Clostridium perfringens the causal agent of necrotic enteritis in poultry

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Abstract

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Clostridium perfringens, an anaerobic Gram-positive bacterium known to be a common pathogen in humans, domestic animals and in wildlife, is the primary cause of clostridial enteric disease in domestic animals. *C. perfringens* can lead to both clinical and subclinical disease in poultry. Due to the diminished use of growth-promoting antibiotics in the European Union, *C. perfringens* associated necrotic enteritis and subclinical diseases have become serious threats to poultry health. The objectives of this PhD project were to learn how *C. perfringens* causes necrotic enteritis in broilers. The genetic diversity of *C. perfringens* in a wider perspective was also determined.

It is of great importance that the plasmid-borne toxin genes are stable, both when the bacteria are transported to the laboratory and at the laboratory, in order that toxin typing of *C. perfringens* isolates should be accurate. Our results demonstrate that the plasmid encoded genes *cpb1*, *cpb2* and *etx* are indeed stable under normal laboratory conditions and under conditions normally applying during transportation.

Pulsed-field gel electrophoresis (PFGE) was used to explore the genetic relatedness of *C. perfringens*. The genetic diversity was found to be wide among *C. perfringens* isolates from different animal species, from food poisoning outbreaks and sewage sludge. Epidemiologically related isolates showed a close genetic similarity, as expected, while isolates with no obvious epidemiological relationship were genetically not so similar. Furthermore, sequence comparison of the *cpb2* gene revealed two genetically different populations.

The genetic relatedness of *C. perfringens* isolates from a broiler flock affected by mild necrotic enteritis (NE) was investigated by PFGE. Generally, a low genetic relatedness was found among *C. perfringens* isolates from these broilers affected by either mild or severe NE.

The uniform susceptibility to narasin indicated that the substance can still be used to control clostridiosis, and that development of the resistance observed is slow. In this study *C. perfringens* also showed a low degree of resistance to most other antimicrobials tested. The exception was the high degree of tetracycline resistance found in *C. perfringens* in Swedish broilers.

Keywords: *Clostridium perfringens*, necrotic enteritis, poultry, genotyping, PFGE, antimicrobial, toxins, plasmid stability

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Sammanfattning

Clostridium perfringens är en välkänd patogen hos människa, domesticerade djur och vilda djur. *C. perfringens* är den vanligaste orsaken till enteriska sjukdomar hos djur orsakade av klostridier. Klostridios är ett multifaktoriellt lidande hos slaktkycklingar som inträffar då *C. perfringens* snabbt tillväxer i tunntarmen och producerar toxiner, främst alfatoxin. Genom minskad användning av tillväxtsbefrämjande antibiotika och jonofora coccidostatika i den Europeiska Unionen, har klostridios blivit ett allvarligt hot mot slaktkycklingproduktionen.

Syftet med detta doktorandprojekt var att få ökad kunskap om *C. perfringens* isolat, som orsakar nekrotiserande enterit hos slaktkyckling, och att undersöka den genetiska diversiteten hos *C. perfringens*.

Det är mycket viktigt att eventuella bakterier, som finns i ett prov, överlever transporten till laboratoriet. Våra resultat visar att *C. perfringens* överlever väl, om proverna transporteras under kortare tid (upp till 44 timmar) och inom ett temperaturintervall av +4°C till +20°C. Dessutom är det av stor vikt att generna är stabila, så att isolaten kan analyseras och klassificeras med molekylärbiologiska metoder. Vidare indikerar resultaten att de plasmidburna generna kan anses vara stabila under normala laboratorieförhållanden och under den simulerade transporten.

Pulsfältgelelektrofores (PFGE) användes i två av delstudierna i denna avhandling för att undersöka de genetiska likheterna hos *C. perfringens*. PFGE visade att det förekom en stor genetisk diversitet hos *C. perfringens* isolerade från olika djurslag, matförgiftningsutbrott och från slamprover. Isolat med en epidemiologisk anknytning visade större genetiska likheter än isolat utan någon epidemiologisk anknytning. Sekvensanalys av beta2 toxin genen (*cpb2*) visade att det fanns två olika evolutionära populationer av denna gen.

De genetiska likheterna hos *C. perfringens* isolerade från en slaktkycklingflock med mild nekrotiserande enterit (NE) undersöktes också med PFGE. Den genetiska likheten hos *C. perfringens* isolerade från kycklingar med mild och klinisk NE var relativt låg. Den relativt låga genetiska likheten mellan isolaten kan sannolikt förklaras med att isolaten, som analyserades, kom från en slaktkycklingflock med låg dödlighet i nekrotiserande enterit.

Ingen resistens gentemot narasin påvisades, vilket indikerar att narasin fortfarande kan användas för att förhindra att sjukdomen klostridios uppkommer hos slaktkyckling. I den här studien visade sig *C. perfringens* också vara känslig mot de flesta andra antibiotika som testades. Undantaget var den höga resistens gentemot tetracyklin som påvisades hos *C. perfringens* isolerade från svenska slaktkycklingar.

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their roman numerals.

I. Johansson, A., Engström, B.E., Frey, J., Johansson, K-E. & Båverud, V. 2005. Survival of *Clostridium perfringens* during simulated transport and stability of some plasmid-borne toxin genes under aerobic conditions. *Acta Veterinaria Scandinavica*, 46(4), 241-247.

II. Johansson, A., Aspan, A., Bagge, E., Båverud, B., Engström, B.E. & Johansson, K-E. Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. Submitted.

III. Johansson, A., Aspan, A., Kaldhusdal, M., Johansson, K-E. & Engström, B.E. Genetic relatedness of *Clostridium perfringens* type A strains isolated from a broiler flock affected by mild necrotic enteritis. Manuscript.

IV. Johansson, A., Greko, C., Engström, B.E., & Karlsson, M. 2004. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry, and distribution of tetracycline resistance genes. *Veterinary Microbiology* 99, 251–257.

Abbreviations

AFLP	amplified fragment length polymorphism
AGP	antimicrobial growth promoter
ATCC	American Type Culture Collection
CCUG	Culture Collection University of Gothenburg
CFU	colony forming units
CPH	*C. perfringens*-associated hepatic change
CIP	Culture Collection of Institute Pasteur
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
IAC	ionophorous anticoccidial
MIC	minimum inhibitory concentration
MLEE	multilocus enzyme electrophoresis
MLVA	multiple-locus variable number tandem repeat analysis
MLS	macrolide-lincosamide-streptogramin
NCCLS	National Committee for Clinical Laboratory Standards
NE	necrotic enteritis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RE	restriction enzyme
RNA	ribonucleic acid
TIGR	The Institute for Genomic Research

Introduction

The bacterial genus *Clostridium* comprises spore-forming, Gram-positive, anaerobic rods, which are present in all kinds of environments. Approximately 2.7 billion years ago, before the initial formation of oxygen, clostridia appeared as a distinct class. Most clostridial species are harmless saprophytes living in nature, in animals and in decaying vegetation. However, up to 25 species are minor pathogens and 13 others can be regarded as major pathogens. These 13 major pathogens produce altogether 59 different toxins. Collectively, the clostridia form the most toxigenic genus known. Based on the number of toxins produced, it may be argued that *Clostridium* is the most important bacterial genus known.

In 1892 the American bacteriologist Welch isolated a Gram-positive anaerobic bacillus from gangrenous wounds. This organism, originally called *Bacillus aerogenes capsulatus,* was later designated *Bacillus perfringens,* then *Clostridium welchii*. The organism is now known as *Clostridium perfringens*. *C. perfringens* is a Gram-positive anaerobic bacterium that is able to form spores. *C. perfringens* is widespread in the environment and is commonly found in the intestines of animals, where under certain circumstances it is pathogenic. As many as 18 toxins have been described in the literature. In humans, *C. perfringens* can cause gangrene and gastrointestinal disorders. It is certainly the most serious cause of clostridial enteric disease in domestic animals.

