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Clostridium difficile in Horses

Viveca Båverud

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

In recent years, several animal hospitals in Sweden have reported an increased frequency of acute and often fatal colitis in mature horses. The most common risk group was horses hospitalized and treated for various non-gastrointestinal diseases. After a few days of hospitalization some horses developed acute diarrhea. Another risk group was mares when their foals were treated orally with erythromycin in combination with rifampicin for *Rhodococcus equi* pneumonia. Some mares developed diarrhea suddenly often after 3-4 days treatment of the foals at an animal hospital.

In human medicine, the bacterium *Clostridium difficile* is since many years a well-known nosocomial pathogen in antibiotic associated diarrhea. This thesis, based on five scientific publications, describes the association of *C. difficile* colonisation with the occurrence of diarrhea, antibiotic treatment and the age of the horses. The occurrence and survival of the bacterium in the environment and its antimicrobial susceptibility were also studied. Furthermore, the role of the antibiotic erythromycin in induction of acute colitis in horses was investigated.

C. difficile is associated with acute colitis in mature horses following treatment with antibiotics, as about 40% of the horses proved positive by culture and 28% in the cytotoxin B test of faeces. No other pathogen was detected in horses affected by antibiotic-associated diarrhea.

C. difficile, and/or its cytotoxin, is also associated with acute colitis in mares when their foals are being treated with erythromycin and rifampicin for *R. equi* pneumonia. The colitis can have resulted from an accidental ingestion of erythromycin by the mares. In an experimental study it was also demonstrated in horses that erythromycin can induce severe colitis associated with proliferation of *C. difficile.*

A new interesting finding was that in healthy foals younger than 14 days, *C. difficile* was isolated from every third foal whereas all older foals except one proved negative. Many asymptomatic carriers were also found among non-diarrheic foals treated with antibiotics.

Antimicrobial susceptibility testing showed that isolates were susceptible to metronidazole (MIC ≤4 μg/ml) and vancomycin (MIC ≤1 μg/ml). The MICs of erythromycin, oxytetracycline, spiramycin and virginiamycin showed a biphasic distribution. All isolates, except three, had uniformly high or low MICs of these antimicrobial agents.

In conclusion, the work described in this thesis is a contribution to increased knowledge of *C. difficile* as an etiological factor in antibiotic-associated diarrhea in horses. Preventive measures to avoid accidental ingestion of erythromycin by mares from the treatment of their foals are recommended.

Keywords: antibiotic-associated, environment, foal, horse, PCR, soil, stable, toxin

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EN VÄNLIG GRÖNSKAS RIKA DRÄKT

En vänlig grönskas rika dräkt har smyckat dal och ängar.
Nu smeker vindens ljumma fläkt de fagra örtesängar,
och solens ljus och lundens sus
och vågens sorl bland viden
förkunna sommartiden.

CD af Wirsén 1889

To Håkan, Hedda, Lisen and Herman

Abstract

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C. difficile is associated with acute colitis in mature horses following treatment with antibiotics, as about 40% of the horses proved positive by culture and 28% in the cytotoxin B test of faeces. No other pathogen was detected in horses affected by antibiotic-associated diarrhea.

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In conclusion, the work described in this thesis is a contribution to increased knowledge of *C. difficile* as an etiological factor in antibiotic-associated diarrhea in horses. Preventive measures to avoid accidental ingestion of erythromycin by mares from the treatment of their foals are recommended.

Keywords: antibiotic-associated, environment, foal, horse, PCR, soil, stable, toxin

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Sammanfattning

Under de senaste åren har man på flera kliniker i Sverige upplevt ett ökande problem med vuxna hästar som drabbats av mycket akuta, ibland dödliga diarréer. Den vanligaste riskgruppen består av vuxna hästar, som inkommer till ett djursjukhus för andra sjukdomstillstånd än diarré. Efter några dagars vistelse på kliniken och oftast i samband med antibiotikabehandling drabbas vissa hästar av en allvarlig akut diarré. En annan riskgrupp består av tidigare helt friska moderston, vars föl behandlas per oralt (i munnen) med erytromycin i kombination med rifampicin för *Rhodococcus equi* infektion. Vissa moderston utvecklar en akut och ibland dödlig diarré ofta efter 3-4 dagars behandling av fölen vid djursjukhusvistelse.

Hos människa är bakterien *Clostridium difficile* sedan många år en välkänd patogen vid antibiotika-associerade diarréer i samband med sjukhusvistelse. När denna studie initierades fanns *C. difficile* endast beskrivet hos föl med diarré och ännu inte som någon patogen hos vuxna hästar. I den här avhandlingen, som bygger på fem vetenskapliga publikationer, beskrivs sambandet mellan *C. difficile* kolonisation med förekomst av diarré, antibiotikabehandling och ålder hos häst. Förekomst och överlevnad av bakterien i miljön och dess antibiotikakänslighet studerades också. Vidare undersöktes också om behandling med erytromycin kan orsaka akut diarré hos häst.

Undersökningarna visar att *C. difficile* är associerad med akut diarré hos vuxna hästar behandlade med antibiotika, då ca 40% av hästarna var positiva i odling och 28% positiva i cytotoxin B test av faeces. Däremot påvisades *C. difficile* ej i faeces från vuxna friska hästar. Andra sjukdomsframkallande tarmbakterier påvisades inte hos hästar med antibiotika-associerad diarré.

C. difficile och/eller dess toxin påvisades också i faeces hos 45% av undersökta moderston med akut diarré när deras föl behandlades med erytromycin och rifampicin för *R. equi* pneumoni. Höga koncentrationer av erytromycin påvisades i faeces hos föl vars moderston utvecklade akut diarré, medan stona till föl med lägre koncentrationer i faeces förblev friska. Diarréen hos moderstona beror sannolikt på ett oavsiktligt upptag av erytromycin från fölens avföring och/eller dess behandling. I en experimentell studie verifierades att mycket små mängder erytromycin kan ge upphov till allvarlig diarré hos häst och framväxt av *C. difficile.*

Ett intressant fynd var att från friska föl yngre än 14 dagar isolerades *C. difficile* från faecesprov från vart tredje föl. Alla äldre föl, utom ett, var negativa. En hög frekvens av asymtomatiska bärare hittades också hos antibiotika-behandlade föl utan diarré. I en experimentell studie visade sig bakterien överleva i naturen och inomhus i minst 4 år i hästfaeces.

Antibiotikabestämning visade att *C. difficile* isolat var känsliga för metronidazol (MIC ≤4 µg/ml) och vancomycin (MIC ≤1 µg/ml). MIC-värdena för erytromycin, oxytetracyclin, spiramycin och virginiamycin visade en bifasisk distribution. Alla isolatat, utom tre, hade antingen höga eller låga MIC-värden för dessa antibiotika.

Rutinundersökning av *C. difficile* och dess cytotoxin rekommenderas då akut diarré uppträder hos vuxna hästar i samband med antibiotikabehandling och vidare hos moderston, som utvecklar akut diarré när deras föl behandlas med erytromycin och rifampicin. Man bör noggrant undvika oavsiktligt upptag av erytromycin hos moderstona vid behandling av deras föl.

Sammanfattningsvis har denna avhandling resulterat i ökade kunskaper om *C. difficile* som en etiologisk faktor vid antibiotika-associerad diarré hos häst.

Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals I-V:

I. Båverud, V., Gustafsson, A., Franklin, A., Lindholm, A. and Gunnarsson, A. 1997. *Clostridium difficile* associated with acute colitis in adult horses treated with antibiotics. *Equine Veterinary Journal 29,* 279-284.

II. 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 Veterinary Journal 30,* 482-488.

III. Gustafsson, A., Båverud, V., Gunnarsson, A., Horn af Rantzien, M., Lindholm, A. and Franklin, A. 1997. The association of erythromycin ethylsuccinate with acute colitis in horses in Sweden. *Equine Veterinary Journal 29,* 314-318.

IV. Båverud, V., Gustafsson, A., Franklin, A., Aspán, A. and Gunnarsson, A. *Clostridium difficile*: prevalence in horses, in environment and antimicrobial susceptibility. *Equine Veterinary Journal,* in press.

V. Båverud, V., Gunnarsson, A., Karlsson, M. and Franklin, A. Antimicrobial susceptibility of equine and environmental isolates of *Clostridium difficile*, manuscript.

Abbreviations

AP-PCR	arbitrarily primed PCR
ATCC	American Type Culture Collection
BHI	brain heart infusion
CCFA	cycloserine, cefoxitin and fructose agar
CFU	colony forming units
CPE	cytopathic effect
erm	erythromycin ribosome methylation (gene)
FAA	fastidious anaerobe agar
FAB	fastidious anaerobe broth
Ig	immunoglobulin
MIC	minimum inhibitory concentration
MLS	macrolide-lincosamide-streptogramin
NCCLS	National Committee for Clinical Laboratory Standards
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMC	pseudomembranous colitis
rRNA	ribosomal RNA
TCCFA	cycloserine, cefoxitin and fructose agar supplemented with taurocholate
TMP	trimethoprim/sulfamethoxazole

Introduction

In 1992, several animal hospitals in Sweden reported an increased frequency of acute and often fatal cases of colitis in mature horses. The most common risk group was horses hospitalized and treated for various diseases other than gastrointestinal. After a few days of hospitalization some horses developed acute and sometimes fatal, life-threatening diarrhoea. Another risk group was mares when their foals had been treated orally with erythromycin in combination with rifampicin for *Rhodococcus equi* pneumonia. Some mares developed sudden and sometimes fatal diarrhea after 3-4 days' treatment of the foals at an animal hospital. The symptoms were similar for both groups. A profuse watery acute diarrhea developed, often together with discoloured mucous membranes, fever and depression. The mortality was high despite intensive therapy.

In human medicine, the bacterium *Clostridium difficile* is since many years a well-known nosocomial pathogen in antibiotic associated diarrhea (Tabaqchali & Jumaa, 1995; Job & Jacobs, 1997). When the present study was initiated, *C. difficile* was only reported in diarrheic foals and not yet as a pathogen in mature horses. Sage (1998) suggested that listening to human experience of nosocomial infections may help the horse.

Historical background

C. difficile was first isolated from faeces of four of ten healthy newborn infants in 1935 (Hall & O'Toole, 1935). The organism was namned *Bacillus difficilis* because of difficulty in isolating and studying these bacteria. The organism produced a toxic culture filtrate that killed guinea pigs and rabbits upon injection (Hall & O'Toole, 1935). Snyder (1940) confirmed and extended these findings and also showed that the toxic activity was neutralized by antisera to *Bacillus difficilis.* As the bacterium was anaerobic endospore-forming and Gram-positive it was later classified as belonging to the genus *Clostridium* (Brazier & Borriello, 2000). In 1974, there were three independent studies that paved the way for understanding its significance in humans. Green (1974) found a cytotoxin in colon of guinea pigs which developed gut disease after receiving penicillin. Secondly, Tedesco *et al.* (1974) found an association between patients receiving clindamycin and the development of pseudomembranous colitis (PMC). Further *C. difficile* and its toxicity were studied in a thesis by Hafiz (1974). It was then demonstated, in a Syrian hamster model, that *C. difficile* was the causative agent of antibiotic-associated diarrhea by Bartlett *et al.* (1977). In 1978, the first human cases were described when *C. difficile* was reported as a cause of PMC (Bartlett *et al.*, 1978; George & Symonds, 1978; George *et al.*, 1978; Larson *et al.*, 1978).

