been published by Autio *et al*. (2003) concerning persistent and sporadic *Listeria monocytogenes* strains, distant relatives of *Clostridium* genus members.

14.2.3.3. Multi-Locus Variable-number tandem repeat Analysis (MLVA)

The most recent development using DNA patterns for characterisation, identification and epidemiology uses the polymorphism of tandem repeats (TR) as informative markers. Although this technique has not yet been used for differentiation of clostridial strains, it should be briefly mentioned here, as its application to clostridia can be anticipated. Originally found in mammalian cells, TR reminiscent of mini- and micro-satellites have also been found in pathogenic prokaryotes (van Belkum *et al*., 1997). This is despite the fact that these species constitute genetically very homogeneous entities. The role of TR in the regulation and translation of genes has been discussed by Le Flèche *et al*. (2001), and the position of TRs has been elucidated by analysis of fully sequenced genomes. The data of these authors indicate firstly, that the density of TR array length (< 100 bp) varies widely in prokaryotic genomes (excess of repeats in *Buchnera* sp., *Ureaplasma urealyticum*). Secondly, it indicates that some species have a very strong excess of TR, with repeat unit lengths comprising a multiple of three (e.g. *Mesorhizobium loti*, *Mycoplasma tuberculosis* and *Pseudomonas aeruginosa*). A TR database is available at (http://minisatellites.u-psud.fr). This locates tandem repeats according to a number of criteria, among others, array length (length of gene fragment enclosed), repeat unit length and number of repeats.

A number of epidemiological molecular subtyping analysis studies have been published, with probably the most well known involving *Bacillus anthracis* (Keim *et al*., 2000). Here, two different methods were used to enable typing of strains of *B. anthracis*, including those involved in bioterrorism. The methods were firstly, sequencing of the protective antigen gene on plasmid pXO1MLVA*pagA* and secondly, Multi-Locus Variable-number tandem repeat Analysis (MLVA). For MLVA analyses, 8 variable number TR markers (6 chromosomal markers and 2 plasmid markers), were combined in 4 multiplex PCR reactions. The PCR products were labelled with fluorescent primers using different coloured dyes, and the amplificates pooled for visualisation on an ABI Genescan. Thus, the location and length of amplificates were easy to detect and to evaluate by applying numerical analyses. The advantage of this system lies in the use of defined DNA regions across

the entire genome. The creation of a cumulative database has enabled the use of standardised amplification and separation methods. The disadvantage of this system lies in the need to have sequence information (preferably of more than a single strain of a species) available prior to the design of PCR primers.

14.2.4. Identification of as yet uncultured clostridia

With the combination of methods covering the isolation of DNA from any environmental sample (PCR amplification of 16S rRNA genes, cloning and sequence analysis), even as yet uncultured bacteria and microbial communities from natural samples have become accessible for identification and phylogenetic analysis. The presence of cells directly in the environment can be detected by *in situ* hybridisation techniques (FISH). These studies have demonstrated the enormous taxonomic, hence biotechnological, potential of hitherto unrecognised clostridia in nature. Some examples of the presence of hitherto uncultured clostridial cells in the environment are listed in Table 14.3.

Table 14.3: Examples of evidence of as yet uncultured clostridial species in environmental samples, as determined by sequence analysis of cloned 16S rRNA genes

Habitat	**Representative 16S rRNA gene accession number**	**Affiliated to *Clostridium* group**	**Reference**
Rice straw in anoxic paddy soil, Italy	AJ289217	I, II, IV, XIVa	Weber *et al*., 2001
Coral surfaces, Curaçao	AF441866	III, XII, XIVa	Frias-Lopez *et al.*, 2002
Lake Fryxell mat, Antarctica	AJ287732	I, II, III, IV, IVb,	Brambilla *et al*., 2001
Bulk soil anoxic rice paddy, Italy	AJ229234	IX, XIVa	Chin *et al*. 1999
Intestine termite *Reticulitermes peratus*	D63599	I, III, XI, XIVa	Ohkuma and Kudo, 1996
Municipal landfill sites, UK	Clones	III, IV, XIVa	Van Dyke and McCarthy, 2002
Trichlorobenzene transforming community, Saale river sediment; Germany	AJ009473	I	Von Wintzingerode *et al*., 1999
Oil field, Canada	U46505	XVIII	Voordouw *et al*., 1996
Marine sediment, Puget Sound, Wash. USA	U43640	XIVa	Gray and Herwig, 1996

14.3. Bibliography

Amann R.I., Ludwig W. and Schleifer K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev*. **59**, 143-169.

Autio T., Keto-Timonen R., Lunden J., Bjorkroth J. and Korkeala H. (2003). Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). *Syst. Appl. Microbiol.* **26,** 539-545.

Brambilla E., Hippe H., Hagelstein A., Tindall B.J. and Stackebrandt E. (2001). 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. *Extremophiles* **5**, 23-33.

Broda D.M., Musgrave D.R. and Bell R.G. (2003). Molecular differentiation of clostridia associated with "blown pack" spoilage of vacuum-packed meats using internal transcribed spacer polymorphism analysis. *Int. J. Food Microbiol*. **84**, 71-77.

Chin K.J., Hahn D., Hengstmann U., Liesack W. and Janssen P.H. (1999). Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. *Appl. Environ. Microbiol*. **65**, 5042-5049.

Collins M.D., Lawson P.A., Willems A., Cordoba J.J., Fernandez-Garayzabal J., Garcia P., Cai J., Hippe H. and Farrow J.A. (1994) The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* **44**, 812-826.

Cordoba M.G., Aranda E., Medina L.M., Jordano R. and Cordoba J.J. (2001). Differentiation of *Clostridium perfringens* and *Clostridium botulinum* from non-toxigenic clostridia, isolated from prepared and frozen foods by PCR-DNA based methods. *Nahrung* **45**, 125-128.

Frias-Lopez J., Zerkle A.L., Bonheyo G.T. and Fouke B.W. (2002). Partitioning of bacterial communities between seawater and healthy black band diseased, and dead coral surfaces. *Appl. Environ. Microbiol.* **68**, 2214-2228.

Gray J.P. and Herwig R.P. (1996). Phylogenetic analysis of the bacterial communities in marine sediments. *Appl. Environ. Microbiol*. **62**, 4049-4059.

Gürtler V., Wilson V.A. and Mayall B.C. (1991). Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J. Gen. Microbiol*. **137**, 2673-2679.

Harmsen H.J., Gibson G.R., Elfferich P., Raangs G.C., Wildeboer-Veloo A.C., Argaiz A., Roberfroid M.B. and Welling G.W. (2000a). Comparison of viable cell counts and fluorescence *in situ* hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. *FEMS Microbiol. Lett*. **183**, 125-129.

Harmsen H.J., Wildeboer-Veloo A.C., Raangs G.C., Wagendorp A.A., Klijn N., Bindels J.G. and Welling G.W. (2000b). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J. Pediatr. Gastroenterol. Nutr.* **30**, 61-67.

Helps C.R., Harbour D.A. and Corry J.E. (1999). PCR-based 16S ribosomal DNA detection technique for *Clostridium estertheticum* causing spoilage in vacuum-packed chill-stored beef. *Int. J. Food. Microbiol*. **52**, 57-65.

Janssen P., Coopman R., Huys G., Swings J., Bleeker M., Vos P., Zabeau M. and Kersters K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacteria taxonomy. *Microbiology* **142**, 1881-1893.

Keim P., Price L.B., Klevytska A.M., Smith K.L., Schupp J.M., Okinaka R., Jackson P.J. and Hugh-Jones M.E. (2000). Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J. Bacteriol*. **182**, 2828-2936

Klijn N., Nieuwenhof FF., Hoolwerf J.D., van der Waals C.B. and Weerkamp A.H. (1995). Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Appl. Environ. Microbiol*. **61**, 2919-2924.

Lane D.J. (1991). 16S/23S rRNA sequencing. Nucleic Acid Techniques. In: *Bacterial Systematics* (Stackebrandt E. and Goodfellow M., Eds.), pp 115-175, Chichester, John Wiley and Sons.

Le Flèche P., Hauck Y., Ontenioente L., Prieur A., Denoeud F., Ramisse V., Sylvestre P., Benson G., Ramisse F. and Vergnaud G. (2001). A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BMC Microbiol.* (http://www.biomedcentral.com/1471-2180/1/2)

McLauchlin J., Salmon J.E., Ahmed S., Brazier J.S., Brett M.M., George R.C. and Hood J. (2002). Amplified fragment length polymorphism (AFLP) analysis of *Clostridium novyi, C. perfringens* and *Bacillus cereus* isolated from injecting drug users during 2000. *J. Med. Microbiol*. **51**, 990-1000.

Ohkuma M. and Kudo T. (1996). Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Appl. Environ. Microbiol*. **62**, 461-468.

Rainey, F.A. and Stackebrandt, E. (1992). rRNA amplification. Application of 16S rDNA-based methods for bacterial identification. In: *Nonradioactive Labelling and Detection of Biomolecules.* (Kessler C., Ed.), pp 396-406, Berlin, Springer.

Rainey F.A., Ward-Rainey N.L., Janssen P.H., Hippe H. and Stackebrandt E. (1996) *Clostridium paradoxum* DSM 7308^T contains multiple 16S rRNA genes with heterogeneous intervening sequences. *Microbiology* **142**, 2087-2095.

Rönner S.G.E. and Stackebrandt E. (1994a) Identification of *Clostridium perfringens* by 16S and 23S rDNA oligonucleotide probes. *Syst. Appl. Microbiol*. **17**, 425-432

Rönner S. and Stackebrandt E. (1994b) Further evidence for the genetic heterogeneity of *Clostridium botulinum* as determined by 23S rDNA oligonucleotide probing. *Syst. Appl. Microbiol.* **17**, 180-188.

Sasaki Y., Yamamoto K., Amimoto K., Kojima A., Ogikubo Y., Norimatsu M., Ogata H. and Tamura Y. (2001). Amplification of the 16S-23S DNA spacer region for rapid detection of *Clostridium chauvoei* and *Clostridium septicum*. *Res. Vet. Sci*. **71**, 227-229.

Sasaki Y., Yamamoto K., Kojima A., Tetsuka Y., Norimatsu M. and Tamura Y. (2000). Rapid and direct detection of *Clostridium chauvoei* by PCR of the 16S-23S DNA spacer region and partial 23S rDNA sequences. *J. Vet. Med. Sci*. **62**, 1275-1281.

Song Y., Liu C., Molitoris D., Tomzynski T.J., Mc Teague M., Read E. and Finegold S.M. (2002). Use of 16S-23S rRNA spacer-region (SR)-PCR for identification of intestinal clostridia. *Syst. Appl. Microbiol*. **25**, 528-535.

Stackebrandt E., Kramer I., Swiderski J. and Hippe H. (1999) Phylogenetic basis for a taxonomic dissection of the genus *Clostridium. FEMS Immunol. Med. Microbiol*. **24**, 253-258.

Stackebrandt E., Tindall B.J., Ludwig W. and Goodfellow M. (1998) Prokaryotic Diversity and Systematics. Diversity and Systematics. In: *Biology of the Prokaryotes* (Lengeler J.W., Schlegel H.G. and Drews G., Eds), pp 674-682, Thieme Verlag, Stuttgart.

Stackebrandt E. and Hippe H. (2001) Taxonomy and systematics. In: *Clostridia, Biotechnology and Medical Applications* (Bahl H. and Dürre P., Eds), pp 19-48, Wiley-VHC, Weinheim

Stackebrandt E., Sproer C., Rainey F.A., Burghardt J., Päuker O. and Hippe H. (1997) Phylogenetic analysis of the genus *Desulfotomaculum*: Evidence for the misclassification *of Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov. comb. nov. *Int. J. Syst. Bacteriol*. **47**, 1134-1139.

Van Belkum A., Scherer S., van Leeuwen W., Willemse D., Van Alphen L. and Verbrugh H. (1997). Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. *Infect. Immun*. **65**, 5017-1527.

Van Dyke M.I. and McCarthy A.J. (2002). Molecular biological detection and characterization of *Clostridium* populations in municipal landfill sites. *Appl. Environ. Microbiol.* **68**, 2049-2053.

von Wintzingerode F., Selent B., Hegemann W. and Göbel U. (1999). Phylogenetic analysis of an anaerobic, thrichlorobenzene-transforming microbial consortium. *Appl. Environ. Microbiol*. **65**, 283-286.

Voordouw G., Armstrong S.M., Reimer M.F., Fouts B., Telang A.J., Shen Y. and Gevertz D. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl. Environ. Microbiol*. **62**, 1623-1629.

Vos P., Hogers R., Bleeker M., Reijans M., Vandelee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. and Zabeau, M. (1995). AFLP: a new technique for DNA-fingerprinting. *Nucleic Acids Res*. **23**, 4407-4414.

Weber S., Stubner S. and Conrad R. (2001). Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. *Appl. Environ. Microbiol*. **67**, 1318-1327.

15. Gas chromatography of metabolic end products and cellular fatty acids for identifying anaerobic bacteria

J.-Ph. Carlier

Conventional methods of identifying anaerobic bacteria are based on morphology observation, culture and classical biochemical tests. In contrast to aerobes, these techniques are often insufficient to permit definitive identification, and additional investigations are required to complete the diagnosis. One of the most established methods consists of the detection and determination of organic compounds.

Two main groups of compounds may serve as support in the identification of anaerobes:

- Metabolites, produced by bacteria as a result of carbohydrate fermentation and/or breakdown of amino acids.
- Long-chain fatty acids, which are the major constituents of lipids (cell membranes), lipopolysaccharides (Gram-Negative bacteria) and lipoteichoic acid (Gram-Positive bacteria).

Gas chromatography (GC) is a well known technique allowing analysis of these metabolic or structural substances. Thus, GC offers 2 possibilities of additional means for the identification of anaerobes. In this chapter we examine the procedures and potential of each one of these approaches in anaerobic bacteriology.

15.1. Metabolic end products

Anaerobic bacteria produce numerous metabolites, which mainly result from sugar fermentation and/or from the breakdown of amino acids by deamination, decarboxylation or Stickland reaction. These metabolites accumulate in the growth medium and are characteristic of bacterial genera and of some species. Most of them have been described in the last 2 decades and consist of 2-oxo-acids (Tsuchiya *et al.*, 1990), 2-hydroxy-acids (Carlier and Sellier, 1987), (methylthio)-acids (Carlier, 1992; Moss and Nunez-Montiel, 1982), aromatic acids (Moss *et al.*, 1970; Van Assche, 1978) and phenolic compounds (Elsden and Hilton, 1976). A list of these molecules is presented in Table 15.1. Their detection by GC is the most common and most documented technique with regard to the identification of anaerobic bacteria. Nevertheless, some other analytical procedures including HPLC (High-Performance Liquid Chromatography) (Guerrant *et al.*, 1982; Paavilainen and Korpela, 1993; Stein *et al.*, 1992), capillary electrophoresis and indirect UV detection (Arellano *et al.*, 2000) have also been developed for the determination of volatile organic acids.

Table 15.1: Microbial metabolites that can be detected by GC from culture media

Compounds	Usual names
Volatile fatty acids and alcohols	
Alcohols	
Ethanol	
2-propanol	Isopropanol
1-propanol	
Butanol	
Hexanol	
Acids	
Ethanoic	Acetic
Propanoic	Propionic
2-methylpropanoic	Isobutyric
Butanoic	Butyric
2-methylbutanoic	*Ante*-isovaleric
3-methylbutanoic	Isovaleric
Pentanoic	Valeric
3-methylpentanoic	*Ante*-isocaproic
4-methylpentanoic	Isocaproic
Hexanoic	Caproic
5-methylhexanoic	Isoheptanoic
Heptanoic	Enanthic
Non-volatile fatty acids and phenolic compounds	
Unsaturated acids	
Trans-butenedioic	Fumaric
4-methyl-2-pentenoic	
2-pentenoic	
4-methyl-2-pentenoic	
Hexenoic	
Saturated	
Butanedioic	Succinic
2-oxo acids	
2-oxopropanoic	Pyruvic
2-oxo-3-methylbutanoic	
2-oxobutanoic	
2-oxo-3-methylpentanoic	
2-oxo-4-methylpentanoic	
Hydroxylated	
2-hydroxypropanoic	Lactic
2-hydroxybutanoic	
2-hydroxy-3-methylbutanoic	
2-hydroxypentanoic	
2-hydroxy-3-methylpentanoic	
2-hydroxy-4-methylpentanoic	
Methylthio acids	
3-(methylthio)-pronanoic	
4-(methylthio)-butanoic	
2-hydroxy-4-(methylthio)-butanoic	
Aromatic acids and phenolic compounds	
2-phenylacetic	
3-phenylpropanoic	Hydrocinnamic
Phenol	
4-methylphenol	*p*-cresol

15.1.1. GC procedures

From an analytical point of view, microbial metabolites are classified as volatile fatty acids (VFAs), also called short-chain fatty acids (SCFAs), and as non-volatile fatty acids (NVFAs). In general, VFAs (acetic to heptanoic), which are the main metabolic products of interest in taxonomy, are directly analysed as free acids after extraction from the growth medium using either diethyl ether (Carlier, 1985; Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002) or methyl *tert*-butyl ether. The latter is a safer solvent, since it is considered less flammable (Thomann and Hill, 1986).

Other methods have been developed, such as headspace chromatography, a complicated technique used for alcohols (C_1–C_4) and VFA analysis (Seifert *et al.*, 1990). Because peak areas are very dependent on analytical conditions (syringe and sample temperature), quantitative analysis is only possible with an automated GC system.

Direct analysis of the supernatants of acidified spent media, faecal or purulent material has also been proposed (Rogosa and Love, 1968; Reed and Sanderson, 1979; Tangerman and Nagengast, 1996). A major drawback of this method is that non-volatile compounds tend to decompose in the injector and column. This problem can be solved by pre-treatment procedures or by use of a glass liner in the injector port. This liner is stopped with glass wool plugs and acts as a pre-column (Tangerman and Nagengast, 1996).

In the last few years, an original extraction technique has been developed, which may be applied to the identification of anaerobic bacteria. Solid-phase micro-extraction (SPME) is a new solvent-free sample preparation technique, which is inexpensive and time efficient (Lord and Pawliszyn, 2000). It utilises a small, fused-silica fibre coated with a thin layer of polymer to extract analytes from the sample matrix. Then the fibre is introduced into the gas chromatograph and the analytes are thermally desorbed into the injector and subsequently chromatographed. The analysis of fatty acids (C_2–C_{10}) with SPME can be carried out directly from liquid (broth culture) or from the headspace above the sample (Abalos *et al.*, 2000; Mills and Walker, 2000). Although this technique has not yet been applied to bacterial cultures, SPME seems to be a promising method for analysis of VFAs produced by anaerobes. Since this method allows for the making of derivatives (Pan and Pawliszyn, 1997), it is certainly also applicable to NVFA analysis. In conclusion, the use of SPME deserves to be developed in anaerobic microbiology.

NVFAs, such as oxo-acids, hydroxylated acids, dicarboxylic acids etc., have a high boiling point, and therefore cannot be directly detected by GC. They must undergo a derivatisation procedure prior to chromatographic analysis. The most commonly used method consists of esterification of analytes by direct incubation of media with methanol-hydrochloric or methanol-sulphuric acid followed by solvent extraction (Carlier, 1985; Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002). However, the methylation reaction is incomplete and the recovery rate of methyl esters varies according to the acid. In addition, 2-oxo acids yield at least 2 products

(Carlier and Sellier, 1989). A typical example is the methylation of pyruvic acid which produces methylpyruvate as expected, and a second component, identified as methyl-2,2-dimethoxypropanoate (Carlier and Sellier, 1989; Hautala and Weaver, 1969). In order to overcome these problems and to obtain reproducible accurate quantitative results, the method has been standardised and a known amount of internal standard (2-methylbenzoic acid) is added in advance to start the methylation reaction (Carlier, 1985).

15.1.2. Other procedures

Esterification of fatty acids can be carried out using different alcohols such as *n*-butanol, 2-butanol, propanol and methanol. A previous method consisted of converting VFAs and NVFAs to butyl esters with 14 % w/v boron trifluoride – butanol reagent (Lambert and Moss, 1972). This method permitted separation of fatty acids (formic acid to heptanoic acid), lactic and succinic acids. Although this technique is no longer used in microbiology, it is always employed for the analysis of fatty acids in dairy products and can be very useful in accurate analysis of VFAs. Indeed, substituting the methyl group with a butyl group improves the flame-ionisation efficiency of fatty acid esters. Moreover, VFA butyl esters have a uniform flame-ionisation detection (FID) response, irrespective of the number of carbon atoms contained in the molecule (Ulberth *et al.*, 1999). Many other derivatisation procedures can be employed, but their description is beyond the scope of this chapter. (Review: Taguchi, 1990).

15.1.3. Application to *Clostridium* genus

The analysis of metabolic end products can be utilised in 2 different ways: firstly, for a rapid presumptive diagnosis of anaerobic infection in the patient, or sometimes for identification by chemical marker of a particular species; secondly, as a tool in the identification of bacteria isolated from a clinical specimen.

15.1.3.1. Rapid presumptive diagnosis

Proteolytic *Clostridium* species degrade amino acids (such as valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan), to iso acids and phenolic compounds. These latter compounds can be considered as microbial markers. Therefore, their detection by headspace analysis or direct injection of body fluids, acidified liquid or solid culture medium (Pankuch and Appelbaum, 1986; Wiggins *et al.*, 1985), indicates the presence of these proteolytic species in the sample. However, a microscopic examination of the sample is needed, since branched chain acids are also produced by *Peptostreptococcus anaerobius* and some Gram-Negative bacilli (*Porphyromonas, Tissierella*). In the same way, a presumptive identification of species can be encouraged by the detection of a specific compound after growth on supplemented broth culture. For example, *C. difficile* produces *p*-cresol on cycloserine-cefoxitin-fructose-egg yolk agar (CCFA) or pre-reduced anaerobically sterilised (PRAS) peptone yeast broth supplemented with tyrosine or *p*-

hydroxyphenylacetic acid (Sivsammye and Sims, 1990). *C. malenominatum, C. tetani, C. tetanomorphum, C. cochlearium* and *C. limosum* produce phenol from tyrosine (Elsden and Hilton, 1976).

15.1.3.2. Bacterial identification

Quantitative analysis of metabolic end products in conjunction with some simple tests such as gas, indole or catalase production, allows rapid identification of many common anaerobes. However, care must be taken to obtain reproducible results. Inoculum size, culture medium and time and condition of incubation must be standardised (Carlier, 1985). The more commonly isolated *Clostridium* may be subdivided into 2 chromatographic groups. The first is composed of species producing one or several straight chain volatile fatty acids, i.e. acetic, or acetic, propionic and butyric acids, whereas the second includes species generating both straight and branched-chain fatty acids.

15.1.3.2.1. Group 1

Table 15.2: Chromatographic profiles of main *Clostridium* producing butyric acid

SPECIES	A*	P	B	C	L	H-B	H-V	Phenol
C. beijerinckii	4.4 (1.9)	0	26.8 (3.4)	0	1.6 (0.5)	tr.	0	0
C. butyricum	14 (4.3)	0	16.0 (3.3)	0	13.2 (3.7)	tr.	0	0
C. innocuum	2.9 (0.7)	0	15.7 (2.9)	0.5 (0.1)	17.4 (4.3)	0.16 (0.1)	0.8 (0.4)	0
C. perfringens	29.9 (6.2)	4.7 (1.9)	12.5 (5.6)	0	4.4 (1.4)	1 (0.9)	0	0
C. paraputrificum	16.6 (3.5)	tr.	10.6 (2.7)	0	8.2 (4.2)	tr.	tr.	0
C. tertium	20.7 (5.6)	0.8 (0.3)	10 (4.8)	0	10.2 (5.4)	0.3 (0.1)	0	0
C. symbiosum	35 (8.4)	0	16.2 (3)	0	10 (7)	0	0	0
C. malenominatum	32.5 (21.9)	5 (3.2)	35.6 (21.5)	0	3.7 (1.1)	2.1 (2.2)	0	0.9 (0.2)
C. tetani	31 (4.8)	5.5 (1.7)	19.8 (5.3)	0	2.2 (0.6)	0.7 (0.4)	0	1 (0.18)

* Mean values expressed in mmol/l; ± standard deviation in parentheses

Abbreviations: A: acetic acid; P: propionic acid; B: butyric acid; C: caproic acid; L: lactic acid; H-B: 2-hydroxybutanoic acid; H-V: 2-hydroxyvaleric acid; tr: trace amount < 0.1 mmol/l.

If acetic acid is the only volatile acid produced, bacteria cannot be identified, since this chromatographic profile is common to many species. Consequently, biochemical tests are required for speciation except for *C.*

clostridioforme, which often stains Gram-Negative and forms characteristic elongated cells. Among the species yielding butyric acid, *C. perfringens* produces propionic and 2-hydroxybutyric acids.

Non-proteolytic *C. botulinum* produces a similar pattern to that shown in Table 15.2 (Lund and Peck, 2000).

It is easy to recognise by its morphology and the presence of a double zone of haemolysis on a sheep-blood agar plate. Other species belonging to group 1 may be tentatively differentiated by some chromatographic characteristics (Table 15.2). *C. beijerinckii*, unlike *C. butyricum*, does not produce acetic acid and only synthesises small amounts of lactic acid. *C. innocuum* exhibits a very specific profile, making its identification very easy without any other test. Two small peaks of acetate and caproate are always detected in addition to prominent peaks of butyric and lactic acids (Carlier, 2003). *C. paraputrificum* and *C. tertium* are very similar and must be identified on the basis of biochemical tests. *C. tertium* is nitrate-positive, whereas *C. paraputrificum* is not. *C. symbiosum,* a species morphologically similar to *C. clostridioforme*, is characterised by the production of butyric acid. Another related species, *C. sphenoides*, does not produce butyrate, but is indole positive (Holdeman *et al.*, 1977). As seen above, 5 species possess chromatographic profiles similar to that of *C. perfringens* in their production of propionic and 2-hydroxybutyric acids. However, they differ in terms of phenol production. Moreover, their cellular morphology is very different. In particular, these bacteria display terminal spores.

15.1.3.2.2. Group 2

Distinctively, on the basis of aromatic and branched-chain acid production, the bacteria belonging to this group form 3 major subgroups (Table 15.3).

The first subgroup includes species producing isocaproate and one aromatic acid, namely 3-phenylpropionate, as differential metabolites. Note the higher production of 3-phenylpropionic acid associated with both *C. sporogenes* and proteolytic *C. botulinum* species. These species are phenotypically similar. In particular, they are lipase positive and proteolytic (milk- digested). Therefore, precise identification must be performed by a toxicity test. Neither related species, *C. bifermentans* nor *C. sordellii*, produces butyric acid, but they are both lecithinase positive and proteolytic. Interspecies differentiation can be carried out using the urease test, which is positive for *C. sordellii*. However, some urease negative strains have been described (Nakamura *et al.*, 1976), and thus, these particular strains cannot be identified by GC pattern alone.

The second subgroup is formed by species producing mainly isocaproic acid and both aromatic acids, i.e. 2-phenylacetic and 3-phenylpropionic acids. *C. mangenoti*, is phenotypically very similar to the *C. sporogenes*/proteolytic *C. botulinum* group and also yields high levels of 3-phenylpropionic acid. However, it is lipase negative and non-toxigenic. This species is rarely present in clinical specimens. *C. difficile* is easily identifiable by its characteristic metabolic end products. Indeed, almost all organic acids usually encountered in anaerobes are

produced by this organism. The particular horse-stable odour, together with non-proteolytic activity complete identification.