C. perfringens is also the causative agent of clostridiosis, one of the most important diseases in broiler and turkey flocks. Due to the withdrawal of growth-promoting antibiotics and ionophorous anticoccidials in the European Union, *C. perfringens*-associated necrotic enteritis and subclinical diseases have become more serious threats to poultry health.

Clostridium spp.

The pathogenic clostridia cause disease in many animal species and in humans. Of the toxigenic clostridia, five species have attracted most attention:
C. botulinum, *C. tetani*, *C. perfringens*, *C. difficile* and *C. chauvoei.*

The most potent toxins known, botulinum toxin (BoNT) and tetanus toxin (TeNT), are produced by bacteria in this genus. For example, the 50% lethal dose (LD_{50}) in mice, humans and horses varies between 0.1 and 1.0 ng of toxin/kg bodyweight. This extreme toxicity is attributable to their absolute neurospecificity and catalytic activity (Hatheway, 1990; Schiavo & Montecucco, 1997). In the current age of global bioterrorist threats, *C. botulinum* has been suggested as a critical biological agent of potential for use in a terrorist attack (Rotz *et al.*, 2002; Wein & Liu, 2005).

Botulism is an intoxication, leading to flaccid neuroparalysis caused by any of seven neurotoxins (BoNTs, serotypes A – G). These are all capable of blocking

acetylcholine release from cholinergic nerve ends, resulting in neuroparalysis (Arnon, 1997; Schiavo & Montecucco, 1997). Botulism is relatively rare in humans; it occurs chiefly in ruminants, horses, mink and fowl, though other carnivores, swine and fish are occasionally affected. The most common toxin types of *C. botulinum* causing disease in animals are C and D, with A, B and E occasionally causing disease (Songer, 1997a).

C. tetani is the causal agent of tetanus, a serious and often fatal disease that leads to spastic paralysis. Tetanus is usually attributed to ingress of the bacterium through an open wound (Arnon, 1997). It is rare in Europe thanks to the widespread use of a highly effective vaccine. Despite the World Health Organization's intention of eradicating tetanus by 1995, 800,000 to 1 million deaths occur worldwide each year due to tetanus, half of these being neonatal tetanus (Goonetilleke & Harris, 2004). Tetanus occurs frequently in horses, less often in other herbivores and occasionally in pigs and carnivores. Cattle may develop tetanus due to growth of *C. tetani* in the rumen (Songer, 1997a).

C. difficile is known to cause antibiotic-associated diarrhea and pseudomembranous colitis. In humans *C. difficile* is a major cause of nosocomial diarrhea, accounting for about 15 – 25% of such cases (Barbut & Petit, 2001).
C. difficile has been associated with typhlocolitis and diarrhea in mature horses treated with antibiotics (Båverud, 2002). Two toxins, the enterotoxin (toxin A) and the cytotoxin (toxin B), produced by *C. difficile*, have been shown to induce all the symptoms of secretory diarrhea, mucosal damage and inflammation of the mucosa in animal models (Barbut & Petit, 2001).

C. chauvoei is a lethal veterinary pathogen causing blackleg, a fatal muscle disease affecting cattle, sheep and other species. *C. chauvoei* produces several toxins, including the necrotizing alpha toxin and the DNAse active beta toxin. Infection mechanisms are not clearly understood, but ingestion of *C. chauvoei* spores is probably the most common route of exposure (Songer, 1997a).

Phylogeny and taxonomy

The genus *Clostridium* currently comprises 181 validly described species (http://www.bacterio.cict.fr/c/clostridium.html; 20-April-2006), most of which have been analysed with 16S rDNA sequencing. Taxonomically the term *Clostridium* is a genus, comprising mainly rod-shaped, anaerobic, endospore-forming and non-sulfur-reducing Gram-positive members of the domain *Bacteria* (Stackebrandt & Rainey, 1997). Genus *Clostridium* is further grouped in the phylum *Firmicutes*, the class *Clostridia*, order *Clostridales* and the family *Clostridiaceae*.

The 16S rDNA based phylogeny of the genus *Clostridium* has been described in detail previously (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999; Stackebrandt & Rainey, 1997). By 16S rDNA analysis, 19 different clusters have been defined (Stackebrandt *et al.*, 1999). Species of cluster I (Collins *et al.*, 1994) (Figure 1), containing the type species *Clostridium butyricum*, are considered core species of

the genus, while those found in other clusters may subsequently be reclassified. *C perfringens* is found in group Ia. Many of the 19 different clusters contain some non-clostridial species (Figure 1).

Phylogenetic analysis based on 16S rDNA sequencing of the genus *Clostridium* has shown it to be a heterogeneous group. The same is true of neighbouring taxa such as the genera *Eubacterium* and *Ruminococcus*. *C. botulinum* is a taxonomic challenge, being a phylogenetically heterogeneous species present in subclusters Ia, Id and Ig (Stackebrandt & Rainey, 1997).

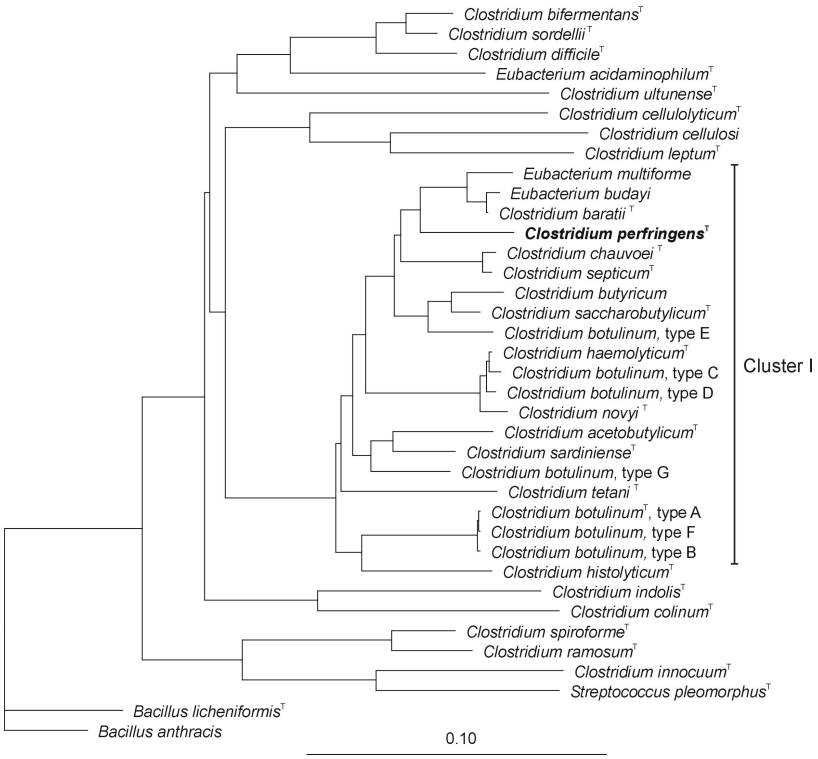

Figure 1. Phylogenetic tree showing 31 approved *Clostridium* species and 6 species of related genera. The tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix that was corrected for multiple substitutions at single locations by the two-parameter method (Kimura, 1980). Prealigned 16S rRNA sequences were retrieved from the Ribosomal Database Project, RDP-II (Cole *et al.*, 2005) and aligned manually. *Bacillus licheniformis* and *Bacillus anthracis* were used as an outgroup. Superscript T indicates type strain.

Clostridium perfringens

Basic characteristics

C. perfringens is a Gram-positive, rod-shaped anaerobe that forms oval subterminal spores. It differs from most other clostridia in that the relatively large rods (0.6–2.4 x 1.3–9.0 µm) are encapsulated and non-motile (Cato, George & Finegold, 1986). The colonies are smooth, round, glistening, surrounded by an inner zone of complete haemolysis caused by the theta-toxin and an outer zone of incomplete haemolysis caused by the alpha-toxin (Quinn *et al.*, 1994).
C. perfringens is classified as an anaerobe, although the bacteria will survive and occasionally grow in the presence of oxygen (Quinn *et al.*, 1994). Growth occurs within the temperature range of 12–50°C, though very slowly below 20°C (Adams & Moss, 1995). Under optimal conditions, 43–47°C, *C. perfringens* grows extremely rapidly, with a generation time of 8–10 min, and growth is accompanied by abundant gas production (Bryant & Stevens, 1997). The bacterium requires 13 essential amino acids for growth (Cato, George & Finegold, 1986). The bacteria grow in the pH range 5–8 and at a substrate water activity of 0.93–0.97.
C. perfringens can survive under extreme conditions, thanks to its differentiation from vegetative cells to highly resistant, dormant endospores (Novak & Juneja, 2002). Bacterial endospores are the most resistant biological cell type known; they can survive under extreme conditions, resisting heat, desiccation, acids and many chemical disinfectants.