Taxonomy

Description of Clostridium difficile

The bacterium *C. difficile* is an obligate anaerobic, large Gram-positive rod, measuring 0.5 x 3–6 μm (Quinn *et al.*, 1994). The cells are usually peritrichous

which means that they are motile in broth cultures because of having flagella distributed over the cell. Some strains produce chains consisting of two to six cells aligned end-to-end. On blood agar the colonies are 2-5 mm in diameter, circular or rhizoid, flat or low convex, opaque, greyish or whitish and have a matt to glossy surface (Cato *et al.,* 1986). The odour of *C. difficile* colonies is likened to that of horse or elephant manure (Lyerly, 1995; Brazier & Borriello, 2000). The optimum temperature for growth *in vitro* is 30–37°C, however growth has also been shown to occur at 25°C and 45°C. *C. difficile* forms oval, subterminal endospores (Cato *et al.,* 1986). The spore is a resting cell, highly resistant to heat, desiccation, oxygen and to chemical agents. When returned to favourable nutritional conditions and activated, the spore germinates to produce a vegetative cell (Brooks *et al.,* 1991). On non-selective agars, colonies usually sporulate after 72 h incubation and hence survive for a prolonged time when exposed to air (Brazier & Borriello, 2000).
C. difficile is a bacterium with low guanine (G) and cytosine (C) content in the genome, 28%, and thus rich in adenine (A) and thymidine (T) (Gottschalk *et al.,* 1981). The *C. difficile* genome is about 4.4 Mb in size (see TIGR databases at http://www.tigr.org).

Clostridium difficile *toxins*

Pathogenic strains of *C. difficile* produce two potent toxins: enterotoxin A and cytotoxin B. These toxins are of major importance in clinical disease (Kelly *et al.,* 1994). The *C. difficile* toxins A and B together with the lethal and haemorrhagic toxin from *C. sordellii* and the α-toxin of *C. novyi,* comprise a group called the large clostridial cytotoxins which are a family of functionally and structurally related toxins (Moncrief & Wilkins, 2000). Toxins A and B are both extremely large, having molecular masses of 308 and 270 kDa, respectively (Just *et al.,* 2000).

Toxin A was designated enterotoxin because it induces fluid accumulation in intestinal loop models (Lyerly *et al.,* 1985). Cytotoxin B does not cause fluid accumulation. Toxin B is about 100 to 1000-fold more cytotoxic to cultured cell lines than toxin A and was therefore called a cytotoxin (Lyerly *et al.,* 1982). Less than a picogram of cytotoxin B causes cells to round up, detach from their support and slowly die (Cato *et al.,* 1986). The cytotoxic effects of the *C. difficile* toxins are reviewed by Thelestam & Chaves-Olarte (2000).

Besides the two 'classic' toxins that can be produced by *C. difficile,* certain strains produce a binary toxin, ADP-ribosyltransferase, which pathophysiological significance is still unclear (Stubbs *et al.,* 2000; Rupnik, 2001). Recently, the genes of the binary toxin were found together with toxin A and B genes in 4 of 17 isolates from horses with various intestinal disorders (Braun *et al.,* 2000).

Natural classification

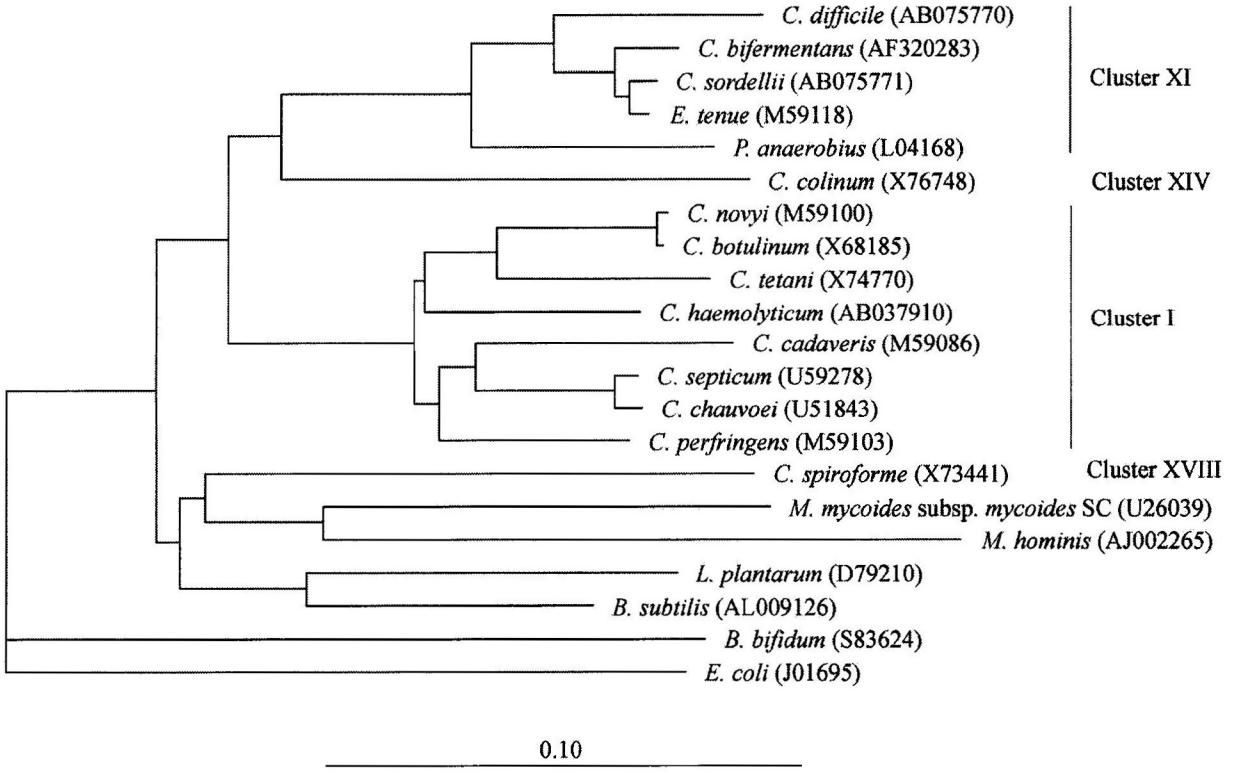
Natural classification reflects natural relations between organisms and can be phenetic or phylogenetic (Priest & Austin, 1993). Phenetic classification is based on a large number of phenotypic and genotypic properties, while phylogenetic

classification is based on the evolutionary history of the organisms. In polyphasic taxonomy, phenetic and phylogenetic classifications are combined to obtain the 'true' relations between the organisms (Vandamme *et al.,* 1996).

The phylum *Firmicutes,* consisting of Gram-positive bacteria with a low G+C content, contains three classes: *Clostridia, Bacilli* and *Mollicutes.* The class *Clostridia* is further divided into orders, families and genera. The genus *Clostridium* belongs to the family *Clostridiaceae,* together with 12 other genera (See; http://www.cme.msu.edu/Bergeys/). The genus *Clostridium* constitutes a phylogenetically heterogeneous group. It arose as an early branch of the Gram-positive bacteria. The formation of endospores was common to the genera *Clostridium* and *Bacillus* and also *Desulfotomaculum, Heliobacterium, Sarcina* and *Sporomusa* (Stackebrandt & Rainey, 1997).

The determination of rRNA relatedness values of 56 *Clostridium* species by Johnson & Francis in 1975 demonstrated that the genus *Clostridium* consists of a wide range of phylogenetically remotely related species.

Clostridium and related genera were divided into clusters I-XIX by Collins *et al.* (1994). Almost all clostridia classified as major pathogens, except *C. difficile* and *C. sordellii,* were included in the 16S rRNA clusters I and II. *C. difficile* was included in the cluster XI which also includes *C. bifermentans, C. sordelli*, 15 other *Clostridium* spp, and also non-*Clostridium* species, such as *Eubacterium tenue* and *Peptostreptococcus anaerobius*. With the new genetic information, taxonomic problems are arising and reclassifications have been considered.


C. difficile (AB075770)
C. bifermentans (AF320283)
C. sordellii (AB075771)
E. tenue (M59118)
P. anaerobius (L04168)
C. colinum (X76748)
C. novyi (M59100)
C. botulinum (X68185)
C. tetani (X74770)
C. haemolyticum (AB037910)
C. cadaveris (M59086)
C. septicum (U59278)
C. chauvoei (U51843)
C. perfringens (M59103)
C. spiroforme (X73441)
M. mycoides subsp. mycoides SC (U26039)
M. hominis (AJ002265)
L. plantarum (D79210)
B. subtilis (AL009126)
B. bifidum (S83624)
E. coli (J01695)
Cluster XI
Cluster XIV
Cluster I
Cluster XVIII
0.10

Special purpose classification of pathogenic Clostridium species

Special purpose classification is not regarded as natural because it is based on very few properties and does not reflect natural relations between organisms (Priest & Austin, 1993). Furthermore, special purpose classifications involve only smaller groups of organisms and are in general useful only within certain fields, for instance clinical bacteriology. The pathogenic *Clostridium* spp. can be divided into four major groups according to the kind of disease they cause in animals (Table 1):

The neurotoxic clostridia (*C. botulinum* and *C. tetani*) produce potent neurotoxins but are non-invasive and colonize the host to a very limited extent.

The histotoxic clostridia (*C. chauvoei, C. septicum, C. novyi, C. haemolyticum, C. sordellii, C. perfringens* type A, and *C. colinum*) produce less potent toxins than the first group, but are invasive.

The clostridia that produce enterotoxaemias are *C. perfringens* types A-E. Enterotoxins are formed in the intestines and are absorbed into the bloodstream, producing a generalized toxaemia.

The clostridia associated with antibiotic-induced disease are *C. difficile* and *C. spiroforme* (Quinn *et al.*, 1994; Carter *et al.*, 1995).

Fig. 1. Evolutionary tree representing the phylogenetic relations of some representatives of the genera *Clostridium*, *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Mycoplasma* and *Peptostreptococcus* based on 16S rRNA sequences. The tree was computed by the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix corrected for multiple nucleotide substitutions by the one-parameter model (Jukes & Cantor, 1969). *Escherichia coli* was used as outgroup. The scale bar shows the distance corresponding to one substitution per 10 nucleotide positions. Clusters or rRNA groups defined according to Collins *et al.* (1994) and Johnson & Francis (1975), respectively.

Table 1. Summary of the hosts and diseases associated with the pathogenic clostridia in animals

***Clostridium* species**	**Hosts**	**Diseases**
NEUROTOXIC CLOSTRIDIA		
Clostridium tetani	Horses, ruminants and other animals	Tetanus
Clostridium botulinum (types A-F)	Many animal species	Botulism
HISTOTOXIC CLOSTRIDIA		
Clostridium chauvoei	Cattle, sheep, (pigs)	Blackleg (Black quarter)
Clostridium septicum	Cattle, sheep, pigs	Malignant edema
	Sheep	Braxy
	Chickens	Necrotic dermatitis
Clostridium novyi		
type A	Sheep	Big-head in rams
	Cattle and sheep	Gas gangrene
type B	Sheep, (cattle)	Black disease (necrotic hepatitis)
type C	Water buffalo	Osteomyelitis reported
Clostridium haemolyticum (*C. novyi* type D)	Cattle, (sheep)	Bacillary haemoglobinuria
Clostridium sordellii	Cattle, sheep, horses	Gas gangrene
Clostridium colinum	Game birds, young chickens and turkey poults	Quail disease (ulcerative enteritis)
ENTEROTOXAEMIAS		
Clostridium perfringens		
type A	Lambs	Enterotoxaemic jaundice
type B	Lambs (under 3 weeks old)	Lamb dysentery
	Neonatal calves and foals	Enterotoxaemia
type C	Piglets, lambs, calves, foals	Haemorrhagic enterotoxaemia
	Adult sheep	Struck
	Chickens, piglets	Necrotic enteritis
Type D	Sheep (all ages except neonates) (goats, calves)	Pulpy kidney disease
Type E	Calves and lambs (rare)	Enterotoxaemia
CLOSTRIDIA ASSOCIATED WITH ANTIBIOTIC-INDUCED DISEASE		
Clostridium spiroforme	Rabbits	Possible role in mucoid enteritis
	Rabbits and guinea-pigs	Spontaneous and antibiotic-induced diarrhea
	Foals and pigs	Enterocolitis (natural)
Clostridium difficile	Hamsters, rabbits, guinea-pigs	Antibiotic-induced enterocolitis
	Dogs, foals, pigs, laboratory animals	Naturally occurring diarrhea

Modified from Quinn, P.J., Carter, M.E., Markey, B. and Carter, G.R. (1994) In: Clinical Veterinary Microbiology, London. Wolfe Publishing, Mosby-Year Book Europe Limited, pp 192.