The third subgroup comprises only 3 species: *C. hastiforme*, *C. argentinense* (formerly *C. botulinum* type G) and its non-toxigenic counterpart, *C. subterminale*. It is defined by the presence of isovaleric acid and the absence of 3-phenylpropionic acid. Also, much more importantly, production of 2-phenylacetic distinguishes this subgroup from the others. Production of propionic acid differentiates *C. hastiforme* from *C. argentinense*/*C. subterminale*.

Table 15.3: Chromatographic profiles of main *Clostridium* producing branched-chain fatty acids

SPECIES	A*	P	iB	B	iV	V	iC	iH	4MeS C4	PhA	PhP
C. sporogenes /proteolytic *C. botulinum*	32 (7.4)	2.1 (0.8)	3.1 (0.9)	8 (2.8)	9.6 (2.8)	0.7 (0.5)	4.4 (3.6)	0.3 (0.2)	0.6 (0.4)	0	10.4 (2.1)
C. bifermentans	42.2 (9.1)	3.8 (1.9)	0.7 (0.5)	0.5 (0.2)	2.2 (1.3)	tr.	10.8 (5)	0.8 (0.5)	0.2 (0.2)	tr.	1.6 (1.3)
C. sordellii	47.8 (8.3)	3 (0.9)	0.7 (0.4)	0.5 (0.1)	1.9 (1.7)	0	6.5 (1.8)	0.7 (0.6)	0.1 (0.1)	0	0.4 (0.3)
C. mangenoti	29.6 (14.1)	5.7 (4.8)	7.6 (3.3)	5.3 (3.3)	9.8 (3.8)	1.2 (0.6)	14.6 (2.4)	2.4 (0.6)	0.8 (0.4)	0.3 (0.2)	6 (3.5)
C. difficile	19.2 (4.2)	0.5 (0.4)	1.8 (1.1)	9 (2.4)	2.8 (1.1)	0.8 (0.7)	15.6 (2.1)	0.6 (0.3)	1 (0.6)	0.4 (0.3)	1 (0.8)
C. hastiforme	50.7 (21)	7.3 (3.8)	4.3 (1.4)	25.4 (14.8)	13.8 (4)	0	0	0	0	4.1 (2.3)	0
C. argentinense /*C. subterminale*	67 (23.3)	0.5 (0.3)	2.9 (1.3)	14.4 (3.9)	11 (3.7)	0	0	0	0	2.5 (1.2)	0

* Mean values expressed in mmol/l; ± standard deviation in parentheses

Abbreviations: A: acetic acid; P: propionic acid; iB: isobutyric acid; B: butyric acid; iV: isovaleric acid; V: valeric acid; iC: isocaproic acid; iH: isoheptanoic acid; 4-MeS C4: 4-(methylthio)-butyric acid; PhA: 2-phenylacetic acid; PhP: 3-phenylpropionic acid; tr: trace amount > 0.1 mmol/l.

15.2. Long-chain fatty acids

Cellular fatty acid (CFA) composition of micro-organisms has been used extensively for bacterial identification and taxonomic purposes. Variability in number of carbon atoms, number and position of double-bonds, *cis* or *trans* isomers and substitution groups are stable parameters, provided that highly standardised analysis and culture conditions are observed. (Review on technology and applicability of CFA analysis: Welch, 1991). The usual method consists of lysing bacterial cells by suspension in a saponification mixture, causing the release of their fatty acids, which are converted to methyl ester derivatives (FAME), and then analysed by gas chromatography. Esterification of fatty acids can be carried out by many other procedures involving acid or base-catalysed transesterification (Review: Eder, 1995). In general, CFA analysis includes saturated, unsaturated, hydroxylated, straight and branched molecules with 9 to 20 carbon atoms. Identification is

performed by comparison of sample retention times with those of commercially prepared FAME standard mixes. Tentative identification of unknown acids is achieved by measuring their equivalent chain length (ECL)[2] against a homolog of the saturated straight-chain monocarboxylic acids.

The amount of each acid is determined via peak areas as a percentage of the total FAME peak areas. However, this technique is not practical for non-specialised laboratories. A major drawback involves the problem of reproducibility and of comparison of multivariate recordings. Besides, many other compounds other than FAMEs may be detected, making the chromatograms difficult to interpret. For example, many anaerobes contain plasmalogens, which are particular phospholipids possessing a vinyl ether bond at the *sn*-1 position, instead of the usual ester bond found in fatty acids. In this case, the methylation procedure leads to the formation of aldehydes, which are converted into dimethylacetals (DMAs). These derivatives are practically impossible to separate from most FAMEs. As a consequence, in addition to the need for a highly standardised procedure, the use of an automated apparatus coupled with a database is strongly recommended for effective interpretation of chromatograms and the accurate identification of micro-organisms.

Today, the only commercially available system for this purpose is the Sherlock® Microbial Identification System (MIS) produced by Microbial ID (MIDI, Newark, DE, USA. http://www.midi-inc.com) and distributed by Agilent Technologies http://www.agilent.com. The MIS requires bacteria to be streaked on agar plates and incubated for 24 h before the derivatisation procedure and gas chromatography. The software takes into account both the presence or absence of FAMEs and DMAs and the ratio of the acids in the sample. The chromatographic profiles are compared with those contained in identification libraries by using a similarity index based upon calculation of the distance between the profile of the unknown strain and the mean profile of the closest library entry. A similarity value of 0.5 or greater is considered an acceptable identification, and a value below 0.5 is taken as an uncertain identification. However, the MIS is not very sensitive and 30–40 mg of cells (wet weight) must be used in the analysis. This is a major drawback for fastidious or slow-growing organisms, requiring a long incubation period. Moreover, because CFA composition of bacteria varies with growth media and culture conditions, strict adherence to the analytical protocol recommended by the manufacturer must be followed to avoid misidentification (Ghanem *et al.*, 1991).

[2] Definition of ECL is as follows: For a specified column and carrier gas, in the range where there is a linear relationship between the molecular weight of the reference saturated straight-chain monocarboxylic methyl esters and logarithm of their retention times, interpolation between the retention times of the reference esters allows determination of an ECL for each compound. This value is independent of experimental conditions (Miwa *et al.*, 1960).

15.2.1. Application to *Clostridium* genus

In general, studies demonstrating the utility of CFA analysis by MIS for identification of *Clostridium* species are lacking. According to Allen *et al*. (1995), among 216 strains representing 18 species, MIS identified 86% of the strains correctly to species level without supplemental tests (*C. perfringens*, *C. difficile*, *C. butyricum*, *C. sporogenes*, *C. septicum*, *C. glycolicum* and *C. symbiosum*) and 6% of the strains were identified to species level with the aid of some complementary tests. 3% of the strains, including *C. ramosum* and *C. clostridioforme* species, were misidentified to genus level. Another study showed that 100% of *C. difficile* strains (n=62) were correctly identified by the system, while misidentification was obtained for one strain of *C. butyricum*, one strain of *C. sordellii* and one strain of *C. septicum* (Anderson *et al.*, 1995). From 686 analyses of 285 *C. botulinum* strains and related organisms, Ghanem *et al*. (1991) found 14 CFA groups for toxigenic strains and 6 other CFA groups for non-toxigenic strains. Depending on the toxin type or CFA group, the MIS system permitted a correct identification for 74 to 100% of the analyses, 89 to 100% for the toxigenic strains, and 60 to 100% for the non-toxigenic strains tested. The authors concluded that CFA analysis offers a reasonable alternative to mouse assays for laboratories that do not have facilities for these toxicity tests.

15.3. Conclusion

Demonstration of the presence of metabolites in spent media or headspace and analysis of CFA are 2 effective applications of GC for identification of bacteria. Detection of metabolic end products is the most used, simplest and least expensive method. Interpretation of chromatograms can be facilitated by comparison with data contained in numerous publications (Carlier and Sellier, 1989; Carlier, 2003; Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002) or by chromatography of reference strains. CFA analysis is an application requiring a highly standardised procedure. Furthermore, it is strongly dependent on the apparatus and software provided by the manufacturer. However, this technique allows the differentiation of more than 140 bacterial compounds (Ghanem *et al.*, 1991), and is considered as an important feature in polyphasic taxonomy (Vandamme *et al.*, 1996).

15.4. Bibliography

Abalos M., Bayona J.M. and Pawliszyn J. (2000). Development of a headspace solid-phase microextraction procedure for the determination of free volatile fatty acids in waste waters. *J. Chromatogr.* A, **873**, 107–115.

Allen S.D., Siders J.A., Riddell M.J., Fill J.A. and Wegener W.S. (1995). Cellular fatty acid analysis in the differentiation of *Clostridium* species in the clinical microbiology laboratory. *Clin. Infect. Dis.* **20** (Suppl. 2), S 198-S 201.

Anderson J., Korver J. and Luchsinger I. (1995). Use of fatty acid analysis (microbial identification system) for the identification of *Clostridium difficile*. *Clin. Infect. Dis.* **20** (Suppl. 2), S202-S203.

Arellano M., Jomard P., El Kaddouri S., Roques C., Nepveu F. and Couderc F. (2000). Routine analysis of short-chain fatty acids for anaerobic bacteria identification using capillary electrophoresis and indirect ultraviolet detection. *J. Chromatogr*. B. **741**, 89-100.

Carlier J.P. (1985). Gas chromatography of fermentation products: its application in diagnosis of anaerobic bacteria. *Bulletin de l'Institut Pasteur* **83**, 57-69.

Carlier J.P. and Sellier N. (1987). Identification by gas chromatography-mass spectrometry of short-chain hydroxy acids produced by *Fusobacterium* species and *Clostridium innocuum*. *J. Chromatogr*. **420**, 121-128.

Carlier J.P. and Sellier N. (1989). Gas chromatographic-mass spectral studies after methylation of metabolites produced by some anaerobic bacteria in spent media. *J. Chromatogr*. **493**, 257-273.

Carlier J.P. (1992). Gas chromatographic detection of sulphur compounds as methyl esters after growth of anaerobic bacteria in broth media. *Lett. Appl. Microbiol*. **14**, 84-87.

Carlier J.P. (2003), The metabolic end products as a tool in identification of anaerobic bacteria. In: *Diagnosis, epidemiology and antibiotic resistance of the genus* Clostridium. Proceedings of the CA QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. and Menozzi M.G., Eds), pp. 22-26, Presses Fac. Méd. vét. Ulg, Liège, Belgium.

Eder K. (1995). Gas chromatographic analysis of fatty acid methyl esters. *J. Chromatogr*. B. **671**, 113-131.

Elsden S.R., Hilton M.G. and Waller J.M. (1976). The end products of the metabolism of aromatic amino-acids by clostridia. *Arch. Microbiol*. **107**, 283-288.

Ghanem F.M., Ridpath A.C., Moore W.E. and Moore L.V. (1991). Identification of *Clostridium botulinum*, *Clostridium argentinense* and related organisms by cellular fatty acid analysis. *J. Clin. Microbiol*. **29**,1114-1124.

Guerrant G.O., Lambert M.A. and Moss C.W. (1982). Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. *J. Clin. Microbiol.* **16**, 355-360.

Hautala E. and Weaver M.L. (1969). Separation and quantitative determination of lactic, pyruvic, fumaric, succinic, malic and citric acids by gas chromatography. *Anal. Biochem*. **30**, 32-39.

Holdeman L.V., Cato E.P. and Moore W.E.C. (1977). In: *Anaerobe Laboratory Manual*, 4th ed., Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Jousimies-Somer H., Summanen P., Citron D.M., Baron E.J., Wexler H.M. and Finegold S.M. (2002). In: *Wadsworth–KTL Anaerobic Bacteriology Manual*, 6th ed., Star Publishing Compagny, Belmont, CA.

Lambert M.A. and Moss C.W. (1972). Gas-liquid chromatography of short-chain fatty acids on Dexsil 300 GC. *J. Chromatogr.* **74**, 335-338.

Lord H. and Pawliszyn J. (2000). Evolution of solid-phase microextraction technology. *J. Chromatogr*. A, **885**, 153-193.

Lund B.M. and Peck M.W. (2000). *Clostridium botulinum*. In: *The microbiological safety and quality of food* (Lund B.M., Baird-Parker A.C. and Gould G.W., Eds), pp. 1057-1109, Aspen, Gaithersburg, USA.

Mills G.A. and Walker V. (2000). Headspace solid-phase microextraction procedures for gas chromatographic analysis of biological fluids and materials. *J. Chromatogr.* A **902**, 267-287.

Miwa T.K., Mikolajczak K.L., Earle F.R. and Wolff I.A. (1960). Gas chromatographic characterization of fatty acids. Identification constants for mono- and dicarboxylic methyl esters. *Anal. Chem.* **32**, 1739-1742.

Moss C.W., Lambert M.A. and Goldsmith D.J. (1970). Production of hydrocinnamic acid by clostridia. *Appl. Microbiol.* **19**, 375-378.

Moss C.W. and Nunez-Montiel O.L. (1982). Analysis of short-chain acids from bacteria by gas-liquid chromatography with a fused-silica capillary column. *J. Clin. Microbiol.* **15**, 308-311.

Nakamura S., Shimamura T. and Nishida S. (1976). Urease-negative strains of *Clostridium sordellii*. *Can. J. Microbiol.* **22**, 673-676.

Paavilainen S. and Korpela T. (1993). Comparison of high-performance liquid and gas chromatography in the determination of organic acids in culture media of alkaliphilic bacteria. *J. Chromatogr.* **634**, 273-280

Pan L. and Pawliszyn J. (1997). Derivatization/Solid-Phase Microextraction: New Approach to Polar Analytes. *Anal. Chem.* **69**, 196-205.

Pankuch G.A. and Appelbaum P.C. (1986). Agar medium for gas-liquid chromatography of anaerobes. *Am. J. Clin. Pathol.* **85**, 82-86.

Rogosa M. and Love L.L. (1968). Direct quantitative gas chromatographic separation of C2-C6 fatty acids, methanol, and ethyl alcohol in aqueous microbial fermentation media. *Appl. Microbiol.* **16**, 285-290.

Reed P.J. and Sanderson P.J. (1979). Detection of anaerobic wound infection by analysis of pus swabs for volatile fatty acids by gas-liquid chromatography. *J. Clin. Pathol.* **32**, 1203-1205.

Seifert H.S.H., Giercke-Sygusch S. and Böhnel H. (1990). Identification of pathogenic bacteria by headspace gas chromtography. In: *Analytical Microbiological Methods* (Fox A., Morgan S.L., Larsson L. and Odham G., Eds), pp. 125-136, Plenum Press, New York.

Sivsammye G. and Sims H.V. (1990). Presumptive identification of *Clostridium difficile* by detection of p-cresol in prepared peptone yeast glucose broth supplemented with p-hydroxyphenylacetic acid. *J. Clin. Microbiol.* **28**, 1851-1853.

Stein J., Kulemeier J., Lembcke B. and Caspary W.F. (1992). Simple and rapid methods for determination of short-chain fatty acids in biological materials by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr.* **576**, 53-61

Taguchi V.Y. (1990). Derivatization techniques. In: *Gas chromatography: Biochemical, Biomedical and Clinical Applications*. (Clement R.E., Ed.), pp 129-179, John Wiley and sons, New York.

Tangerman A. and Nagengast F.M. (1996). A gas chromatographic analysis of fecal short-chain fatty acids, using the direct injection method. *Anal. Biochem*. **236**, 1-8.

Thomann W.R. and Hill G.B. (1986). Modified extraction procedure for gas-liquid chromatography applied to the identification of anaerobic bacteria. *J. Clin. Microbiol*. **23**, 392-394.

Tsuchiya H., Sato M., Yamamoto K., Yamauchi M., Tani H., Namikawa I. and Takagi N. (1990). High-performance liquid chromatographic analysis of alpha-keto acids produced from amino acid metabolism in oral *Bacteroides*. *J. Appl. Bacteriol*. **69**, 125-133.

Ulberth F., Gabernig R.G. and Schrammel F. (1999). Flame-ionization detector response to methyl, ethyl, propyl, and butyl esters of fatty acids. *J. Amer. Oil Chem. Soc*. **76**, 263-266.

Vandamme P., Pot B., Gillis M., De Vos P, Kersters K. and Swings J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev*. **60**, 407-438.

Van Assche P.F.D. (1978). Differentiation of *Bacteroides fragilis* species by gas chromatographic detection of phenylacetic acid. *J. Clin. Microbiol*. **8**, 614-615

Welch D.F. (1991). Applications of cellular fatty acid analysis. *Clin. Microbiol. Rev.* **4**, 422-438.

Wiggins R.J., Wilks M. and Tabaqchali S. (1985). Analysis by gas liquid chromatography of production of volatile fatty acids by anaerobic bacteria grown on solid medium. *J. Clin. Pathol.* **38**, 933-936.

16. Toxintyping by phenotypic and genetic assays

M. Popoff and R.W. Titball

16.1. Glucosyltransferases

Glucosylating toxins are also referred to as large clostridial toxins (Table 16.1) and are single chain proteins ranging in size from 250 to 300 kDa. The large clostridial toxin family encompasses *Clostridium difficile* toxins A and B (ToxA and ToxB), *Clostridium sordellii* lethal toxin (LT), and haemorrhagic toxin (HT) and *Clostridium novyi* α toxin.

ToxB and LT are highly related (76% amino acid sequence identity) and are more distantly related to ToxA and α toxin of *C. novyi* (48-60% identity) (Richard *et al.*, 1999).

Table 16.1: Enzymatic activity and substrates of the large clostridial toxins

Clostridium **species**	**Toxins**	**Enzymatic reaction**	**Substrates**
C. difficile	ToxA	Glucosylation	Rho, Rac, Cdc42
	ToxB(VPI10463)	(UDP-glucose)	Rho, Rac, Cdc42
	ToxB(8864)		Rac, Rap, Ral
C. novyi	α toxin	(UDP-*N*-AGlu)	Rho, Rac, Cdc42
C. sordellii	LT82	(UDP-glucose)	Rac, Ras, Rap, Ral
	LT9048		Rac, Cdc42, Ras, Rap
	HT		Rho, Rac, Cdc42

C. difficile is the aetiological agent of pseudomembranous colitis and is responsible for about 30% of cases of post-antibiotic diarrhoea. ToxA, which experimentally induces necrotic and haemorrhagic intestinal lesions, seems to be the main virulence factor in naturally-occurring disease. In addition, ToxB and ToxA appear to participate in the recruitment of inflammatory cells, which are abundant in

lesions. *C. sordellii* and *C. novyi* are also the aetiological agents of gangrene, and *C. sordellii* is also an agent of haemorrhagic enteritis and enterotoxaemia in cattle.

The large clostridial toxins catalyse the glucosylation of 21 kDa G-proteins from UDP-glucose, except for *C. novyi*, which uses UDP-*N*-acetylglucosamine as co-substrate. ToxA and ToxB glucosylate Rho, Rac and Cdc42 at Thr-37, whereas LT glucosylates Ras at Thr-35, Rap, Ral and Rac. The large clostridial toxins cleave the co-substrate and transfer the glucose moiety to the acceptor amino acid of the Rho proteins (Just *et al.*, 1995a, 1995b; Popoff *et al.*, 1996). The conserved Thr, which is glucosylated, is located in switch I. Thr37/35 is involved in the coordination of Mg^{++} and subsequently in the binding of the β and γ phosphates of GTP. The hydroxyl group of Thr37/35 is exposed on the surface of the molecule in its GDP-bound form, which is the only accessible substrate of glucosylating toxins. The nucleotide binding of glucosylated Ras by LT is not grossly altered, but the GEF activation of GDP forms is decreased (Hermann *et al.*, 1998). Glucosylation of Thr35 completely prevents the recognition of the downstream effector, blocking the G-protein in the inactive form (Hermann *et al.*, 1998).

The large clostridial toxins, by modifying Rho proteins, induce cell rounding with loss of actin stress fibres, reorganisation of the cortical actin and disruption of the intercellular junctions. ToxA and ToxB disrupt apical and basal actin filaments and subsequently disorganise the ultrastructure and component distribution (ZO-1, ZO-2, occludin, claudin) of tight junctions, whereas E-cadherin junctions show little alteration (Chen *et al.*, 2002; Nusrat *et al.*, 2001). In contrast, LT which modifies only Rac among the Rho proteins, alters the permeability of intestinal cell monolayers, causing a redistribution of E-cadherin, while ZO-1 labelling is not significantly affected (Richard *et al.*, 1999).

The toxins are produced in the late exponential growth phase and early stationary phase of growth. Glucose and other rapidly metabolisable sugars are inhibitors of ToxA and ToxB synthesis in *C. difficile* (Dupuy and Sonenshein, 1998; Mani and Dupuy, 2001). When grown in liquid medium containing peptones and yeast extract, the toxins are produced in 2 to 4 days.

16.1.1. Biological methods for large clostridial toxin detection

16.1.1.1. Mouse lethality test

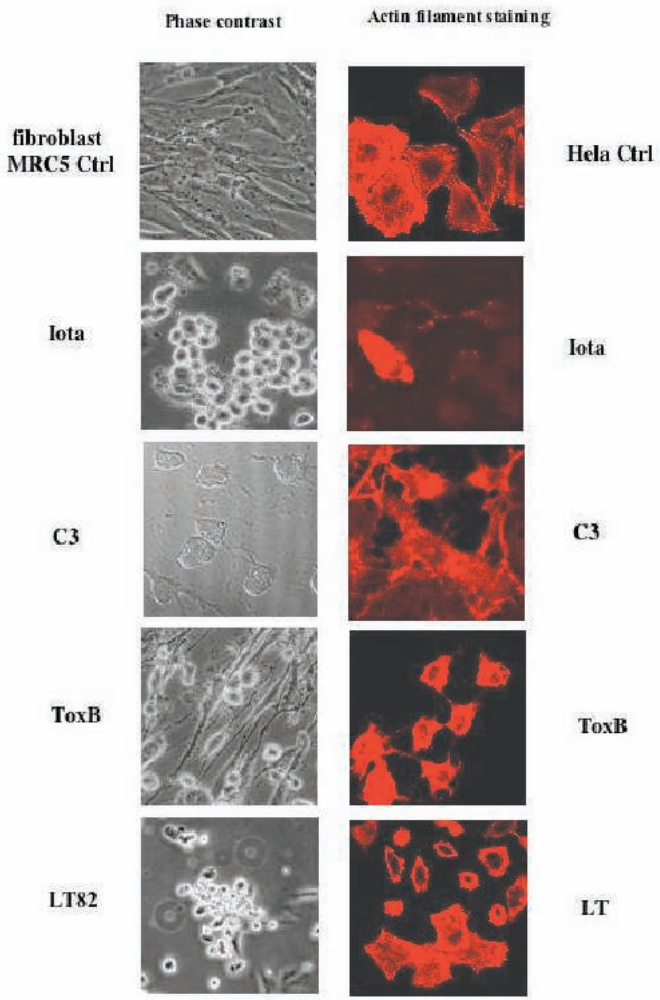
The toxins are lethal to mice upon intraperitoneal injection, but the toxicity varies for each toxin. The lethal dose in mice (LD50) is estimated to be 10-26 ng for ToxA and 1.5-4.4 μg for ToxB and LT (Lyerly *et al.*, 1988). Specific neutralising antibodies are required for the identification of the toxins with the mouse lethality test, but such sera are not commercially available.

16.1.1.2. Cytotoxicity

By modifying Rho proteins, the large clostridial toxins induce cell rounding, with loss of actin stress fibres, reorganisation of the cortical actin, and disruption of the intercellular junctions. ToxB induces loss of actin stress fibres and reorganisation of focal adhesions accompanied by cytoplasmic retraction and cell rounding, whereas

long protrusions radiating around the cell give a particular morphology (Ottlinger and Lin, 1988), which is called actinomorphic or arborising (Fig. 16.1)

Figure 16.1: Cytopathic effects induced by toxins modifying the actin cytoskeleton. MRC5 fibroblasts or Hela cells have been treated with *C. perfringens* E iota toxin, *C. botulinum* C3 enzyme, *C. difficile* Toxin B or *C. sordellii* lethal toxin from IP82 (LT82). Control cells and cells treated with toxins have been observed by phase contrast microscopy, or actin filaments have been stained with fluorescent phalloidin.



ToxA and *C. novyi* α toxin cause similar cell alterations, but LT produces cell rounding without arborisation. ToxB is a very potent cytotoxin (the minimal cytotoxic dose is about 0.2-1 pg), whereas ToxA is less active (minimal cytotoxic dose 10-40 ng) (Lyerly *et al.*, 1998). Many cell lines are sensitive to large clostridial toxins; Vero cells and fibroblasts such as MRC5 are among the most used for the detection of *C. difficile* toxins. In *C. difficile*-dependent colitis, toxins are present in patients' stool specimens. Most of the toxigenic *C. difficile* strains produce both ToxA and ToxB. Since ToxB is extremely cytotoxic, tissue culture assay is highly sensitive and is considered as the reference test to detect *C. difficile* toxins in stool samples and in bacteria cultured *in vitro*. Faecal specimens or culture supernatants are diluted in Hanks solution or PBS and filtered. A 100 µl portion and serial 2-fold dilutions are added to confluent cell monolayers in 96-well microtitre plates. Plates are incubated at 37 °C in 5% CO_2, and then examined over 48 h for cytopathic effects characteristic of *C. difficile* toxins. The ability of antisera to bind the toxin in order to neutralise the cytotoxic effects can provide an identification of the toxin. However, ToxB and LT show immunological cross-reactivity, and antiserum against LT is able to neutralise the cytopathic effects induced by ToxB.

16.1.2. Immunological methods, ELISA

Numerous immunoassays have been developed for the rapid detection of ToxA and ToxB in clinical samples, and many of them are commercially available (Table 16.2).

These assays use polyclonal or monoclonal antibodies against ToxA or ToxB. The advantage of immunoassays is their rapidity, about 90 min versus 18-24 h for the cytotoxicity test. In addition, immunoassays do not require equipment, such as cell culture facilities, and certain manipulations can be automated. Immunoassays are less sensitive than the cytotoxicity test, but most of the tests range between 85 and 95% for sensitivity (Table 16.2). The best diagnostic strategy is to detect the toxin in faecal samples by immunoassay for a rapid presumptive diagnosis and then to confirm this result using tissue culture assay. Most of the *C. difficile* strains involved in colitis produce both ToxA and ToxB, but approximately 5% of toxigenic strains are A^-B^+ and were shown to occur in about 25% of hospitals surveyed by Wilkins and Lyerly (2003). For these reasons, immunoassays directed against ToxA and ToxB are preferred to those only detecting ToxA.