Genetic properties

Among the toxigenic clostridial species, *C. perfringens* is the paradigm species for genetic studies, because of its tolerance of oxygen, high growth rate, and ability to genetic manipulation (Rood, 1998). In 2002 the complete genome of *C. perfringens* strain 13 was published (Shimizu *et al.*, 2002). The chromosome of *C. perfringens* strain 13 consists of 3,031,430 base pairs, 2,660 protein-coding regions and 10 rRNA genes, showing low overall G + C content (28.6%).

When *C. perfringens* genome is compared with the genome of the non-pathogenic bacterium, *C. acetobutylicum* (Nolling *et al.*, 2001), the most obvious difference is that virulence-related genes are present in the former, but not in the latter. In addition to the known virulence genes, Shimizu found many virulence-associated genes in the *C. perfringens* genome (Shimizu *et al.*, 2002). Five putative haemolysin genes were identified, based on their similarity to haemolysins previously described in other bacterial species. Two types of putative fibronectin-binding protein genes sharing similarities with genes from *Listeria monocytogenes* (Gilot, Jossin & Content, 2000) and *Bacillus subtilis* (Kunst *et al.*, 1997) were also detected, indicating involvement in pathogenesis as colonization factors (Shimizu *et al.*, 2002).

The genome sequencing of *C. perfringens* strain 13 revealed that the virulence genes do not cluster in pathogenicity islands. Only a few mobile genetic elements can be detected and signs of horizontally acquired genes are hardly detectable in the *C. perfringens* genome. Therefore, it is conceivable that the different toxin

types of *C. perfringens* have evolved from type A by acquiring extrachromosomal elements such as plasmids and transposons. The genome sequencing of two other *C. perfringens* strains is in progress (strains ATCC 13124 and strain SM101) at the Institute for Genomic Research (TIGR).
(http://www.tigr.org/tdb/mdb/mdbinprogress.html; 20-April-2006).

Whereas the genes encoding alpha-toxin and theta-toxin are located on the chromosome, many other toxin-encoding genes are located on large plasmids (Table 1). The enterotoxin gene (*cpe*) can be either chromosomal or plasmid encoded. In food poisoning isolates, the *cpe* gene is located on the chromosome (Brynestad & Granum, 1999). However, non-food-poisoning human gastrointestinal disease isolates and veterinary isolates carry the *cpe* gene on large plasmids (Cornillot *et al.*, 1995).

Expression of alpha-toxin and theta-toxin is regulated by a two-component signal transduction system (VirR/VirS) consisting of two components, a sensor histidine kinase, VirS, and a response element, VirR. Regulation occurs at the transcriptional level and mutations can alter the productions of both alpha-toxin and theta-toxin (Rood, 1998).

Clostridium perfringens toxins

Members of the species *C. perfringens* can be subtyped into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha, beta, epsilon and iota (Songer, 1996). *C. perfringens* is unique not only in terms of the number of toxins produced, but also in terms of their toxicity and lethal activity (Table 1).

Table 1. The most important *C. perfringens* toxins (Modified from Petit, Gibert & Popoff, 1999).

Toxin	Gene	Gene location	Biological activity
Alpha toxin	*plc*	Chromosome	Cytolytic, haemolytic, dermonecrotic, lethal
Beta toxin	*cpb1*	Plasmid	Cytolytic, dermonecrotic, lethal
Epsilon toxin	*etx*	Plasmid	Oedema in various organs: liver, kidney and central nervous system
Iota toxin Ia and Ib	*iap/ ibp*	Plasmid	Disruption of actin cytoskeleton and cell barrier integrity
Beta2 toxin	*cpb2*	Plasmid	Cytolytic, lethal
Enterotoxin	*cpe*	Chromosome/ plasmid	Cytotoxic, lethal, causes diarrhea by leakage of water and ions
Theta toxin	*pfoA*	Chromosome	Lyses red blood cells and modulates the host inflammatory response

In 1941 MacFarlane and Knight (MacFarlane & Knight, 1941) showed that the alpha-toxin of *C. perfringens* possessed phospholipase C enzymatic properties.
Alpha-toxin, the main lethal toxin of *C. perfringens*, is a multifunctional phospholipase, produced in varying amounts by almost all *C. perfringens* isolates.

It causes hydrolysis of membrane phospholipids in different cells, resulting in lysis or other forms of cytotoxicity (Songer, 1997b; Titball, 1993). Alpha-toxin displays haemolytic, necrotic, vascular permeabilization and platelet aggregating properties. One *C. perfringens* isolate with an atypical alpha-toxin gene (*plc*) which does not produce alpha-toxin has been identified (Justin *et al.*, 2002)

The beta-toxin is a highly trypsin-sensitive protein that causes mucosal necrosis and possibly central nervous signs observed in domestic animals. As little as 2 ng of beta-toxin produces dermonecrosis in guinea pigs (Sakurai & Duncan, 1977; Sakurai & Fujii, 1987). Although gross pathological effects of the beta-toxin have been described in humans and domestic animals, the biological activities responsible for these effects are not understood (Leary & Titball, 1997).

Epsilon-toxin is a potent toxin responsible for lethal enterotoxaemia in livestock (Songer, 1996). Epsilon toxin is necrotizing and lethal and the most potent clostridial toxin after botulinum and tetanus neurotoxins, although its precise biological activity has not been identified (Tamai *et al.*, 2003).

The iota-toxin consists of two proteins, one active component (Ia) and a binding protein (Ib). Iota-toxin has been shown to increase vascular permeability and is dermonecrotic and lethal for mice at higher doses (Petit, Gibert & Popoff, 1999). With the exception of iota-toxin, which acts intracellularly, the *C. perfringens* toxins interact with the cell membrane (Petit, Gibert & Popoff, 1999).

The major toxins, together with enterotoxin and beta2 toxin, play the major role in several severe diseases (Petit, Gibert & Popoff, 1999; Songer, 1996). The beta2 toxin was described fairly recently (Gibert, Jolivet-Reynaud & Popoff, 1997) and has been associated with enteric diseases in domestic animals, especially piglets (Garmory *et al.*, 2000; Klaasen *et al.*, 1999; Waters *et al.*, 2003) and horses (Herholz *et al.*, 1999). A recent study (Fisher *et al.*, 2005) suggests that beta2 toxin could be an additional virulence factor in *C. perfringens* enterotoxin-associated antibiotic-associated diarrhea and sporadic diarrhea.

Enterotoxin is the best understood of the *C. perfringens* toxins (Granum, 1990). It interacts with epithelial cell tight junction proteins, leading to diarrhea and intestinal cramping caused by leakage of water and ions (McClane, 2001). Production of enterotoxin is co-regulated with sporulation, the toxin is released when vegetative cells undergo lysis.

Another important extracellullar protein is theta-toxin or perfringolysin O, a pore-forming cytolysin that can lyse red blood cells (Tweten, 1997). Together with alpha-toxin, theta-toxin modulates the host inflammatory response, causing leukocyte accumulation within blood vessels and preventing the normal influx of phagocytic cells into infected host tissue (Awad *et al.*, 2001).