Pathogenesis

The normal intestinal microflora is one of the first-line of defence against infection (Borriello, 1998; Cleary, 1998). The normal microflora is capable of preventing colonisation by exogenous bacteria and limits the concentration of endogenous, potentially pathogenic bacteria. This protective effect of the normal microflora is frequently referred to as colonization resistance (Vollaard & Clasener, 1994).

C. difficile is usually a harmless environmental bacterium (Brazier & Borriello, 2000). The normal intestinal microflora has to be disturbed before *C. difficile* can become established and produce toxins (Borriello, 1998). By antibiotic treatment there is an initial disruption of the normal colonic bacterial flora, allowing *C. difficile* from endogenous or exogenous origins to establish itself in the colon and proliferate. If the isolate is toxigenic, toxins A and B are produced simultaneously in most cases. These protein toxins bind to specific receptors on the luminal aspect of the colonic epithelium and are then, by receptor-mediated endocytosis, transported into the cytoplasm (Farrel & LaMont, 2000). *C. difficile* toxins produce mucosal injury in the colon, as a result of damage to the cytoskeleton and inhibition of the functioning of tight junctions (Hecht *et al.*, 1992; Riegler *et al.*, 1995). The toxins cause fluid secretion, inflammation and mucosal damage, leading to diarrhea or pseudomembranous colitis (Barbut & Petit, 2001). The pathogenesis above is described in general terms; specific studies in horses have not yet been performed.

Antibiotic therapy
⇓
Alteration of colonic microflora
⇓
***C. difficile* exposure and colonization**
⇓
Release of toxins A and B
⇓
Binding to receptors on intestinal epithelial cells
⇓
Colonic mucosal injury and acute inflammation
⇓
Diarrhea and colitis

Fig. 2. Pathogenesis of *C. difficile*-induced diarrhea and colitis (modified from Farrell & LaMont, 2000)

Epidemiology

The reservoir

For the development of *C. difficile* diarrhea, exposure to a toxigenic isolate of *C. difficile* is a prerequisite. The organism or its spores may originate from other diarrheic horses that are shedding the organism, from the horse itself by asymptomatic carriage, from the environment or from the hands of staff.

Carriage rates

C. difficile is rarely isolated from faecal samples of mature horses, having an isolation rate of 0-1% (Jones *et al.*, 1987; Al Saif & Brazier, 1996; Weese *et al.*, 2001a). Furthermore, *C. difficile* was found in 0-3% of faecal samples from healthy foals (Jones *et al.*, 1987; Beier *et al.*, 1994; Magdesian *et al.*, 1999; Weese *et al.*, 2001a).
In humans, carriage rates of *C. difficile* in adults vary from 0-3% up to 15% in Japan (Larson *et al.*, 1978; Nakamura *et al.*, 1981; Möllby *et al.*, 1985; Aronsson *et al.*, 1985). Carriage rates in neonates and infants are much higher with rates up to 52% (Larson *et al.*, 1982).

Environment

In several studies, samples from the environment have been analysed for *C. difficile.* The first was by Hafiz (1974) who isolated the organism from various sites such as sand and soil in Pakistan. Others have also isolated the organism from the soil (Blawat & Chylinski, 1958; Al Saif & Brazier, 1996). Interestingly, a high percentage of river waters (87.5%) and lake waters (50%) as well as swimming pools tested positive for *C. difficile* (Al Saif & Brazier, 1996).

In animal hospitals in Australia, Britain and Canada, *C. difficile* was isolated from various sites of environmental samples (Riley *et al.*, 1991; Al Saif & Brazier, 1996; Weese *et al.*, 2000a). Examples of positive sites were postmortem room floor, scales, thermometers, dog walk entry, stalls and medical staff (Weese *et al.*, 2000a).

Infection with *C. difficile* in humans is usually nosocomial (i.e. acquired during hospitalization). Several investigations of the hospital environment have been made. It was demonstrated early that *C. difficile* or its spores could be isolated from the human hospital environment of patients with antibiotic associated diarrhea (Mulligan *et al.*, 1979; Fekety *et al.*, 1981; Kim *et al.*, 1981; McFarland *et al.*, 1989). Spores were found to persist in the environment despite routine cleaning of rooms (McFarland *et al.* 1989). *C. difficile* has also been found in environments outside hospitals, such as in family homes and student residences (Al Saif & Brazier, 1996).

Transmission

Transmission of *C. difficile* is thought to occur via ingestion of the organism or its spores via the oro-faecal route (Jumaa *et al.*, 1996; Cleary, 1998; Worsley, 1998). When there are outbreaks in human hospitals, transmission is usually suggested to

occur via the hands of the hospital staff, or by direct contact with *C. difficile*-positive patients or contaminated surfaces (McFarland *et al.*, 1989; Worsley, 1998).

Several factors may facilitate transmission. The spores are resistant to the most commonly used disinfectants and antiseptics and can therefore survive for several months in the hospital environment (Barbut & Petit, 2001). However, the use of hypochlorite disinfectant was found to reduce the incidence of *C. difficile* infections (Mayfield *et al.*, 2000).

Risk factors

Most adult horses with *C. difficile* diarrhea have been treated with antibiotics prior to infection (Divers, 2002). Certain antibiotics have in particular been associated with *C. difficile* diarrhea in horses. These are erythromycin, trimethoprim/sulfonamides, gentamicin and β-lactam antibiotics (Madewell *et al.*, 1995; Magdesian *et al.*, 1997; Divers, 2002). Other antibiotics associated with diarrhea in horses are tetracyclines (Andersson *et al.*, 1971), lincomycin (Raisbeck *et al.*, 1981; Staempfli *et al.*, 1992), trimethoprim/sulphadiazine (Ensink *et al.*, 1996) and ceftiofur and metronidazole (Magdesian *et al.*, 1997). Orally administered antibiotics and those that undergo enterohepatic circulation or excretion into the intestine (macrolides, lincosamides, tetracyclines, and some cephalosporins) are more likely to cause diarrhea than parenterally administered antibiotics that do not gain access to the lumen of the intestine in an active form (Jones, 2000).

Various stress factors may have had a secondary influence on the colonic bacterial flora, such as transportation to the clinic, the stay at the animal hospital, presurgery fasting and surgery or medical treatment (Paper I). A risk factor for developing *C. difficile* disease in horses is the withholding of roughage. The volatile fatty acids produced in the colon by normal fibre fermentation are protective against disruption of the intestinal microflora (Divers, 2002).

Prevention

To prevent *C. difficile* diarrhea in horses it is important to isolate infected horses and foals and routine hand washing by all staff should be performed (Divers, 2002). Thorough cleaning with detergents to reduce the spores in the environment is essential (Worsley, 1998) and surface disinfection with hypochlorite may kill the spores (Divers, 2002).

The dams, of foals treated orally with erythromycin and rifampicin for *Rhodococcus equi* pneumonia should be fed from a container raised off the ground to prevent ingestion of erythromycin from the faeces of the foals. Foals treated orally with erythromycin should not be allowed to drink water from a shared bucket directly after treatment, in order to avoid ingestion of the antibiotic by the mare (Divers, 2002).

Laboratory diagnosis

The same methods as used for laboratory diagnosis of *C. difficile* in humans are also used in horses.

The specimen

An optimal laboratory investigation should be performed on a freshly taken faecal sample, directly submitted to the laboratory for investigation (Brazier & Borriello, 2000). However, due to practical circumstances, delay in arrival of the samples is not unusual. Some studies have been performed on the survival of the organism. According to Brazier & Borriello (2000) the organism sporulates 'readily' and the organism survives well in faeces for a long time at 4°C or frozen at –70°C. In a personal observation by Brazier, the organism survived in faecal samples at –70°C for a decade and at 4°C for many months. In several studies with positive cultures for *C. difficile*, faecal samples from horses were frozen before processing (Ehrich *et al.*, 1984; Weese *et al.*, 2001a). However, in its vegetative form the organism does not survive well in aerobically stored faecal samples (Weese *et al.*, 2000b). Our experience is that after 4 years *C. difficile* can still be isolated from faecal samples stored at –20°C (paper IV).

Storage of faecal samples at ambient temperature for a prolonged time may lead to denaturation of faecal toxin. Bowman & Riley (1986) demonstrated a 100-fold decrese of the cytotoxin titre of specimens stored at room temperature for 2 days. However, the toxins were stable for at least 60 days at 4°C according to experimental studies by Weese *et al.* (2000b). In conclusion, specimens that are not processed directly are recommended to be stored at 4°C or –20°C (Brazier & Borriello, 2000) or –70°C (Jones, 2000).

Culture

A selective agar medium, containing cycloserine (500 µg/ml), cefoxitin (16 µg/ml) and fructose agar (CCFA), was developed by George *et al.* (1979a). Later, an increased isolation rate, was reported by Levett (1985), with half of the antibiotic concentrations described by George *et al.*, (1979a). The antibiotics are of importance for reduction of contaminating intestinal flora. The addition of bile salts such as taurocholate to a medium was found to enhance the germination of spores (Wilson *et al.*, 1982; Buggy *et al.*, 1983).

Enrichment cultures have been tried in different studies (Beier *et al.*, 1994; Levett, 1984). However, it is generally accepted that for culture of faecal samples from diseased patients, it is not necessary (Brazier, 1998). Beier *et al.* (1994) used the enrichment technique after a spore selection procedure (5 min at 80°C) in faecal samples from horses. Selective enrichment may be useful in environmental studies, according to Buchanan (1984). Recently, contact plates have been used, with good results in environmental studies (Al Saif & Brazier, 1996; Weese *et al.*, 2000a). Various groups have reported the efficacy of alcohol (ethanol)-shock treatment of stool specimens to select for *C. difficile* spores (Borriello & Honour, 1981; Bartley & Dowell, 1991; Al Saif & Brazier, 1996; Brazier & Borriello, 2000).

In recent years, the value of culture in the diagnosis of *C. difficile* infection in humans has been discussed. According to several authors, toxin detection in faecal samples is sufficient for diagnosis (Lyerly *et al.*, 1998).