Table 16.2: Performance of immunoassays compared with tissue culture assay for the detection of *C. difficile* toxins in faeces specimens. PPV, positive predictive value; NPV, negative predictive value

Immunoassay	Toxin	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	References
Cytoclone	A and B	79.6	97.3	86.7	95.7	Barbut *et al.*, 1993
Premier	A	72.5	99.5	97.4	94.3	
Vidas	A	68	100	100	93.4	
Premier	A	84	97	75	98	Whittier *et al.*, 1993
Vidas	A	71	98	76	97	
Bartels	A	94	92	56	99	
TechLab ToxA	A	93	92	56	99	
Bartels Prima	A	87	96	65	99	Merz *et al.*, 1994
Cambridge cytoclone	A and B	89	99	90	99	
Meridian Premier	A	87	98	77	99	
TechLab	A and B	87	95	60	99	
TechLab	A and B	83.3-95.7	100	100	96-99	Lyerly *et al.*, 1988
ImmunoCard	A	52	100	100	83	O'Connor *et al.*, 2001
Oxoid Toxin A	A	49	99	96	82	
TechLab A/B	A and B	80	99	97	92	
Premier Toxin	A and B	82	99	97	93	
Vidas	A	80.6	96.8	96.7	81.1	Lipson *et al.*, 2003
Triage	A	93	66			Alfa *et al.*, 2002
TechLab	A and B	89	99			
Cytoclone	A and B	73.3	99.1	90.2	97.1	Turgeon *et al.*, 2003
ICard	A	56.4	99.7	95.0	95.3	
Prima A	A	82.2	98.3	84.7	98.0	
Techlab	A and B	77.2	99.4	94.0	97.5	
Triage	A	59.4	99.6	93.8	95.6	
Vidas	A	70.3	98.9	87.7	96.8	

16.1.3. Biochemical methods: glucosylation

The large clostridial toxins are glucosyltransferases, the substrates of which are indicated in Table 16.1. The activity of these toxins can be evidenced using a glucosylation assay. Toxin preparation is incubated with the recombinant appropriate substrates, UDP-[^{14}C]glucose, in HEPES buffer 20 mM, (pH 7.4), containing 2 mM $MgCl_2$, 100 mM KCl, 0.1 mM GDP and protease inhibitors. After incubation (30 min, 37 °C), the proteins are separated by SDS-gel electrophoresis (PAGE). Gels are then stained, dried and autoradiographed. Glucosylation activity can be detected using cell lysate instead of the recombinant substrates, but this method does not permit identification of each substrate. For α-novyi toxin, UDP-[^{14}C]-*N*-acetylglucosamine is used instead of UDP-[^{14}C]glucose (Just *et al.*, 1997).

16.1.4. Detection of toxin encoding genes

The *C. difficile toxA* and *toxB* genes have been characterised and PCR-based methods have been developed for the toxinotyping of *C. difficile* isolates and detecting this bacteria directly in stool samples (Alonso *et al.*, 1999; Arzese *et al.*, 1995; Boondekhun *et al.*, 1993; Kato *et al.*, 1993; Wolfhagen *et al.*, 1994; Wren *et al.*, 1993). The PCR-based methods are more rapid than the standard cultures in anaerobic conditions and in the characterising of isolates from stool. Commercial extraction kits, such as the QIAmp DNA Stool kit, enable easy purification of DNA from stool samples.

An evaluation of a PCR method for the detection of the *toxB* gene has shown that of 118 stool samples, all the specimens negative in the tissue culture assay were also shown to be negative by the PCR method (specificity of 100%). Moreover, of the 59 cytotoxin-positive samples, 54 were PCR positive (91.5% sensitivity). The detection limit obtained by PCR was estimated at 10^6 *C. difficile* cells/g of stool (Guilbault *et al.*, 2002). A rapid method by real time PCR directed against *toxA* and *toxB* genes was able to give the results in about 20 min with a limit level of detection of *C. difficile* in stool samples of 5×10^4 bacteria/g. The specificity of this method was 100% and the sensitivity 97% in the case of 59 faecal specimens from hospitalised patients (Bélanger *et al.*, 2003).

Epidemiological studies have shown that *C. difficile* strains are highly polymorphic. Several methods have been developed to differentiate the *C. difficile* strains from different outbreaks and to trace strains which could be more virulent. For example the following have been used in *C. difficile* typing: pulsed-field gel electrophoresis of total DNA, restriction with enzymes having infrequent DNA recognition sites and ribotyping (Clabots *et al.*, 1993; Gürtler, 1993; Kato *et al.*, 1994; Kristjansson *et al.*, 1994; Stubbs *et al.*, 1999). Amplified fragment length polymorphism technique showed equivalent results with pulsed-field gel electrophoresis. Moreover, this technique is easier to perform even in the presence of partial DNA degradation, which has the effect of impairing pulsed-field gel electrophoresis, leading to inconclusive results with that method (Klaassen *et al.*, 2002). Interestingly, a new method of PCR (based on the amplification of repetitive non-coding sequences through the genome [rep-PCR]) gave comparable results for differentiating *C. difficile* isolates to those obtained with pulsed-field gel electrophoresis and was more discriminatory than PCR ribotyping (Spigaglia and Mastrantonio, 2003).

A pioneering method of *C. difficile* typing was based on serogrouping by slide agglutination with rabbit antisera, together with the protein profiles obtained by polyacrylamide gel electrophoresis. In this way, 20 groups and subgroups were differentiated corresponding to somatic antigens and common flagellar antigen (Delmée *et al.*, 1986; Toma *et al.*, 1998). A novel toxinotyping scheme was developed involving PCR amplification of *toxA* and *toxB* genes in 3 fragments for each gene, and 4 other fragments (1 intergenic, 2 upstream of *toxB* gene, and 1 downstream of *toxA* gene), thus covering the entire pathogenicity locus (PaLoc). Analysis of the PCR products by restriction enzymes (PCR-RFLP, restriction

fragment length polymorphism) individualises at least 20 toxinotypes (noted I to XX) (Table 16.3). This toxinotyping correlates with ribotyping and slide agglutination typing (Rupnik *et al.*, 1998, 1997, 2001, 2003). Most toxigenic *C. difficile* strains contain both *toxA* and *toxB* genes. However, variations occur in the *toxA* and *toxB* genes that can be detected by restriction profiles of PCR fragments. Some strains (mainly from serogroup F) contain a *toxB* gene, which is a hybrid between typical *C. difficile toxB* and *C. sordellii* LT genes. The resultant ToxB variant glucosylates a RhoGTPase set intermediate between the 2 classical toxins (Chaves-Olarte *et al.*, 1999; Depitre *et al.,* 1993). A proportion of *C. difficile* strains (0.2 to 12% according to various investigations) isolated from patients with pseudomembranous colitis or antibiotic-associated diarrhoea only produce ToxB (Alfa *et al.*, 2000; Barbut *et al.*, 2002; Brazier *et al.*, 1999; Johnson *et al.*, 2003; Kato *et al.*, 1999, 1998; Limaye *et al.*, 2000; Lyerly *et al.*, 1988; Moncrief *et al.*, 2000; Rupnik *et al.*, 2003; Sambol *et al.*, 2000). These A^-/B^+ strains contain variable deletions in ToxA gene or nonsense mutations leading to the lack of detection of the toxin (Barbut *et al.*, 2002; Rupnik *et al.*, 2003; von Eichel-Streiber *et al.*, 1999). In addition, some *C. difficile* strains (6-8%) contain a binary toxin called CDT (see below), the pathogenic significance of which is still poorly understood (Perelle *et al.*, 1997; Rupnik *et al.*, 2003; Stubbs *et al.*, 2000). A PCR strategy is also used to routinely identify the presence of the genes encoding both CDT components. Most of the strains that harbour CDT genes also produce ToxA and ToxB. However, a low percentage of strains (2%) only produce CDT and are ToxA- and ToxB-negative (Geric *et al.*, 2003; Stubbs *et al.*, 2000).

Table 16.3: Main toxinotypes of *C. difficile* based on *toxA, toxB* and *cdt* gene detection and restriction fragment length polymorphism (RFLP) of *toxA* and *toxB* genes

cdt gene detection	*toxA* and *toxB* gene detection	*toxA* and *toxB* RFLP
CDT+	A+ B+	**toxinotypes I to XX**
CDT-	A+ B+	
CDT+	A- B+	
CDT-	A- B+	
CDT+	A- B-	
CDT-	A- B-	

16.2. Actin-ADP ribosyltransferases

The clostridial actin-ADP ribosyltransferases have a common structure consisting of 2 independent protein components linked by a covalent or a disulfide bridge, one being the binding component (~100 kDa), and the other the enzymatic component (~ 45 kDa). Both components are required for biological activity. Two families can be distinguished: firstly, the Iota family, which encompasses ι toxin produced by *C. perfringens* type E, *C. spiroforme* toxin and *C. difficile* ADP-

ribosyltransferase (CDT) synthesised by some strains of *C. difficile* and secondly, the C2 family, which corresponds to the C2 toxins from *C. botulinum* C and D. At the amino acid sequence level, the components of the Iota family are highly related (80-85% identity), whereas C2 toxin shows 31-40% identity with the Iota family proteins. Components from Iota and C2 families show no immunological cross-reaction and no functional cross-complementation (review: Popoff, 2000). These toxins are also related to the vegetative insecticidal proteins (VIP) produced by *Bacillus cereus* and *Bacillus thuringiensis* (Warren *et al.*, 1996).

The toxin components are encoded by two genes, which are closely located and are organised into operon. The Iota family components are synthesised during the exponential growth phase and are secreted as inactive proteins by means of a signal peptide. They are proteolytically activated by removal of a 20 kDa N-terminal peptide from the binding component (80 kDa for the active form) and 9 to 11 N-terminal residues from the enzymatic component (Gibert *et al.*, 2000). In contrast, C2 toxin components are produced during the sporulation phase and are released during bacterial lysis. Signal peptides are absent from the C2 toxin proteins. The C2 toxin binding component also requires proteolytic processing resulting in the removal of a 20 kDa N-terminal peptide (Ohishi, 2000).

16.2.1. Biological methods

Actin ADP-ribosyltransferases are lethal for mice. Classically, a mouse lethality test has been used to identify these toxins. Note that, in order to be active, C2 toxin from *C. botulinum* requires trypsinisation prior to injection into the mouse. Polyclonal antibodies against crude supernatant of *C. perfringens* type E have been prepared for the toxinotyping of *C. perfringens*. Anti-ι toxin antibodies cross-react with *C. spiroforme* toxin (Stiles and Wilkins, 1986). Iota toxin induces a circular white necrosis upon intradermal injection in guinea pigs, which is neutralised by incubating the toxin with serum against *C. perfringens* type E. In the past, this method has been used for *C. perfringens* toxinotyping (Sterne and Batty, 1975).

Actin ADP-ribosyltransferases are cytotoxic, causing cell rounding and complete disorganisation of the actin cytoskeleton. Confluent cells, such as 3T3, Vero and MRC5 are incubated with preparations containing binary toxins. Observation under phase contrast microscopy reveals the rounding up of cells. Fixation and staining of the cells with fluorescent phalloidin, which specifically labels actin filaments, is used to reveal the actin-dependent actin depolymerisation (Fig. 16.1) (Aktories *et al.*, 1997). Effects on the cell can be neutralised with the corresponding antibody. A quantitative assay of actin filament depolymerization is based on fixation of control and toxin-treated cells, staining with rhodamine-phalloidin and analysis with spectrofluorimetry.

16.2.2. Immunological methods

Antibodies against each component of the binary toxins have been prepared, but are not commercially available. Cross-reactions occur between the enzymatic and binding components of iota, *C. spiroforme* toxin and CDT, whereas anti-C2-I and anti-C2-II only recognise homologous antigens. Western blotting of crude

Clostridium culture supernatant has been used to identify the toxin components. ELISA tests have not been developed for diagnostic purposes.

16.2.3. Biochemical methods

Actin ADP-ribosylation assay consists of incubating the toxin preparation with globular actin or crude cell lysate and [^{32}P]-NAD in 50 mM triethanolamine buffer (pH 7.5) containing 1 mM $MgCl_2$, 1 mM dithiotreitol, 10 mM thymidine and protease inhibitors. After 30 min incubation at 37 °C, the proteins are separated by SDS-PAGE, and the gel is dried and analysed by autoradiography. The actin band becomes labelled by the incorporation of [^{32}P]-ADP ribose in the presence of the clostridial binary toxin. Iota toxin, *C. spiroforme* toxin and CDT recognise both muscular and non-muscular actin, whereas C2 toxin is reactive only with non-muscular actin. A quantitative assay is based on actin ADP-ribosylation with serial dilutions of toxin preparation. Alternatively, ADP-ribosylation reactions are precipitated with trichloracetic acid, filtered on cellulose filters, washed and counted for radioactivity (Aktories *et al.*, 1997).

16.2.4. Detection of toxin encoding genes

Genes encoding the clostridial actin ADP-ribosylating toxins have all been cloned and sequenced. PCR-based methods have been developed for the detection of these genes in clostridia. In particular, this method is used for *C. perfringens* typing and for the detection of the CDT gene in *C. difficile*.

16.3. Rho-ADP ribosyltransferases

In addition to C2 toxin, *C. botulinum* C and D produce another ADP-ribosyltransferase, which is termed C3 enzyme. C3 related enzymes are also produced by *Clostridium limosum*, *Bacillus cereus, Bacillus thuringiensis* and *Staphylococcus aureus* (epidermal cell differentiation inhibitor, EDIN), and are specific to Rho proteins.

C3 enzyme (28 kDa) possesses only a catalytic domain and lacks the binding and translocation domains permitting the entry of toxins into cells. Thus, C3 enzyme is not cytotoxic. The crystal structure shows that C3 consists of a core structure of 5 antiparallel β-strands packed against a three-stranded antiparallel β-sheet flanked by four consecutive α-helices (Han *et al.*, 2001). Interestingly, the C3 structure is similar to that of the catalytic domain of the enzymatic components VIP2 and Ia (Han *et al.*, 1999; Tsuge *et al.*, 2003). C3 enzyme ADP ribosylates Rho protein at Asn-41. ADP-ribosylation of Rho-Asn41 by C3 does not impair the exchange of GDP with GTP, the intrinsic and GAP-stimulated GTPase activity, or the interaction with its effectors (Ren *et al.*, 1996; Sehr *et al.*, 1998) It does, however, prevent Rho translocation to the membrane, where it is required for activation and interaction with its effectors (Fujihara *et al.*, 1997). This results in disorganisation of actin filaments and cell rounding. C3 recognises RhoA, B and C, whereas EDIN also

modifies RhoE. The C3 gene is located on phage DNA in *C. botulinum* C and D, which also harbour the neurotoxin genes C1 and D, respectively. C3 enzyme is produced during the exponential growth phase and is secreted in the culture supernatant by means of a signal peptide.

16.3.1. Biological methods

Since C3 enzyme lacks a binding and translocation domain, it cannot enter cells efficiently and is not biologically active. C3 enzyme, even at high concentration, is not lethal for mice. To evidence the cytotoxic effects of C3, confluent cells, such as 3T3 or Vero cells are incubated with high concentrations of enzyme (50 to 100 µg/ml) for 24-48 h at 37 °C. The cells are then fixed and the actin filaments stained with fluorescent phalloidin. ADP-ribosylation of Rho by C3 induces rounding of the cells and disappearance of actin stress fibres (Fig. 16.1). An alternative method uses C3 microinjected into cells (Nobes and Hall, 1997).

16.3.2. Immunological methods

Specific antibodies have been raised against C3 enzyme, and Western blotting with crude *Clostridium* culture supernatant has been used to evidence this protein. No ELISA test has been developed for the identification of C3 and related enzymes.

16.3.3. Biochemical methods

C3 enzymatic reaction is performed by incubating C3 preparation with [^{32}P]-NAD and recombinant Rho protein or crude cell homogenate in a 50 mM Triethanolamine (pH 7.5) buffer containing 1 mM dithiotreitol, 5 mM $MgCl_2$ and 10 mM thymidine. After 1 h incubation, the mixture is loaded on an SDS-PAGE. The gel is then dried and autoradiographed (Saito and Narumiya, 1997).

16.3.4. Detection of toxin encoding genes

The C3 gene has been characterised in *C. botulinum* C and D, *C. limosum*, *Staphylococcus aureus* (EDIN) and *Bacillus cereus*, and can be detected by PCR.

16.4. Metalloproteases

Clostridial neurotoxins, botulinum neurotoxins (BoNTs) (responsible for flaccid paralysis called botulism) and tetanus toxin (TeTx), (by contrast producing a spastic paralysis, tetanus) are all zinc-dependent proteases.

C. tetani forms a homogeneous bacterial species, which produces only one type of TeTx, whereas botulinum neurotoxin (BoNT)-producing strains are heterogeneous. *C. botulinum* is divided into 4 groups, which, on the basis of phenotypic and genotypic parameters, correspond to different species (Stackebrandt, 2002). In addition, some strains of other species, such as *C. butyricum* and *C. baratii,* can produce a related BoNT type E and F respectively. Seven toxinotypes of BoNT (A, B, C1, D, E, F and G) are individualised according to their antigenic properties.

BoNTs and TeTx share a common structure. They are synthesised as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor, which does not contain signal peptide, is released from the bacteria possibly by a cell-wall exfoliation mechanism (Call *et al.*, 1995). The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases, such as digestive proteases in the intestinal content. The active neurotoxin consists of a light chain (L, about 50 kDa) and a heavy chain (H, about 100 kDa), which remain linked by a disulfide bridge. The structure of BoNT shows three distinct domains: the L-chain containing α-helices and β-strands and including the catalytic zinc binding motif, the N-terminal part of the H-chain forming two unusually long and twisted α-helices and the C-terminal part of H-chain consisting of two distinct subdomains involved in the recognition of the receptor (Emsley *et al.*, 2000; Lacy and Stevens, 1999; Lacy *et al.*, 1998; Umland *et al.*, 1997).

Although BoNTs and TeTx are toxic by different routes, they display a similar intracellular mechanism of action. BoNTs enter the host by the oral route with transcytosis across the digestive mucosa (Maksymowych *et al.*, 1999). After diffusion in extracellular fluid and circulation in the bloodstream, BoNTs target motoneuron endings. In contrast, TeTx is formed in wounds colonised by *C. tetani*. TeTx diffuses in the extracellular fluid and can be internalised in all types of nervous endings (sensory, adrenergic and motor neurons).

Molecular genetic studies revealed that clostridial neurotoxins contain a conserved zinc- dependent proteolytic site (His-Glu-x-x-His) in the middle of the L chain (Niemann, 1991; Popoff and Marvaud, 1999). A variety of studies have demonstrated that the clostridial neurotoxins are zinc endopeptidases; the toxins are inhibited by metalloprotease-specific inhibitors and specific antibodies to the zinc-binding motif. In addition, zinc ions are found in the neurotoxins and mutation of the zinc-binding motif abolishes activity. These toxins cleave previously identified synaptic proteins. TeTx, BoNT/B, D, F and G cleave VAMP/synaptobrevin, a membrane protein from synaptic vesicles. BoNT/A and E cleave SNAP25, a presynaptic membrane protein and BoNT/C1 cleaves both SNAP25 and syntaxin, which is also a presynaptic membrane protein. VAMP, SNAP25 and syntaxin are called SNARE proteins for SNAP receptors. They form a large protein family, since many isoforms have been identified in almost all the cells. Each clostridial neurotoxin recognises its substrate at a specific binding site (SNARE motifs, 2 in VAMP and syntaxin, and 4 in SNAP25), and cut at a specific cleavage site. When an individual SNARE protein is cleaved by a clostridial neurotoxin, the SNARE complex formation is not inhibited, but its stability is reduced and the release of neurotransmitter is blocked. Even the physiological properties induced by the cleavage of either VAMP, SNAP25 or syntaxin are not equivalent at the neuromuscular junctions. All the clostridial neurotoxins cause a blocking of the regulated neurotransmission, which varies in intensity and duration according to each neurotoxin type (review: Humeau *et al.*, 2000; Meunier *et al.*, 2002).

16.4.1. Samples

In humans or animals suspected of suffering from botulism, BoNTs can be found in serum samples, in faeces, in intestinal content or sometimes in samples of organs, such as the liver. In cases of botulism consequent to infection, such as infant botulism, BoNT is recovered from faeces or from serum. In adult humans and birds suffering from botulism, BoNT is commonly detected in serum. Cattle occasionally show the presence of BoNT in blood circulation (25-30% of the cases) (Popoff, 1989). BoNT has also to be investigated in suspected food to determine the source of the contamination.

BoNTs and TeTx are synthesised during the late growth phase and early stationary phase of culture. They are exported in the external medium by an unknown mechanism. Classically, clostridial neurotoxins are identified in supernatant of 1 to 4-day cultures.

16.4.2. Biological methods

16.4.2.1. Mouse test

The mouse test consists of the intraperitoneal injection of toxin-containing samples into mice: (18-22 g) culture supernatant or filtrates from clinical or environmental samples (Schantz and Kautter, 1978). A volume of up to 1 ml may be inoculated. Culture supernatants from non-proteolytic (Group II) C. *botulinum* E and B must first be treated with trypsin (200 µg/ml for 20 min at room temperature) to activate the neurotoxin. The mice are observed for the appearance of characteristic symptoms of flaccid paralysis (difficulty of mobility, severe generalised weakness, contraction of the abdomen or wasp-shaped abdomen, respiratory distress) and for death up to 4 days post-injection. The confirmation of the presence of BoNT and its typing is achieved by incubating the samples with specific neutralising antibodies against each BoNT type before injection into mice. The neutralising antisera against each type of BoNT are specific. Partial cross-neutralisation is, however, observed between types C and D and between types E and F. Until recently, the mouse lethality test has been the most sensitive diagnostic test for BoNTs. The BoNT/A lethal dose for mice after intraperitoneal injection is estimated to be 2-4 pg.

The quantitative determination of the toxin is usually performed by the intraperitoneal injection of 4 mice with 0.5 ml of 10-fold or 2-fold serial dilutions in gelatine-phosphate buffer. After four days, the LD50 is determined, often by the Reed and Münch method. Toxin can also be given by the intravenous route. Mice injected intravenously die within some minutes according to a definite and reproducible dose-survival time relationship (Boroff and Fleck, 1966). Standard curves have been prepared for different types of toxins (Sakaguchi *et al.*, 1968).

16.4.2.2. Effects on isolated organs

An isolated hemidiaphragm from a rat, guinea pig or mouse can be used to measure the flaccid paralytic effects of BoNT. Muscle contraction is monitored in response to electrical stimulation of the phrenic nerve. BoNT inhibits muscular

contraction. This model is less sensitive than the mouse test and cannot be used routinely in the laboratory.

16.4.2.3. Cell test

Neuroblastoma cells and adrenal medullary cells, such as the PC12 line, have been used with BoNT. These cells secrete catecholamines and/or acetylcholine upon stimulation, for example, by high K^+ concentrations. The cells are loaded with radioactive neurotransmitters and the blocking of their release by BoNT measured (Habermann, 1989). The primary nerve cell or chromaffin cell cultures are the most sensitive, but the cell models are less sensitive than the animal tests and are not appropriate for routine identification of BoNTs.

16.4.3. Immunological methods

The development of immunoassays for the detection of BoNTs was pioneered by Notermans. Polyclonal and monoclonal antibodies have been developed against the various purified BoNTs and have been tested in immunoassays (Kozaki *et al.*, 1989; Notermans and Nagel, 1989). Using culture supernatants from various proteolytic *C. botulinum* B strains and standard sandwich ELISA, polyclonal antibodies detect about 8 to 90 mouse ip LD50/ml and monoclonal antibody between 600 and 700 mouse ip LD50/ml. Polyclonal antibodies were less effective with non-proteolytic *C. botulinum* strains (limit detection of 700-800 mouse ip LD50/ml), reflecting differences in the antigenicity of BoNT/B from proteolytic and non-proteolytic strains (Notermans and Nagel, 1989).

In order to improve the sensitivity of ELISA tests, amplifying techniques have been developed. A system proposed by Shone et al. is based on enzymatic transformation of $NADP^+$ to NAD^+ by alkaline phosphatase that is coupled to the IgG anti-BoNT. The NAD^+ molecules produced are used in another enzymatic reaction, leading to the formation of several hundred coloured formazan molecules. This system increases the sensitivity of the immunoassay by about 50 to 100 times (Shone *et al.*, 1986).

A modified ELISA, derived from the coagulation assay is called an enzyme-linked-coagulation assay (ELCA). A coagulation activating enzyme (RVV-XA) isolated from the venom of Russel's viper is combined with affinity-purified horse antibodies specific for BoNT/A, BoNT/B or BoNT/E. Plates are coated with affinity-purified antibodies and incubated with the toxin samples. Detection is achieved with anti-toxin antibodies labelled with RVV-XA, with washing and with the addition of a modified plasma substrate, which contains all the coagulation factors mixed with alkaline phosphatase-fibrinogen and solid phase fibrinogen coated on silicone rubber nubs. In a second phase, the nubs are removed from the plate, washed three times with saline in beakers, and placed on a second plate containing an alkaline phosphate substrate and incubated for 30 min. The coloured reaction is then measured with a plate reader. ELISA-ELCA allows detection of about 0.1 pg/ml of BoNT using culture filtrates (Doellgast *et al.*, 1994, 1993). This method has been used to detect BoNT/E production in fish inoculated with *C. botulinum* E (Roman *et al.*, 1994).

A sensitive ELISA has been proposed for the titration of BoNT/A in therapeutic preparations. Toxin-specific antibodies coated on plate are used to capture BoNT/A, and the detection is achieved with affinity-purified antitoxin and species-specific immunoglobulins labelled with horseradish peroxidase. Ekong *et al.* (1994) found the limit of detection to be 4-8 pg of purified toxin/ml, equivalent to 1-2 mouse bioassay units/ml. Using affinity-purified horse polyclonal antibodies directed against the C fragment (Fc) of the heavy chain of BoNT/A or BoNT/B, the sensitivity of the detection was 20 pg per well (200 pg/ml). Szilagyi *et al.* (2000) used the purified antibodies as capture, and the same antibodies labelled with biotin enabled the detection of the toxin in association with neutravidin-linked alkaline phosphatase.

The sensitivity of an ELISA directed against BoNT/C1 and BoNT/D is typically 70% and the specificity 96% on samples from animals with botulism, compared to the mouse bioassay (Thomas, 1991).

16.4.4. Biochemical methods.

A biochemical test has been proposed for the detection of the BoNTs. BoNTs are proteases, which specifically cleave an intracellular target in the neuronal cells. BoNT/B, C, D, F and G cleave synaptobrevin, BoNT/A and E cleave SNAP25 and BoNT/C1 cleave both syntaxin and SNAP25. These substrates are called SNARE proteins and have an essential role in neuroexocytosis. They can be obtained as recombinant proteins and used for *in vitro* reaction with BoNTs. The test developed for the detection of BoNT/A consists of the coating of SNAP25 in wells of plastic plate, incubation with samples containing the neurotoxin and washing. BoNT/A releases a small C-terminal fragment of SNAP25 and the cleaved form, which remains attached to the bottom of the well, is detected with a specific antibody recognising only the proteolysed SNAP25 and not the intact molecule. The assay is developed with a conjugate linked to peroxidase or alkaline phosphatase and directed against the first antibody. Concentration of the toxin with an immunoaffinity column increases the sensitivity of the method. This assay allows detection of type B toxin at a concentration of 5 pg/ml (0.5 mouse 50% lethal dose per ml) (Wictome *et al.*, 1999a, 1999b). A variant method uses synthetic cleavable oligopeptide labelled with fluorescein and covalently attached to multiwell plates. Solubilised products after incubation with botulinum neurotoxin are measured with a spectrofluorometer. The sensitivity of this method ranges from 10 to 100 ng/ml (Schmidt *et al.*, 2001). A similar fluorescence-based assay has been proposed for the determination of tetanus toxin and BoNT/B protease activity (Anne *et al.*, 2001; Soleilhac *et al.*, 1996).

16.4.5. Detection of toxin encoding genes

The routine identification of neurotoxin-producing *Clostridium* is hampered by the need for anaerobic conditions for growth and the variable bacteriological characteristics of the different groups. The molecular characterisation of *bont* genes offers the possibility of using genetic methods, such as polymerase chain reaction (PCR) and DNA-DNA hybridisation.