Sampling, culturing and identification

Diagnosis of a clostridial disease requires isolating the pathogen or detecting which toxins are involved in the infection. Specimens should be taken from recently dead animals as clostridia and other facultative anaerobes multiply rapidly post mortem (Quinn *et al.*, 1994). Rapid transport of specimens is not so crucial as *C. perfringens* can survive in swabs up to 2 weeks and the temperature during transport is not a critical factor for the survival of *C. perfringens* in clinical samples under conditions normally applying (Österblad *et al.*, 2003). The ability of *C. perfringens* to form spores must also been taken into consideration when survival of *C. perfringens* is discussed. Their presence in a clinical sample can significantly influence survival time.

C. perfringens can be isolated on blood agar plates incubated anaerobically at 37°C overnight. *C. perfringens* colonies are surrounded by an inner zone of complete haemolysis caused by the theta-toxin and an outer zone of incomplete haemolysis caused by the alpha-toxin. A spore selection procedure with subsequent enrichment in broth can also be used to detect the bacteria in environmental samples and in samples with an overgrowth of other bacteria. To further identify *C. perfringens* in a specimen, Gram staining, biochemical tests and nagler reaction are often used (Quinn *et al.*, 1994).

Besides biological assays, *C. perfringens* toxins can be detected by immunological methods. Enzyme-linked immunosorbent assays (ELISAs) for detection of *C. perfringens* major toxins and enterotoxin from faeces and other biological fluids are commercially available. Previously, laboratory animal tests and serological methods were used for toxin typing of *C. perfringens*. Nowadays a multiplex PCR can be used in which all the major toxin genes: alpha (*plc*), beta (*cpb1*), epsilon (*etx*) and iota (*iap*) can be detected (Yoo *et al.*, 1997).

Antimicrobial susceptibility

In vitro studies of the antimicrobial susceptibility of *C. perfringens* are numerous. Penicillins are known to be particularly active against *C. perfringens*. Resistance to penicillin is very rare and B-lactamase has not been demonstrated in *C. perfringens*. A low degree of resistance to ampicillin has been reported in several studies (Sasaki *et al.*, 2001; Watkins *et al.*, 1997). Tetracycline resistance is the most common antibiotic resistance trait found in *C. perfringens* (Lyras & Rood, 1996). It has been reported that *C. perfringens* can carry the tetracycline-resistance genes *tetA(P)*, *tetB(P)*, *tetA408(P)*, *tetM* and *tetQ* (Kather, Marks & Foley, 2006; Lyras & Rood, 1996; Martel *et al.*, 2004; Sasaki *et al.*, 2001).

Resistance to MLS antibiotics is also a common trait. It is caused by methylation of the 23S rRNA gene and mediated by erm proteins (Berryman, Lyristis & Rood, 1994). Chloramphenicol resistance in *C. perfringens* is not common, though several resistant isolates have been identified (Bannam & Rood, 1991). No resistance to the ionophorous anticoccidial (IAC) narasin, an important substance

used to control clostridiosis in the Nordic countries, has been found (Martel *et al.*, 2004; Watkins *et al.*, 1997).

Genotyping of *Clostridium perfringens*

The relatedness of bacterial isolates has in the past been determined by testing for one or several phenotypic properties. They reflect the bacteria's production of certain proteins, which may not be expressed under certain environmental or culture conditions. In contrast, some of the newer molecular typing methods involving the analysis of DNA offer many advantages over traditional techniques. Genotyping, which is based on a more stable marker, DNA, is not dependent on gene expression. Another advantage of genotyping methods is that the discriminatory power of DNA-based methods is generally superior to that of phenotypic methods.

The ability to distinguish between genomes is important to several disciplines of microbiological research, for example in studies on population genetics and microbial epidemiology (van Belkum *et al.*, 2001). Of great importance when choosing a method for genotyping are the typability, reproducibility, discriminatory power and also the ease and cost of performing the analysis.

With polymerase chain reaction (PCR), selected segments of any DNA molecule can be amplified exponentially. The PCR technique is a powerful tool to detect and identify minimal numbers of microorganisms. A multiplex PCR, which can detect all *C. perfringens* major toxin genes has been developed (Engström *et al.*, 2003; Yoo *et al.*, 1997). PCR systems for detection of two other important toxin genes, *cpe* and *cpb2*, are also available (Herholz *et al.*, 1999; Kadra *et al.*, 1999).

For more detailed characterization of *C. perfringens* isolates a variety of methods have been used: plasmid profiling (Schalch *et al.*, 1999), multilocus enzyme electrophoresis (MLEE) (Pons, Combe & Leluan, 1994), ribotyping (Schalch *et al.*, 2003; Schalch *et al.*, 1999), amplified fragment length polymorphism (AFLP) (Engström *et al.*, 2003; McLauchlin *et al.*, 2000), pulsed field gel electrophoresis (PFGE) (Engström *et al.*, 2003; Lukinmaa, Takkunen & Siitonen, 2002; Maslanka *et al.*, 1999) and multiple-locus variable number tandem repeat analysis (MLVA) (Sawires & Songer, 2005).

Pulsed-field gel electrophoresis (PFGE)

In 1984, Schwartz and Cantor (Schwartz & Cantor, 1984) described pulsed-field gel electrophoresis (PFGE) for the first time, introducing a new way to separate large DNA fragments. Applications of PFGE are numerous and the method has been used successfully for subtyping over 50 bacterial species (Tenover, 1998). With PFGE one can determine the relatedness of bacterial strains of the same species. Reproducibility and discriminatory power are generally high.

In PFGE analysis, chromosomal DNA is cleaved by restriction enzymes. By using restriction enzymes having only few recognition sites in the genome, a pattern with large DNA fragments can be obtained. To prevent shearing and degradation of DNA, the cells are protected in agar plugs. The cleaved DNA is then separated by PFGE, in which the orientation of the electric field across the gel is shifted periodically (Schwartz & Cantor, 1984). By altering the electric field, large DNA fragments can effectively be separated. After separation, the gel is stained with ethidium bromide and analysed by visual inspection or by software such as GelcompareII. The pattern obtained by PFGE usually consists of 5 – 20 fragments.

In *C. perfringens* research, PFGE has been used to examine food-borne outbreaks (Lukinmaa, Takkunen & Siitonen, 2002; Maslanka *et al.*, 1999), and to investigate the diversity of *C. perfringens* causing clostridiosis in poultry (Engström *et al.*, 2003; Gholamiandekhordi *et al.*, 2006; Nauerby, Pedersen & Madsen, 2003). PFGE has also been used to study the genome composition (Canard & Cole, 1989) and location of the toxin genes *cpe* and *cpb2* (Brynestad, Synstad & Granum, 1997; Fisher *et al.*, 2005). The general consenus regarding *C. perfringens* strains previously investigated by PFGE is that strains originating from the same outbreak have similar PFGE patterns and that non-outbreak strains have lower genetic relatedness. These patterns have been observed when isolates from poultry (Engström *et al.*, 2003; Gholamiandekhordi *et al.*, 2006; Nauerby, Pedersen & Madsen, 2003) and food-borne outbreaks (Lukinmaa, Takkunen & Siitonen, 2002; Maslanka *et al.*, 1999) have been investigated.

Clostridium perfringens diseases in poultry

Although *C. perfringens* is often found in the intestinal tract of healthy birds it can cause outbreaks of disease, especially in broilers and turkey flocks. Clostridiosis occurs both as an acute clinical disease, necrotic enteritis (NE), in 2–4-week-old chickens, causing high mortality, but also as a subclinical disease with focal necrosis in the intestines or as *C. perfringens*-associated hepatitic change (CPH) with cholangiohepatitis or fibrinoid necrosis in the liver. In broilers, outbreaks of clinical NE have been reported from many countries (Ficken & Wages, 1997), periodically causing serious problems. In the 1960s, NE was first described and experimentally reproduced by Parish (Parish, 1961a; Parish, 1961b; Parish, 1961c). It appeared to be particularly prevalent in countries developing intensive broiler production (Ficken & Wages, 1997). Necrotic enteritis has even been found in wild geese (Wobeser & Rainnie, 1987), wild crows (Asaoka *et al.*, 2004), ostriches (Kwon, Lee & Mo, 2004) and in capercaillies reared in captivity (Hofshagen & Stenwig, 1992).