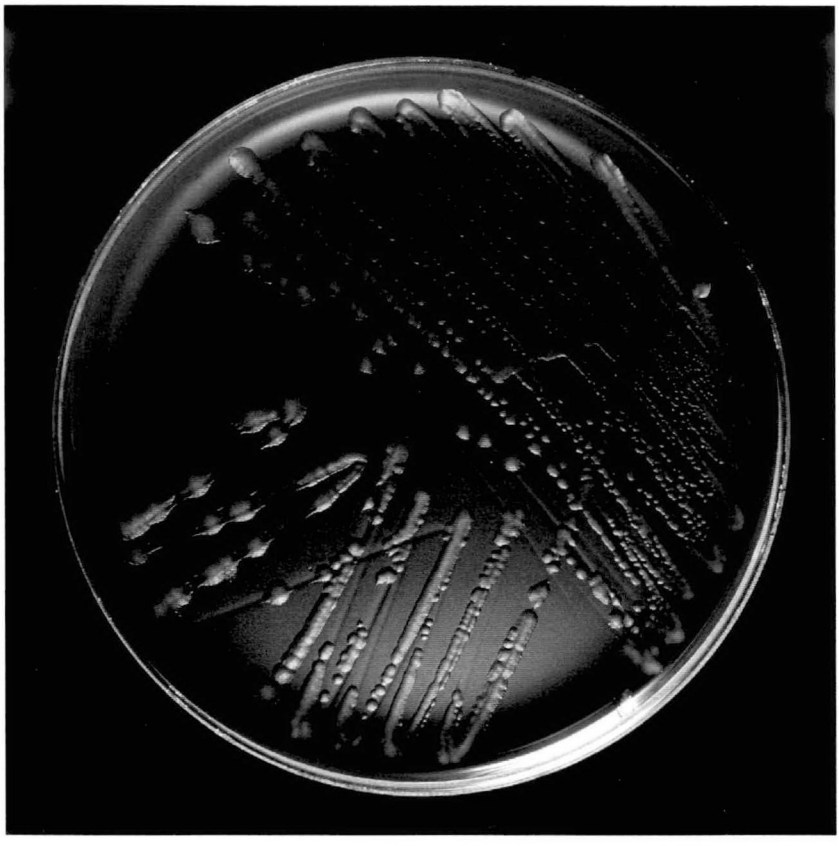


Fig. 3. *Clostridium difficile* ATCC 9689 colonies on FAA plates with defibrinated horse blood. Photo: Bengt Ekberg

Identification

To identify *C. difficile,* its distinctive odour on agar plates may be of good help, together with colony appearance and Gram stain. Another characteristic is the ability of colonies, after 48 h incubation on non-selective blood-based agar, to produce a pale green to chartreuse fluorescence under long-wave length ultraviolet light (Cato *et al.*, 1986; Perrin *et al.*, 1993a; Brazier, 1998). The following tests have been used for typing of equine isolates: Culturette CDT latex agglutination test (Becton Dickinson, Cockeysville, Md, USA) (Madewell *et al.*, 1995), detection of L-proline aminopeptidase production (ProDisc, Carr-Scarborough Microbiological, Decatur, Ga, USA) (Weese *et al.*, 2001a), gas-liquid chromatography with a large peak of isocaproic acid and biochemical testing (Jones *et al.*, 1989; Perrin *et al.*, 1993a).

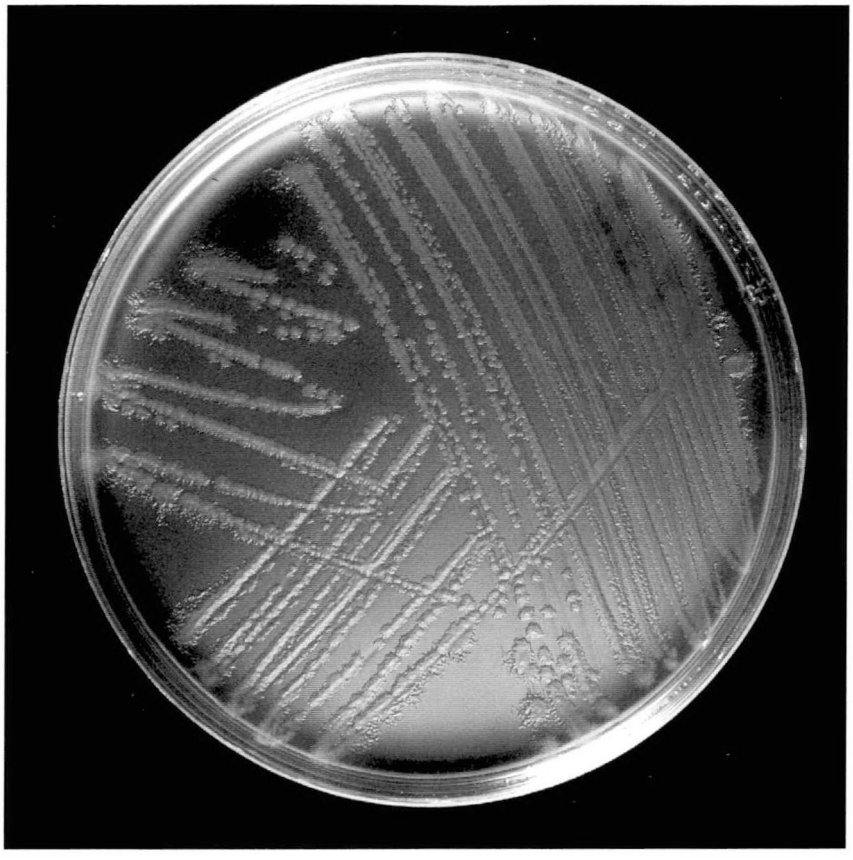


Fig. 4. *Clostridium difficile* ATCC 9689 colonies on TCCFA plates. Photo: Bengt Ekberg

Demonstration of Clostridium difficile *toxins*

Weese *et al.* (2001a) have suggested that demonstration of toxin in faecal samples from horses showing symptoms is required for a positive diagnosis of *C. difficile* diarrhea. In humans, detection of *C. difficile* toxins is considered the standard for diagnosis (Lyerly *et al.*, 1998). However, Delmée (2001) also considered culture-positive but toxin-negative faecal samples with *in vitro* toxin production of the isolate as probable *C. difficile* diarrhea.

Cytotoxin B assay by tissue culture

Both enterotoxin A and cytotoxin B are cytotoxic. However, the use of tissue culture to demonstrate *C. difficile* toxins in faecal samples has become synonymous with the detection of toxin B. The reason for this is that toxin B is more potent that toxin A for most cell lines. Also, before toxin A was known, toxic effects on tissue culture were studied (Brazier, 1998). The method consists of inoculating a filtrate of a faecal specimen into a cell culture. A cytopathic effect (CPE) is observed after 24-48 h incubation at 37°C, as a consequense of disruption of the cytoskeleton; which may result in cell rounding in many cell lines. Confirmation of the specificity is obtained by neutralization with antitoxin. *C. difficile* or *C. sordellii,* which share the same antigens, are recommended (Delmée, 2001).

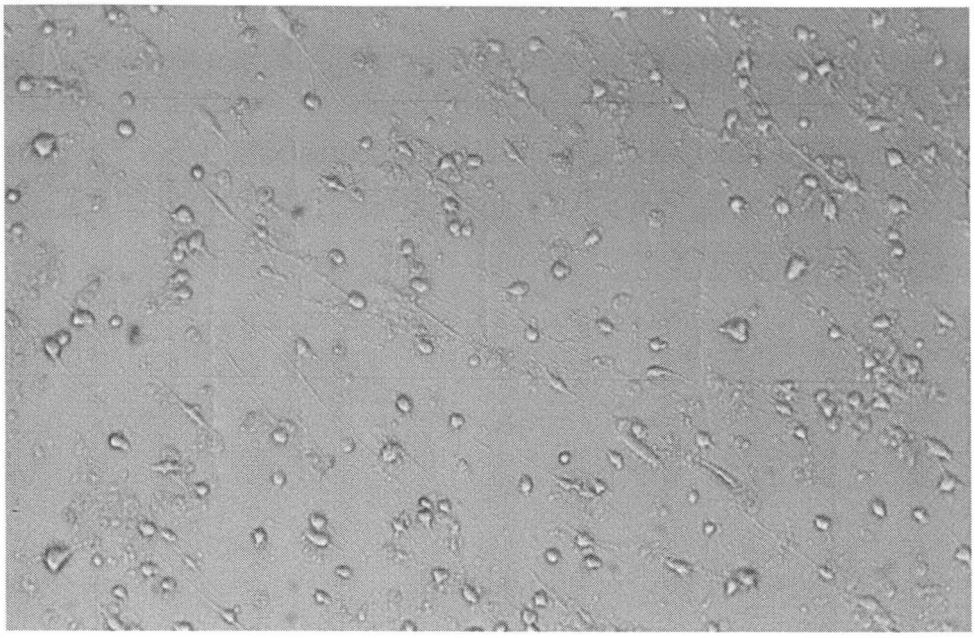


Fig. 5. Cytotoxin B positive assay (MRC-5 cells). Photo: Viveca Båverud

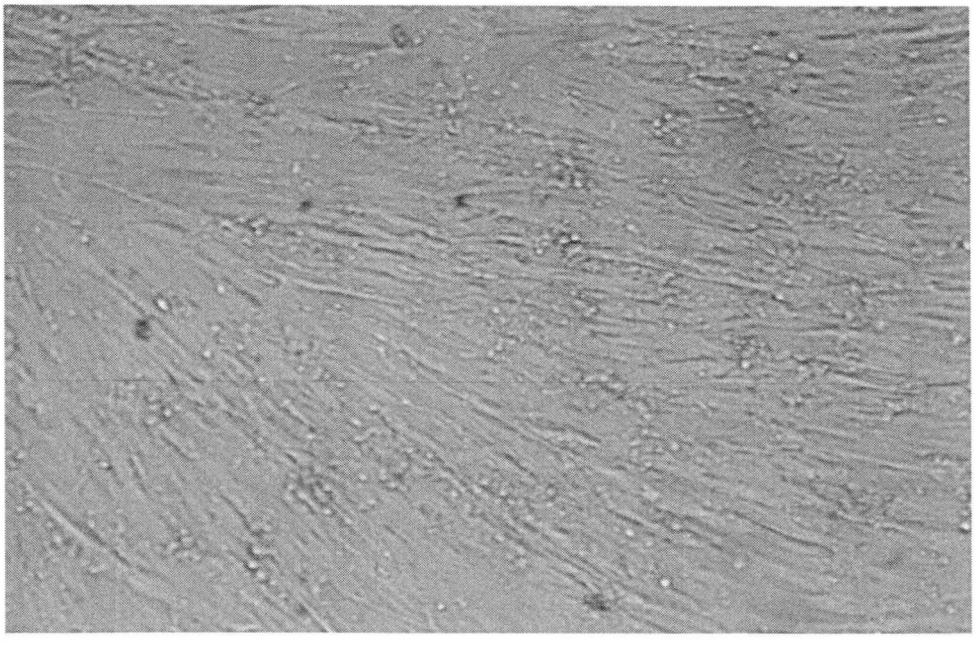


Fig. 6. Cytotoxin B negative assay (MRC-5 cells). Photo: Viveca Båverud

The cytotoxin assay for detection of toxin B in faecal specimens is considered to be the gold standard method by virtue of its high sensitivity and specificity. As the toxin is labile, false-negative results may occur due to inactivation during transport (Fekety, 1995). Other disadvantages are technical complexity and slow

turnaround time (24-48 h). The need to maintain cell lines is both time-consuming and expensive, especially if only a small number of specimens are processed (Delmée, 2001).