The sequences of the different BoNT types are distinct. The overall similarity between the available neurotoxin sequences ranges from 34 to 97% identity at the amino acid level. The neurotoxin sequences from the same toxinotype and from the same physiological groups are almost identical, except for BoNT/A1 and BoNT/A2, which are highly related (90% identity) but differ in 129 residues (Binz *et al.*, 1990; Thompson *et al.*, 1990; Titball, 1993). The strains isolated from infant botulism in Japan fall into the A2 group, the strains from foodborne and infant botulism from the USA and the UK fall into the A1 and A2 groups (Cordoba *et al.*, 1995). BoNTs of the same toxinotype, but produced by strains from different *C. botulinum* groups or *Clostridium* species, show a high level of identity (70 to 97%). BoNT sequences from *C. botulinum* E and *C. butyricum* have 97% identity (Fujii *et al.*, 1993; Poulet *et al.*, 1992). BoNT/B sequences from proteolytic and non-proteolytic *C. botulinum* B share 93% identity (Hutson *et al.*, 1994; Whelan *et al.*, 1992), but BoNT/F from *C. botulinum* F and *C. baratii* have only 70% identity (East *et al.*, 1992; Thompson *et al.*, 1993).

C. botulinum A, B, E, F and G identification using specific primers from each toxin gene has been described by several authors (Ferreira *et al.*, 1993; 1994; Franciosa *et al.*, 1994; Hielm *et al.*, 1996; Kakinuma *et al.*, 1997; Szabo *et al.*, 1992; 1993; 1994; Takeshi *et al.*, 1996).

In order to minimise the number of PCR reactions, a multiplex PCR consisting of a mix of primers specific to the neurotoxin types (A, B, E, and F) involved in human botulism has been designed (Henderson *et al.*, 1997). We have developed a primer pair, partially degenerated on their 3' end, able to amplify a conserved region of *bont* genes from type A, B, E, F and G. Internal probes specific to each type, allow typing by hybridisation. Where this method was used in contaminated food samples, it showed a 95.6% correlation with the standard method (Fach *et al.*, 1995). A similar strategy was used by other authors (Aranda *et al.*, 1997; Campbell *et al.*, 1993). A single internal probe was designed to recognise the amplification products, without distinction of type, in the work of Aranda *et al.* (1997). A double PCR procedure has also been proposed for identification of *C. botulinum* C and D (Fach *et al.*, 1996).

Quantification of toxigenic *Clostridium* is required for certain biological or food samples. For example, monitoring levels of *C. perfringens* is important in the food industry, since certain levels (50-200 per gram) of these bacteria are tolerated in some food products. The standard detection by PCR, as described above, is not quantitative. A quantitative technique based on the most probable number method, consisting of inoculating serial dilutions of food samples into an enrichment medium and performing PCR with each dilution culture, has been proposed for enumeration of *C. botulinum* (Hielm *et al.*, 1996), and could be used for other toxigenic clostridia. A new technology of quantitative PCR based on detection by fluorescent probe has been introduced (TaqMan assay). This method has been developed for quantitative detection of *C. botulinum* E (Kimura *et al.*, 2001). It allows the enumeration of *C. botulinum* in the 10^2 to 10^8 CFU/g range within 1 or 2 h.

Evidence of a toxin gene does not mean that the toxin is actually produced. A toxin gene can be silent. This is the case for *botulinum* neurotoxin B gene in some *C. botulinum* type A strains (Hutson *et al.*, 1996). A possible means of detecting toxin genes, which are effectively expressed, consists of the PCR method from mRNA. A reverse transcription-PCR has been described to monitor *botulinum* neurotoxin E production (McGrath *et al.*, 2000).

16.5. Clostridial collagenases

C. histolyticum is responsible for dramatic myonecrosis and gangrene. The main secreted toxins are collagenases (also called α-clostripain), which exist in at least 6 different forms with molecular masses ranging from 68 to 125 kDa. They are divided into 2 classes, ColG and ColH, based on amino acid sequence similarities and specificities toward peptide substrates. The collagenases are encoded by 2 genes, *colG* and *colH*. Both genes, which probably derive from duplication of an ancestral gene, encode for a 116 kDa precursor, which is processed in the C-terminal part to yield the multiple forms (Matsushita *et al.*, 1999). ColH consists of 4 segments, S1, S2a, S2b, and S3, while S2a and S2b are homologous; ColG also possesses 4 segments, S1, S2, S3a and S3b. All collagenase forms contain in the N-terminal part (segment 1) the consensus active site HExxH of zinc metalloprotease (Jung *et al.*, 1993). The other segments are involved in substrate recognition and binding. Thus, S3 is required for binding to collagen (Matsushita *et al.*, 1998; Matsuchita and Okabe, 2001).

C. histolyticum collagenases are specific to collagen and gelatine. They cleave peptide bonds on the amino side of the glycine residue in the PXGP sequence, and they degrade their substrates in small dialysable peptides. Collagenases play an important role in the degradation of the connective tissue.

C. perfringens produces a collagenase, ColA, which is related to ColG from *C. histolyticum* and contains the characteristic motif, HExxH (Matsushita *et al.*, 1999; 1994). ColA has an additional effect to the phospholipase α toxin in the destruction of tissues.

A gene encoding a collagenase (Col-T) related to *C. histolyticum* collagenases has been identified on the same plasmid, which contains the tetanus toxin (TeTx) gene. ColT could be involved in the destruction of tissue, permitting invasion by *C. tetani* (Brüggemann *et al.*, 2003).

16.5.1. Biological methods

C. histolyticum collagenases are lethal to mice upon intraperitoneal injection, and a neutralisation test in mice is used to identify *C. histolyticum*. An antiserum against crude supernatant of this bacterium is commercially available. Production of myonecrotic lesions upon intramuscular injections to guinea pigs has also been also used to characterise the toxigenic *C. histolyticum* strains.

16.5.2. Biochemical methods

A collagenase assay is based on the digestion of collagen from bovine Achilles' tendon. Collagen (5 mg/ml) is swollen in Tris-HCl (pH 7.5) containing 0.36 mM $CaCl_2$. One unit of enzyme activity equals 1 µmol of L-leucine equivalents liberated from collagen during 5 h at 37 °C (Jung *et al.*, 1999). Another collagenase assay consists of using the Pz peptide (Sigma) (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg). One unit of enzyme activity is defined as the amount of enzyme causing an increase of 0.1 A_{320} unit per min. (Jung *et al.*, 1999). A qualitative test uses photographic film in culture medium. The presence of gelatinase induces a deposition of silver nitrate particles in the bottom of the culture tube.

16.6. Clostridial phospholipases

Two types of phospholipase C are produced by the clostridia. A phosphatidylinositol hydrolysing enzyme is produced by *Clostridium novyi* (β toxin) (Taguchi and Ikezawa, 1978), but enzymes with similar substrate specificity produced by other clostridia have not been reported. The *C. novyi* β toxin is relatively poorly characterised and is not discussed further in this section. In contrast, a wide range of clostridia produce zincmetallophospholipases C that are exported from the bacterial cell into the culture medium (Titball, 1993) (Table 16.4).

The best characterised of these phospholipases C is the *C. perfringens* α-toxin, and much of the information on assay systems has arisen from work with this protein over the past 50 years. All the clostridial enzymes contain zinc ions in the active site cleft. These ions not only play a role in catalytic activity, but also stabilise the tertiary structure of the protein (Naylor *et al.*, 1998; Titball, 1993). The enzymes are produced maximally during the late logarithmic phase of growth, and early stationary phase cultures are usually used for the isolation of the proteins from culture medium (Jolivet-Reynaud *et al.*, 1988; Krug and Kent, 1984; Mollby, 1978). Providing the growth medium contains zinc ions these enzymes are all relatively stable in culture fluid (Sato *et al.*, 1978). However, although the zinc-metallophospholipases C can be isolated and purified from culture medium, they are not the only exported proteins. This has always presented the potential problem of co-purification of additional proteins, since some of these are also toxins. Therefore, more recently, recombinant forms of the zinc-metallophospholipases C, produced in *Escherichia coli*, have been used for many experimental studies (Clark *et al.*, 2003; Titball *et al.*, 1989; Tso and Siebel, 1989).

Table 16.4: Characterised phosphatidylcholine hydrolysing Phospholipases C produced by clostridia

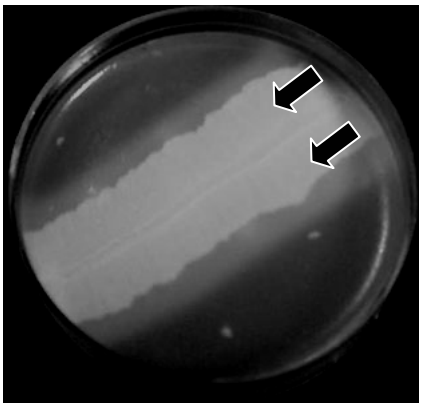
Enzyme	Substrate specificity	Properties	Reference
C. perfringens α toxin	PC, SM and PS	toxic, haemolytic	Krug and Kent, 1984
Clostridium absonum PLC	PC, SM	toxic, haemolytic	Clark *et al.*, 2003
Clostridium baratii PLC	NR	NR	
Clostridium bifermentans PLC	PC, SM	weakly toxic, weakly haemolytic	Jepson *et al.*, 1999 Tso and Siebel, 1989
Clostridium novyi γ toxin	PC, SM, PE, PS, PG	toxic, haemolytic	Taguchi and Ikezawa, 1975
Clostridium haemolyticum PLC	PC, SM	toxic, haemolytic	
Clostridium sordellii PLC	PC	non-toxic, weakly haemolytic	Karasawa *et al.*, 2003

PC: phosphatidylcholine, SM: sphingomyelin, PS: phosphatidylserine,
PE: phosphatidylethanolamine, PG: phosphatidylglycerol, NR: not reported

16.6.1. Detection of phospholipase C activity on solid media

Because the clostridial phospholipases C are enzymes, detection of their activity on solid media is relatively straightforward. The most common assay involves the growth of bacteria on medium (such as Brain-Heart Infusion agar), which contains, typically, 10% (v/v) egg yolk emulsion (Gubash, 1991; MacFarlane and Knight, 1941). The egg yolk emulsion is added to molten media, which has been cooled to approximately 50 ^{o}C, before pouring. Suitable egg yolk emulsion for use in such plates is available from Oxoid Ltd (Basingstoke, UK), but must be centrifuged before use (for example, at 10,000 x g for 20 minutes) to remove particulate matter. After growth of the bacteria at 37 ^{o}C on egg-yolk agar, typically for 18 h, phospholipase C activity is evidenced as a zone of turbidity around bacterial colonies (Fig 16.2) (MacFarlane and Knight, 1941; Titball *et al.*, 1989). In the case of *C. perfringens*, a refinement of this test involves the addition of antiserum to part of the agar plate (for example, by swabbing antiserum across part of the plate before inoculation). The resultant absence of a zone of turbidity in the area swabbed with antitoxin (the so-called Nagler reaction) is considered to be diagnostic for the bacterium (MacFarlane and Knight, 1941; Willis and Gowland, 1962). However, it is known that many other clostridial phospholipases are also partially or fully neutralised by antitoxin to *C. perfringens* α toxin. Therefore, more accurately, this test may indicate the presence of a clostridial species rather than *C. perfringens*.

Figure 16.2: *C. perfringens* growing on agar containing egg yolk. The diagonal line of growth is flanked by zones of turbidity (arrowed)



Because some clostridial phospholipases are also haemolytic, it is also possible to detect their activities on blood-agar plates (Gubash, 1980). In order to produce such plates, either sheep or mouse erythrocytes are added to molten agar at 50 ^{o}C before pouring. The zones of haemolysis caused by clostridial phospholipases are typically less clear than the zones caused by other haemolytic toxins produced by these bacteria. For this reason, the unequivocal identification of phospholipase C activity on the basis of zones of haemolysis around bacterial colonies is difficult.

16.6.2. Detection of biologically active phospholipase C activity in culture medium

A wide range of assays have been described to measure phospholipase C activity in culture supernatant fluid. These different assays have advantages and disadvantages which reflect the relative importance of the head group and carbonyl group for substrate recognition. Fatty acyl chain lengths of between C6 and C14 are necessary for efficient phospholipid hydrolysis (El-Sayed *et al.*, 1985; Nagahama *et al.*, 1996). Phospholipase C activity also appears to be dependent on calcium ions for substrate binding (but apparently not for hydrolysis *per se*), and this has been elegantly demonstrated using α toxin and phospholipid monolayers (Moreau *et al.*, 1988). In some of the older literature it has been suggested that these calcium ions balance the charge on phospholipid head groups, but it is now known that calcium ions play a more direct role in substrate binding (Naylor *et al.*, 1999). Therefore, all assays which involve phospholipids require the addition of calcium ions (typically at 5mM final concentration). It is also desirable to have excess zinc ions (typically at 2mM final concentration) in the reaction buffer; one of the zinc ions in the active site cleft is relatively loosely bound and the enzymes show a low level of activity in the absence of this ion.

16.6.2.1. Simple phospholipid-hydrolysis assays

One of the simplest assays for clostridial phospholipases involves incubating a suspension of egg yolk emulsion (which is rich in phosphatidylcholine linked to protein) with either culture supernatant or purified enzyme at 37 ^{o}C. The resultant increase in turbidity of the mixture can be monitored spectrophometrically at, for example, 540nm (Takahashi *et al.*, 1981). Kinetic studies are possible using this assay system, but most researchers use a simple 50% end-point assay, when the turbidity of the suspension is measured after incubation with dilutions of the enzyme (Jepson *et al.*, 1999; Titball *et al.*, 1989). Microtitre-tray based assays have proved to be simple and robust for carrying out such end-point assays (Titball *et al.,* 1989). Using such assays, it is possible to detect as little as 15µg of α toxin and 25µg of the *C. bifermentans* phospholipase C (Jepson *et al.,* 1999).

The use of "pure" phospholipids in assays can provide an indication of substrate specificity. However, for solution-based assays the phospholipid must be below the critical micellar concentration, or alternatively used at higher concentrations in the presence of detergents. Hydrolysis can be visualised after separation of the reaction products by thin layer chromatography (Taguchi and Ikezawa, 1975; Titball *et al.,* 1991), but the precise quantification of hydrolysis often requires the use of radiolabelled substrates or the measurement of released phosphate. In an attempt to resolve the problems of solubilising phospholipids for use in these assays, various researchers have proposed the use of synthetic derivatives which lack the hydrophobic tail groups. Water-soluble phospholipid analogues, such as in ρ-nitrophenolphosphorylcholine (ρNPPC) have the phospholipid tail groups replaced with the chromophore ρ-nitrophenol (Kurioka and Matsuda, 1976). Assays utilising ρNPPC are relatively simple to perform, involving the spectrophotometric monitoring of the release of the chromophore (Kurioka and Matsuda, 1976). Although, the K_m value for ρNPPC is high, reflecting the importance of the fatty acyl chains for substrate recognition, a microtitre tray assay using ρNPPC is reportedly able to detect as little as 4 µg of *C. perfringens* α toxin (Jepson *et al.*, 1999). Other researchers have devised water-soluble substrates, such as dioctanoylthiophosphatidylcholine, where the head group is modified and short fatty acyl chains are present (Synder, 1987). This compound may be more similar to the natural substrate, but it is unlikely that these substrates fully reflect the interaction of the enzyme with membrane phospholipids. Despite these concerns, phospholipid derivatives, such as ρNPPC and dioctanoylthiophosphatidylcholine do have a place in some studies, especially when the interaction of the active site with substrate is being probed.

16.6.2.2. Activity towards artificial and natural membranes

Some clostridial phospholipases are active towards membrane-packed phospholipids, whether in natural or artificial membranes. The use of liposomes composed of a single phospholipid type, and containing an entrapped dye, such as carboxyfluorescein or calcein, allows phospholipids to be presented in a form similar to that found in cell membranes (Jepson *et al.*, 1999; Lim *et al.*, 1997). Within the

liposome, the natural fluorescence of these dyes is quenched. The incubation of these liposomes with clostridial phospholipases results in the release of the entrapped dye, which then fluoresces and can be measured using a fluorimeter (Lim *et al.,*1997).

The activity of clostridial phospholipases towards natural membranes is often measured as cytolytic or cytotoxicity activity (Taguchi and Ikezawa, 1976). One of the simplest assays involves the use of erythrocytes in suspension (typically at 5% v/v) and the measurement of lysis as a decrease in turbidity of the cell suspension at 540nm. Simple 50% end-point assays, where dilutions of the enzyme mixed with erythrocytes in microtitre trays are often used (Titball *et al.*, 1989). Erythrocytes from different species differ widely in their susceptibility to lysis. Many researchers use sheep erythrocytes, in which case complete lysis is seen only when the mixture is first incubated at 37 ^{o}C and then cooled to 4 ^{o}C (Taguchi and Ikezawa, 1976). The authors have found mouse erythrocytes to be highly susceptible to lysis without the requirement for the additional cooling step after incubation with phospholipase at 37 ^{o}C (Titball *et al.,* 1989). Using a microtitre tray assay with mouse erythrocytes it is possible to detect as little as 4µg of *C. perfringens* α toxin (Jepson *et al.*, 1999).

A range of cell types is susceptible to the cytotoxic effects of clostridial phospholipases, such as the *C. perfringens* α toxin, including lymphocytes (Titball *et al.*, 1993) and Chinese hamster cells (Flores-Diaz *et al.*, 1998). As an alternative to measuring cell death, some researchers have measured the release of cell metabolites as evidence of membrane damage (Bryant *et al.*, 2003; Bunting *et al.*, 1997; Gustafson and Tagesson, 1990). These types of assay are complex, and their use is not warranted for routine purposes.

16.6.2.3. *In vivo* toxicity studies

The level of toxicity of different clostridial phospholipases in animals is not similar. The most potent, such as *C. perfringens* α toxin, have LD_{50} doses in the range of 0.1-1.0 µg after intraperitoneal (i.p.) inoculation into mice (Titball, 1999; Titball *et al.*, 1989). Others, such as the *C. bifermentans* enzyme, are relatively non-toxic, with LD_{50} doses of greater than 10 µg (Jepson *et al.*, 1999; Titball and Rood, 2000; Tso and Siebel, 1989). For toxic enzymes, death usually occurs within 24 hours of challenge (Schoepe *et al.*, 1997; Titball *et al.*, 1989). The ability of toxin-specific antiserum (or monoclonal antibodies) to neutralise toxicity in mice has, in the past, been used as one of the tests for typing strains of *C. perfringens*. However, the use of such assays is not encouraged nowadays, and there are now immunological or genetic tests able to detect the production of toxin or the presence of toxin-encoding genes. Moreover, the LD_{50} appears broadly to correlate with the haemolytic or cytotoxic activities of the enzymes. For some purposes, therefore, these assays provide alternatives to toxicity studies involving animals.

16.6.3. Immunological detection and identification of clostridial phospholipases C

A number of researchers have reported the development of enzyme-linked immunosorbent assay (ELISA) tests that are able to detect *C. perfringens* α toxin

(Aschfalk and Muller, 2001; Hale and Stiles, 1999; Hauer and Clough, 1999; Holdsworth and Parratt, 1994; Naylor *et al.*, 1997). The full protocols for these assays are detailed in these reports. These assays use either polyclonal or monoclonal antibodies developed against the toxin. It is not clear whether these tests also react with other clostridial phospholipases. However, since there is a degree of immunological cross-reactivity between these proteins (Nakamura *et al.*, 1973; Willis, 1977), it is possible that tests using polyclonal antibody would react with all clostridial phospholipases. These tests often form one element of a panel of ELISA assays, which are used to detect the production of the major toxins produced by *C. perfringens* and, therefore, to type strains.

16.6.4. Detection of toxin encoding genes

One of the major applications of tests to detect the presence of toxin genes is in the typing of strains. For example, *C. perfringens* can be typed into one of 4 biotypes, depending on the differential production of 4 major toxins (Table 16.5). The phospholipase C is produced by all biotypes of *C. perfringens*, and a range of PCR tests to detect the presence of the α toxin-encoding gene have been reported (Aschfalk and Muller, 2002; 2001; Garmory *et al.*, 2000; Gkiourtzidis *et al.*, 2001; Miserez *et al.*, 1998; Waters *et al.*, 2003).

Table 16.5: Typing of *C. perfringens* strains based on toxin production or toxin gene presence

***C. perfringens* type**	**α toxin**	**β toxin**	**ε toxin**	**ι toxin**	**Disease association**
A	+	-	-	-	gas gangrene of humans and animals, fowl and porcine necrotic enteritis, bovine and ovine enterotoxaemia, food poisoning in humans, colitis in horses, canine haemorrhagic gastroenteritis
B	+	+	+	-	lamb dysentery, enterotoxaemia of foals, sheep and goats
C	+	+	-	-	pig-bel (necrotic enteritis) in humans, enterotoxaemia of sheep (struck), calves, lambs and piglets
D	+	-	+	-	enteroxaemia of lambs and sheep (pulpy kidney), goats and cattle
E	+	-	-	+	rabbit enteritis, enterotoxaemia of calves and lambs

The full methodologies for carrying out such PCR tests are described in these reports. In some cases, multiplex PCR tests have been devised allowing the simultaneous identification of all 4 major toxin genes, although PCR-based tests are now considered to be the simplest and most reliable for the typing of *C. perfringens* (Meer and Songer, 1997; Petit *et al.*, 1999). However, it is important to note that these tests do not necessarily indicate that the gene is functional (Petit *et al.*, 1999). It may therefore be valuable to carry out, in parallel, immunological typing tests.

The genes encoding clostridial phospholipases are generally sufficiently dissimilar for PCR tests, devised to detect the phospholipase C gene in one species, not to amplify gene fragments from other species. However, in the case of *C. perfringens*, there is also increasing evidence of significant differences in the sequence of the α toxin gene from atypical strains (Justin *et al.*, 2002). Therefore, although a positive PCR test for the phospholipase C from *C. perfringens* might be interpreted as diagnostic for the species, care should be taken when interpreting negative results.

16.7. Cholesterol-dependent cytolysin toxins

A wide range of clostridia produce cholesterol-dependent cytolysins (Table 16.6; also previously known as thiol-activated or oxygen labile cytolysins) but only 3, perfringolysin (Hauschild *et al.*, 1973; Smyth, 1975; Yamakawa *et al.*, 1977), tetanolysin (Alving *et al.*, 1979; Mitsui *et al.*, 1980) and botulinolysin (Haque *et al.*, 1992) have been purified and characterised. The cholesterol-dependent cytolysins are synthesised with signal peptides and are exported from the bacteria as soluble extracellular proteins Tweten, 1988). The proteins are relatively easy to isolate from culture supernatant, but many researchers now use purified recombinant protein produced in *E. coli* for experimental studies (Shepard *et al.*, 1998).

Table 16.6: Known cholesterol-dependent cytolysins produced by the clostridia

cholesterol dependent cytolysin	**Origin**
Bifermentolysin	*C. bifermentans*
Botulinolysin	*C. botulinum*
Chauveolysin	*C. chauvoei*
Histolyticolysin	*C. histolyticum*
Novyilysin	*C. novyi*
Perfringolysin (θ toxin)	*C. perfringens*
Septicolysin	*C. septicum*
Sordellilysin	*C. sordellii*
Tetanolysin	*C. tetani*

The most striking feature of the cholesterol-dependent cytolysins is the apparent requirement for reduction for full biological activity (Alouf, 1999). Initially, it was believed that this reflected the critical role of the single cysteine residue. However, it is now known that this residue does not play a major role in the cytolytic or cytotoxic activity of theses toxins (Alouf, 1999). Rather, it is now thought that this residue is close to a functionally critical region of the molecule, and that other cysteine-containing molecules, which become complexed with toxin, markedly reduce the activity of the toxin. Irrespective of the function of the single cysteine residue, there has always been a need to reduce samples containing toxin before measuring their activity. Usually this involves the addition of a reducing agent, such as dithiotreitol, 2-mercaptoethanol or sodium thioglycolate (Smyth and Duncan, 1978).

16.7.1. Detection of cholesterol-dependent cytolysins on solid media

The cholesterol-dependent cytolysins form large pores (250-300 Å) in host cell membranes that allow the passage of most proteins (Alouf, 2000). The cytolytic activity is most easily demonstrated towards erythrocytes, and these toxins are usually responsible for the clear zone or β-haemolysis that surrounds colonies of bacteria on blood agar plates (Alouf, 2000). The toxins are active towards a wide range of erythrocyte types (Table 16.7), and human, rabbit, horse or sheep erythrocytes are often used to detect cytolytic activity.

Table 16.7: Susceptibility (relative to rabbit) of erythrocytes from various species, to lysis by *C. perfringens* θ toxin (Smyth and Duncan, 1978)

Erythrocyte source	Relative susceptibility
baboon	100
cat	50
chicken	50-100
cow	200-400
dog	200-400
gerbil	100
human	100
horse	100
mouse	<12.5
rabbit	100
rat	50-100
sheep	100

16.7.2. Detection of cholesterol-dependent cytolysins in culture medium

The presence of cholesterol-dependent cytolysins in culture supernates can be determined using haemolysis assays with the erythrocytes identified above. Prior reduction of the sample is necessary before testing. Care needs to be taken when testing crude culture supernates because many other exoproducts of the clostridia are also able to cause haemolysis. This problem can be overcome to some extent by selecting erythrocytes and testing conditions that provide maximum sensitivity to cholesterol-dependent cytolysins. For example, horse erythrocytes are relatively insensitive to *C. perfringens* α toxin, but highly sensitive to *C. perfringens* θ toxin. Also, the finding that haemolytic activity increases on reduction of the sample, provides a good indication of the presence of a cholesterol-dependent cytolysin.

16.7.3. *In vivo* toxicity

Only some of the clostridial cholesterol-dependent cytolysins have been tested for toxicity in mice. However, in general the cholesterol-dependent cytolysins have LD_{50} doses of approximately 1μg/mouse (Smyth and Duncan, 1978).

16.7.4. Immunological detection and identification of cholesterol-dependent cytolysins

Immunoassays for the detection and/or identification of clostridial cholesterol-dependent cytolysins have not been reported.

16.7.5. Detection of toxin-encoding genes

There is one report that a PCR test has been used to screen isolates of *C. perfringens* from foals for the θ toxin-encoding gene in addition to the genes encoding the 4 other major toxins (Netherwood *et al.*, 1998). However, PCR tests for the clostridial cholesterol-dependent cytolysins are generally not widely used.

16.8. Aerolysin-like pore-forming toxins

Both the *C. septicum* α toxin and the *C. perfringens* ε toxin are believed to form pores in host cell membranes and may be related to the pore-forming toxin, aerolysin. Both toxins are produced as relatively non-toxic prototoxins, which are exported from the bacterial cell and accumulate in culture medium. The toxins can be purified from culture medium, and the yield can differ markedly from strain to strain. (Ballard *et al.*, 1992; Cortinas *et al.*, 1997; Hunter *et al.*, 1992; Parreiras *et al.*, 2002). The genes encoding these prototoxins have been expressed in *E. coli* (Goswami *et al.*, 1996; Hunter *et al.*, 1992; Imagawa *et al.*, 1994) and recombinant proteins are now used for many studies (Diep *et al.*, 1999; Oyston *et al.*, 1998).

Both prototoxins are activated on exposure to proteases. In the case of *C. septicum* α toxin, trypsin or furin treatment removes a carboxy-terminal peptide (Ballard *et al.*, 1995; Gordon *et al.*, 1997). Treatment of *C. perfringens* ε toxin with proteases, such as trypsin, chymotrypsin or *C. perfringens* λ-protease removes both amino- and carboxy-terminal peptides (Minami *et al.*, 1997). However, it is the removal of the carboxy-terminal peptide that is believed to be responsible for toxin activation (Minami *et al.*, 1997). Depending on the time of harvesting and the level of co-produced protease, the proteins isolated from culture filtrates may be prototoxin, toxin or, more likely, a mixture of both. Whilst this may not be important when immunological identification tests are used, full biological activity is dependent on exposure to an appropriate protease. For some assays, activation can result from exposure to cell surface proteases.