Pathogenesis, clinical signs and pathology

The occurrence of NE is associated with proliferation of *C. perfringens* in the intestinal tract followed by increased toxin production. In dead and clinically sick birds affected by NE, large numbers of *C. perfringens* can be found, up to 10^7 to

10^8 CFU/g of intestinal content, whereas in healthy broilers, counts of 0 to 10^5 CFU/g of intestinal content are normal (Long, Pettit & Barnum, 1974).
C. perfringens type A, from soil, dust and contaminated feed and litter, has been implicated as a source of infection (Craven *et al.*, 2001). Although more than one toxin produced by *C. perfringens* might be involved in causing NE, alpha-toxin is the most important, as clearly shown by Al-Sheikhly & Truscott (Al-Sheikhly & Truscott, 1977a; Al-Sheikhly & Truscott, 1977b). They reproduced typical field lesions using crude alpha-toxin from a type A strain, and observed reduced pathological changes when inactivated alpha-toxin was used instead.

The environment in the intestinal tract is of vital importance for the growth of *C. perfringens* and there probably has to be a disturbance in the jejunum before *C. perfringens* can start to proliferate in this part of the gut. Important factors are nutrition, pH, oxygen and the microflora in the jejunum. Different types of stress experienced by the birds may also cause disturbances in the gut.

The most important known predisposing factor for NE is damage to the intestinal mucosa, caused by coccidial pathogens. In particular, *Eimeria* species that colonize the small intestine, such as *Eimeria maxima* and *Eimeria acervulina*, are known to predispose to necrotic enteritis (Al-Sheikhly & Al-Saieg, 1980; Williams *et al.*, 2003). Measures to control coccidiosis have therefore an indirect prophylactic effect. Diet also influences the incidence of NE. Diets with a high content of indigestible, water-soluble non-starch polysaccharides, e.g. rye, wheat and barley, predispose to NE (Kaldhusdal & Hofshagen, 1992; Riddell & Kong, 1992).

The complex pathogenesis of NE and other clostridial infections in birds is highlighted when experimental models are designed. Experimental reproduction of NE by oral administration of *C. perfringens* has produced varying results (Al-Sheikhly & Truscott, 1977a; Al-Sheikhly & Truscott, 1977b; Kaldhusdal *et al.*, 1999; Olkowski *et al.*, 2006; Pedersen *et al.*, 2003). One problem observed in these studies is that *C. perfringens* in the natural flora might cause lesions and not the test strain. One finding is that there are considerable difficulties involved in experimentally reproducing the disease. Consequently it is difficult to perform and evaluate studies on putative risk factors and preventive measures.

Birds affected by severe NE show marked to severe depression, loss of appetite, reluctance to move, ruffled feathers, and drooping wings and head (Al-Sheikhly & Truscott, 1977b; Helmboldt & Bryant, 1971). Mortality may sometimes exceed 1% daily, and the duration of the disease is normally one week. The birds often become dehydrated and emit a foul smell. The most evident gross pathological findings can be seen in the small intestine, but lesions can also occur in other organs such as caecum, liver and kidney. The duodenum, jejunum and ileum are thin walled and filled with gas (Broussard *et al.*, 1986). Diffuse mucosal necrosis of large parts of the small intestine, covered by a yellow-brown or bile-stained pseudomembrane, is the predominant gross pathological finding in cases of severe NE (Figure 2a)(Helmboldt & Bryant, 1971; Long, Pettit & Barnum, 1974).

Figure 2a. Severe necrotic lesions in the jejenum.
Photo: Bengt Ekberg.

Figure 2b. Liver with cholangiohepatitis.
Photo: Bengt Ekberg.

Besides the severe form of NE already described, a mild subclinical form has been found in the field, leading to decreases in performance. In these cases, ulcers arise in the form of a depression in the mucosal surface or pseudomembrane, with discoloured, amorphous material adhering to the mucosal surface (Kaldhusdal & Hofshagen, 1992). *C. perfringens* also causes a liver-associated disease *C. perfringens* associated hepatic change (CPH). Two types of gross liver lesions have been described earlier: a distinct pattern characterized by small, pale islets in the parenchyma, occasionally with discolouration of the extrahepatic bile tree (Figure 2b) (Randall, 1991) and focal, subcapsular nodules (including fibrinoid necroses) (Lovland & Kaldhusdal, 1999; Randall, 1991). CPH is found during meat inspection at slaughter, often without any sign of disease in the flock, CPH has been reported from several countries and has periodically been a problem in

the Nordic countries, Britain and Canada (Lovland & Kaldhusdal, 1999). In Norway, gizzard lesions with large numbers of *C. perfringens* have been found in healthy broiler chickens. Gizzard lesions in broilers have in most cases been caused by adenoviruses and associated with increased flock mortality (Abe *et al.*, 2001; Nakamura *et al.*, 2002).

Prophylactic measures

Antimicrobial growth promoters (AGP) have been used for many years in broiler flocks to increase weight gain and decrease the feed conversion ratio. In Europe, clostridiosis is currently controlled by routine use of a combination of AGP and ionophorous anticoccidials (IAC), which both have an antibiotic effect on Gram-positive bacteria such as *C. perfringens* (Elwinger *et al.*, 1994; Prescott, Sivendra & Barnum, 1978). A ban on the use of these products in Europe is foreseen in the near future. In countries that have already stopped the use of AGPs, problems with *C. perfringens*-associated disease in broilers have arisen (Grave *et al.*, 2004).

In the Nordic countries, AGPs are no longer used; in Sweden since 1986 (SOU1997:132, 1997), Norway 1995 (Kruse & Simonsen, 2003), Denmark 1998 (Emborg *et al.*, 2001). Clostridiosis is nowadays controlled with IAC (e.g. narasin), good hygiene management and a modified diet (Elwinger & Teglöf, 1991; Lovland & Kaldhusdal, 2001; Wierup, 2001). Modern poultry production still depends on antimicrobials to prevent or treat clostridiosis. Since the withdrawal of AGP, narasin has been by far the most commonly used antimicrobial in commercial poultry production in Sweden. In the present situation, responsiveness to narasin and other IACs is important for the prevention of clostridiosis.

Necrotic entertis in other animals can be prevented by vaccination (Songer, 1996). So far there is no available vaccine to prevent NE in broilers. However, some recent studies have shown promising results, which may in the future give rise to a vaccine. Heier and colleagues (Heier *et al.*, 2001) established that flocks with high titres of maternal antibodies to alpha-toxin had lower mortality during the production period than flocks with low titres. Lovland and colleagues (Lovland *et al.*, 2004) have experimentally vaccinated broiler with vaccines based on *C. perfringens* type A and type C toxoids in order to protect the progeny via maternal antibodies. The results were promising for future protection against subclinical necrotic enteritis in broilers. A recent study by Thompson and colleagues (Thompson *et al.*, 2006), showed that alpha-toxin-negative mutants given to broilers protected birds from experimental NE, which indicates that immunogens other than alpha-toxin might be important for the prophylactic immunity. As coccidiosis is a factor predisposing to NE, vaccination against coccidiosis might also have a preventive effect against the development of NE in broilers. For poultry production, possibly a mix of NE vaccine and coccidiosis vaccine would be the best solution.

Other prophylactic measures to control NE have been proposed. Oral administration of a mix of bacteria from healthy adult birds (competitive exclusion)

to newly hatched chicks, reduced the incidence of NE and a decreased colonization was observed (Craven *et al.*, 1999; Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 2001). Probiotics, i.e. live microbial feed supplement that beneficially affects the host by improving the intestinal balance, have been shown to reduce gross lesions of NE (Hofacre *et al.*, 1998). Diets with high levels of indigestible, water-soluble non-starch polysaccharides, such as rye, wheat and barley, predispose to NE, while diets with maize (corn) reduce the incidence and severity of NE (Kaldhusdal & Hofshagen, 1992; Riddell & Kong, 1992). Enzyme preparations are evidently able to decompose indigestible polysaccharides, thereby reducing the occurrence of NE (Elwinger & Teglöf, 1991).