Enzyme immunoassays for testing of *C. difficile* toxin A and/or B

Commercial products for rapid immunological detection of *C. difficile* toxin have been developed. Most assays use monoclonal antitoxin A antibodies, whereas a few are designed to detect both toxins (Lyerly *et al.,* 1998; Delmée, 2001; O'Connor *et al.,* 2001). The tests have been performed on faecal specimens from horses (Weese *et al.,* 2001a; Donaldson & Palmer, 1999) but are not validated or compared with the cytotoxin assay. The advantages of using the rapid immunoassays are that they are relatively simple to perform and provide the possibility of testing samples the same day even for single specimens. When compared with faecal cytotoxin detection on cell lines, the different enzyme immunoassays show a slightly lower sensitivity (O'Connor *et al.,* 2001).

Detection of toxin A and B genes by PCR in faecal samples

Direct detection of toxin A or B genes in faecal samples by the Polymerase Chain Reaction (PCR) has been used (Kato *et al.,* 1993; Gumerlock *et al.,* 1993; Wolfhagen *et al.,* 1994; Arzese *et al.,* 1995) but the results so far have not proved significantly better than with the classic methods for toxin demonstration (Delmée, 2001).

Detection of toxin A and B genes by PCR on isolates

Several PCR-based methods have been developed for detection of the toxin A and B genes in *C. difficile* isolates (Wren *et al.,* 1990; Kato *et al.,* 1991; McMillan *et al.,* 1992; Karasawa *et al.,* 1999; Kato *et al.,* 1998). Toxins A and B are encoded by separate genes located in close proximity on the chromosome (Braun *et al.,* 1996). The PCR test shows if the genes are present, but gives no information about their expression.

Methods of subtyping C. difficile

Various methods have early been developed to understand the epidemiology of nosocomial outbreaks of *C. difficile* infections in humans. At first, phenotypic typing methods were developed to study the epidemiology of *C. difficile* infection at a local level (reviewed by Brazier, 2001), e.g. antibiograms. Further, Delmée *et al.* (1985) developed a method where isolates of *C. difficile* could be grouped into at least 10 different serotypes based on slide agglutination with rabbit antisera. This method is frequently used as the standard by which other typing methods are compared.

Recently, many molecular typing methods have been developed and used (reviewed by Brazier, 1998, 2001). Pulsed-field gel electrophoresis (PFGE) has been widely used as a molecular fingerprinting technique for subtyping of clinical isolates. However, in different studies, some isolates have been found non-typable by PFGE because of DNA degradation (Samore *et al.,* 1996; Kristjansson *et al.,* 1994; Klaassen *et al.,* 2002). PCR-ribotyping, based on the spacer region between the 16S and 23S rRNA regions, has frequently been used (O'Neill *et al.,* 1996;

Clostridium difficile in humans

According to Fekety (1995) as many as 5-10% of hospitalized patients given antibiotics develop diarrhea. Antibiotic-associated diarrhea is associated with prolonged hospitalization, higher costs and, furthermore an increase in mortality (Frost *et al.*, 1998; Spencer, 1998a). *C. difficile* accounts for about 20-25% of antibiotic-associated diarrhea cases in humans and causes the majority of antibiotic-associated colitis and pseudomembranous colitis (PMC) (Aronsson *et al.*, 1981; Möllby *et al.*, 1985; Lyerly *et al.*, 1988; Bartlett, 1990; McFarland *et al.*, 1990; Fekety, 1995). *C. difficile* is known to be the most common nosocomial enteric pathogen in humans (Silva, 1994; Melcher & Moyer, 1995; Tabaqchali & Jumaa, 1995; Job & Jacobs, 1997). The infection is typically acquired in hospital. A high proportion of the patients are treated with antibiotics in an environment where *C. difficile* is highly prevalent (Farrell & LaMont, 2000). Especially cephalosporins, ampicillin/amoxicillin, and clindamycin predispose to *C. difficile*-induced enteric disease in humans (Aronsson *et al.*, 1985; Mitty & LaMont, 1994; Spencer, 1998b; Johnson *et al.*, 1999). However, many antibiotics have been associated with predisposition to *C. difficile* infection (Spencer, 1998b; Möllby *et al.*, 1980).

C. difficile disease varies from mild diarrhea, which is the most common form, to life threatening PMC (Jumaa *et al.*, 1996). PMC, when pseudomembranes are formed, may cause development of a paralytic ileus and colonic dilatation which can result in a paradoxical decrease in the diarrhea. Colonic perforation and peritonitis may occur (Tabaqchali & Jumaa, 1995). Recurrent *C. difficile* associated disease, through relapse or reinfection, is not uncommon (Fekety, 1995; Alcantara & Guerrant, 2000).

Infants are very often asymptomatic carriers of *C. difficile*, even with toxigenic isolates (Merida *et al.*, 1986; Tullus *et al.*, 1989). Infection with *C. difficile* is uncommon in this age group. However, it has recently been reported that *C. difficile* was causing disease in infants (Kelly *et al.*, 1994; McGowan & Kader, 1999).

Prevention

To prevent *C. difficile* infections in humans, it is important to institute regulated and directed antibiotic treatment (Spencer, 1998b). Isolation and treatment of infected patients are also important. Thorough handwashing by all staff after contact with patients and their environment is important, as also is daily cleaning to reduce level of environmental spores (Tabaqchali & Jumaa, 1995).

Clostridium difficile in animals other than horses

Dogs

Isolation rates varying between 0% and 40% have been reported in mature dogs (Borriello *et al.*, 1983; Weber *et al.*, 1989; Riley *et al.*, 1991; Martirossian *et al.*, 1992; Perrin *et al.*, 1993b; Struble *et al.*, 1994; Buogo *et al.*, 1995; Al Saif &

Brazier, 1996; Weese *et al.*, 2001b). In most of these investigations, samples were taken from dogs at animal clinics or animal hospitals. In-patients (overnight hospitalization) were found to be at increased risk of carrying the organism (Struble *et al.*, 1994). In some studies the carriage rates were higher when dogs were treated with antibiotics (Martirossian *et al.*, 1992; Riley *et al.*, 1991).

Neonatal puppies may be asymptomatic carriers of *C. difficile,* as the organism was isolated from 46% (Buogo *et al.*, 1995) and 94.3% of the puppies during the first 10 weeks of life and from 42.9% of their dams (Perrin *et al.*, 1993b).

The role of *C. difficile* in diarrheic dogs is not clear. Recently, *C. difficile* toxins A and/or B were demonstrated in diarrheic dogs, but also in a small number of healthy dogs (Weese *et al.*, 2001b). In a report by Berry & Levett (1986) *C. difficile* was suggested to play an etiological role in chronic diarrhea of dogs. Further studies are needed to clarify the significance of *C. difficile* in diarrheic dogs.

Cats

Most studies on *C. difficile* carriage in cats have been performed at veterinary clinics or hospitals. The reported isolation rates were 2–38.1% (Borriello *et al.*, 1983; Weber *et al.*, 1989; Riley *et al.*, 1991; Al Saif & Brazier, 1996; Madewell *et al.*, 1999). Several studies confirm the prescence of *C. difficile* in cats at animal hospitals.

In a study by Madewell *et al.* (1999) *C. difficile* was found in 9.4% of the patients at the hospital, whereas none of the healthy cats examined were carrriers. Interestingly, cats that harboured toxigenic isolates of *C. difficile* were all (except one) treated with antibiotics for various diseases. The significance of *C. difficile* in diarrheic cats remains to be studied.

Other animals

Diarrhea caused by *C. difficile* was first described in hamsters treated with clindamycin (Bartlett *et al.*, 1977). Several animal models, particularly involving hamster, were used to better understand the development of diarrhea in humans. Antibiotic-associated diarrhea caused by *C. difficile* was described in rabbits and guinea pigs (Chang *et al.*, 1978; Fekety *et al.*, 1979; Rehg & Lu, 1981; Rehg & Pakes, 1981; Rothman, 1981). Enteritis due to *C. difficile* has also been reported in rats and mice (Lyerly *et al.*, 1985) and further in captive ostriches (Frazier *et al.*, 1993).

C. difficile has been isolated from faeces of various non-diarrheic animal species such as sheep and poultry (Al Saif & Brazier, 1996), camel and donkey (Hafiz & Oakley, 1976).

Acute colitis of other bacteriological etiology than *C. difficile*

Acute colitis (an inflammation of the colon) in mature horses is often a very severe disease with high mortality. Often the etiology is not clear (Vaughan, 1973; Mair *et al.,* 1990; Staempfli *et al.,* 1991; Murray, 1992; Palmer, 1992; Cohen & Woods, 1999). Various bacterial pathogens have been suggested to be etiological agents.

Salmonella spp.

In many countries, *Salmonella* spp. is the most common cause of infectious colitis (Prescott *et al.,* 1988; Smith, 1991; Murray, 1992). However, in many intensive care veterinary hospitals, *C. difficile* is more frequently isolated from mature horses than is *Salmonella* spp. (Divers, 2002). In Sweden, *Salmonella* spp. rarely causes infections in horses, with 1-5 outbreaks annually over a decade (Eld *et al.,* 1991; Malmqvist *et al.,* 1995).

Ehrlichia risticii

Equine ehrlichial colitis is usually called Potomac horse fever, since the disease was first reported in 1979 along the Potomac River in Maryland, USA. This disease, which is caused by the bacterium *Ehrlichia risticii,* has been confirmed also in Canada and Europe (Divers, 2002). The disease, a monocytic ehrlichiosis, has never been diagnosed in Sweden.

Clostridium spp.

Clostridial diarrhea in mature horses may result from infections with toxigenic isolates of *C. difficile* or *C. perfringens* (Divers, 2002), but even other clostridia have been implicated. *C. septicum* has been isolated from faecal samples from horses with colitis (Jones & Wilson, 1993) and *C. sordellii* from diarrheic foals (Hibbs *et al.,* 1977). Further, *C. cadaveris* was found in horses treated with lincomycin (Staempfli *et al.,* 1992).

Clostridium perfringens

In a doctoral thesis by Wierup (1977) diarrheal disease was reported to be associated with high intestinal counts of *C. perfringens* type A. Acute colitis has also been induced experimentally by *C. perfringens* type A (Ochoa & Kern, 1980). In foals, *C. perfringens* types B, C and D have been associated with severe haemorrhagic enterocolitis (Stubbens, 1990; Traub-Dargatz & Jones, 1993). *C. perfringens* is classified according to the major toxins into five different types (type A-E). Some *C. perfringens* isolates of type A produce an enterotoxin, also a cause of food poisoning in humans. *C. perfringens* enterotoxin was demonstrated in 16% and 19%, respectively, of faecal samples from diarrheic horses (Donaldson & Palmer, 1999; Weese *et al.,* 2001a). Recently, the gene of a novel toxin β_2 produced by certain strains of *C. perfringens* was found together with the gene for toxin α in isolates from horses with typhlocolitis. No β_2-toxigenic *C. perfringens* was found in faecal samples from control horses (Herholz *et al.,* 1999).

Antimicrobial susceptibility

Early studies on the antimicrobial susceptibility of *C. difficile* demonstrated susceptibility to metronidazole, penicillin and ampicillin, whereas all strains were resistant to aminoglycosides (Fekety *et al.,* 1979; George *et al.,* 1979b; Aronsson *et al.,* 1981; Nakamura *et al.,* 1982). Furthermore, strains were highly resistant to cefoxitin and cycloserine (Aronsson *et al.,* 1981; Nakamura *et al.*, 1982), the antibiotics that are widely used in selective agar plates. Susceptibility of *C. difficile* strains to erythromycin, rifampicin, clindamycin, lincomycin, chloramphenicol and tetracycline varied widely, with either very high or low minimum inhibitory concentrations (MIC) (Nakamura *et al.,* 1982; Delmé & Avesani, 1988; Wüst and Hardegger, 1988). In studies by Delmé & Avesani (1988) a correlation was demonstrated between susceptibility profiles and serogroups. Most isolates belonging to serogroup C were resistant to erythromycin, rifampicin, clindamycin, tetracycline and chloramphenicol.