16.8.1. Detection of toxins on solid media

There are no simple methods for the detection of production of *C. perfringens* ε toxin on solid media. However, *C. septicum* α toxin is haemolytic (Ballard *et al.*, 1992; Imagawa *et al.*, 1994) and can therefore be detected as a zone of β-haemolysis surrounding colonies of bacteria on agar containing rat or human erythrocytes. It is important to use unwashed cells for this assay, since washing can remove cell surface proteases, which are required to fully activate the toxin.

16.8.2. Detection of biologically active toxins in culture medium

The haemolytic activity of *C. septicum* α toxin can be measured relatively easily using a 0.5- 1% (v/v) suspension of rat (Diep *et al.*, 1999; Gordon *et al.*, 1997) or human erythrocytes (Ballard *et al.*, 1992; 1993; Diep *et al.*, 1999) in phosphate- or Tris-buffered saline. Toxin and erythrocytes are typically incubated together for anywhere between 1 and 18 h. The activity towards human erythrocytes is reported to lie in the range $1.5x10^6$ to $2x10^7$ haemolytic units (HU)/mg protein (Ballard *et al.*, 1992; 1993). Rat erythrocytes appear to be less susceptible to lysis (Diep *et al.*, 1999). Microtitre tray assays, which are simple and easy to use, have been described, but some researchers use more complex assays (Ballard *et al.*, 1992). For example, released haemoglobin can be separated from cell debris by centrifuging, and the concentration of released haemoglobin estimated from the OD540nm of the supernatant fluid (Gordon *et al.*, 1997).

Both toxins are active towards cultured cells, but in the case of ε toxin only a limited range of cell types are susceptible to the toxin. Of these, only Madin Darby Canine Kidney cells (MDCK) are considered to be susceptible enough to form the basis of a cytotoxicity assay (Payne *et al.*, 1994; Shortt *et al.*, 2000). The *C. septicum* α toxin shows cytotoxic activity towards a much wider range of cell types, including CHO cells (Gordon *et al.*, 1997), FD11 cells (Gordon *et al.*, 1997) 13 ng/ml and EL4 T-cells (Diep *et al.*, 1999).

16.8.3. *In vivo* toxicity

The toxicity of *C. septicum* α toxin can be measured after the i.p. injection of toxin into mice with monitoring for 48 h (Ballard *et al.*, 1992). Typically, the LD_{50} dose of the toxin is 10μg/kg (Ballard *et al.*, 1992). In comparison, the *C. perfringens* ε toxin is highly potent after intravenous administration into mice, with an LD_{50} dose in the 16-50 ng/kg range (Habeeb, 1969; Miyata *et al.*, 2001)

16.8.4. Immunological detection and identification of toxins

Tests for *C. perfringens* ε toxin are not only important for the typing of isolates of the bacterium, but also form an important part of the test for the potency of clostridial vaccines, as outlined in monograph 363 of the European Pharmacopoeia (Eur. Ph.). In the current potency test, neutralising antibodies against *C. perfringens* ε toxin are determined in a mouse neutralisation test (MNT). The lack of reliable and simple assays for the measurement of *C. perfringens* ε toxin may be one of the main drivers for the development of immunoassays to detect this toxin. These assays may use either monoclonal or polyclonal antibodies and are frequently configured as simple ELISA tests (Aschfalk and Muller, 2002; 2001; Hauer, 1993). Some derivatives of these immunoassays are used to determine the antibody responses to toxoid vaccines, and these assays are valuable replacements for the MNT (Ebert *et al.*, 1999; 1998). Immunoassays for the detection or identification of *C. septicum* α toxin have not been reported.

16.8.5. Detection of toxin-encoding genes

There are no reports of the wide-scale use of PCR tests for the *C. septicum* α toxin gene, but PCR based tests for the *C. perfringens* ε toxin gene have been reported by a number of researchers (Ashfalk and Muller, 2002; 2001; Garmory *et al.*, 2000; Gkiourtzidis *et al.*, 2001; Goswami *et al.*, 1996; Meer and Songer, 1997; Miserez *et al.*, 1998; Netherwood *et al.*, 1998). These tests are now widely used for the typing of strains of *C. perfringens*. Although the ε toxin is produced by 2 biotypes of *C. perfringens* (types B and D), the sequences of the encoding genes appear to be very similar (Havard *et al.*, 1992).

16.9. Hla-like pore-forming toxins

The only known Hla-like toxin produced by the clostridia is the β toxin of *C. perfringens*. The β toxin is one of the 4 major toxins that may be produced by the bacterium (Table 16.5), and the detection of this toxin, or the toxin-encoding gene, forms one of the important tests for the typing of strains of *C. perfringens* (Petit *et al.*, 1999). The toxin is synthesised with an N-terminal signal sequence, and is exported across the bacterial cell wall (Hauer, 1993; Hunter *et al.*, 1993). However, the detection of the toxin in culture supernatant is not easy because the toxin is extremely labile (Lawrence *et al.*, 1979; Pal *et al.*, 1990; Sakurai and Duncan, 1977; Worthington and Mulders, 1975). It may, therefore, be necessary to test culture fluid taken at various points in the late logarithmic or early stationary phase of growth for the presence of toxin. Like tests for *C. perfringens* ε toxin, tests for β toxin are important for the typing of isolates and form an important part of the European Pharmacopoeia (Eur. Ph.) test for efficacy of clostridial vaccines. In the currently used potency test, neutralising antibodies against *C. perfringens* β toxin are determined using an MNT.

16.9.1. Detection of β toxin on solid media

There are no simple methods for the detection of β toxin on solid media.

16.9.2. Detection of biologically active β toxin in culture medium

There are no simple tests for detecting biologically active β toxin in culture medium. However, there are some reports that the toxin is active towards certain cell types, such as Chinese hamster ovary cells (Jolivet-Reynaud *et al.*, 1986) and guinea pig monocytes (Allan, 1963). Assays that exploit these observations have not yet been devised.

16.9.3. *In vivo* toxicity studies

Until relatively recently, the measurement of toxicity in the mice that could be neutralised by antiserum or antibody has been the most frequently used test for the presence of β toxin. The 50% lethal dose of the toxin in mice by the i.p. route is reported to be in the 0.68-1.87 μg range (Sakurai and Fujii, 1987; Worthington and Mulders, 1975). The toxicity of β toxin for mice has been exploited in tests for the potency of antisera raised against toxoids that are to be used in veterinary vaccines.

However, the use of this type of test for the presence of β toxin (or antibody to β toxin) is no longer encouraged, and immunological or genetic tests provide excellent alternatives (see below).

16.9.4. Immunological detection and identification of β toxin

Because of the difficulties associated with the detection of β toxin in culture filtrates, and because of the need to replace the MNT with tests that do not use animals, a number of researchers have reported the development of immunological tests for the detection and identification of β toxin. The simplest of these involve the use of an ELISA, with either monoclonal or polyclonal antibodies (Aschfalk and Muller, 2002; 2001; Hauer and Clough, 1999). Variations of this assay, which are configured to allow the capture and quantification of antibodies to β toxin, have also been reported (Ebert *et al.*, 1998). These assays are of particular value for the assessment of the potency of veterinary vaccine, which contains β toxoid (Ebert *et al.*, 1999, 1998). A more elegant immunoassay involves the use of surface plasmon resonance (SPR), where the SPR chip is coated with monoclonal antibody and the sample (which can be fermenter broth) is passed over the chip, with the subsequent capture of β toxin (Hsieh *et al.*, 1998). This assay can reportedly detect toxin within 20 minutes.

16.9.5. Detection of toxin-encoding genes

PCR tests are now the method of choice for typing strains of *C. perfringens* and are often used in conjunction with other tests for the detection of the other major toxins (Garmory *et al.*, 2000; Gkiourtzidis *et al.*, 2001; Meer and Songer, 1997; Miserez *et al.*, 1998; Walters *et al.*, 2003). These tests have been widely used to genotype *C. perfringens* isolates from diseased animals, though currently it is not possible to carry out these tests without first isolating and culturing the bacterium. Details of the PCR primers and amplification conditions are detailed in the relevant publications, and many researchers have used the protocol originally reported by Meer and Songer (1997).

16.10. Enterotoxin

The clostridial enterotoxin of major significance is produced by *C. perfringens*. Methods for the isolation of this toxin and for the identification using cell culture, biochemical, immunological or genetic methods are described in Chapter 3.

16.11. Bibliography

Aktories K., Prepens U., Sehr P. and Just I. (1997) Probing the actin cytoskeleton by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin In: *Bacterial toxins* (Aktories K., Ed), pp 129-139, Chapman and Hall, Weinheim.

Alfa M., Kabani J., Lyerly A.D., Moncrief S., Neville L.M., Al-Barrak A., Harding G.K.H., Dyck B., Olekson K. and Embil J.M. (2000) Characterization of toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **38**, 2706-2714.

Alfa M.J., Swan B., VanDekerkhove B., Pang P. and Harding G.K.M. (2002) The diagnosis of *Clostridium difficile*-associated diarrhea: comparison of Triage *C. difficile* panel, EIA for ToxA/B and cytotoxin assays. *Diag. Microbiol. Infect. Dis.* **43**, 257-263.

Allan D. (1963) Cytotoxic effects produced by the beta-toxin of *Clostridium wechiii* on guinea pig monocytes. *Immunol.* **6**, 3-14.

Alonso R., Munoz C., Gros S., Garcia de Viedma D., Pelaez T. and Bouza E. (1999) Rapid detection of toxigenic *Clostridium difficile* from stool samples by a nested PCR of toxin B gene. *J. Hosp. Infect.* **41**, 145-149.

Alouf J.E. (2000) Cholesterol-binding cytolytic protein toxins. *Int. J. Med. Microbiol.* **290**, 351-356.

Alouf J.E. (1999) Introduction to the family of the structurally related cholesterol-binding cytolysins (sulfhydryl-activated toxins) In: *The comprehensive sourcebook of bacterial proteins toxins* (Freer J.H., Ed), pp 443-456, Academic Press, London SanDiego Boston New York Sydney Tokyo Toronto.

Alving C.R., Habig W.H., Urban K.A. and Hardegree M.C. (1979) Cholesterol-dependent tetanolysin damage to liposomes. *Biochem. Biophys. Acta.* **551**, 224-228.

Anne C., Cornille F., Lenoir C. and Roques B.P. (2001) High-throughput fluorogenic assay for determination of botulinum type B neurotoxin protease activity. *Anal. Biochem.* **291**, 253-261.

Aranda E., Rodriguez M.M., Asensio M.A. and Cordoba J.J. (1997) Detection of *botulinum* types A, B, E, and F in foods by PCR and DNA probe. *Letts. Appl. Microbiol.* **25**, 186-190.

Arzese A;, Trani G;, Riul L. and Botta G.A. (1995) Rapid polymerase chain reaction method for specific detection of toxigenic *Clostridium difficile. Eur. J. Clin. Microbiol. Infect. Dis.* **14**, 716-719.

Aschfalk A. and Muller W. (2002) *Clostridium perfringens* toxin types from wild-caught Atlantic cod (*Gadus morhua* L.), determined by PCR and ELISA. *Can. J. Microbiol.* **48**, 365-368.

Aschfalk A. and Muller W. (2001) *Clostridium perfringens* toxin types in hooded seals in the Greenland Sea, determined by PCR and ELISA. *J. Vet. Med. B Infect. Dis. Vet. Public Health.* **48**, 765-769.

Ballard J., Bryant A., Stevens D. and Tweten R.K. (1992) Purification and characterization of the lethal toxin (Alpha- Toxin) of *Clostridium septicum. Infect. Immun.* **60**, 784-790.

Ballard J., Crabtree J., Roe B.A. and Tweten R.K. (1995) The primary structure of *Clostridium septicum* alpha-toxin exhibits similarity with that of *Aeromonas hydrophila* aerolysin. *Infect. Immun.* **63**, 340-344.

Ballard J., Sokolov Y., Yuan W.L., Kagan B.L. and Tweten R.K. (1993) Activation and mechanism of *Clostridium septicum* alpha-toxin. *Mol. Microbiol.* **10**, 627-634.

Barbut F., Kaizer C., Planas N. and Petit J.C. (1993) Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **31**, 963-967.

Barbut F., Lalande V., Burghoffer B., Vu Thien H., Grimpel E. and Petit J.C. (2002) Prevalence and genetic characterization of toxin A variant strains of *Clostridium difficile* among adults and children with diarrhea in France. *J. Clin. Microbiol.* **40**, 2079-2083.

Bélanger S.D., Boissinot M., Clairoux N., Picard F.J. and Bergeron M.G. (2003) Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J. Clin. Microbiol.* **41**, 730-734.

Binz T., Kurazono H., Wille M., Frevert J., Wernars K. and Niemann H. (1990) The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. *J. Biol. Chem.* **265**, 9153-9158.

Boondekhun H.S., Gurtler V., Odd M.L., Wilson V.A. and Mayall B.C. (1993) Detection of *Clostridium difficile* enterotoxin gene in clinical specimens by the polymerase chain reaction. *J. Med. Microbiol.* **38**, 384-387.

Boroff D.A. and Fleck U. (1966) Statistical analysis of a rapid *in vivo* method for the titration of the toxin of *Clostridium botulinum. J. Bacteriol.* **97**, 1580-1581.

Brazier J.S., Stubbs S.L. and Duerden B.I. (1999) Prevalence of toxin A negative/B positive *Clostridium difficile* strains. *J. Hosp. Infect.* **42**, 248-249.

Brüggemann H., Bäumer S., Fricke W.F., Wiezr A., Liesagang H., Decker I., Herzberg C., Martinez-Arias R., Henne A. and Gottschalk G. (2003) The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc. Ntl. Acad. Sci. (USA).* **100**, 1316-1321.

Bryant A.E., Bayer C.R., Hayes-Schroer S.M. and Stevens D.L. (2003) Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. *J. Infect. Dis.* **187**, 408-417.

Bunting M., Lorant D.E., Bryant A.E., Zimmerman G.A., McIntyre T.M., Stevens D.L. and Prescott S.M. (1997) Alpha-toxin from *Clostridium perfringens* induces proinflammatory changes in endothelial cells. *J. Clin. Invest.* **100**, 565-574.

Call J.E., Cooke P.H. and Miller A.J. (1995) *In situ* characterization of *Clostridium botulinum* neurotoxin synthesis and export. *J. Appl. Bacteriol.* **79**, 257-263.

Campbell K.D., Collins M.D. and East A.K. (1993) Gene probes for identification of the botulinal neurotoxin gene and specific identification of neurotoxin types B, E and F. *J. Clin. Microbiol.* **31**, 2255-2262.

Chaves-Olarte E., Low P., Freer P., Norlin T., Weidmann M., von Eichel-Streiber C. and Thelestam M. (1999) A novel cytotoxin from *Clostridium difficile*

serogroup F is a functional hybrid between two other large clostridial toxins. *J. Biol. Chem.* **274**, 11046-11052.

Chen M.L., Pothoulakis C. and LaMont J.T. (2002) Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to *Clostridium difficile* toxin A. *J. Biol. Chem.* **277**, 4247-4254.

Clabots C.R., Johnson S., Bettin K.M., Mathie P.A., Mulligan M.E., Schaberg D.R., Peterson L.R. and Gerding D.N. (1993) Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. *J. Clin. Microbiol.* **31**, 1870-1875.

Clark C., Briggs D., Karasawa T., Wang X.M., Jayasekeera P., Cole A., Maegawa T., Naylor C.E., Moss D.S., Miller J., Nakamura S., Basak A. and Titball R.W. (2003) *Clostridium absonum* a-toxin: new insights into Clostridial alpha-toxin substrate binding and specificity. *J. Mol. Biol*. submitted.

Cordoba J.J., Collins M.D. and East A.K. (1995) Studies on the genes encoding botulinum neurotoxin type A of *Clostridium botulinum* from a variety of sources. *System. Appl. Microbiol.* **18**, 13-22.

Cortinas T.I., Mattar M.A. and deGuzman A.M.S. (1997) Alpha-toxin production by *Clostridium septicum* at different culture conditions. *Anaerobe*. **3**, 199-202.

Delmée M., Laroche Y., Avesani V. and Cornelis G. (1986) Comparison of serogrouping and polyacrylamide gel electrophoresis for typing of *Clostridium difficile*. *J. Clin. Microbiol.* **24**, 991-994.

Depitre C., Delmée M., Avesani V., L'Haridon R., Roels A., Popoff M.R. and Corthier G. (1993) Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J. Med. Microbiol.* **38**, 434-441.

Diep D.B., Nelson K.L., Lawrence T.S., Sellman B.R., Tweten R.K. and Buckley J.T. (1999) Expression and properties of an aerolysin - *Clostridium septicum* alpha toxin hybrid protein. *Mol. Microbiol.* **31**, 785-794.

Doellgast G.J., Beard G.A., Bottoms J.D., Cheng T., Roh B.H.., Roman M.G., Hall P.A. and Triscott M.X. (1994) Enzyme-linked immunosorbent assay and enzyme-linked coagulation assay for detection of *Clostridium botulinum* neurotoxins A, B, and E and solution-phase complexes with dual-label antibodies. *J. Clin. Microbiol.* **32**, 105-111.

Doellgast G.J., Triscott M.X., Beard G.A., Bottoms J.D., Cheng T., Roh B.H., Roman M.G., Hall P.A. and Brown J.E. (1993) Sensitive enzyme-linked immunosorbent assay for detection of *Costridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. *J. Clin. Microbiol.* **31**, 2402-2409.

Dupuy B. and Sonenshein A.L. (1998) Regulated transcription of *Clostridium difficile* toxin genes. *Mol. Microbiol.* **27**, 107-120.

East A.K., Richardson P.T., Allaway D., Collins M.D., Roberts T.A. and Thompson D.E. (1992) Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum. FEMS Microbiol. Letts.* **75**, 225-230.

Ebert E., Oppling V., Werner E. and Cussler K. (1999) Development and prevalidation of two different ELISA systems for the potency testing of *Clostridium perfringens* beta and epsilon-toxoid containing veterinary vaccines. *FEMS Immunol. Med. Microbiol.* **24**, 299-311.

Ebert E., Oppling V.V., Werner E. and Cubetaler K. (1998) Development and prevalidation of alternative methods for the potency testing of *C. perfringens* vaccines. *ALTEX* **15**, 59-61.

Ekong T.A.N., McLellan K. and Sesardic D. (1994) Immunological detection of *Clostridium botulinum* toxin type A in therapeutic preparations. *J. Immunol. Meth.* **180**, 181-191.

El-Sayed M.Y., DeBose C.D., Coury L.A. and Roberts M.F. (1985) Sensitivity of phospholipase C (*Bacillus cereus*) activity to phosphatidylcholine structural modifications. *Biochim. Biophys. Acta.* **837**, 325-335.

Emsley P., Fotinou C., Black I., Fairweather N.F., Charles I.G., Watts C., Hewitt E. and Isaacks N.W. (2000) The structures of the Hc fragment of Tetanus Toxin with carbohydrate subunit complexes provide insight into ganglioside binding. *J. Biol. Chem.* **275**, 8889-8894.

Fach P., Gibert M., Grifais R., Guillou J.P. and Popoff M.R. (1995) PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. *Appl. Environ. Microbiol.* **61**, 389-392.

Fach P., Gibert M., Griffais R. and Popoff M.R. (1996) Investigation of animal botulism outbreaks by PCR and standard methods. *FEMS Immunol. Med. Microbiol.* **13**, 279-285.

Ferreira J.L., Baumstark B.R., Hamdy M.K. and McCay S.G. (1993) Polymerase chain reaction for detection of type A *Clostridium botulinum* in food samples. *J. Food Prot.* **56**, 18-20.

Ferreira J.L., Hamdy M.K., McGay S.G., Hemphill M., Kirma N. and Baumstark B.R. (1994) Detection of *Clostridium botulinum* type F using the polymerase chain reaction. *Mol. Cell. Probes.* **8**, 365-373.

Flores-Diaz M., Alape-Giron A., Titball R.W., Moos M., Guillouard I., Cole S., Howells A.M., von Eichel-Streiber C., Florin I. and Thelestam M. (1998) UDP-glucose deficiency causes hypersensitivity to the cytotoxic effect of *Clostridium perfringens* phospholipase C. *J. Biol. Chem.* **273**, 24433-24438.

Franciosa G., Ferreira J.L. and Hatheway C.L. (1994) Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. *J. Clin. Microbiol.* **32**, 1911-1917.

Fujihara H., Walker L.A., Gong M.C., Lemichez E., Boquet P., Somlyo A.V. and Somlyo A.P. (1997) Inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. *Mol. Biol. Cell.* **8**, 2437-2447.

Fujii N., Kimura K., Yokosawa N., Yashiki N., Tsuzuki K. and Oguma K. (1993) The complete nucleotide sequence of the gene encoding the nontoxic

component of *Clostridium botulinum* type E progenitor toxin. *J. Gen. Microbiol.* **139**, 79-86.

Garmory H.S., Chanter N., French N.P., Bueschel D., Songer J.G. and Titball R.W. (2000) Occurrence of *Clostridium perfringens* beta 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol. Infect.* **124**, 61-67.

Geric B., Johnson S., Gerding D.N., Grabnar M. and Rupnik M. (2003) Frequency of binary toxin genes among *Clostridium difficile* strains that do not produce large clostridial toxins. *J. Clin. Microbiol.* **41**, 5227-5232.

Gibert M., Petit L., Raffestin S., Okabe A. and Popoff M.R. (2000) *Clostridium perfringens* iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. *Infect. Immun.* **68**, 3848-3853.

Gkiourtzidis K., Frey J., Bourtzi-Hatzopoulou E., Iliadis N. and Sarris K. (2001) PCR detection and prevalence of alpha-, beta-, beta 2-, epsilon-, iota- and enterotoxin genes in *Clostridium perfringens* isolated from lambs with clostridial dysentery. *Vet. Microbiol.* **82**, 39-43.

Gordon V.M., Benz R., Fujii K., Leppla S.H. and Tweten R.K. (1997) *Clostridium septicum* alpha-toxin is proteolytically activated by furin. *Infect. Immun.* **65**, 4130-4134.

Goswami P.P., Rupa P., Prihar N.S. and Garg L.C. (1996) Molecular cloning of *Clostridium perfringens* epsilon-toxin gene and its high level expression in *E. coli. Biochem. Biophys. Res. Commun.* **226**, 735-740.

Gubash S.M. (1991) Improved egg-yolk agar plate medium for the detection of Clostridial phospholipase-C activity. *Res. Microbiol.* **142**, 87-93.

Gubash S.M. (1980) Synergistic haemolysis test for presumptive identification and differentiation of *Clostridium perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens. J. Clin. Pathol.* **33**, 395-399.

Guilbault C., Labbé A.C., Poirier L., Busque L., Béliveau C. and Laverdière M. (2002) Development and evaluation of a PCR method for detection of the *Clostridium difficile* toxin B gene in stool specimens. *J. Clin. Microbiol.* **40**, 2288-2290.

Gürtler V. (1993) Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J. Gen. Microbiol.* **139**, 3089-3097.

Gustafson C. and Tagesson C. (1990) Phospholipase-C from *Clostridium perfringens* stimulates phospholipase-A2-mediated arachidonic acid release in cultured intestinal epithelial cells (Int-407). *Scand. J. Gastroenterol.* **25**, 363-371.

Habeeb A.F. (1969) Studies on epsilon-prototoxin of *Clostridium perfringens* type D. I. Purification method: evidence for multiple forms of epsilon-prototoxin. *Arch. Biochem. Biophys.* **130**, 430-440.

Habermann E. (1989) Clostridial neurotoxins and the central nervous system: functional studies on isolated preparations In: *Botulinum neurotoxins and Tetanus toxin* (Simpson L.L., Ed), pp 255-279, Academic Press, San Diego.

Hale M.L. and Stiles B.G. (1999) Detection of *Clostridium perfringens* alpha toxin using a capture antibody ELISA. *Toxicon.* **37**, 471-484.

Han S., Arvai A.S., Clancy S.B. and Tainer J.A. (2001) Crystal structure and novel recognition motif of Rho ADP-ribosylating C3 exoenzyme from *Clostridium botulinum*. Structural insights for recognition specificity and catalysis. *J. Mol. Biol.* **305**, 95-107.

Han S., Craig J.A., Putnam C.D., Carozzi N.B. and Tainer J.A. (1999) Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nature Struct. Biol.* **6**, 932-936.

Haque A., Sugimoto N., Horiguchi Y., Okabe T., Miyata T., Iwanaga S. and Matsuda M. (1992) Production, purification, and characterization of botulinolysin, a thiol-activated hemolysin of *Clostridium botulinum. Infect. Immun.* **60**, 71-78.

Hauer P.J. (1993) Purification and characterization of *Clostridium perfringens* type-C beta-toxin. *Vet. Microbiol.* **37**, 410-410.

Hauer P.J. and Clough N.E. (1999) Development of monoclonal antibodies suitable for use in antigen quantification potency tests for clostridial veterinary vaccines. *Dev. Biol. Stand.* **101**, 85-94.

Hauschild A.H., Lecroisey A. and Alouf J.E. (1973) Purification of *Clostridium perfringens* type C theta toxin. *Can. J. Microbiol.* **19**, 881-5.

Havard H.L., Hunter S.E.C. and Titball R.W. (1992) Comparison of the nucleotide sequence and development of a PCR test for the epsilon toxin gene of *Clostridium perfringens* Type-B and Type-D. *FEMS Microbiol. Lett.* **97**, 77-81.

Henderson I., Davis T., Elmore M. and Minton N. (1997) The genetic basis of toxin production in *Clostridium botulinum* and *Clostridium tetani In: The Clostridia: Molecular Biology and Pathogenesis* (Rood I., Ed), pp 261-294, Academic Press, New York.

Hermann C., Ahmadian M.R., Hofmann F. and Just I. (1998) Functional consequences of monoglucosylation of Ha-Ras at effector domain amino acid threonine 35. *J. Biol. Chem.* **273**, 16134-16139.

Hielm S., Hyyttia E., Ridell J. and Korkeala H. (1996) Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. *Int. J. Food Microbiol.* **31**, 357-365.

Holdsworth R.J. and Parratt D. (1994) Development of an ELISA assay for *Clostridium perfringens* phospholipase C (alpha toxin). *J. Immunoassay.* **15**, 293-304.

Hsieh H.V., Stewart B., Hauer P., Haaland P. and Campbell R. (1998) Measurement of *Clostridium perfringens* beta-toxin production by surface plasmon resonance immunoassay. *Vaccine.* **16**, 997-1003.

Humeau Y., Doussau F., Grant N.J. and Poulain B. (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie.* **82**, 427-446.

Hunter S.E.C., Brown J.E., Oyston P.C.F., Sakurai J. and Titball R.W. (1993) Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals

sequence homology with alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus. Infect. Immun.* **61**, 3958-3965.

Hunter S.E.C., Clarke I.N., Kelly D.C. and Titball R.W. (1992) Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli. Infect. Immun.* **60**, 102-110.

Hutson R.A., Collins M.D., East A.K. and Thompson D.E. (1994) Nucleotide sequence of the gene coding for non-proteolytic *Clostridium botulinum* type B neurotoxin: comparison with other Clostridial neurotoxins. *Curr. Microbiol.* **28**, 101-110.

Hutson R.A., Zhou Y., Collins M.D., Johnson E.A., Hatheway C.L. and Sugiyama H. (1996) Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences. *J. Biol. Chem.* **271**, 10786-10792.