Clostridium perfringens diseases in animals other than poultry

Lamb enterotoxaemia (yellow lamb disease) is caused by *C. perfringens* type A (Table 2) and is seen primarily in spring, when the population of suckling lambs is high (McGowan, Moulton & Rood, 1958). Adult horses with "intestinal clostridiosis" are occasionally seen and the disease is characterized by profuse watery diarrhea and high mortality, with large numbers of *C. perfringens* type A in the gut (Wierup, 1977; Wierup & DiPietro, 1981). *C. perfringens* type A isolates have also been associated with enteric disease in suckling and fattening pigs with mild necrotic enterocolitis (Collins *et al.*, 1989; Waters *et al.*, 2003).

Table 2. Major toxin types of *C. perfringens* and associated diseases in animals and humans

Toxin type	**Major toxins**	***C. perfringens* associated diseases**
A	Alpha	Gangrene and gastrointestinal diseases in humans. Necrotic enteritis in fowl, intestinal clostridiosis in horses, necrotic enteritis in piglets and enterotoxaemia in lambs
B	Alpha, beta, epsilon	Dysentery in lambs, chronic enteritis in lambs
C	Alpha, beta	Haemorrhagic or necrotic enterotoxaemia in neonatal pigs, lambs, calves, goats and foals. Necrotic enteritis (pigbel, darmbrand) in humans
D	Alpha, epsilon	Enterotoxaemia in sheep (pulpy kidney disease)
E	Alpha, iota	Enterotoxemia in lambs, calves and rabbits
A (*cpb2*)	Alpha	Typhocolitis in horses, necrotic enteritis in piglets
A (*cpe*)	Alpha	Food poisoning in humans

C. perfringens type B (Table 2) is the causal agent of dysentery in newborn lambs (Songer, 1996). The enterotoxaemia results in enteritis and extensive haemorrhage of the small intestine (Frank, 1956), and the primary sign is sudden death. Chronic disease in older lambs with chronic abdominal pain but without diarrhea has also been reported (Songer, 1996).

C. perfringens type C (Table 2) infects pigs, cattle, sheep, horses, chickens and dogs (Songer, 1996). *C. perfringens* type C is the causal agent of haemorrhagic or necrotic enterotoxaemia in neonatal pigs, lambs, calves, goats and foals. Newborn animals are more susceptible, due to lack of competitive gut flora. Piglets display diarrhea and dysentery, with blood and necrotic debris in faeces (Songer, 1996). In piglets, 1 – 2 days old, up to 10^9 CFU/ml of *C. perfringens* can be found in the intestinal content. Morbidity rates of 30–50% and case fatality rates of 50–100 % are not uncommon (Niilo, 1988). A similar disease in piglets may occur in neonatal calves, lambs and goats (Griner & Bracken, 1953; Griner & Johnson, 1954).

C. perfringens type D (Table 2) causes enterotoxaemia in sheep (pulpy kidney). Type D is also the main causal agent of death in weaning animals up to nearly 1 year of age, often in combination with overeating (Popoff, 1984).

C. perfringens type E (Table 2) is an uncommon cause of enterotoxaemia in lambs, calves and rabbits (Songer, 1997a).

Clostridium perfringens diseases in humans

Enterotoxin is present in fewer than 5% of all *C. perfringens* isolates, though enterotoxin-positive type A strains are significant enteric pathogens. In humans, they cause *C. perfringens* type A food poisoning, which is one of the most common food-borne illnesses in industrially developed countries, and 5 to 20% of all cases of non-food-borne human gastrointestinal illness, including antibiotic-associated diarrhea and sporadic diarrhea (Adams & Moss, 1995; Collie & McClane, 1998).

C. perfringens type A is frequently isolated from patients with trauma-induced gas gangrene. The syndromes of gas gangrene are related to the expression of potent toxins, especially alpha-toxin and theta-toxin (Awad *et al.*, 2001; Bryant, 2003). Invasion and destruction of healthy tissue, advancing several inches per hour despite appropriate antibiotic therapy, has been reported and removal of infected tissues is many times necessary for survival of the patient (Maclennan, 1962).

C. perfringens type C is the cause of necrotic enteritis (pigbel, darmbrand), which is characterized by haemorrhagic, inflammatory necrosis of the jejunum. The disease occurs in humans in developing countries but is rare in developed countries (Devitt & Stamp, 1983).

Aims

The main objective of the present work was to study *C. perfringens* and its significance in necrotic enteritis in broilers, and to elucidate the genetic diversity of *C. perfringens* in a wider perspective.

The aims of the study were as follows:

• To evaluate how *C. perfringens* survives during a simulated transport and to investigate the stability of some plasmid-borne toxin genes.

• To investigate, using PFGE, the genetic diversity of *C. perfringens* isolated from healthy and diseased domestic animals, wild animals, food-borne outbreaks and sludge. A further aim was to investigate the distribution of the *cpb2* and *cpe* genes. The former was also partially sequenced. Nucleotide sequences of *cpb2* deposited in Genbank were also analysed to ascertain if any differences were present.

• To establish the genetic relatedness of *C. perfringens* isolated from one broiler flock affected by mild NE.

• To determine the *in vitro* susceptibility of *C. perfringens*, isolated from poultry to antimicrobials used in poultry production, especially to narasin. Due to a high resistance of tetracycline, the distribution of tetracycline resistance genes was also examined.

Comments on material and methods

Detailed descriptions of the materials and methods used are presented in each paper.

Bacterial isolates, culturing and identification

All Swedish isolates of *C. perfringens* used in papers I, II and IV were retrieved from the collection of anaerobic isolates at the Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden. In paper IV, *C. perfringens* isolated from poultry in Norway and Denmark was also included. Some of the food isolates in paper II were provided from Norway. The isolates in paper III were all collected in Norway during a field trail.

Type and reference strains were purchased from the Culture Collection University of Gothenburg (CCUG) and the Culture Collection of Institute Pasteur (CIP). Type and reference strains used were *C. perfringens* typestrain CCUG 1795 (ATCC 13124) (A) and reference strains: CIP 106526 (A), CCUG 2035 (B), CCUG 2036 (C), CCUG 2037 (D) and CCUG 44727 (E).

Isolates were grown on fastidious anaerobe agar (FAA) (LabM, Bury, Lancashire, England) with 10% defibrinated horse blood and incubated anaerobically in jars at 37°C for 24 h. The isolates were identified as *C. perfringens* by applying standard biochemical tests, Gram staining, lecithinase test and the toxin type was established by multiplex-PCR (Engström *et al.*, 2003).

Genotyping of *C. perfringens* isolates with PFGE

One important parameter when setting up a PFGE experiment is the choice of restriction enzyme (RE). The REs used in PFGE are rare-cutting enzymes producing few but large fragments. Other parameters that can affect the final results of PFGE are: buffer system, temperature, agar composition and other separation parameters. The advantages of this method are its high reproducibility and high discriminatory power. Limitations of the method are the rather time consuming procedure and that only a limited number of samples can be processed simultaneously. Problems with non-typable strains that can arise can be due to the absence of cleavage sites for the restriction enzyme used. Problems with endonuclease activity are also known, and has been reported for clostridial species (Maslanka *et al.*, 1999; Schalch *et al.*, 1999; Sperner *et al.*, 1999).

In the present studies (papers II and III) *C. perfringens* DNA was cleaved with *Sma*I (Figure 3), which proved to be a well suited RE for *C. perfringens* in previous studies (Engström *et al.*, 2003; Lukinmaa, Takkunen & Siitonen, 2002; Nauerby, Pedersen & Madsen, 2003). Two molecular weight standards were used: the conventional lambda ladder with a size range of 0.13 to 194 kb (Low Range PFG Marker, New England Biolabs Inc.) and *Salmonella* serotype Braenderup

(H9812) digested with *Xba*I, as described in greater detail by Hunter and colleagues (Hunter *et al.*, 2005). The use of *Salmonella* serotype Braenderup (H9812) digested with *Xba*I as a size marker made it easier to normalize tiff images than by using the commercially available lambda ladder, due to its more stable concentrations of DNA in the prepared plugs.