Few antimicrobial susceptibility tests have been performed on *C. difficile* isolates from horses. In the study by Weese *et al.* (2001a) where the Etest was used, all isolates were susceptible to metronidazole (MIC $\leq$1.5 μg/ml) and vancomycin (MIC $\leq$2 μg/ml), the antibiotics used to treat *C. difficile* diarrhea. However, Jang *et al.,* (1997) found metronidazole resistance (MIC >8 μg/ml) in 19% of 105 investigated horse isolates, whereas all isolates had low MICs to vancomycin (MIC $\leq$2 μg/ml).

Aims of the present investigations

In 1992 several animal hospitals in Sweden reported an increased frequency of acute and often fatal colitis affecting mature horses treated for various diseases other than gastrointestinal. The etiology was unknown. *C. difficile* was an interesting possibility as, in human medicine, the organism had for many years been a well-known nosocomial pathogen in antibiotic-associated diarrhea. When work on the present investigations concerning horses began, *C. difficile* was reported to be found only in diarrheic foals but not yet as a pathogen in mature horses.

The overall aim of this work was to study ***C. difficile* and its significance in horses with acute colitis**. The aim can be further specified as studies on:

- the association of *C. difficile* colonization with the occurrence of diarrhea, antibiotic treatment, and age of the horses;
- the impact of oral dosage of erythromycin and rifampicin in mature horses;
- the occurrence and survival of *C. difficile* and its spores in the environment;
- the antimicrobial susceptibility of *C. difficile.*

Comments on Materials and Methods applied

A brief introduction and some additional information to Materials and Methods used in the thesis are presented here. Further details are given in Papers I-V.

Animals (Papers I-IV)

The diseased mature horses and foals in Papers I-II and IV were sampled at three animal hospitals, three large animal clinics and in general practice in order to obtain samples from horses at different animal hospitals and clinics. The mature horses and foals without enteric disorders were sampled at their home stables in the Uppsala region and at the Faculty of Veterinary Medicine. The purpose was to get samples from horses of different breeds and age and from numerous different stables and also horses that were used in different types of sports, e.g. riding, trotting, and for breeding.
For the experimental study reported in Paper III, horses belonging to the Faculty of Veterinary Medicine were used. The study was approved by the Ethical Committee for Animal Experimentation, Uppsala, Sweden.

Faecal samples (Papers I-IV)

The faecal samples were taken from the rectum of mature horses and diseased foals and packed in thick plastic bags or plastic tubes. Excess air was pressed out. Most of the healthy foals were sampled with rectal swabs which were transported in Amies' medium with charcoal (Venturi Transystem, Copan, Brescia, Italy) in order to ensure anaerobic condition during transport.

In Papers I, II and IV the faecal samples were cultured within 48 h and the majority within 24 h. Samples from horses in Paper III were cultured within 4 h (except one sample from each horse, which was processed within 24 h). Samples from horses at the animal hospitals were stored in the refrigerator until forwarding to the laboratory. On arrival at the laboratory, the samples were frozen at –20°C for later cytotoxin B assay.

Environmental samples (Paper IV)

The purpose of obtaining indoor and outdoor environmental samples was to get specimens from different horse environments and also from public places. The indoor surface samples were collected at the Faculty of Veterinary Medicine, on stud farms, and at stables with mature horses and public places. The samples were taken with swabs that were moistened with a NaCl solution and then placed in Amies' medium with charcoal.
The soil samples were taken from paddocks and enclosed pastures at stud farms and stables with mature horses and also from public parks, gardens, cultivated fields and playgrounds.

Bacterial isolates (Papers I-V)

All isolates used in the antimicrobial susceptibility tests in Papers IV and V were isolated from faecal samples from horses further described in Papers I-IV and from environmental sites described in Paper IV.
Reference strains were purchased from the American Type Culture Collection (ATCC). For the quality control of the antibiotic panels, two anaerobic and four aerobic strains recommended by The National Committee for Clinical Laboratory Standards ([NCCLS] 1999, 2001) were used: *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29 741, *Enterococcus faecalis* ATCC 29 212, *Escherichia coli* ATCC 25 922, *Pseudomonas aeruginosa* ATCC 27 853 and *Staphylococcus aureus* ATCC 29 213.

Culture of *Clostridium difficile* (Papers I-IV)

All faecal and environmental samples throughout the study were cultured on a selective agar containing cycloserine, cefoxitin, fructose, egg yolk and taurocholate, TCCFA (George *et al.,* 1979a; Wilson *et al.,* 1982). Early in the studies the antibiotic concentrations of cycloserine and cefoxitin were 500 µg/ml and 16 µg/ml respectively. Later in the study and for the majority of samples, the concentrations of the antibiotics were reduced to 250 and 8 µg/ml for cycloserine and cefoxitin, respectively (Levett, 1985; Brazier, 1993).

In Papers I and II faeces were also inoculated onto *Clostridium difficile* (CD) agar. The composition of the medium was the same as for TCCFA except that taurocholate was not included and the egg yolk emulsion was replaced with blood. The *C. difficile* colonies appeared yellow on the TCCFA plates and were therefore easier to find. The CD agar was later excluded because *C. difficile* was not isolated more often on CD agar than on TCCFA agar.

At the beginning of the studies, faeces were also inoculated into an enrichment broth; BHI broth 37.0 g/l, supplemented with yeast extract 5.0 g/l, Resazurin solution 4.0 ml/l, Bacto agar 0.5 g/l, L-cysteine HCl 0.5 g/l and vitamin K1-haemin solution 10.0 ml/l. and incubated for 48 h at 37°C. The broth was then inoculated onto TCCFA and CD plates. The isolation frequency was no higher after cultivation in enrichment broth than with primary isolation on selective agar, possibly due to absence of selective enrichment. The broth was therefore not included further in the investigation.

All incubations were performed in an anaerobic chamber or anaerobic jars at 37°C for 48 and 96 h. The environmental samples were incubated for 5 days according to Al Saif & Brazier (1996).

Identification of *Clostridium difficile* (Papers I-IV)

All isolates were typed by the following tests: characteristic smell from colonies of horse odour, colony morphology, Gram stain, biochemical tests and gas-liquid chromatography. In order to obtain material for the identification, all isolates were

subcultured on fastidious anaerobe agar (FAA) (LabM, Bury, Lancashire, England) with 5% defibrinated horse blood. Biochemical tests and gas-liquid chromatography were performed from prereduced anaerobically sterilized medium (Holdeman *et al.*, 1977) in Papers I-III and from fastidious anaerobe broth (FAB) (LabM, Bury, Lancashire, England) in Paper IV due to new routines at the anaerobic diagnostic laboratory.

Biochemical tests (Papers I-IV)

The biochemical tests used in the routine diagnostics of anaerobic bacteria at our laboratory were used to identify *C. difficile.* The following biochemical tests were applied to all isolates: esculin, fructose, glucose, lactose, maltose, mannitol, sucrose, indole, nitrate, starch and urea.

Gas-liquid chromatography (Papers I-IV)

Gas-liquid chromatography is a well-established method for typing of anaerobic bacteria. The bacteria produce short-chain fatty acids as a result of carbohydrate metabolism (Holdeman *et al.* 1977). The volatile fatty acids are analysed. An ether extract is prepared from a culture and the gas-liquid chromatography was performed according to Holdeman *et al.* (1977) with ether.

Storing of isolates

All isolates were stored at –70°C.

Clostridium difficile cytotoxin B assay (Papers I-IV)

A cytotoxin B assay was performed on faecal specimens. A suspension of the faecal sample was made in phosphate-buffered saline. After centrifugation, the supernatant was passed through a filter and the filtrate was inoculated onto the cell layer. After incubation at 37°C the cells were examined by microscopy for cytopathic effects after 4 and 18 h. Later in the study the cells were also examined after 2 days. Positive toxin B samples were confirmed by neutralization with *C. difficile* antitoxin B.

Early in the studies the test was performed at the Karolinska Institute, Stockholm and human embryonal intestinal cells, ATCC CCL 6 (HEIC) were used. Later, and for the majority of samples, the test was performed at the National Veterinary Institute with human diploid lung fibroblast cells (ECACC, European collection of animal cell cultures 84101801 [MRC-5]). First the cells were obtained from the Department of Clinical Microbiology, University Hospital, Uppsala and later produced at the Department of Bacteriology, National Veterinary Institute. According to Delmée (2001) almost every cell line used in clinical microbiology laboratories can be used to detect faecal *C. difficile* cytotoxin.

In vitro amplification of toxin A and B gene fragments by PCR (Paper IV)

Faecal samples from healthy foals could not be tested for cytotoxin B as the amounts of faeces were too small and the environmental samples most probably contain spores with no growth of *C. difficile* and thus would not produce toxins. The isolates from foals and environment were therefore tested for possession of toxin A and B genes. A duplex PCR system was designed to amplify fragments of a 1217-bp toxin A gene and a 1050-bp toxin B gene, according to McMillin *et al.* (1992), with some modifications further described in Paper IV.

Anaerobic culture (Papers I-III)

Anaerobic cultures for anaerobic bacteria other than *C. difficile* were performed from faecal samples from diarrheic horses on FAA plates with 5% defibrinated horse blood according to Holdeman *et al.* (1977). The plates were read for growth of *Clostridium* spp. other than *C. difficile* with special attention to *C. perfringens*. To isolate *C. perfringens* an encrichment technique was also used after a spore selection procedure (30 min at 65°C) but only as a complement when it was difficult to recover from the primary isolation. Typical colonies of *C. perfringens* were identified by colony morphology with a characteristic haemolysis, Gram stain and positive lecithinase test. As *C. perfringens* has been associated with diarrhea in horses (Wierup, 1977; Ochoa & Kern, 1980; Donaldson & Palmer, 1999; Weese *et al.*, 2001a; Herholz *et al.*, 1999) it was important to culture for this organism.

Bacteriological examination of faecal flora (Papers I-III)

A bacteriological examination of the faecal flora of diarrheic horses in Papers I-III was made according to Wierup (1977) and Wierup & DiPietro (1981). Faeces had to be cultured within 4 h of collection according to the method (Wierup & DiPietro, 1981). Counts of colony forming units (CFU) per gram faeces of lecithinase-positive clostridia, coliform bacteria, *Bacillus* spp. and moulds were performed. The pH was also measured. Due to practical circumstances a bacteriological examination was not performed on all diarrheic faecal samples. This investigation has commonly been performed in Sweden on faecal samples from horses with intestinal disorders. Wierup (1977) demonstrated that acute diarrhea was associated with high counts (up to 10^7 CFU/g faeces) of lecithinase-positive clostridia. Divers (2002) suggested $>10^5$ CFU/g faeces if *C. perfringens* is to be blamed as the causal agent of the diarrhea.

Antimicrobial susceptibility of *Clostridium difficile* (Papers II-V)

The method recommended by the NCCLS (2001) for susceptibility testing of anaerobes is agar dilution. However, this method is tedious and inconvenient to perform in routine laboratories, especially for a small number of isolates. Recently, broth microdilution was recommended for susceptibility testing of the *Bacteroides fragilis* group as an alternative to agar dilution (NCCLS, 2001).