Imagawa T., Dohi Y. and Higashi Y. (1994) Cloning, nucleotide sequence and expression of a hemolysin gene of *Clostridium septicum. FEMS Microbiol. Lett.* **117**, 287-292.

Jepson M. Howells A., Bullifent H.L., Bolgiano B., Crane D., Miller J., Holley J., Jayasekera P. and Titball R.W. (1999) Differences in the carboxy-terminal (putative phospholipid binding) domains of *Clostridium perfringens* and *Clostridium bifermentans* phospholipases C influence the hemolytic and lethal properties of these enzymes. *Infect. Immun.* **67**, 3297-3301.

Johnson S., Sambol S.P., Brazier J.S., Delmée M., Avesani V., Merrigan M.M. and Gerding D.N. (2003) International typing study of toxin A-negative, toxin B-positive *Clostridium difficile variants. J. Clin. Microbiol.*, **41**, 1543-1547.

Jolivet-Reynaud C., Moreau H. and Alouf J.E. (1988) Purification of alpha toxin from *Clostridium perfringens* phospholipase-C. *Method Enzymol.*, **165**, 91-94.

Jolivet-Reynaud C., Popoff M.R., Vinit M.-A., Ravisse P., Moreau H. and Alouf J.E. (1986) Enteropathogenicity of *Clostridium perfringens* beta-toxin and other clostridial toxins In: *Bacterial Protein Toxins*; 2nd European Workshop (Falmagne P. Ed), pp 145-151, Gustav Fischer Verlag, Stuttgart.

Jung C.M., Matsushita O., Katayama S., Minami J., Sakurai J. and Okabe A. (1999) Identification of metal ligands in the *Clostridium histolyticum* ColH collageanse. *J. Bacteriol.* **181**, 2816-2822.

Jung M., Just I., Van Damme J., Vandekerckhove J. and Aktories K. (1993) NAD-binding site of the C3-like ADP-ribosyltransferase from *Clostridium limosum. J. Biol. Chem.* **268**, 23215-23218.

Just I., Selzer J., Hofmann F. and Aktories K. (1997) *Clostridium difficile* toxin B as a probe for Rho GTPases In: *Bacterial toxins* (Aktories K., Ed), pp 159-168, Chapman and Hall, Weinheim.

Just I., Selzer J., Wilm M., von Eichel-Streiber C., Mann M. and Aktories K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature.* **375**, 500-503.

Just I., Wilm M., Selzer J., Rex G., von Eichel-Streiber C., Mann M. and Aktories K. (1995) The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J. Biol. Chem.* **270**, 13932-13936.

Justin N., Walker N., Bullifent H.L., Songer G., Bueschel D.M., Jost H., Naylor C., Miller J., Moss D.S., Titball R.W. and Basak A.K. (2002) The first strain of *Clostridium perfringens* isolated from an avian source has an alpha-toxin with divergent structural and kinetic properties. *Biochemistry.* **41**, 6253-6262.

Kakinuma H., Maruyama H., Yamakawa K., Nakamura S. and Takahashi H. (1997) Application of nested polymerase chain reaction for the rapid diagnosis of infant botulism type B. *Acta Paed. Jap.* **39**, 346-348.

Karasawa T., Wang X.M., Maegawa T., Michiwa Y., Kita H., Miwa K. and Nakamura S. (2003) *Clostridium sordellii* phospholipase C: Gene cloning and comparison of enzymatic and biological activities with those of *Clostridium perfringens* and *Clostridium bifermentans* phospholipase C. *Infect. Immun.* **71**, 641-646.

Kato H., Kato N., Katow S., Maegawa T., Nakamura S. and Lyerly D.M. (1999) Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol. Lett.* **175**, 197-203.

Kato H., Kato N., Watanabe K., Iwai N., Nakamura H., Yamamoto T., Suzuki K., Kim S.M., Chong Y. and Wasito E.B. (1998) Identification of toxin A-negative, toxin B-positive *Clostridium diffiicle* strains by PCR. *J. Clin. Microbiol.* **36**, 2178-2182.

Kato H., Kato N., Watanabe K., Ueno K., Ushijima H., Hashira S. and Abe T. (1994) Application of typing by pulse-field gel electrophoresis to the study of *Clostridium difficile* in a neonatal intensive care unit. *J. Clin. Microbiol.* **32**, 2067-2070.

Kato N., Ou C.Y., Kato H., Bartley S.L., Luo C.C., Killgore G.E. and Ueno K, (1993) Detection of toxigenic *Clostridium difficile* in stool specimens by the polymerase chain reaction. *J. Infect. Dis.* **167**, 455-458.

Khul S.J., Tang Y.J., Navarro L., Gumerlock P.H. and Silva J. (1993) Diagnosis and monitoring of *Clostridium difficile* infections with the polymerase chain reaction. *Clin. Infect. Dis.* **16**, S234-S238.

Kimura B., Kawasaki S., Nakano H. and Hujii T. (2001) Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. *Appl. Environ. Microbiol.* **67**, 206-218.

Klaassen C.H.W., van Haren H.A. and Horrevorts A.M. (2002) Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *J. Clin. Microbiol.* **40**, 101-104.

Kozaki S., Kamata Y., Takahashi M., Shimizu T. and Sakaguchi G. (1989) Antibodies against Botulinum Neurotoxin In: *Botulinum Neurotoxin and Tetanus Toxin* (Simpson L.L., Ed), pp 301-318, Academic Press, San Diego.

Kristjansson M., Samore M.H., Gerding D.N., DeGirolami P.C., Bettin K.M., Karchmer A.W. and Arbeit R.D. (1994) Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. *J. Clin. Microbiol.* **32**, 1963-1969.

Krug E.L. and Kent C. (1984) Phospholipase C from *Clostridium perfringens*: preparation and characterisation of homogenous enzyme. *Arch. Biochem. Biophys.* **231**, 400-410.

Kurioka S. and Matsuda M. (1976) Phospholipase C assay using p-nitrophenolphosphorylcholine and its application to studying the metal and detergent requirement of the enzyme. *Anal. Biochem.* **75**, 281-289.

Lacy D.B. and Stevens R.C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol.* **291**, 1091-1104.

Lacy D.B., Tepp W., Cohen A.C., Das Gupta B.R. and Stevens R.C. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nature Struct. Biol.* **5**, 898-902.

Lawrence G., Watt S. and Basten A. (1979) Purification and some effects of *Clostridium welchii* type C beta toxin. *P N G Med J.* **22**, 79-86.

Lim S.J., Koh U.C., Lee E.O. and Kim C.K. (1997) Liposome-based assay for phospholipase C. *Bull. Korean Chem. Soc.* **18**, 761-766.

Limaye A.P., Turgeon D.K., Cookson B.T. and Fritsche T.R. (2000) Pseudomebranous colitis caused by a toxin A^-B^+ strain of *Clostridium difficile. J. Clin. Microbiol.* **38**, 1696-1697.

Lipson S.M., Tortora G., Tempone A., Fedorko D.P. and Spitzer E.D. (2003) Rapid detection of *Clostridium difficile* in stool using the VIDAS^R *C. difficile* toxin A II assay. *Diag. Microbiol. Infect. Dis.* **45**, 117-121.

Lyerly D.M., Barroso L.A., Wilkins T.D., Depitre C. and Corthier G. (1992) Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect. Immun.* **60,** 4633-4639.

Lyerly D.M., Krivan H.C. and Wilkins T.D. (1988) *Clostridium difficile*: its disease and toxins. *Clin. Microbiol. Rev.* **1**, 1-18.

Lyerly D.M., Neville L.M., Evans D.T., Fill J., Allen S., Green W., Sautter R., Hantuck P., Torpey D.J. and Schwalbe R. (1998) Multicenter evaluation of the *Clostridium difficile* Tox A/B test. *J. Clin. Microbiol.* **36**, 184-190.

MacFarlane M.G. and Knight B.C.J.G. (1941) The biochemistry of bacterial toxins I Lecithinase activity of *Cl welchii* toxins. **35**, 884-902.

Maksymowych A.B., Rienhard M., Malizio C.J., Goodnough M.C., Johnson E.A. and Simpson L.L. (1999) Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. *Infect. Immun.* **67**, 4708-4712.

Mani N. and Dupuy B. (2001) Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. *Proc. Ntl. Acad. Sci. (USA).* **98**, 5844-5849.

Matsushita O., Jung C.M., Katayama S., Minami J., Takahashi Y. and Okabe A. (1999) Gene duplication and multiplicity of collagenases in *Clostridium histolyticum. J. Bacteriol.* **181**, 923-933.

Matsushita O., Jung C.M., Minami J., Katayama S., Nishi N. and Okabe A. (1998) A study of the collagen-binding domain of a 116 kDa *Clostridium histolyticum* collagenase. *J. Biol. Chem.* **273**, 3643-3648.

Matsushita O. and Okabe A. (2001) Clostridial hydrolytic enzymes degrading extracellular components. *Toxicon.* **39**, 1769-1780.

Matsushita O., Yoshihara K., Katayama S., Minami J. and Okabe A. (1994) Purification and characterization of a *Clostridium perfringens* 120-kilodalton collagenase and nucleotide sequence of the corresponding gene. *J. Bacteriol.* **176**, 149-156.

McGrath S., Dooley J.S.G. and Haylor R.W. (2000) Quantification of *Clostridium botulinum* toxin gene expression by competitive reverse transcription PCR. *Appl. Environ. Microbiol.* **66**, 1423-1428.

Meer R.R. and Songer J.G. (1997) Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens. Am. J. Vet. Res.* **58**, 702-705.

Merz C.S., Kramer C., Forma M., Gluck L., Mills K., Senft K., Steiman I., Wallace N. and Charache P. (1994) Comparison of four commercially available rapid enzyme immunoassays with cytotoxin assay for detection of *Clostridium difficile* toxin(s) from stool samples. *J. Clin. Microbiol.* **32**, 1142-1147.

Meunier F.A., Herreros J., Schiavo G., Poulain B. and Molgo J. (2002) Molecular mechanism of action of botulinal neurotoxins and the synaptic remodeling they induce in vivo at the skeletal neuromuscular junction In: *Handbook of Neurotoxicology* (Massaro J., Ed), pp 305-347, Humana Press, Totowa, NJ.

Minami J., Katayama S., Matsushita O., Matsushita C. and Okabe A. (1997) Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. *Microbiol. Immunol.* **41**, 527-535.

Miserez R., Frey J., Buogo C., Capaul S., Tontis A., Burnens A. and Nicolet J. (1998) Detection of alpha- and epsilon-toxigenic *Clostridium perfringens* type D in sheep and goats using a DNA amplification technique (PCR). *Lett. Appl. Microbiol.* **26**, 382-386.

Mitsui N., Mitsui K. and Hase J. (1980) Purification and some properties of tetanolysin. *Microbiol. Immunol.* **24**, 575-84.

Miyata S., Matsushita O., Minami J., Katayama S., Shimamoto S. and Okabe A. (2001) Cleavage of a C-terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon-toxin in the synaptosomal membrane. *J. Biol. Chem.* **276**, 13778-13783.

Mollby R. (1978) Bacterial phospholipases In: *Bacterial toxins and cell membranes* (Wadstrom T., Ed), pp 367-424, Academic Press, London.

Moncrief J.S., Zheng L., Neville L.M. and Lyerly D.M. (2000) Genetic characterization of Toxin A-negative, Toxin B-positive *Clostridium difficile* isolates by PCR. *J. Clin. Microbiol.* **38**, 3072-3075.

Moreau H., Pieroni G., Jolivet-Reynaud C., Alouf J.E. and Verger R. (1988) A new kinetic approach for studying phospholipase-C (*Clostridium perfringens* alpha toxin) activity on phospholipid monolayers. *Biochemistry.* **27**, 2319-2323.

Nagahama M., Michiue K. and Sakurai J. (1996) Membrane-damaging action of *Clostridium perfringens* alpha-toxin on phospholipid liposomes. *Biochim. Biophys. Acta-Biomembr.* **1280**, 120-126.

Nakamura S., Shimamura T., Hayase M. and Nishida S. (1973) Numerical taxonomy of saccharolytic clostridia particularly *Clostridium perfringens*-like strains: descriptions of *Clostridium absonum* sp n and *Clostridium paraperfringens. Int. J. Syst. Bacteriol.* **23**, 419-429.

Naylor C.E., Eaton J.T., Howells A., Justin N., Moss D.S., Titball R.W. and Basak A.K. (1998) Structure of the key toxin in gas gangrene. *Nat. Struct. Biol.* **5**, 738-746.

Naylor C.E., Jepson M., Crane D.T., Titball R.W., Miller J., Basak A.K. and Bolgiano B. (1999) Characterisation of the calcium-binding C-terminal domain of *Clostridium perfringens* alpha-toxin. *J. Mol. Biol.* **294**, 757-770.

Naylor R.D., Martin P.K. and Barker L.T. (1997) Detection of *Clostridium perfringens* alpha toxin by enzyme-linked immunosorbent assay. *Res. Vet. Sci.* **63**, 101-102.

Netherwood T., Wood J.L.N., Mumford J.A. and Chanter N. (1998) Molecular analysis of the virulence determinants of *Clostridium perfringens* associated with foal diarrhoea. *Vet. J.* **155**, 289-294.

Niemann H. (1991) Molecular biology of Clostridial neurotoxins In: *Sourcebook of Bacterial Protein Toxins* (Freer J., Ed.), pp 299-344, Academic Press, New York.

Nobes C.D. and Hall A. (1997) *Clostridium botulinum* C3 exoenzyme and studies on Rho proteins In: *Bacterial toxins* (Aktories K., Ed.), pp 71-83, Chapman and Hall, Weinheim.

Notermans S. and Nagel J. (1989) Assays for botulinum and tetanus toxins In: *Botulinum Neurotoxin and Tetanus Toxin* (Simpson L.L., Ed.), pp 319-331, Academic Press, San Diego.

Nusrat A., von Eichel-Streiber C., Turner J.R., Verkade P., Madara J.L. and Parkos C.A. (2001) *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infect. Immun.* **69**, 1329-1336.

O'Connor D., Hynes P., Cormican M., Collins E., Corbett-Feeney G. and Cassidy M. (2001) Evaluation of methods for detection of toxins in specimens of feces submitted for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **39**, 2846-2849.

Ohishi I. (2000) Structure and function of actin-adenosine-diphosphate-ribosylating toxins In: *Bacterial Protein Toxins* (Just I., Ed.), pp 253-273, Springer, Berlin.

Ottlinger M.E. and Lin S. (1988) *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultured cells. *Exp. Cell Res.* **174**, 215-229.

Oyston P.C.F., Payne D.W., Havard H.L., Williamson E.D. and Titball R.W. (1998) Production of a non-toxic site-directed mutant of *Clostridium perfringens* epsilon-toxin which induces protective immunity in mice. *Microbiology-(UK)* **144,** 333-341.

Pal K.K., Harbola P.C., Sikdar A. and Kumar A.A. (1990) Different physicochemical factors on the beta toxin production of *Clostridium perfringens* Type-B. *Indian J. Anim. Sci.* **60**, 424-425.

Parreiras P.M., Lobato F.C.F., Heneine C., Assis R.A., Balsamao G.M. and Nascimento R.A.P. (2002) Production and purification of epsilon prototoxin produced by *Clostridium perfringens* type D. *Arq. Bras. Med. Vet. Zootec.* **54**, 328-330.

Payne D.W., Williamson E.D., Havard H., Modi N. and Brown J. (1994) Evaluation of a new cytotoxicity assay for *Clostridium perfringens* type D epsilon toxin. *FEMS Microbiol. Lett.* **116**, 161-167.

Perelle S., Gibert M., Bourlioux P., Corthier G. and Popoff M.R. (1997) Production of a complete binary toxin (actin-ADP-ribosylating toxin) by *Clostridium difficile* CD196. *Infect. Immun.* **65**, 1402-1407.

Petit L., Gibert M. and Popoff M.R. (1999) *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol.* **7**, 104-110.

Popoff M.R. (2000) Molecular Biology of Actin-ADP-Ribosylating Toxins In: *Bacterial Protein Toxins* (Just I., Ed.), pp 275-302, Springer, Berlin.

Popoff M.R. (1989) Revue sur l'épidémiologie du botulisme bovin en France et analyse de sa relation avec les élevages de volailles. *Rev. Sci. Techn. Off. Int. Epiz.* **8**, 129-145.

Popoff M.R., Chaves-Olarte E., Lemichez E., Von Eichel-Streiber C., Thelestam M., Chardin P., Cussac D., Antonny B., Chavrier P., Flatau G., Giry M., de Gunzburg J. and Boquet P. (1996) Ras, Rap, and rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J. Biol. Chem.* **271**, 10217-10224.

Popoff M.R. and Marvaud J.C. (1999) Structural and genomic features of clostridial neurotoxins In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (Freer J.H., Ed.), pp 174-201, Academic Press, London.

Poulet S., Hauser D., Quanz M., Niemann H. and Popoff M.R. (1992) Sequences of the botulinal neurotoxin E derived from *Clostridium botulinum* type E (strain Beluga) and *Clostridium butyricum* (strains ATCC43181 and ATCC43755). *Biochem. Biophys. Res. Commun.* **183**, 107-113.

Ren X.D., Bokoch G.M., Traynor-Kaplan A., Jenkins G.H., Anderson R.A. and Schwartz M.A. (1996) Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol. Biol. Cell.* **7**, 435-442.

Richard J.F., Petit L., Gilbert M., Marvaud J.C., Bouchaud C. and Popoff M.R. (1999) Bacterial toxins modifying the actin cytoskeleton. *Internatl. Microbiol.* **2**, 185-194.

Roman M.G., Humber J.Y., Hall P.A., Reddy N.R., Solomon H.M., Triscott M.X., Beard G.A., Bottoms J.D., Cheng T. and Doellgast G.J. (1994) Amplified immunoassay ELISA-ELCA for measuring *Clostridium botulinum* type E neurotoxin in fish fillets. *J. Food Protect.* **57**, 985-990.

Rupnik M., Avesani V., Janc M., von Eichel-Streiber C. and Delmée M. (1998) A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J. Clin. Microbiol.* **36**, 2240-2247.

Rupnik M., Braun V., Soehn F., Jane M., Hofstetter M., Laufenberg-Feldmann R. and von Eichel-Streiber C. (1997) Characterization of polymorphisms in the toxin A and B genes of *Clostridium difficile*. *FEMS Microbiol. Lett.* **148**, 197-202.

Rupnik M., Brazier J.S., Duerden B.I., Grabnar M. and Stubbs S.L.J. (2001) Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiol.* **147**, 439-447.

Rupnik M., Kato N., Grabnar M. and Kato H. (2003) New types of Toxin A-negative, Toxin B-positive strains among *Clostridium difficile* isolates from Asia. *J. Clin. Microbiol.* **41**, 1118-1125.

Saito Y. and Narumiya S. (1997) Preparation of *Clostridium botulinum* C3 exoenzyme and application of ADP-ribosylation of Rho proteins in biological systems In: *Bacterial toxins* (Aktories K., Ed.), pp 85-92, Chapman and Hall, Weinheim.

Sakaguchi G., Sakaguchi S. and Kondo H. (1968) Rapid bioassay for *Clostridium botulinum* type E toxins by intravenous injection into mice. *Jpn. J. Med. Sci. Biol.* **21**, 369-378.

Sakurai J. and Duncan C.L. (1977) Purification of beta-toxin from *Clostridium perfringens* type C. *Infect Immun.* **18**, 741-745.

Sakurai J. and Fujii Y. (1987) Purification and characterization of *Clostridium perfringens* beta toxin. *Toxicon.* **25**, 1301-1310.

Sambol S.P., Merrigan M.M., Lyerly D., Gerding D.N. and Johnson S. (2000) Toxin A gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. *J. Clin. Microbiol.* **68**, 5480-5487.

Sato H., Yamakawa Y., Ito A. and Murata R. (1978) Effect of zinc and calcium ions on the production of alpha-toxin and proteases by *Clostridium perfringens*. *Infect. Immun.* **20**, 325-333.

Schantz E.J. and Kautter D.A. (1978) Microbiological methods: Standardized assay for *Clostridium botulinum* toxins. *J. Assoc. Anal. Chem.*, **61**, 96-99.

Schmidt J.J., Stafford R.G. and Millard C.B. (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. *Anal. Biochem.* **296**, 130-137.

Schoepe H., Wieler L.H., Bauerfeind R., Schlapp T., Potschka H., Hehnen H.R. and Baljer G. (1997) Neutralization of hemolytic and mouse lethal activities of

C. perfringens alpha-toxin need simultaneous blockade of two epitopes by monoclonal antibodies. *Microb. Pathog.* **23**, 1-10.

Sehr P., Gili J., Genth H., Just I., Pick E. and Aktories K. (1998) Glucosylation and ADP ribosylation of Rho proteins: effects on nucleotide binding, GTPase activity, and effector coupling. *Biochem.* **37**, 5296-5304.

Shepard L.A., Heuck A.P., Hamman B.D., Rossjohn J., Parker M.W., Ryan K.R., Johnson A.E. and Tweten R.K. (1998) Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: An alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. *Biochemistry.* **37**, 14563-14574.

Shone C., Appleton N., Hambleton P., Modi N., Gatley S. and Melling J. (1986) *In vitro* assay for botulinum toxin and antitoxins. *Dev. Biol. Standard.* **64**, 141-145.

Shortt S.J., Titball R.W. and Lindsay C.D. (2000) An assessment of the in vitro toxicology of *Clostridium perfringens* type D epsilon-toxin in human and animal cells. *Hum. Exp. Toxicol.* **19**, 108-116.

Smyth C.J. (1975) The identification and purification of theta-haemolysin (theta-toxin) of *Clostridium perfringens* type A. *J. Gen. Microbiol.* **87**, 219-238.

Smyth C.J. and Duncan J.L. (1978) Thiol-activated (oxygen-labile) cytolysins In: *Bacterial toxins and cell membranes* (Wadstom T., Ed.), pp 129-183, Academic Press, London, New York, San Francisco.

Soleilhac J.M., Cornille F., Martin L., Lenoir C., Fournie-Zaluski M.C. and Roques B.P. (1996) A sensitive and rapid fluorescence-based assay for determination of tetanus toxin peptidase activity. *Anal. Biochem.* **241**, 120-127.

Spigaglia P. and Mastrantonio P. (2003) Evaluation of repetitive element sequence-based PCR as a molecular typing method for *Clostridium difficile*. *J. Clin. Microbiol.* **41**, 2454-2457.

Stackebrandt E. (2002). Systematics of the clostridia: waiting for the grand solution. In: *Molecular genetics, classification, pathology and ecology of the genus* Clostridium. Proceedings of the meeting of the CA QLK2-CT2001-01267 (Duchesnes C. and Mainil J., Eds), p 24, Presses de la Fac. Méd. Vét. ULg, Liège, Belgium.

Sterne M. and Batty I. (1975). Pathogenic Clostridia.

Stiles B.G. and Wilkins T.D. (1986) Purification and characterization of *Clostridium perfringens* iota toxin: dependence on two nonlinked proteins for biological activity. *Infect. Immun.* **54**, 683-688.

Stubbs S., Rupnik M., Gibert M., Brazier J., Duerden B. and Popoff M.R. (2000) Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol. Lett.* **186**, 307-312.

Stubbs S.J., Brazier J.S., O'Neil G.L. and Duerden B.I. (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. **37**, 461-463.

Synder W.R. (1987) A continuous spectrophotometric assay for the *Bacillus cereus* phospholipase C using a thiophosphate analog: evaluation of assay conditions and chromogenic agents. *Anal. Biochem.* **164**, 199-206.

Szabo E.A., Pemberton J.M. and Desmarchelier P.M. (1992) Specific detection of *Clostridium botulinum* type B by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **58**, 418-420.

Szabo E.A., Pemberton J.M. and Desmarchellier P.M. (1993) Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**, 3011-3020.

Szabo E.A., Pemberton J.M., Gibson A.M., Eyles M.J. and Desmarchellier P.M. (1994) Polymerase chain reaction for detection of *Clostridium botulinum* types A, B and E in food, soil and infant faeces. *J. Appl. Bacteriol.* **76**, 539-545.

Szilagyi M., Rivera V.R., Neal D., Merril G.A. and Poli M.A. (2000) Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes A and B. *Toxicon.* **38**, 381-389.

Taguchi R. and Ikezawa H. (1978) Phosphatidyl inositol specific phospholipase C from *Clostridium novyi* Type A. *Arch. Biochem. Biophys.* **186**, 196-201.

Taguchi R. and Ikezawa H. (1975) Phospholipase C from *Clostridium novyi* type A.I. *Biochim. Biophys. Acta.* **409**, 75-85.

Taguchi R. and Ikezawa H. (1976) Studies on the hemolytic and hydrolytic actions of phospholipases against mammalian erythrocyte membranes. *Arch. Biochem. Biophys.* **173**, 538-545.

Takahashi T., Sugahara T. and Ohsaka A. (1981) Phospholipase C from *Clostridium perfringens. Methods Enzymol.* **71**, 710-725.

Takeshi K., Fujinaga Y., Inoue K., Nakajima H., Oguma K., Ueno T., Sunagawa H. and Ohyama T. (1996) Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction. *Microbiol. Immunol.* **40**, 5-11.

Thomas R.J. (1991) Detection of *Clostridium botulinum* types C and D toxin by ELISA. *Aust. Vet. J.* **68**, 111-113.

Thompson D.E., Brehm J.K., Oultram J.D., Swinfield T.J., Shone C.C., Atkinson T., Melling J. and Minton N.P. (1990) The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. *Eur. J. Biochem.* **189**, 73-81.

Thompson D.E., Hutson R.A., Easy A.K., Allaway D., Collins M.D. and Richardson P.T. (1993) Nucleotide sequence of the gene coding for *Clostridium barati* type F neurotoxin: comparison with other clostridial neurotoxins. *FEMS Microbiol. Letts.* **108**, 175-182.

Titball R.W. (1993) Bacterial phospholipases-C. *Microbiol. Rev.* **57**, 347-366.

Titball R.W. (1999) Membrane-damaging and cytotoxic phospholipases. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (Freer J.H., Ed.), pp 310-329, Academic Press, London.

Titball R.W., Fearn A.M. and Williamson E.D. (1993) Biochemical and immunological properties of the C-terminal domain of the alpha-toxin of *Clostridium perfringens. FEMS Microbiol. Lett.* **110**, 45-50.

Titball R.W., Hunter S.E.C., Martin K.L., Morris B.C., Shuttleworth A.D., Rubidge T., Anderson D.W. and Kelly D.C. (1989) Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase-C) of *Clostridium perfringens. Infect. Immun.* **57**, 367-376.

Titball R.W., Leslie D.L., Harvey S. and Kelly D. (1991) Hemolytic and sphingomyelinase activities of *Clostridium perfringens* alpha-toxin are dependent on a domain homologous to that of an enzyme from the human arachidonic acid pathway. *Infect. Immun.* **59**, 1872-1874.

Titball R.W. and Rood J.I. (2000) Bacterial phospholipases In: *Handbook of Experimental Pharmacology: Bacterial Protein Toxins* (Just I., Ed.), Springer Verlag.

Toma S., Lesiak G., Magus M., Lo H.L. and Delmée M. (1998) Serotyping of *Clostridium difficile. J. Clin. Microbiol.* **26**, 426-428.

Tso J.Y. and Siebel C. (1989) Cloning and expression of the phospholipase-C gene from *Clostridium perfringens* and *Clostridium bifermentans. Infect. Immun.* **57**, 468-476.