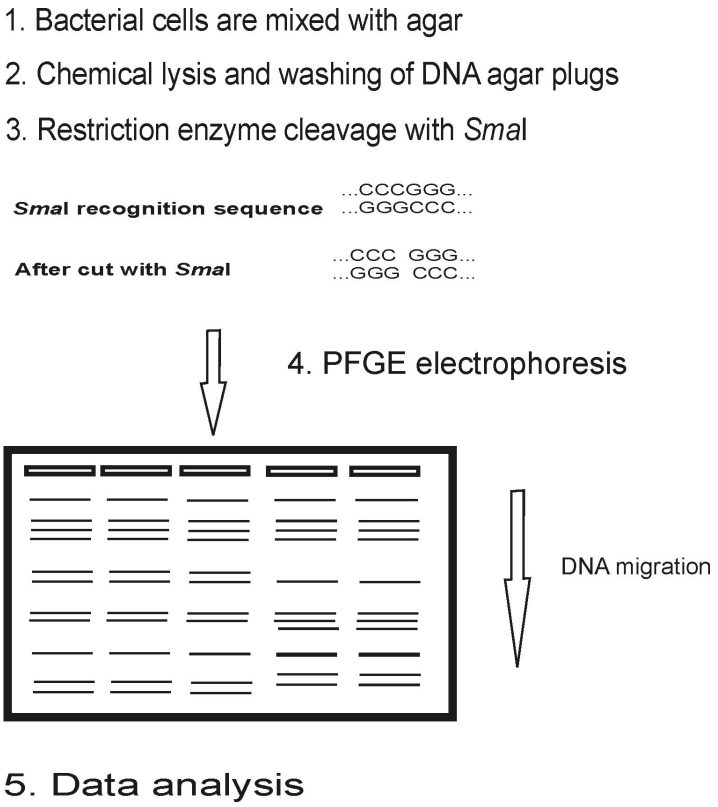

Figure 3. Schematic illustration of PFGE for *C. perfringens*.

PCR

Polymerase chain reaction (PCR) is a technique that is used to amplify a specific region of DNA, in order to produce sufficient DNA for further analysis.
In this study PCR were used in all papers (I – IV), for different purposes. For diagnosis, multiplex PCR was used to establish which toxin type (A – E), the isolates belonged to. PCR was also used to detect the enterotoxin gene and beta2 toxin gene. Tetracycline resistance genes were also detected by PCR. PCR was also used to generate amplicons for cycle sequencing analysis of the *cpb2* gene and the 16S rRNA gene.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of fastidious anaerobic, bacteria can be attended by several problems. It may prove difficult to obtain satisfactory and reproducible results. High initial concentrations of bacteria and long incubations may be needed (Wexler, 1991). The use of an anaerobic control strain is therefore very important when testing anaerobes. For susceptibility testing of *C. perfringens*, both agar dilution and broth microdilution have been used successfully (Alexander *et al.*, 1995; Devriese *et al.*, 1993), thanks to the fact that *C. perfringens* has a very fast generation time and is not overly sensitive to oxygen.

In paper IV, commercially available 96-well panels (VetMIC^{TM}, National Veterinary Institute, Uppsala) were used for monitoring the following eight antimicrobials: narasin, oxytetracycline, virginiamycin, bacitracin, vancomycin, ampicillin, avilamycin and erythromycin. All the strains were tested twice and the reference strain *C. perfringens* (ATCC 13124) was included in every test batch. Initially we tested two different broths (Mueller Hinton and Wilkins Chalgren) and two different incubation times (24 h and 48 h). When using Wilkins Chalgren Broth and incubating the isolates for 24 h, optimal growth was obtained.

Either a microbiological or a clinical breakpoint can be used to separate resistant from susceptible isolates. The microbiological breakpoint, only takes into account the distribution of MICs, whereas the clinical breakpoint is based on MIC, pharmacokinetic data and clinical efficacy.

In this study, MIC distributions were used to establish breakpoints between susceptible and resistant isolates. The MIC was determined as the lowest concentration of antimicrobial agent that inhibited visible growth.

Results and discussion

Paper I

The survival of *C. perfringens* was independent of temperature (4°C and 20°C) and duration (24h and 44h) of the simulated transport of the bacteria on cotton-tipped swabs. The prolonged storage of *C. perfringens* for 44 h at 4°C did not affect survival. However, as demonstrated by Österblad and colleagues (Österblad *et al.*, 2003), temperature has an impact on the survival of *C. perfringens* when stored for a long time.

The three plasmid-borne genes *cpb1*, *cpb2* and *etx* were stable when exposed repeatedly to oxygen in the laboratory and after simulated transport. Survival of *C. perfringens* seems not to be affected by temperature when transportation time is short. Anecdotal data imply that *C. perfringens* plasmid-borne genes might be unstable, though no significant data are available to prove this. It has also been discussed previously whether the plasmid-borne genes *cpb1, etx* and *iap* may be lost when strains are stored for extensive periods (Yamagishi *et al.*, 1997). One possible explanation for the reported apparent loss of the plasmid-borne genes could be that one isolate contained different clones bearing different plasmid-borne genes. One way to avoid this is to pick several colonies and to ensure that the isolates are pure (Buogo *et al.*, 1995).

It is of great importance that the plasmid-borne toxin genes are stable both when the bacteria are transported to the laboratory and at the laboratory, for a correct toxin typing of *C. perfringens* isolates. In this study the plasmid-borne genes were found to be stable both when exposed to ambient atmosphere and after simulated transport. Our results show that *C. perfringens* plasmid-borne genes are stable under normal laboratory conditions and under conditions normally applying during transportation. Consequently, *C. perfringens* toxin typing by PCR can be performed reliably, as the risk of plasmid loss seems to be a minor consideration.

Paper II

In this study the genetic diversity of 95 *C. perfringens* type A isolates from eight different sources (poultry, pigs, horses, sheep, roe deer, wild birds, food poisoning outbreaks and sewage sludge samples) was examined. The genetic relatedness of the isolates ranged from 58% to 100% and 56 unique PFGE patterns were identified. In this study 13% of the isolates were non-typable due to endogenous endonuclease activity, which has previously been reported as a problem for *C. perfringens* (Maslanka *et al.*, 1999; Schalch *et al.*, 1999). The result reveals a wide genetic diversity and there is no definite relationship between sources of isolates and the PFGE pattern. However, the wide diversity found by PFGE analysis was not reflected in the 16S rRNA sequences, which had high sequence similarity. Most of the isolates from food poisoning outbreaks obviously cluster together in the dendrogram. The *cpb2*-positive pig isolates are also genetically related.

The *cpe* gene was detected in all isolates originating from food poisoning outbreaks and in one isolate each from horse and roe-deer. The results are consistent with data from other studies (Sparks *et al.*, 2001; Tschirdewahn *et al.*, 1991). A high prevalence of the *cpb2* gene were found in isolates from pigs, horses and sheep, which is also in agreement with previously published articles (Herholz *et al.*, 1999; Klaasen *et al.*, 1999).

By sequence analysis of our 28 *cpb2* sequences and 60 *cpb2* sequences previously deposited in Genbank (National Center for Biotechnology, Bethesda, Md, USA), two main groups of the *cpb2* gene were found (I and II). A sequence similarity of 73.8% was observed between the two groups. Groups I and II were both subdivided into three subclusters a, b, c. The *cpb2* sequences in this study all belong to group I and were divided into three subclusters: porcine isolates (Ia), animal isolates of non-porcine origin (Ib), and isolates from food poisoning outbreaks (Ic). One of the horse isolates AN 5036/01, clusters together with the food isolates. In the study by Vilei and colleagues (Vilei *et al.*, 2005), the same subdivision was observed; porcine isolates were found in group Ia and non-porcine isolates in group Ib. Most isolates in group II have an American origin (Jost *et al.*, 2005). However, isolates containing *cpb2* isolated from humans with gastrointestinal diseases were distributed in both groups (Ic and IIa), irrespective of geographical origin (Fisher *et al.*, 2005).