Etest (Paper II-IV)

The Etest (AB Biodisk, Solna, Sweden) is a commercially available agar diffusion susceptibility test, which was found to be a convenient and simple method to determine MIC of certain antimicrobial agents for *C. difficile.* The method is not approved by the NCCLS but is frequently used in clinical laboratories. Plastic Etest strips are coated with a gradient of an antimicrobial agent. The test was performed on prereduced Wilkins Chalgren agar with 5% defibrinated horse blood. A suspension of each *C. difficile* isolate was streaked on the agar plate and the strip was applied to the surface of the plate according to the manufacturer's instruction. The plates were incubated at 37°C in anaerobic atmosphere for 2 days. The MIC value was read at the point of intersection between the inhibition ellipse edge and the Etest strip. Two quality control strains of *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29 741 were included in each test run. The purity of each suspension was checked in an anaerobic atmosphere and an anaerobic indicator strip (Oxoid Limited, Basingstoke, Hampshire, England) was used to check the anaerobic atmosphere.

Broth microdilution (Paper V)

Commercially available 96-well panels for monitoring of antibiotic resistance of Gram-positive bacteria were used (VetMIC™, National Veterinary Institute, Uppsala, Sweden). The panels had small volumes of antimicrobial agents dried in two-fold dilutions. Each well was inoculated with a suspension of the isolate. The panels were incubated for 2 days at 37°C in anaerobic atmosphere. The MIC was determined as the lowest concentration where no visible growth, or the most significant reduction of growth, was observed.

Before the study, various broths were tested for growth of *C. difficile,* such as: FAB (LabM, Bury, Lancashire, England), BHI broth, BHI broth supplemented with 10% fetal calf serum, BHI supplemented with 10% horse serum, Haemophilus Test Medium Broth, Mueller Hinton broth and BHI broth 37.0 g/l, supplemented with yeast extract 5.0 g/l, Resazurin solution 4.0 ml/l, L-cysteine HCl 0.5 g/l and vitamin K1-haemin solution 10.0 ml/l. For our study we chose BHI, supplemented with 10% fetal calf serum, which supported growth well and gave correct MICs for the reference strains.

In their recommendations of 1997, the NCCLS reported that several broth media had been used successfully. However, in their latest version (NCCLS, 2001), supplemented Brucella broth was reported to be optimal for growth of most anaerobic bacteria when prepared aerobically, but incubated anaerobically. As our study was performed earlier, we did not test this recently recommended medium.

When performing susceptibility testings it is very important to conduct quality controls to ascertain if the method is reliable. In this study the following quality control measures were performed: two anaerobic reference strains were included in each test run, the purity of each suspension was checked in aerobic and anaerobic atmospheres and an anaerobic indicator strip (Oxoid Limited, Basingstoke, Hampshire, England) was placed in each box. Furthermore, in each panel there were two wells without antimicrobial agents containing dried dilution

buffers, used as growth checks and the inoculum density (1 x 10^8 CFU/ml) was checked by viable counts.

At first, one reference strain, *C. difficile* ATCC 9689 was included. Despite several tests, this reference strain did not grow well in the control wells and was therefore excluded from the investigation. For future studies it is important to include a reference strain.

Results and Discussion (Papers I-V)

Clostridium difficile in mature horses (Papers I, IV)

It was shown that *C. difficile* was associated with acute colitis in mature horses that were treated with antibiotics for other diseases than gastrointestinal. *C. difficile* was also reported by others to be associated with diarrhea in horses treated with antibiotics (Beier *et al.*, 1994; Cosmetatos *et al.*, 1994; Madewell *et al.*, 1995; Magdesian *et al.*, 1997). However, some reports did not state whether or not the horses had been treated with antibiotics (Jang *et al.*, 1997; Donaldson & Palmer, 1999; Weese *et al.*, 2001a).

Most of horses proving positive for *C. difficile* had been treated with β-lactam antibiotics, either alone or often in combination with other antibiotics. The high representation of β-lactam antibiotics may be attributed to the fact that penicillin is the most frequently used antibiotic for horses in Sweden. In humans, most of the currently available antibiotics have been implicated in *C. difficile* diarrhea (Worsley, 1998). In horses, the antibiotics that in particular have been associated with *C. difficile* diarrhea are β-lactam antibiotics, erythromycin, gentamicin and trimethoprim/sulfonamides (Madewell *et al.*, 1995; Magdesian *et al.*, 1997; Divers, 2002). Other antibiotics associated with diarrhea in horses are lincomycin (Raisbeck *et al.*, 1981), tetracyclines (Andersson *et al.*, 1971), ceftiofur and metronidazole (Magdesian *et al.*, 1997).

It was noteworthy that β-lactam antibiotics can lead to overgrowth of *C. difficile* in the colon despite the susceptibility of this organism to these antibiotics *in vitro*. The concentrations of antibiotics in the intestinal lumen generally are not known for horses. Most probably the concentration of penicillin in the colon is too low to inhibit growth.

In humans, the multiple use of antibiotics increases the risk of *C. difficile* infection (Gerding *et al.*, 1986). In horses, it would be interesting to study if horses treated with a combination of antibiotics run a greater risk of developing *C. difficile* diarrhea than horses treated with a single antibiotic.
Other factors than antibiotic treatment may also be of importance and may have a secondary influence on the colonic bacterial flora. Hospitalized horses are often exposed to several stress factors such as transportation to the animal hospital, the hospitalization itself, presurgery fasting, surgery and medical treatment.

In Paper IV *C. difficile* was also isolated from a few (4/72) diarrheic horses not treated with antibiotics. *C. difficile* has been isolated from untreated diarrheic horses by others, too (Beier *et al.*, 1994; Cosmetatos *et al.*, 1994; Magdesian *et al.*, 1997). *C. difficile* was not found in faeces of any healthy mature horses, from non-diarrheic hospitalized horses treated with antibiotics nor from untreated horses with colic.

Two horses were culture negative but cytotoxin B positive. Weese *et al.* (2000b) demonstrated that lack of anaerobic storage can reduce the incidence of positive

cultures. If there are no spores in a sample it may be difficult for the organism to survive.

In humans, *C. difficile* is a nosocomial pathogen. In horses too, *C. difficile* infection may be nosocomial (Cosmetatos *et al.,* 1994; Madewell *et al.,* 1995). In paper I, 8 of 10 and in Paper IV, 12 of 18 *C. difficile* positive horses were or had recently been hospitalized.

In the present study, *C. perfringens* was not demonstrated to be involved in the etiology of antibiotic-associated colitis. In paper I, 4 diarrheic horses, not treated with antibiotics, had high numbers of lecithinase-positive clostridia other than *C. difficile.* Demonstration of toxins in the faecal samples is recommended for future studies. No other investigated bacterial pathogen was found in horses with antibiotic-associated diarrhea, except that *Salmonella* typhimurium was isolated from one horse (Paper IV). However, in the majority of cases of antibiotic associated colitis, the etiology still remains unclear. In humans, about 20-25% of antibiotic-associated diarrhea cases are caused by *C. difficile* (Möllby *et al.,* 1985; Lyerly *et al.,* 1988; Bartlett, 1990; McFarland, 1990; Fekety, 1995). In Paper IV, cytotoxin B was demonstrated in 28% of the faecal samples from horses with antibiotic-associated diarrhea.

Clostridium difficile associated with acute colitis in mares when their foals are treated with erythromycin and rifampicin for *Rhodococcus equi* pneumonia (Paper II)

In 1992, acute colitis was reported in previously healthy mares when their foals were treated orally with erythromycin in combination with rifampicin for *Rhodococcus equi* pneumonia. A few mares developed sudden and sometimes fatal diarrhea often after 3-4 days' treatment of their foals at an animal hospital or rarely at a stud farm. The problem had earlier been observed in USA by Wilson (1992) but the etiology had not been established.

One theory was that accidental ingestion of an antibiotic by the mares from the treatment of their foals might have disturbed the intestinal flora and resulted in acute diarrhea. Two different erythromycin formulas were used in Sweden for treatment of *R. equi* infection in foals. Erythromycin base in tablet form was used at one animal hospital and erythromycin ethylsuccinate in oral suspension was used at the other animal hospitals. Both formulas caused acute colitis in the mares. Wilson (1992) reported that erythromycin base was used at their American clinic.

In the present study it was demonstrated that faeces from foals whose dams had acute colitis also had high concentrations of erythromycin, while faeces from foals whose dams remained healthy had low concentrations of erythromycin. There must have been an accidental ingestion of erythromycin by the mares from the faeces of the foals and/or treatment of the foals. Materials in the foal's environment may have been contaminated with erythromycin. The problem of colitis in mares was considered very serious and within a year, the Swedish

animal hospitals included in the study replaced erythromycin by gentamicin for treatment of *R. equi* infection. Since this change in treatment regime, the number of cases of fatal colitis in these mares is now virtually nil. The number of foals investigated in the study was therefore low. Foals treated with gentamicin in combination with rifampicin were also included.

C. difficile or its cytotoxin was demonstrated in faecal samples of 45% of the diarrheic mares. Probably due to accidental ingestion of antibiotics by the mares, their gastrointestinal flora became disturbed, which may have enhanced growth, colonization and toxin production by *C. difficile.* As foals treated with erythromycin combined with rifampicin were often asymptomatic carriers of *C. difficile,* they may have served as a potential reservoir. Some mares may also have carried the organism in such low numbers that it was not detectable by routine methods. Another source of infection could be the environment of the animal hospital. In faecal samples of mares that remained healthy, *C. difficile* was not demonstrated even though their foals often were asymptomatic carriers. It seems as if certain concentration levels of erythromycin in the intestinal lumen are required as a predisposing factor. The etiology of the other 55% of the diseased mares still remains unclear.

Faecal samples from 3 mares proved culture positive, but cytotoxin B negative. To prove that *C. difficile* is the cause of infection, toxin needs to be demonstrated in the faecal sample (Weese *et al.,* 2001a). Due to transportation and/or storage of the samples, the cytotoxin may have become inactivated or present at an undetectably low concentration (McFarland & Stamm 1986).

Association of erythromycin with acute colitis (Paper III)

An experimental study, approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden, was performed. The purpose was to establish whether low oral doses of erythromycin and rifampicin could induce acute colitis in mature horses. To simulate an accidental intake by the mare, a very small dose was given at the same intervals as to foals for treatment. Erythromycin or rifampicin was given alone or in combination. Erythromycin has been described as a risk antibiotic by others (Burrows, 1980; Whitlock, 1986; Roberts, 1990; Murray, 1992; Prescott & Baggot, 1993).

Of 4 horses treated with erythromycin, alone or in combination with rifampicin, 2 developed severe colitis which was fatal in one. One of the diarrheic horses proved positive for *C. difficile* both in culture and in the cytotoxin B test. From the other diarrheic horse, no investigated pathogen was found. Furthermore, *C. perfringens* was isolated in very high numbers (2×10^5 CFU/g faeces) on several consecutive days from the faecal samples of one horse treated with erythromycin only. This horse had loose and foul-smelling faeces. Neither the faecal samples nor the isolates were tested for *C. perfringens* toxins. *C. difficile* and its cytotoxin B were also demonstrated in the fourth horse given 1/10 (0.125 mg/kg) of the

dose given to the horses that developed acute colitis. This horse did not develop diarrhea but had signs of anorexia and was uneasy.

It was amazing that such small amounts of erythromycin (1.25 mg/kg x 3) could induce acute colitis in mature horses. Foals are usually treated with erythromycin 25 mg/kg x 3 in combination with rifampicin 5 mg/kg x 2 for *R. equi* pneumonia. None of the horses treated with rifampicin only developed colitis. Nor was there any detectable change in their intestinal flora. Rifampicin has not been associated with diarrhea (Prescott & Baggot, 1993).