Tsuge H., Nagahama M., Nishimura H., Hisatsune J., Sakaguchi Y., Itogawa Y., Katunuma N. and Sakurai J. (2003) Crystal structure and site-directed mutagenesis of enzymatic components from *Clostridium perfringens* iota-toxin. *J. Mol. Biol.* **325**, 471-483.

Turgeon D.K., Novicki T.J., Quick J., Carlson L., Miller P., Ulness B., Cent A., Ashley R., Larson A., Coyle M., Limaye A.P., Cookson B.T. and Fritsche T.R. (2003) Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J. Clin. Microbiol.* **41**, 667-670.

Tweten R.K. (1988) Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. *Infect. Immun.* **56**, 3235-3240.

Umland T.C., Wingert L.M., Swaminathan S., Furey W.F., Schmidt J.J. and Sax M. (1997) The structure of the receptor binding fragment H_c of tetanus neurotoxin. *Nature Struct. Biol.* **4**, 788-792.

von Eichel-Streiber C., Zec-Pirnat I., Grabnar M. and Rupnik M. (1999) A nonsense mutation abrogates production of a functional enterotoxin A in *Clostridium difficile* toxinotype VIII strains of serogroups F and X. *FEMS Microbiol. Lett.* **178**, 163-168.

Warren G., Koziel M., Mullins M.A., Nye G., Carr B., Desai N., Kostichka K., Duck N. and Estruch J.J. (1996) Novel pesticidal proteins and strains. WO 96/10083**.**

Waters M., Savoie A., Garmory H.S., Bueschel D., Popoff M.R., Songer J.G., Titball R.W., McClane B.A. and Sarker M.R. (2003) Genotyping and phenotyping

of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J. Clin. Microbiol.* **41**, 3584-3591.

Whelan S.M., Elmore M.J., Bodsworth N.J., Brehm J.K., Atkinson T. and Minton N.P. (1992) Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. *Appl. Environ. Microbiol.* **58**, 2345-2354.

Whittier S., Shapiro D.S., Kelly W.F., Walden P., Wait K.J., McMillon L.T. and Gilligan P.H. (1993) Evaluation of four commercially available enzyme immunoassays for laboratory diagnosis of *Clostridium difficile*-associated diseases. *J. Clin. Microbiol.* **31**, 2861-2865.

Wictome M., Newton K., Jameson K., Hallis B., Dunnigan P., Mackay E., Clarke S., Taylor R., Gaze P., Foster K. and Shone C. (1999) Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay. *Appl. Environ. Microbiol.* **65**, 3787-3792.

Wictome M., Newton K.A., Jameson K., Dunnigan P., Clarke S., Gaze J., Tauk A., Foster K.A. and Shone C.C. (1999) Development of in vitro assays for the detection of botulinum toxins in foods. *FEMS Immunol. Med. Microbiol.* **24**, 319-323.

Wilkins T.D. and Lyerly D.M. (2003) *Clostridium difficile* testing: after 20 years, still challenging. *J. Clin. Microbiol.* **41**, 531-534.

Willems A., East A.K., Lawson P.A. and Collins M.D. (1993) Sequence of the gene coding for the neurotoxin of *Clostridium botulinum* type A associated with infant botulism: comparison with other clostridial neurotoxins. *Res. Microbiol.* **144**, 547-556.

Willis A.T. and Gowland G. (1962) Some observations on the mechanism of the Nagler reaction. *J. Pathol. Bacteriol.* **83**, 219-226.

Willis T.A. (1977). Anaerobic Bacteriology: Clinical and Laboratory Practice.

Wolfhagen M.J., Fluit A.C., Torensma R., Poppelier M.J. and Verboef J. (1994) Rapid detection of toxigenic *Clostridium difficile* in fecal samples by magnetic immuno PCR assay. *J. Clin. Microbiol.* **32**, 1629-1633.

Worthington R.W. and Mulders M.S. (1975) The partial purification of *Clostridium perfringens* beta toxin. *Onderstepoort J. Vet. Res.* **42**, 91-98.

Wren B.W., Heard S.R., Al-Saleh A.I. and Tabaqchaali S. (1993) Characterization of *Clostridium difficile* strains by polymerase chain reaction with toxin A- and B-specific primers. *J. Med. Microbiol.* **38**, 109-113.

Yamakawa Y., Ito A. and Sato H. (1977) Theta-toxin of *Clostridium perfringens*; purification and some properties. *Biochim. Biophys. Acta.* **494**, 301-13.

PART 3: Food poisoning organisms: *C. botulinum* and *C. perfringens*

17. Methods for use with foodpoisoning clostridia

P.E. Granum, M.W. Peck

17.1. Introduction

17.1.1. Clostridia of importance in relation to foodborne illness

The two clostridia of most significance with respect to foodborne illness are *Clostridium botulinum* and *C. perfringens*. These organisms are responsible for two very different diseases. Foodborne botulism is an intoxication due to consumption of preformed neurotoxins in foods. Although relatively rare, foodborne botulism is a serious disease that is often fatal, and treatment usually involves long-term hospitalisation. Infant botulism is an infection of the gut of infants less than 12 months of age, and can be associated with consumption of honey. This disease is also severe, but rare. On the other hand, *C. perfringens* type A is the cause of one of the most common foodborne diseases (infections) in the Western world, but is mostly self-limited diarrhoea and normally lasts for less than 24 hours (Peck *et al*., 2004). Except for occasional dehydration in the infirm and elderly, treatment is not necessary. *C. perfringens* type C food poisoning (infection) is rare in the industrialised world today and has not been recorded in Europe during the last decade. Symptoms start with an acute sudden onset of severe abdominal pain and diarrhoea (often bloody), sometimes with vomiting, followed by necrotic inflammation of the small intestine. Untreated, the disease is often fatal and has a mortality rate of 15-25%, even with treatment. Further information on these diseases is given in our scientific booklet (Peck *et al*., 2004).

17.1.2. Outbreaks of foodborne illness associated with clostridia

Outbreaks of clostridial foodborne diseases are almost always through contamination of spores in the foods. Since the bacteria are anaerobic only spores will survive in food production environments. The final product must allow the spores to germinate and outgrow, and anaerobic conditions are created after cooking. When such products are cooled to below the maximum growth temperature, germination followed by growth will start. With the short generation time of *C. perfringens* (8 min) multiplication of the bacterium is fast (Brynestad and Granum, 2002). This is also reflected by the types of products that are usually involved in food poisoning incidents (Labbe, 2000). Outbreaks of foodborne botulism are only rarely associated with commercial foods, although when this does happen the consequences can be serious. More commonly outbreaks of foodborne botulism involve home-prepared foods when known control measures have failed to be implemented. Examples include, the home-canning of vegetables, the home-preparation of meats, and the home-preparation of fish (Lund and Peck, 2000; Peck *et al*., 2004).

In order to ensure the safety of foods with respect to *C. botulinum* and *C. perfringens*, various recommendations and guidelines have been drawn up. For *C. perfringens*, products of concern include meats mishandled during catering

operations or in the home, with slow cooling, holding at room temperature or for extended periods prior to serving. Control of cooling rates has therefore been the major target for reduction of *C. perfringens* risks, and there are various regulations and recommended cooling criteria in different countries. These often aim to restrict the possibility of growth (e.g. less than a 1 log increase in the finished product (USDA, 1999)). To achieve this, chilling from 54.4ºC to 26.6ºC should be achieved in less than 90 min, and from 26.6°C to 4.4°C in less than 5 hours (USDA, 1999). Alternatively predictive models may be used for guidance (e.g. Perfringens Predictor (www.ifr.ac.uk/Safety/GrowthPredictor/default.html), Pathogen Modeling Program (www.arserrc.gov/mfs/PMP6_Start.htm). Methods of controlling *C. botulinum* in foods have been reviewed (Hauschild, 1989; Lund and Peck, 2000). One example is the F_o3 process (121°C/3min) that is given to low acid canned foods to reduce by a factor of 10^{12} the number of viable spores of proteolytic *C. botulinum.* Procedures to ensure the safety of chilled foods with respect to non-proteolytic *C. botulinum* have also been reviewed elsewhere (Lund and Peck, 2000; Martens, 1997; Peck and Stringer, 2005). A committee of the European Food Safety Authority (EFSA) has recently considered the risk presented by foodborne clostridia (EFSA, 2004).

17.1.3. Incidence of *C. botulinum* and *C. perfringens* in the environment

C. perfringens is a spore-forming bacterium and a natural inhabitant of soil and the intestinal tract of many warm-blooded animals and humans. The ubiquitous nature of this bacterium and its spores makes it a frequent problem for the food industry and establishments where large amounts of foods are prepared (Andersson *et al*., 1995). Spores of *C. botulinum* are also ubiquitous in the environment, albeit generally in low numbers (Lund and Peck, 2000).

17.1.4. Working with *C. botulinum* and *C. perfringens*

Since clostridia are anaerobic microorganisms, skills in anaerobic techniques are necessary to work with these organisms. Both *C. botulinum* and *C. perfringens* can be handled in laboratories without anaerobic chambers, although difficulties may be encountered (e.g. achieving sporulation for some strains of *C. perfringens*).

17.2. Methods for use with *Clostridium botulinum*

In view of the danger presented by *C. botulinum* and its potent neurotoxins, work on this pathogen is restricted to containment laboratories offering the appropriate degree of protection. It should be ensured, before work is started, that all appropriate safety guidelines are followed. In most countries, work with *C. botulinum* is restricted to a small number of highly specialised laboratories.

17.2.1. Detection, isolation and quantification of *C. botulinum*

Six different clostridia are capable of producing a botulinum neurotoxin. Two of these organisms, proteolytic *C. botulinum* and non-proteolytic *C. botulinum*

are responsible for most cases of foodborne botulism. Since, these two clostridia differ physiologically, a single method cannot be relied on for both organisms. Conditions that are optimum for enrichment and isolation of proteolytic *C. botulinum* are often not the best for non-proteolytic *C. botulinum*, and vice-versa. For example, heating samples at 80°C for 10 min to inactivate competing, vegetative bacteria is valuable for isolation from spores of proteolytic *C. botulinum*, but would probably inactivate spores of non-proteolytic *C. botulinum.* Culture at 35°C is suitable for isolation of proteolytic *C. botulinum*, while a lower temperature of 26°C is recommended for isolation of non-proteolytic *C. botulinum* (Solomon *et al.*, 1995).

In attempts to detect and isolate *C. botulinum* from the environment or foods it is important to assess the adequacy of the method or methods used, by demonstrating that strains of all relevant groups of *C. botulinum* can be isolated following addition of low numbers to test samples (Sugiyama *et al.*, 1970; Del Torre *et al.*, 2004). The failure to include such controls may be a limitation of some previous studies.

Isolation may be required for studies of the incidence of *C. botulinum* in the environment or in foods, for detection of the organisms in foods suspected of being implicated in outbreaks of botulism or from clinical samples from patients and for investigation of the properties of isolates. The various types of sample present different problems of isolation. Heat treatment of samples is often used to eliminate competing vegetative bacteria. Heating at 75°-80°C for 10-15 min is useful in culturing from spores of proteolytic *C. botulinum*, while 60°C is more appropriate for use during isolation of non-proteolytic *C. botulinum* and the addition of lysozyme to the enrichment medium can increase recovery of the heated spores (Peck *et al.*, 1993; Sebald and Petit, 1994). There may also be merit in tested unheated samples in order to isolate vegetative bacteria or spores that are not fully heat-resistant. Treatment with ethanol may be an alternative to heat treatment for isolation of spores of non-proteolytic *C. botulinum* (Smith and Sugiyama, 1988; Solomon *et al.*, 1995). Media for enrichment must be fully anaerobic (Hatheway, 1988; Kautter *et al.*, 1992; Sebald and Petit, 1994; Solomon *et al.*, 1995), and the redox dye resazurin can be a useful indicator (Lund and Wyatt, 1984). To enrich for proteolytic *C. botulinum*, samples should be inoculated into cooked meat medium (CMM) and incubated at 35°C, while for non-proteolytic *C. botulinum* CMM glucose medium, chopped meat glucose starch medium (Hauschild, 1989) or trypticase-peptone-glucose-yeast extract broth containing trypsin (TPGYT) can be used with incubation at 26°C (Solomon *et al.*, 1995). Filter sterilised trypsin is included to inactivate bacteriocins (boticins) that may be produced by closely related clostridia. In a previous study, it was found that sediment samples from the Fox river in the USA were highly inhibitory to non-proteolytic *C. botulinum* type E and it was necessary to add 10^6 spores of this bacterium to one gram of sediment before type E toxin could be detected in culture (Kautter *et al.*, 1966; Sugiyama et al, 1970). Cultures should be incubated for seven days, or longer if necessary, to allow growth, sporulation and toxin formation. After enrichment a portion of the culture can be used to test for botulinum neurotoxin and a further portion plated onto a suitable solid medium. Before plating, the sample of

enrichment culture may be treated by heating or treatment with ethanol in order to eliminate competing vegetative bacteria (Solomon *et al.*, 1995). A suitable non-selective solid medium is egg-yolk agar on which colonies of proteolytic *C. botulinum* and non-proteolytic *C. botulinum* have a typical appearance associated with their lipase activity. Several selective plating media have been used including Botulinum Selective Medium (BSM) (Mills *et al.*, 1985) and *C. botulinum* Isolation (CBI) agar (Dezfulian et al, 1981). However, the trimethoprin in these media may inhibit non-proteolytic *C. botulinum* (Hatheway, 1988). Colonies suspected to be *C. botulinum* are characterized by toxin formation and physiological characteristics (Hatheway, 1988; Smith and Sugiyama, 1988).

17.2.2. Detection and quantification of *C. botulinum* neurotoxins and their genes

17.2.2.1. Detection and assay of neurotoxins by injection into mice

Intraperitoneal injection into mice has been the standard method for detection and identification of botulinum neurotoxin (Hatheway, 1988). The procedure, controls necessary and the interpretation have been described (Solomon *et al.*, 1995). In order to detect toxin formed by non-proteolytic *C. botulinum* treatment of samples with trypsin is necessary to convert the single chain toxin to the more toxic dichain form. A collaborative study showed that this method was repeatable and reproducible for the detection of toxin produced by proteolytic *C. botulinum* and non-proteolytic *C. botulinum* in foods (Kautter and Solomon, 1977). The specificity of the test is achieved by the use of specific antisera and by observations of typical symptoms of botulism in the mice prior to death. Assays for quantitative determination of toxin have been developed and a stable reference standard preparation of type A toxin was prepared, enabling the response of laboratory mice under a given set of conditions to be assessed and the toxin content of an unknown sample to be expressed in terms of the equivalent of weight of type A toxin per gram or ml (Schantz and Kautter, 1978). Intraperitoneal injection will detect as little as 5-10 pg of type A toxin and provides a measure of the biological activity of the toxin (Hatheway, 1988). Alternative methods involving a range of animals have been described (Pearce *et al.*, 1997), these include subcutaneous injection into mice resulting in flaccid paralysis of muscles rather than death, which is as sensitive as intraperitoneal injection but may be less convenient. Further details of methods for the detection and assay of neurotoxins by injection into mice are given in section 16.4. Advantages of these biological tests are their extreme sensitivity and their ability to detect previously undescribed toxins, atypical toxins and antigenic variants. Disadvantages are the undesirability of using animals and the need to wait several days before a sample can be judged negative.

17.2.2.2. Detection and assay of neurotoxins by ELISA and other immunological techniques

Immunological methods for the detection of botulinum neurotoxins include enzyme-linked immunosorbent assays (ELISAs) and other tests (Pearce *et al.*, 1997). Some of the ELISAs have the same sensitivity and specificity as the mouse test and are cheap and easy to use. The ELISA tests have the following limitations: (i) some may react with biologically inactive toxin; (ii) some may react differently with toxins of a specific type produced by different strains, since these toxins may differ in antigenicity (Doellgast *et al.*, 1993; Ekong *et al.*, 1995; Gibson *et al.*, 1987; Gibson *et al.*, 1988; Huhtanen *et al.*, 1992) (iii) some of the tests use antibodies that were raised to preparations containing a mixture of antigens, so that the tests are not specific for neurotoxins (Huhtanen *et al.*, 1992; Potter *et al.*, 1993; Sakaguchi, 1979); (iv) many ELISAs need a complex and expensive amplification system to achieve the sensitivity of the mouse test (e.g. Doellgast *et al.*, 1993; Flemmig and Stojanowic, 1980; Modi *et al.*, 1986; Shone *et al.*, 1985). Despite these limitations, ELISAs have been widely used, some of which are described in Table 17.1. An ELISA developed by Ferreira and colleagues (Ferreira and Crawford, 1998; Ferreira , 2001; Ferreira *et al.*, 2001; Ferreira *et al.*, 2004) offers independent detection of types A, B, E and F toxins. This assay has been used to detect toxin in food associated with botulism outbreaks (Ferreira *et al.*, 2001; 2004). The sensitivity is about ten-time less than the mouse bioassay. A similar ELISA and standards are commercially available from Metabiologics (www.metabiologics.com/default.htm). Immunological techniques for detection and assay of neurotoxins are also discussed in section 16.4.

Immunological techniques have also been used to identify colonies of proteolytic *C. botulinum* types A and B (Goodnough *et al.*, 1993) and non-proteolytic *C. botulinum* type E (Goodnough *et al.*, 1993; Dezfulian, 1993).

Recently, highly specific *in vitro* assay for botulinum neurotoxins have been developed that are based on a combination of their endopeptidase activities and an immunological reaction. The advantages of these methods over ELISA procedures include (i) the tests measure the biological activity of the light chain of the toxin (but not of the heavy chain); (ii) variations in antigenicity of toxins of a specific type do not influence the response; (iii) the problem resulting from antibodies having been raised to a mixture of antigens is eliminated. In these tests, a fragment of the target protein (SNAP-25 for type A toxin, VAMP for type B toxin) is attached to a microtitre plate, and serves as the substrate. The sample containing neurotoxin is then added and, after a period of incubation, specific antibodies are added that bind to the cleaved target protein, followed by secondary antibodies and detection system.

Table 17.1: Examples of some of the more sensitive ELISAs developed for detection of *C. botulinum* neurotoxins

Toxin type detected (minimum detection limit [MLD_{50}/ml])	**Comments**	**Reference**
A (5-10)	Failed to detect toxin produced by one type A strain. No cross reaction with, other clostridia, denatured toxin, or other toxin types. Complex amplification system. Used with foods.	(Shone *et al.*, 1985; Gibson *et al.*, 1987)
B (20)	Failed to detect toxin produced by one type B strain. No cross reaction with other clostridia or other toxin. Used with foods. Complex amplification system.	(Modi *et al.*, 1986; Gibson *et al.*, 1988)
F (10)	No cross reaction with other clostridia or type A, B or C toxin, slight cross reaction with type D and E toxin. Tested with foods.	(Ferreira *et al.*, 1990)
A (1-32), B (<1-16)	ELISA may respond to antigens with no neurotoxicity. Correlation between the response from ELISA and mouse bioassay not always consistent. Used with foods.	(Huhtanen *et al.*, 1992)
A (9), B (<1), E (<1)	Cross reaction with other clostridia. Used extensively to measure formation of type A, B, and E toxin in meat and in vegetable preparations. Also reacted with type F toxin.	(Potter *et al.*, 1993; Carlin and Peck, 1995; Fernandez and Peck, 1999; Stringer *et al.*, 1999)
A (1), B (1), E (1)	Weak reaction with toxin from some strains, suggesting the presence of biologically inactive, but immunochemically reactive neurotoxin. Used to measure toxin production by non-proteolytic *C. botulinum* in fish fillets. Complex amplification system used.	(Doellgast *et al.*, 1993; Doellgast *et al.*, 1994; Roman *et al.*, 1994)
A (1-20)	Developed for therapeutic preparations.	(Ekong *et al.*, 1995)
E (1-10)	No cross reaction with other toxins or other clostridia. Used with foods.	(Wong, 1996)
A, B, E, F (10 for each)	Tested in a ring trial. Used to quantify toxin present in food samples associated with botulism outbreaks	(Ferreira and Crawford, 1998; Ferreira , 2001; Ferreira *et al.*, 2001; Ferreira *et al.*, 2004)

Endopeptidase assays have been developed for type A toxin and type B toxin that were specific, and did not cross react with other neurotoxins (Hallis *et al.*, 1996), the mean detection limits were 260 MLD_{50}/ml for type A toxin and 380 MLD_{50}/ml for type B toxin. Sensitivity was increased approximately ten-fold by an

amplification system. An endopeptidase assay developed for therapeutic preparations of type A toxin correlated well with the mouse assay and was more sensitive, with a detection limit of 0.2-1.0 MLD_{50}/ml (Ekong *et al.*, 1997). A combination of endopeptidase and ELISA has many advantages but possible problems include: (i) interference by other proteases; (ii) positive reaction with toxin that is inactive *in vivo*, this is because the endopeptidase assays relate to the biological activity of the light chain and would not be affected by inactivation of the biological activity of the heavy chain. Assays are also developed that capture the toxin on an immuno-affinity column, prior to an endopeptidase assay (Wictome and Shone, 1998). Further information about endopeptidase assays is given in section 16.4.

17.2.2.3. Detection of neurotoxin genes using the polymerase chain reaction (PCR) and gene probes

Probes have been constructed that enable PCR tests for the non-specific detection of genes for all botulinum toxin types (Campbell *et al*., 1993) and for the specific detection of genes for each of the toxins produced by *C. botulinum* and other clostridia species (e.g. Fach *et al.*, 1995; Ferreira and Hamdy, 1995; Franciosa *et al.*, 1994; Szabo *et al.*, 1993; Alsallami and Kotlowski, 2001; Carlin *et al.*, 2004; Fach *et al.*, 2002; Wu *et al.*, 2001). A multiplex PCR for simultaneous detection of type A, B, E and F toxin genes was described by Lindstrom and colleagues (Lindstrom *et al.*, 2001). Following a cultural enrichment these methods correlated well with tests for toxin using mice and, with the inclusion of an MPN series of dilutions in the cultural enrichment, have been used for quantitative detection of bacteria containing these genes in aquatic sediments, fish and other samples (Aranda *et al.*, 1997; Hielm *et al.*, 1998; Hielm *et al.*, 1996; Lindstrom *et al.*, 2001; Carlin *et al.*, 2004). The use of a cultural enrichment provides a relatively high number of target bacteria, improves sensitivity and minimises possible problems due to the presence of extracellular DNA or to dead bacteria. Further details of molecular techniques to detect neurotoxin genes are given in section 16.4.

17.2.3. Procedures for sporulation

It can be difficult to get a substantial number of spores of *C. botulinum*. For proteolytic *C. botulinum*, the Anellis broth appears suitable for most strains (Anellis *et al.*, 1972; Del Torre *et al.*, 2004). One difficulty encountered is that of spores retained within cell. Mild sonication may be useful here. A biphasic medium has been found to give good sporulation for many strains of non-proteolytic *C. botulinum* (Peck *et al.*, 1992; Plowman and Peck, 2002).

17.3. Methods for use with *Clostridium perfringens*

17.3.1. Detection, isolation and quantification of *C. perfringens*

In water there is usually too much oxygen for vegetative cells to survive so tests are generally made for spores. The presence of spores of *C. perfringens* in water is used as indicator of faecal contamination (Labbe, 2000), although it might be

argued that soil and sediments may contaminate water as well. In foods it is important not to add oxygen by mixing or stirring the sample prior to testing. Frequently only the core of a food sample contains viable *C. perfringens* cells, especially if the food has been sampled long before analysis. Sometimes the cells can be visualised in the microscope even if no growth occurs after enrichment from the food samples, and it might be possible to culture *C. perfringens* from such samples. Freezing and thawing of food samples containing *C. perfringens* is not recommendable since the cells tolerate freezing poorly. Diluting food samples should preferably be carried out in freshly boiled solutions with as little oxygen as possible, although even vegetative cells tolerate oxygen for a short while. Once seeded the agar plates should be incubated anaerobically as quickly as possible.

Several media have been described for detection and quantification of *C. perfringens*. The most commonly used medium is the Tryptose-Sulfite-Cycloserine (TSC) agar (commercially available) where black colonies will be visible after 24 hours. A top layer of the same agar after seeding will result in faster and more reliable detection. Without the top layer the sulphite reduction and development of black colonies may take more the 24 hours. If the samples are seeded on blood agar plated (bovine) at the same time typical round and slightly opaque and shiny colonies on the surface of agar plates are observed. Colonies usually show a double-zone of haemolysis on blood agar plates with a clear inner θ toxin zone and a hazy outer zone caused by α toxin production (Brynestad and Granum, 2002). *C. perfringens* can grow between 15°C and 52°C with an optimum of about 45°C for most strains. The incubation temperature used is 37ºC.

Confirmation of foodborne outbreaks does not generally involve isolation of the organism, instead it involves detection of the enterotoxin gene or enterotoxin, either by PCR or immunological methods (see below). Enterotoxin negative cells will not cause food poisoning (Brynestad and Granum, 2002).

For water samples (and now also for food samples) a relatively new medium, mCP-agar, is frequently used (EU, 1998; NMKL, 2003) The medium is commercially available and consists of: tryptose (30g/l), yeast extract (20g), sucrose (5.0g/l), L-cysteine hydrochloride (1.0g/l), $MgSO_4 \cdot 7H_2O$ (0.1g/l), Bromocresol purple (40mg/l) and agar (15g/l), plus a supplement of D-cycloserine (400mg/l), indoxyl-β-D-glucoside (60mg/l), phenolphtalein diphosphate (20ml/l) and 4.5% $FeCl_3 \cdot 6\ H_2O$ (2.0ml/l). The plates are incubated anaerobically for 24±3h at 37.0±1.0°C. Colonies of *C. perfringens* are yellow to grey on mCP-agar, 3-6mm in diameter, and produce a yellow colour in the agar around the colonies. An acid phosphatase test is done with ammonia, and a positive reaction is visualised as a red colour (Schallehn and Brandis, 1973).

17.3.2. Procedures for sporulation, and detection and quantification of enterotoxin and enterotoxin genes

Since the enterotoxin from *C. perfringens* (Cpe) is produced only during sporulation, the strains have to grow on specific sporulation media prior to testing for

duringenterotoxin activity. Not all strains will sporulate in the described sporulation media in normal open laboratories (probably due to small amounts of oxygen). However stools after foodborne outbreaks always contain free spores and enterotoxin that can be detected directly by immunological methods. Alternatively PCR methods may be used to determine if the strains harbour the *cpe* gene (see below). Although rare, possible silent genes are then also detected. Several sporulation media for *C. perfringens* have been published (Meyer and Tholozan, 1999; de Jong, 2002) but we here describe one that we are commonly using. This is the Duncan and Strong (DS) medium (Duncan and Strong 1968), and is improved by the addition of raffinose (Labbe and Rey, 1979). Detection of the relative amount of *C. perfringens* enterotoxin in a crude "sporulated extract" is easily carried out using a commercially available RPLA test (Oxoid). Although this kit also reacts with a protein that is produced during vegetative growth (Brynestad and Granum, 2002) it does not matter if using extracts from sporulating cells, because it contains so much enterotoxin. However, if the extract is only positive after 2-8 fold dilution the result might be a false positive. Usually, enterotoxin containing cell extracts from sporulating cells are positive after dilution from 64 to over 1000-fold by this kit.