In this study, none of the isolates from poultry, horse and pigs were degraded. Consequently, PFGE is a very suitable method for epidemiological investigation of enteric diseases in poultry, horses and pigs caused by *C. perfringens*. Two genetically different populations of the *cpb2* gene were found by sequence analysis. Furthermore, isolates causing enteric diseases in animals and humans seem not to have a close genetic relatedness, according to PFGE analysis.

Paper III

In this study *C. perfringens* was isolated from broilers on four different occasions during the growing period and at slaughter. Litter samples were also collected on each sampling occasion. Three flocks from three different farms were studied and the most common lesions in the 120 chickens examined were gizzard lesions. Because the largest number of birds with NE lesions was found in flock B, the isolates from this flock were chosen for further investigation by PFGE. In flock B, the accumulated mortality at slaughter was normal.

The genetic relatedness of 90 *C. perfringens* isolates from flock B was examined by PFGE and 88 were successfully characterized. Two isolates were degraded by endogenous endonucleases. The genetic relatedness of these isolates ranged between 70% and 100%. A total of 32 PFGE genotypes with unique patterns were observed.

The genetic relatedness among broilers with mild NE and broilers with no signs of NE was quite low. Often there were different genotypes of *C. perfringens* in

different tissues in the same bird, but occasionally the same genotype was found in both jejunum and caecum. During the rearing period, different genotypes of *C. perfringens* were found at different times. Those genotypes found in the early stages disappeared during the following weeks but some found at 30 days of age also appeared at slaughter a few days later. The isolates from the 4 broilers affected by severe NE or clinical CPH also shared a low genetic relatedness with five different genotypes found among them.

The relatively low genetic relatedness observed might be explained with this: a flock affected by mild NE with low mortality was studied, resulting in a wider distribution of different *C. perfringens* genotype clones. Other studies have shown that *C. perfringens* isolated from clinical outbreaks of NE with a high mortality rate in the flock had a close genetic relatedness and that *C. perfringens* from healthy broilers have a low genetic relatedness (Engström *et al.*, 2003; Gholamiandekhordi *et al.*, 2006; Nauerby, Pedersen & Madsen, 2003). By contrast, isolates in the present study were obtained from broilers affected by mild NE and only sporadic cases of severe NE with low mortality in the flock were observed.

Isolates with several different genotypes caused the mild or severe NE or gizzard lesions observed. The low mortality rate recorded in the investigated flock might be one explanation for the quite low genetic relatedness found among broilers affected by both mild and severe NE.

Paper IV

The broth microdilution method proved reproducible within one doubling dilution when using *C. perfringens* ATCC 13124 as control strain in 14 independent experiments. The isolates were classified as susceptible or resistant according to MIC distribution. In this study all *C. perfringens* isolates were classified as susceptible to the following five antimicrobials: narasin, ampicillin, vancomycin, avilamycin and erythromycin. A small proportion of resistance to virginiamycin and bacitarcin was found.

However, a high resistance to tetracycline was found in isolates from all the three countries: Sweden (76%), Norway (29%) and Denmark (10%). In 80% of the tetracycline-resistant isolates, both the resistance genes *tetA(P)* and *tetB(P)* were detected, while in 20% only *tetA(P)* was found. The high level of tetracycline resistance observed in the Swedish *C. perfringens* isolates cannot be attributed to greater use of tetracycline in Sweden than in Denmark and Norway (Bager, Emborg & Heuer, 2002; Bengtsson, Greko & Wallén, 2003; Kruse & Simonsen, 2003). Tetracycline resistance is the most commonly observed antibiotic resistance phenotype found in *C. perfringens*; other studies have also revealed a high level of resistance (Kather, Marks & Foley, 2006; Martel *et al.*, 2004; Sasaki *et al.*, 2001).

Despite the intensive use of narasin in broiler flocks in the Nordic countries (Bager, Emborg & Heuer, 2002; Bengtsson & Wallén, 2001; Kruse & Simonsen,

2003), all isolates were susceptible to narasin. Uniform susceptibility to narasin has also been reported in other studies (Martel *et al.*, 2004; Watkins *et al.*, 1997). Reduced susceptibility to narasin in both *E. faecium* and *E. faecalis* isolated from broilers has been reported from Sweden, Belgium and Norway (Bengtsson, Greko & Karlsson, 2004; Bengtsson, Greko & Karlsson, 2005; Butaye, Devriese & Haesebrouck, 2000; Sorum *et al.*, 2004). This phenomenon has not been observed so far in *C. perfringens* isolated from poultry.

In conclusion, the results of the present study show that narasin, which has a good preventive effect on clostridiosis (Elwinger *et al.*, 1994), is still an effective antimicrobial against *C. perfringens* and that the development of resistance is slow in *C. perfringens* isolated from poultry.

Concluding remarks

Clostridium perfringens is an important cause of clostridial enteric disease in domestic animals. In poultry *C. perfringens* can lead to both clinical and subclinical disease. The objectives of the project were to gain knowledge of *C. perfringens* causing necrotic enteritis in broilers, and to study the genetic diversity of *C. perfringens* in a broader perspective.

• Neither temperature nor duration of the simulated transport seemed to affect the survival of *C. perfringens*. Furthermore, it was demonstrated that three of the *C. perfringens* plasmid-borne toxin genes (*cpb1*, *cpb2* and *etx*) were stable both after transportation and after repeated exposure to aerobic conditions.

• The results presented in this thesis show that PFGE is a reliable and robust method for studies on the genetic diversity of *C. perfringens*, though DNA degradation may occur. Considerable genetic diversity and many distinct PFGE genotypes were found when *C. perfringens* isolates from a variety of sources were analysed by PFGE. No definite relationship between the source of the isolate and the positions in the dendrogram could be established. Epidemiologically related isolates showed close genetic similarity, while isolates with no obvious epidemiological relationship were not so genetically similar. Sequence comparison of the *cpb2* gene revealed two genetically different populations with a sequence similarity of 73.8%.

• A weak genetic relatedness was found between *C. perfringens* isolated from a broiler flock affected by mild NE. The relatedness among strains isolated from broilers with subclinical NE and broilers not showing signs of NE was also weak. The genetic relatedness was weak even between isolates obtained from dead broilers affected by severe NE and clinical CPH. This relatively weak genetic relatedness might have to do with the low mortality registered in this flock, which resulted in a wider distribution of different *C. perfringens* PFGE genotypes.

• The uniform susceptibility to narasin observed indicates that the substance can still be used to control clostridiosis. Despite the small amounts of tetracycline used in poultry production, a considerable degree of resistance to tetracycline was found in *C. perfringens* isolates from Swedish broilers.

Future perspectives

The main virulence factor in necrotic enteritis is the *C. perfringens* alpha-toxin. Further studies on the regulation and expression of this toxin are needed to learn more about the pathogenesis of necrotic enteritis in broilers. Improvement of quantitative methods for measuring the amounts of alpha-toxin expressed would be useful. Furthermore, it would be intresting to evaluate the production of alpha-toxin under different conditions such as temperature, pH, growth medium and other growth conditions. Knowledge gained from such experiments might give further insight into the pathogenesis of necrotic enteritis and give some additional information on how to avoid necrotic enteritis in broilers raised without IACs or AGPs.

The genome sequencing of *C. perfringens* strain 13 revealed a number of putative virulence genes; it would be of great interest to evaluate and characterize these virulence factors in greater detail. Studies on the five putative haemolysins would be of great interest and might add some more information on *C. perfringens* virulence. Hitherto, *C. perfringens* virulence has been explained in terms of toxin activity and the adhesion of the bacteria to eukaryotic cells has not been studied. The finding that *C. perfringens* strain 13 carries two putative fibronectin-binding protein genes is very intresting and merits further studies.

As previously mentioned, sequencing of two additional *C. perfringens* genomes (*C. perfringens* strain ATCC 13124 and *C. perfringens* strain SM101) is in progress at TIGR. Comparative genomic studies of these genomes and *C. perfringens* strain 13 would be very intresting and could hopefully reveal more insight into *C. perfringens* virulence and pathogenicity.

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