An important finding was that cytotoxin B was demonstrated one day later than the positive cultivation of faecal samples from both *C. difficile* positive horses. Consequently it is important to take samples on consecutive days from horses with *C. difficile* diarrhea.

Clostridium difficile in foals (Paper IV)

An interesting new finding was that *C. difficile* was isolated from 29% of healthy foals, 1-13 days old, whereas all older foals except one proved negative. The foals were asymptomatic carriers as has also been observed in puppies and human infants (Merida *et al.*, 1986; Tullus *et al.*, 1989; Perrin *et al.*, 1993b). A high frequency of asymptomatic carriers was also found in non-diarrheic older foals treated with antibiotics. Cytotoxin B was demonstrated in faecal samples of foals treated with antibiotics. Healthy foals could not be tested for cytotoxin B as the faecal samples were too small. Instead, the isolates were tested for toxin A and B genes. Forty percent of the isolates of healthy foals had both toxin A and B genes. These isolates can serve as a reservoir of toxigenic *C. difficile.*

In studies of outbreaks of diarrhea, *C. difficile* has been associated with diarrhea in young foals (Jones *et al.*, 1987; 1988b; Magdesian *et al.*, 1999). Weese *et al.* (2001a) also found *C. difficile* in faecal samples from diarrheic foals. However, the ages of these foals were not reported (Weese *et al.*, 2001a). In this thesis, a high frequency of asymptomatic carriers of *C. difficile* was found both in healthy foals younger than 14 days and in non-diarrhoiec older foals treated with antibiotics. In the present investigation, the common occurrence of *C. difficile* is not associated with disease. For further studies, it is important to know the ages of diarrheic and healthy foals and whether the foals were treated with antibiotics to clarify its role in diarrheic disease.

Occurrence and survival of *Clostridium difficile* in the environment (Paper IV)

C. difficile was more commonly isolated from soil samples originating from stud farms than from farms with mature horses. This is consistent with the high carrier rate in foals in comparison with mature horses. The positive samples were from paddocks where the youngest foals were kept together with their dams and also from outside the stable doors. The positive samples were taken both in the spring

and in the autumn. As demonstrated by PCR, some isolates had toxin A and B genes.

At the Veterinary Faculty only 3% of environmental samples were positive. Other studies reported a higher isolation frequency (Riley *et al.*, 1991; Al Saif & Brazier, 1996; Weese *et al.*, 2000a). According to studies in human hospitals it is very difficult to get rid of spores. As there had been horses with *C. difficile* diarrhea at the clinic it was expected to find more positive samples. One reason for the low isolation rate could be the good cleaning regime.

Survival of a sporulated isolate of *C. difficile* in equine faeces was studied experimentally. The spores survived for at least 4 years in nature at ambient temperature and in a jar at room temperature under aerobic conditions. Further testing was not possible prior to the publishing of this thesis.

Antimicrobial susceptibility of *Clostridium difficile* (Papers IV,V)

Etest (Paper IV)

Antimicrobial susceptibility testing of *C. difficile* isolates from horses has been performed mainly by Etest (AB Biodisk, Solna, Sweden) (Jang *et al.*, 1997; Weese *et al.*, 2001a; Paper II; III; IV) and in one study by agar dilution (Jang *et al.*, 1997).

The Etest is simple to perform. However, it was sometimes difficult to read the MICs due to sparse growth. The MIC endpoints of clindamycin and tetracycline for some isolates could not be determined which is why these antibiotics were excluded from the study. The Etest could be a useful tool for the clinician as it is possible in a short time to obtain information as to whether a clinical isolate is susceptible or resistant. In comparison with agar dilution, the MICs of a number of antimicrobial agents for *C. difficile* are reliable, although MICs of metronidazole are often underestimated by the Etest (Rosenblatt & Gustafson, 1995; Barbut *et al.*, 1999; Poilane *et al.*, 2000).

Fourteen of 52 isolates were uniformly resistant to erythromycin (MIC >256 µg/ml) and rifampicin (MIC >256 µg/ml). These isolates were from faecal samples from horses at two animal hospitals. At one hospital, three isolates showed this resistance pattern. These were isolated from horses that had been treated with erythromycin combined with rifampicin. The isolates with low MICs (n=10) were isolated from horses treated with other antibiotics. At the other animal hospital all ten isolates proved resistant to erythromycin and rifampicin irrespective of which antimicrobial agent was used. Most probably, treatment with these antibiotics selected for the spread of this resistance pattern, especially as erythromycin-resistance genes have been shown to reside on highly mobile genetic elements in *C. difficile* (Mullany *et al.*, 1995).

All isolates included in the study were susceptible to metronidazole and vancomycin. In human medicine, metronidazole is the preferred drug for treatment of *C. difficile* infections. However, metronidazole-resistant isolates have been found in humans and in American horses (Kelly *et al.*, 1994; Jang *et al.*,

1997). Studies by Norén *et al.* (1998) and Svenungsson *et al.* (2001) have also demonstrated susceptibility to metronidazole and vancomycin of most Swedish human isolates.

Broth microdilution (Paper V)

Antimicrobial susceptibility testing of equine and environmental *C. difficile* isolates was performed by broth microdilution. All 50 isolates were tested in duplicate, showing close agreement between tests done in duplicate. For all antimicrobial agents, the MICs did not differ by more than 2 twofold dilutions and for most isolates the MICs were within one twofold dilution.

In the panel there was only one antimicrobial agent, clindamycin, with accepted ranges according to NCCLS (2001). In all tests the MICs of clindamycin for the quality control strains were within recommended ranges (NCCLS, 2001). The MICs of the other antimicrobial agents for the quality control strains were also reproducible in all tests. Thus it was concluded that the test method was reliable. The broth microdilution test was easy to perform and the MIC endpoints were easily read. Furthermore, many antimicrobial agents could be tested in the same panel.

The MICs of erythromycin, oxytetracycline, spiramycin and virginiamycin showed a biphasic distribution. Eighteen isolates had uniformly higher MICs of erythromycin, oxytetracycline, spiramycin and virginiamycin than the other isolates. Interestingly, the isolates with high MICs of erythromycin, spiramycin, virginamycin and tetracycline were mainly isolated from horses at animal hospitals and further from environmental samples at a stud farm. On the other hand, all isolates, except one, from healthy foals and from the soil in public parks had low MICs of these antimicrobial agents.

The elevated MICs of erythromycin and spiramycin may have been due to possession of an *erm* gene. Recently, the macrolide–lincosamide–streptogramin B (MLS_B) resistance determinant from *C. difficile* strain 630 was reported to contain *erm* B genes (Farrow *et al.,* 2000). The resistance to erythromycin, oxytetracycline, spiramycin and virginiamycin seemed to be linked, possibly because of localization on the same genetic element. The transfer of an MLS resistance gene, mediated by a conjugative transposon Tn*5398* from *C. difficile,* between *C. difficile* strains and also to and from *Bacillus subtilis,* was reported by Mullany *et al.* (1995). The high mobility of this or a similar transposon, taken together with the impact of the selection pressure from the treatment of foals with erythromycin, may explain the common occurrence of this resistance pattern described above.

The isolates in this study with high MICs of erythromycin, spiramycin and virginiamycin also had high MICs of oxytetracycline and chloramphenicol, an association also described by Delmée & Avesani (1988) and Mullany *et al.* (1995). Furthermore, Delmée and Avesani (1988) found this resistance pattern in toxigenic strains belonging to serogroup C.

Different MICs of trimethoprim/sulfamethoxazole (TMP) for the same *C. difficile* isolates were obtained in Papers IV and V. All isolates analysed by the Etest had high MICs (>32 μg/ml) of TMP, whereas in broth microdilution the MICs for the majority of isolates were lower (1–4 μg/ml) and for five isolates above the concentration range tested (>4 μg/ml). The MICs in broth microdilution had a normal distribution and seemed more reliable than the MICs in the Etest study. The problem is further discussed in Paper V.

In the Etest, Wilkins Chalgren agar with 5% defibrinated horse blood was used. Falsely resistant results of TMP may occur due to excessive amounts of thymine or thymidine and various folates in the media (Indiveri & Hirsch, 1991). Addition of thymidine-phosphorylase or lysed horse blood which contains thymidine-phosphorylase, may improve the results (NCCLS, 1999; Prescott, 2000). The *C. difficile* type strain ATCC 9689 was tested by Etest with Wilkins Chalgren agar supplemented with 5% defibrinated horse blood and with 5% lysed horse blood, respectively. At initial testing, elevated levels of MICs of TMP were obtained with both defibrinated and lysed blood. The reason(s) for the discrepant results reported when determining MICs of TMP need to be further investigated.

General summary

The work described in this thesis is a contribution to increased knowledge of *C. difficile* as an etiological factor in antibiotic-associated diarrhea in horses.

It has been shown that:

- *C. difficile* is associated with acute colitis in mature horses, following treatment with antibiotics;
- most of the horses positive for *C. difficile* were treated with β-lactam antibiotics, alone or often in combination with other antibiotics;
- *C. difficile* and/or its cytotoxin is associated with acute colitis in mares when their foals are being treated with erythromycin and rifampicin for *R. equi* infection;
- the colitis in the mares is most likely due to accidental ingestion of erythromycin by the mares.
- in an experimental study, it was demonstrated that low oral intake of erythromycin can induce severe colitis in horses, associated with major changes of the intestinal microflora;
- there is a high frequency of *C. difficile* carriers among healthy neonatal foals and in non-diarrheic older foals treated with antibiotics;
- *C. difficile* was more common in soil samples from stud farms than from farms with mature horses;
- the presence of toxin A and B genes in isolates of *C. difficile*, as demonstrated by PCR, in both healthy foals and the environment, shows that they can serve as potential reservoirs of toxigenic *C. difficile*;
- the bacteria survived for at least 4 years in nature;
- antimicrobial susceptibility testing demonstrated that isolates were susceptible to metronidazole (MIC ≤4 μg/ml) and vancomycin (MIC ≤1 μg/ml);
- the MICs of erythromycin, oxytetracycline, spiramycin and virginiamycin had a biphasic distribution. All isolates, except three, had either uniformly high or low MICs of these antimicrobial agents;
- routine examination for *C. difficile* and its cytotoxin are recommended in mature horses when diarrhea occurs in combination with antibiotic treatment and further in mares with acute colitis when their foals are treated orally with erythromycin and rifampicin;

- preventive measures to avoid accidental ingestion of erythromycin by mares from the treatment of their foals are recommended;
- the etiology of the main part of acute colitis remains unclear.

Suggestions for future studies

- Study experimental infection of horses with a clinical toxigenic isolate of *C. difficile* to ascertain if diarrhea can be induced by this challenge.
- Evaluate rapid immunoassays for detection of *C. difficile* toxins in faecal samples from horses with diarrhea.
- Conduct epidemiological studies on isolates by molecular fingerprinting methods, e.g. PFGE.
- Study possible variation in subtypes of *C. difficile* in individual horses by molecular methods.
- Study pathological changes at necropsy in the intestine of horses with *C. difficile* infection.
- Conduct further study of healthy young foals that are asymptomatic carriers of *C. difficile,* to establish if they can develop diarrhea later.
- Investigate faecal samples from *C. difficile*-positive foals for cytotoxin B.
- Identify and characterize antimicrobial resistance genes in *C. difficile* and their potential linkage with toxin genes.

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