17.3.2.1. Method for sporulation and detection of the enterotoxin

A *C. perfringens* strain is transferred from an agar culture into 10ml of freshly boiled Robertsons cooked medium (RCM) and incubated at 37°C until sporulation (usually 2-7 days). The sporulated *C. perfringens* strain is then heat shocked at 77°C ± 2°C for 20 min. One ml of this medium is then inoculated into 10ml of FTG (Fluid Thioglycollate) medium, and incubated at 37°C ± 2°C for about 16 hours. A 1ml aliquot from the overnight FTG culture of *C. perfringens* is then transferred into 100ml DS-medium (preferably containing 0.4% raffinose). The DS-medium should be freshly autoclaved and rapidly cooled to 37°C before inoculation. The flasks of DS-medium are then incubated at 37°C for 7-8 hours. Providing that at least 1% of the cells have sporulated (as judged by phase contrast microscopy), then the culture is suitable for testing for enterotoxin. If sporulation has been successful, the flasks should then be held at 4°C for about 16 hours in order to lyse sporulated cells. The culture is then centrifuged at about 10,000g for 25 min, and the supernatant used for enterotoxin detection using the Oxoid RPLA kit, according to the manufacturer's instructions.

17.3.2.2. Method for purification and detection of enterotoxin

Once samples of sporulated *C. perfringens* cells have been produced it is relatively easy to detect the enterotoxin. Either the cells can be allowed to lyse (as described above) or the sporulated cells can be disrupted by sonication, French press or by other physical tools. It is not necessary to purify the enterotoxin, since it is usually produced in huge quantities by food poisoning strains during sporulation (Labbe, 2000). Because of the high hydrophobicity of the enterotoxin (319 amino acids, MW of 35 kDa), the protein is also easy to purify to at least 95% purity using a

double ammonium sulphate precipitation (Granum and Whitaker, 1980). Cell extracts after sporulation and sonication are centrifuged at about 10,000g for about 20 min, and the supernatant containing the enterotoxin (up to 10-20% of the protein) is precipitated with 40% ammonium sulphate. The precipitated protein can be harvested by another centrifugation step (as above). The precipitate (pellet) is then dissolved in 10% of the volume used the first time and subjected to 15% ammonium sulphate precipitation. After a further centrifugation, the pellet is dissolved in buffer with a pH above 6.8. The enteroroxin is now close to purity. Further purification is possible, by passing a sample down a column that separates on the basis of molecular weight (i.e. G-100 column). The purified protein should be stored below 1 mg/ml to avoid aggregation. The crude or pure enterotoxin can be tested for biological activity by a variety of methods, but the most common involves tissue culture, like Vero cells that are relatively easy to grow and maintain (Granum, 1982).

17.3.2.3. Method for detection of *C. perfringens* enterotoxin using Vero cell tissue culture

The growth medium for Vero cells comprises; MEM (500 ml), foetal calf serum (50 ml), and penicillin-streptomycin solution (5 ml). In order to culture the Vero cells, a single flask with growing Vero cells is taken, and the growth medium removed. The cells are then removed using a cell scraper, and resuspended in 15 ml of fresh growth medium. A 5 ml aliquot of the cell suspension is then transferred to each of three new 250 ml flasks, and 15 ml of fresh growth medium added. The flasks are incubated under 5% CO_2 at 37°C.

The Vero cells are then used in a cytotoxicity test. The cells are loosened from one 250 ml flask with a cell scraper and added to 20 ml of fresh growth medium. After mixing, a further 30 ml of fresh growth medium is added. This suspension contains enough Vero cells for a single 24 well plate. An aliquot of 1 ml of cell suspension is transferred to each of the wells in the multi-well plate and incubated under 5% CO_2 at 37°C for two days. Before the start of the toxicity test, it should be checked that the wells are completely covered with Vero cells. The growth medium is then removed from the wells, and each well is washed once with MEM (1 ml). An addition of 1 ml of pre-warmed (37°C) MEM without leucine is made to each well, and 50 µl of enterotoxin solution is added immediately. The Vero cells are incubated with the enterotoxin at 37°C for 2 hours. The medium is then removed, and the cells washed once with 1 ml of pre-warmed (37°C) MEM without leucine. An addition of 16 µl ^{14}C-leucine is then made to 8 ml of MEM without leucine (this is enough for one 24 well plate), and 300 µl of this solution is added to each well. The plates are incubated at 37°C without CO_2 for 1 hour, and then the medium is removed, and 1 ml 5% TCA is added to each well. After incubation at room temperature for 10 min, the TCA is removed and each well washed twice with 1 ml 5% TCA. An addition of 300µl 0.1M KOH is then made to each well, and incubated at room temperature for a further 10 min. All of the solutions from each well are transferred into individual scintillation tubes, and 2 ml of scintillation liquid added.

After shaking, counts are made in a scintillation counter for 1 min. For a positive control, cells incubated with 50 µl supernatant from a positive *C. perfringens* strain are used (see enterotoxin preparation). For calculation, the average of two samples of the same enterotoxin test sample is used. The calculation of percentage inhibition of ^{14}C-leucine uptake is made by; (i) subtraction of the value for background counts (which is usually 30-60 cpm [counts per minute]); (ii) [cpm for Vero cells without toxin added – cpm test sample] x 100/ cpm for Vero cells without toxin added.

17.3.2.4. Method for detection of *C. perfringens* enterotoxin gene (*cpe*)

The sequence of gene transcribing the enterotoxin has been known for many years, and since it has little homology with other known genes, it is easy to detect by PCR. The high AT content of the gene is the only small problem. The presence of the enterotoxin gene (*cpe*) in the *C. perfringens* genome can be shown by PCR according to the following protocol (From: Norwegian Reference Laboratory for Spore-forming Food Pathogens):

1. DNA is isolated from one colony on blood agar (or a selective medium). The DNA is extracted from the cells by using ADVAMAX™ Beads from AGTC (Advanced Genetic Technologies Corp.) according to the protocol from the suppliers. The DNA-pellet is suspended in 50 µl 10 mM Tris-HCl pH 7.5-8.5.
2. The following primers are used:
   Forward: CAAGTCAAATTCTTAATCCT
   Reverse: CATCACCTAAGGACTGTTCT
3. A master mix is made for x number of samples (including a positive and negative control) + 1 extra = n number of samples of 50 µl:

10x PCR buffer		5 µl x n
dNTP-mix (10 mM each)		1 µl x n
primer 1	(10 pmol/µl)	1 µl x n
primer 2	(10 pmol/µl)	1 µl x n
DNA polymerase (2U/µl)		1 µl x n
dH2O		40 µl x n

4. The following PCR program is used: First 92°C for 3 min, followed by 30 cycles of 92°C for 1 min, 50°C for 1 min and 72°C for 1 min, end by 72°C for 7 min.
5. The product size is 700 bp. Run the PCR products in a 1% agarose gel containing 1 µg/ml ethidium bromide, at 70 V for 30 min. Visualise under UV light. Use known size markers on the gel, and always run a *cpe*-positive *C. perfringens* on the gel.

17.3.3. Methods for detection of the *C. perfringens* β toxin

The description of the methods in use for detection of the β toxin responsible for the *C. perfringens* type C disease is described in section 16.4.

17.4. Bibliography

Alsallami A.A. and Kotlowski R. (2001). Selection of primers for specific detection of *Clostridium botulinum* types B and E neurotoxin genes using PCR method. *Int. J. Food Microbiol.* **69**, 247-253

Andersson A., Rönner U. and Granum P.E. (1995) What problems does the food industry have with the sporeforming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int. J. Food Microbiol*. **28**, 145-155.

Anellis A., Berkowitz D., Kemper D. and Rowley D.B. (1972). Production of types A and B spores of *Clostridium botulinum* by the biphasic method: effect on spore population, radiation resistance and toxigenicity. *Appl. Microbiol*. **23**, 734-739.

Aranda E., Rodriguez M.M., Asensio M.A. and Córdoba J.J. (1997) Detection of *Clostridium botulinum* types A, B, E and F in food by PCR and DNA probe. *Lett. Appl. Microbiol.* **25**, 186-90.

Brynestad S. and Granum P.E. (2002) *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol*. **74**, 195-202.

Campbell K.D., Collins M.D. and East A.K. (1993). Gene probes for identification of the botulinal neurotoxin gene and specific identification of neurotoxin types B, E and F. *J. Clin. Microbiol.* **31**, 2255-62.

Carlin F. and Peck M.W. (1995). Growth and toxin production by non-proteolytic and proteolytic *Clostridium botulinum* in cooked vegetables. *Lett. Appl. Microbiol*. **20**, 152-156.

Carlin F., Broussolle V., Perelle S., Litman S. and Fach P. (2004). Prevalence of *Clostridium botulinum* in food raw materials used in REPFEDs manufactured in France. *Int. J. Food Microbiol*. **91**, 141-145

de Jong A.E., Beumer R.R. and Rombouts F.M. (2002). Optimizing sporulation of *Clostridium perfringens*. *J. Food Prot.* **65**, 1457-62.

Del Torre M., Stecchini M.L., Braconnier A. and Peck M.W. (2004). Prevalence of *Clostridium* species and behaviour of *Clostridium botulinum* in gnocchi, a REPFED of Italian origin. *Int. J. Food Microbiol*. **96**, 115-131

Dezfulian M., McCroskey L.M., Hatheway C.L. and Dowell V.R. (1981). Selective medium for isolation of *Clostridium botulinum* from human faeces. *J. Clin. Microbiol*. **13**, 526-31.

Dezfulian M. (1993). A simple procedure for identification of *Clostridium botulinum* colonies. *World J. Microbiol. Biotechnol*. **9**, 125-27.

Doellgast G.J., Triscott M.X., Beard G.A., Bottoms J.D., Cheng T., Roh B.H., Roman M.G., Hall P.A. and Brown J.E. (1993). Sensitive enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* neurotoxins A, B and E using signal amplification via enzyme-linked coagulation assay. *J. Clin. Microbiol*. **31**, 2402-09.

Doellgast G.J., Beard G.A., Bottoms, J.D., Cheng T., Roh B.H., Roman M.G., Hall P.A. and Triscott M.X. (1994). Enzyme-linked immunosorbent assay and enzyme-linked coagulation assay for detection of *Clostridium botulinum*

neurotoxins A, B and E and solution-phase complexes with dual-label antibodies. *J. Clin. Microbiol*. **32**, 105-11.

Duncan C.L. and Strong D.H. (1968). Improved medium for sporulation of *Clostridium perfringens. Appl. Microbiol*. **16**, 82-89.

EFSA (2004). Opinion of the scientific panel on biological hazards on a request from the Commission related to *Clostridium* spp in foodstuffs. *The EFSA J.* **199**, 1-65.

Ekong T.A.N., Feavers I.M. and Sesardic D. (1997). Recombinant SNAP-25 is an effective substrate for *Clostridium botulinum* type A toxin endopeptidase activity *in vitro*. *Microbiol.* **143**, 3337-3347.

Ekong T.A.N., McLellan K. and Sesardic D. (1995). Immunological detection of *Clostridium botulinum* toxin type A in therapeutic preparations. *J. Immunol. Methods* **180**, 181-191.

EU (1998). Directive 98/83/CE of Council of 3 November 1998 on the quality of water intended for human consumption. *Off. J. Eur. Commun., L* **330**, pp. 32–54.

Fach P., Gibert M., Griffais R., Guillou J.P. and Popoff M.R. (1995). PCR and gene probe identification of botulinum neurotoxin A-,B-,E-, F-, and G-producing *Clostridium* spp. and evaluation of food samples. *Appl. Environ. Microbiol.* **61**, 389-392.

Fach P., Perelle S., Dilasser F., Grout J., Dargaignaratz C., Botella L., Gourreau J.M., Carlin F., Popoff M.R. and Broussolle V. (2002). Detection by PCR-enzyme-linked immunosorbent assay of *Clostridium botulinum* in fish and environmental samples from a coastal area in northern France. *Appl. Environ. Microbiol.* **68**, 5870-5876

Fernandez P.S. and Peck M.W. (1999). A predictive model that describes the effect of prolonged heating at 70 to 90°C and subsequent incubation at refrigeration temperatures on growth and toxigenesis by non-proteolytic *Clostridium botulinum* in the presence of lysozyme. *Appl. Environ. Microbiol*. **65**, 3449-3457.

Ferreira J.L. (2001). Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinal toxins A, B, E, and F. *J. AOAC Int*. **84**, 85-88.

Ferreira J.L. and Crawford R.G. (1998). Detection of type a botulinal toxin-producing organisms subcultured from cheese using an amplified ELISA system. *J. Rapid Methods Automation Microbiol.* **6**, 289-296.

Ferreira J.L., Eliasberg S.J., Harrison M.A. and Edmonds P. (2001). Detection of preformed type A botulinal toxin in hash brown potatoes by using the mouse bioasssay and a modified ELISA test. *J. AOAC Int*. **84**, 1460-1464.

Ferreira J.L., Eliasberg S.J., Edmonds P. and Harrison M.A. (2004). Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinal toxin in food. *J. Food Prot.* **67**, 203-206.

Ferreira J.L. and Hamdy M.K. (1995). Detection of botulinal toxin genes: types A and E or B and F using the muliplex polymerase chain reaction. *J. Rapid Methods Automation Microbiol.* **3**, 177-83.

Ferreira J.L., Hamdy M.K., McCay S.G. and Zapatka F.A. (1990). Monoclonal antibody to type F *Clostridium botulinum* toxin. *Appl. Environ. Microbiol*. **56**, 808-811.

Flemmig R. and Stojanowic V. (1980). Untersuchungen von Bienenhonig auf *Clostridium botulinum* sporen. *Arch. Lebensmittelhyg*. **31**, 179-180.

Franciosa G., Ferreira J.L. and Hatheway C.L. (1994). Detection of type A, B and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. *J. Clin. Microbiol*. **32**, 1911-1917.

Gibson A.M., Modi N.K., Roberts T.A., Shone C.C., Hambleton P. and Melling J. (1987). Evaluation of a monoclonal antibody-based immunoassay for detecting type A *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. *J. Appl. Bacteriol*. **63**, 217-26.

Gibson A.M., Modi N.K., Roberts T.A., Hambleton P. and Melling J. (1988). Evaluation of a monoclonal antibody-based immunoassay for detecting type B *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. *J. Appl. Bacteriol*. **64**, 285-291.

Goodnough M.C., Hammer B., Sugiyama H. and Johnson E.A. (1993). Colony immunoblot assay of botulinal toxin. *Appl. Environ. Microbiol*. **59**, 2339-42.

Granum P.E. (1982). Inhibition of protein synthesis by a tryptic polypeptide of *Clostridium perfringens* type A enterotoxin. *Biochim. Biophys. Acta* **708**, 6-11.

Granum P.E. and Whitaker J.R. (1980). Improved method for purification of enterotoxin from *Clostridium perfringens* type A. *Appl. Environ. Microbiol*. **39**, 1120-1122.

Hallis B., James B.A.F. and Shone C.C. (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. *J. Clin. Microbiol.* **34**, 1934-38.

Hatheway C.L. (1988), Botulism. In: *Laboratory diagnosis of infectious diseases; principles and practice* (Balows A., Hausler Jr. W.J., Ohashi M. and Turano A., Eds), pp 111-133, Springer-Verlag, London.

Hauschild A.H.W. (1989), *Clostridium botulinum*. In *Foodborne bacterial pathogens* (Doyle M.P., Ed), pp 112-189, Marcel Dekker Inc., New York.

Hielm S., Hyytiä E., Andersin A-B. and Korkeala H. (1998). A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. *J. Appl. Microbiol*. **84**, 133-37.

Hielm S., Hyytia E., Ridell J. and Korkeala H. (1996) Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. *Int. J. Food Microbiol.* **31**, 357-365

Huhtanen C.N., Whiting R.C., Miller A.J. and Call J.E. (1992) Qualitative correlation of the mouse neurotoxin and enzyme-linked immunoassay for determining *Clostridium botulinum* types A and B toxins. *J. Food Saf.* **12**, 119-27.

Kautter D.A. and Solomon H.M. (1977) Collaborative study of a method for the detection of *Clostridium botulinum* and its toxins in foods. *J. Assoc. Off. Anal. Chem.* **60**, 541-545

Kautter D.A., Harmon R.K., Lynt R.H. and Lilly T. (1966) Antagonistic effect on *Clostridium botulinum* type E by organisms resembling it. *Appl. Microbiol.* **14**, 616-622.

Kautter D.A., Solomon H.M., Lake D.E. *et al.* (1992) *Clostridium botulinum* and its toxins. In *Compendium of methods for the microbiological examination of foods* (3rd ed) (Vanderzant C. and Splittstoesser D.F., Eds), pp. 605-621, American Public Health Association, Washington, DC.

Labbe R.G. and Rey D.K. (1979) Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* type A. *Appl. Environ. Microbiol.* **37**, 1196-1200.

Labbe R.G. (2000) *Clostridium perfringens*. In: *The Microbiological Safety and Quality of Food* (Lund B.M., Baird-Parker T. and Gould G.W., Eds.), pp 1110-1135, Aspen Publishers MD, USA.

Lindstrom M., Keto R., Markkula A., Nevas M., Hielm S. and Korkeala H. (2001). Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Appl. Environ. Microbiol.* **67**, 5694-5699.

Lund B.M. and Peck M.W. (2000). *Clostridium botulinum*. In: *The microbiological safety and quality of food* (Lund B.M., Baird-Parker A.C. and Gould G.W., Eds), pp. 1057-1109, Aspen, Gaithersburg, USA.

Lund B.M. and Wyatt G.M. (1984) The effect of redox potential, and its interaction with sodium chloride concentration, on the probability of growth of *Clostridium botulinum* type E from spore inocula. *Food Microbiol.* **1**, 49-65.

Martens T. (1997) *Harmonization of safety criteria for minimally processed foods*. Inventory report FAIR Concerted Action. FAIR-CT96-1020. EC. Brussels.

Meyer M. and Tholozan J.L. (1999) A new growth and in vitro sporulation medium for *Clostridium perfringens*. *Lett. Appl. Microbiol.* **28**, 98-102.

Mills D.C., Midura T.F. and Arnon S.S. (1985) Improved selective medium for the isolation of lipase-positive *Clostridium botulinum* from feces of human infants. *J. Clin. Microbiol.* **21**, 947-950.

Modi N.K., Shone C.C., Hambleton P. and Melling J. (1986) Monoclonal antibody based enzyme-linked-immunosorbent-assays for *Clostridium botulinum* toxin types A and B. In *Proceedings of the 2nd World Congress Foodborne Infections and Intoxications. Vol. II.* Berlin (West), pp. 1184-1188.

NMKL (2003) Nordic Committee on Food Analysis, No 95, 4th ed.

Pearce L.B., First E.R., MacCallum R.D. and Gupta A. (1997) Pharmacologic characterization of botulinum toxin for basic science and medicine. *Toxicon* **35**, 1373-1412.

Peck M.W., Fairbairn D.A. and Lund B.M. (1992). The effect of recovery medium on the estimated heat-inactivation of spores of non-proteolytic *Clostridium botulinum*. *Lett. Appl. Microbiol.* **15**, 146-151.

Peck M.W., Fairbairn D.A. and Lund B.M. (1993) Heat-resistance of spores of non-proteolytic *Clostridium botulinum* estimated on medium containing lysozyme. *Lett. Appl. Microbiol*. **16**, 126-131.

Peck M. W., Granum P. E., Mainil J. and Gould G.W. (2004) *Foodborne clostridia and sporulation.* Scientific booklet of the CA QLK2-CT2001-01267, pp 1-50, Presse Fac. Méd. Vét. ULg, Liège, Belgique (http://www.genusclostridium.net).

Peck M.W. and Stringer S.C. (2005). The safety of pasteurised in-pack chilled meat products with respect to the foodborne botulism hazard - a review. *Meat Science* **70**, 461-475.

Plowman J. and Peck M.W. (2002). Use of a novel method to characterise the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. *J. Appl. Microbiol*. **92**, 681-694.

Potter M.D., Meng J. and Kimsey P. (1993) An ELISA for detection of botulinal toxin types A, B and E in inoculated food samples. *J. Food Prot.* **56**, 856-61.

Roman M.G., Humber J.Y., Hall P.A. *et al*. (1994) Amplified immunoassay ELISA-ELCA for measuring *Clostridium botulinum* type E neurotoxin in fish fillets. *J. Food Protect*. **57**, 985-90.

Sakaguchi G. (1979) Botulism. In: *Foodborne infections and intoxications.* 2nd ed. (Riemann H. and Bryan F.L., Eds), pp. 389-442, New York. Academic Press.

Schallehn G. and Brandis H. (1973). Phosphatase-reagent for quick identification of *Clostridium perfringens*. *Zbl. Bakt. I Abt. Orig. A* **225**, 343–345

Schantz E.J. and Kautter D.A. (1978) Standardized assay for *Clostridium botulinum* toxins. *J. Assoc. Off. Anal. Chem*. **61**, 96-99.

Schmidt J.J. and Bostian K.A. (1995) Proteolysis of synthetic peptides by type A botulinum neurotoxin. *J. Protein Chem.* **14**, 703-08.

Sebald M. and Petit J.C. (1994) *Méthodes de laboratoire bacteréries anaérobes et leur identification*. Institut Pasteur, Paris.

Shone C., Wilton-Smith P., Appleton N., Hambleton P., Modi N., Gatley S. and Melling J. (1985) Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* toxin is comparable to the mouse bioassay. *Appl. Environ. Microbiol*. **50**, 63-7.

Smith L.DS. and Sugiyama H. (1988) *Botulism*. Charles C. Thomas, Springfield, Illinois, USA

Solomon H.M., Rhodehamel E.J. and Kautter D.A. (1995) *Clostridium botulinum.* In: *Bacteriological Analytical Manual*. 8th ed., FDA, Washington, D.C.

Stringer S.C., Haque N. and Peck M.W. (1999). Growth from spores of non-proteolytic *Clostridium botulinum* in heat treated vegetable juice. *Appl. Environ. Microbiol*. **65**, 2136-2142.

Sugiyama H., Bott T.L. and Foster E.M. (1970) *Clostridium botulinum* type E in an inland bay (Green Bay of Lake Michigan). In: *Toxic Microorganisms*. Proceedings 1st U.S.-Japan Conference on Toxic Microorganisms, pp 287-291, Honolulu, HI.

Szabo E.A., Pemberton J.M. and Desmarchelier P.M. (1993) Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. *Appl. Environ. Microbiol*. **59**, 3011-3020.

USDA-FSIS (1999) Performance standards for the production of certain meat and poultry products. *Fed. Regist*. **64**, 732-749.

Wictome M. and Shone C.C. (1998) Botulinum neurotoxins: mode of action and detection. *J. Appl. Microbiol*. **84**, 87s-97s.

Wong P.C.K. (1996) Detection of *Clostridium botulinum* type E toxin by monoclonal antibody enzyme immunoassay. *J. Rapid Methods Automation Microbiol.* **4**, 191-206.

Wu H.C., Huang Y.L., Lai S.C., Huang Y.Y. and Shaio M.F. (2001). Detection of *Clostridium botulinum* neurotoxin type A using immuno-PCR. *Lett. Appl. Microbiol.* **32**, 321-325

Index

Appendix

Some official and non-official reference laboratories for the diagnosis of clostridial infections

Belgium
Institut/Instituut Pasteur Bruxelles, Dr M. Turneer
Rue d'Angleterre/Engelandstraat, 642 – 1180 Bruxelles/Brussel – Belgium
Tel: +32 2 373 33 10 Fax: +32 2 373 33 15
mturneer@pasteur.be
http://www.pasteur.be/botnl.htm;

Denmark
For humans:
Statens Serum Institute
Artillerivej, 5 – 2300 Copenhagen S – Denmark
Tel: +45 32 68 32 68 Fax: +45 32 68 38 68
serum@ssi.dk http://www.ssi.dk/

For animals and food:
Danish Institute for Food and Veterinary Research
Bülowsvej, 27 – 1790 Copenhagen V – Denmark
Tel: +45 72 34 60 00 Fax: +45 72 34 60 61
http://www.dfvf.dk/

Finland
For humans:
National Public Health Institute – KTL – Anaerobe Reference Laboratory
Mannerheimintie, 166 – 00300 Helsinki – Finland
Tel: +358 9 47 44 82 48 Fax: +358 9 47 44 82 38

For animals and food:
National Veterinary and Food Research Institute – EELA
PO Box 45 (Hameentie 57) - 00581 Helsinki – Finland
Tel: +358 9 393 1827 Fax: +358 9 393 1907

France
Institut Pasteur, Dr M. Popoff
Centre national de Référence des Anaérobies
Rue du Docteur Roux, 28 – 75724 – Paris cedex 15 – France
Tél: +33 1 45 68 83 10 Fax: +33 1 40 61 31 23
mpopoff@pasteur.fr
http://www.pasteur.fr/actu/presse/dossiers/cnr/ana.html

Germany
Institut für Medizinische Mikrobiologie une Infektionsepidemiologie
Liebigstr., 24 – 04103 Leipzig – Germany
Tel: +49 3 41 971 52 00 Fax: +49 3 41 971 52 09
e-mail: acr@medizin.uni-leipzig.de
http://www.uni-leipzig.de/~mikrob/index.htm

Italy
National Reference Centre for Botulism - National Centre for food quality and risk assesment – Istituto Superiore di Sanità – Dr Paolo Aureli
Viale Regina Elena, 299 – 00161 Rome – Italy
Tel: +39 06 4990 3418
E-mail: cola@iss.it http://www.iss.it/cnra/

Department for Intectious, parasitic and immunomediated diseases
Anaerobic Bacteria unit – Istituto Superiore di Sanità – Dr Antonio Cassone
Viale Regina Elena, 299 – 00161 Rome – Italy
Tel: +39 06 4990 3219 Fas: +39 06 4938 7183
e-mail: mipisegr@iss.it http://www.iss.it/mipi/

The Netherlands
Central Institute for animal Disease Control-Lelystad
PO Box 2004 – 8203 AA Lelystad – The Netherlands
Tel: +31 320 23 81 53
http://www.cidc-lelystad.nl/

Norway
Norwegian Reference Laboratory – Norwegian School of Veterinary Science
Food Hygiene – PO Box 8146 Dep – Ullevalsveien, 72 – 0033 Oslo
Tel: +47 22 59 70 52 Fax: +47 22 96 48 50
http://oslovet.veths.no/

Poland
Department of Medical Microbiology – Medical University of Warsaw
Chalubinski street, 5 – 02 004 Warsaw – Poland
e-mail: hanna.pituch@ib.amwaw.edu.pl

Slovakia
State veterinary and food institute
Dr Maria Kantikova
026 01 Dolny Kubin
kantikova@svpudk.sk http://www.svssr.sk/

Switzerland
Animal clostridia reference center - ZOBA
Institute of Veterinary Bacteriology – University of Bern
Dr. R. Miserez
Laenggassstrasse, 122 – 3001 Bern – Switzerland
Tel: +41 31 631 2484 Fax: +41 31 631 2634
e-mail: raymond.miserez@vbi.unibe.ch

Human *Clostridium botulinum* reference center - NENT/NANT
Institute of Veterinary Bacteriology – University of Bern
Dr. H. Haechler
Laenggassstrasse, 122 – 3001 Bern – Switzerland
Tel: +41 31 631 2484 Fax: +41 31 631 2634
e-mail: herbert.haechler@vbi.unibe.ch

United Kingdom
HPA – Colindale – Food Safety Microbiology Laboratory
Dr. Jim McLauchlin
61 Colindale Avenue – London NW9 5HT – UK
Tel: +44 20 8200 4400 Fax: +44 20 8200 8264
http://www.hpa.org.uk/srmd/qualitystandards.htm

European Commission

EUR 21463 — **Clostridia in medical, veterinary and food microbiology - Diagnosis and typing**

Luxembourg: Office for Official Publications of the European Communities

2006 — 214 pp. —14.8 x 21 cm

ISBN 92-79-00422-3