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PRESS

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CONTENTS

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III Naaber P., Klaus K., Sepp E., Björksten B., Mikelsaar M. Colonization of infants and hospitalized patients with *Clostridium difficile* and lactobacilli. Clinical Infectious Diseases. 1997; 25; Suppl 2 (In press).

IV Naaber P., Mikelsaar M. Antibiotic-compromised murine model of *Clostridium difficile* infection. Microecology and Therapy. 1995; 25: 201–205.

V Naaber P., Salminen S., Mikelsaar M. Bacterial translocation, intestinal microflora and morphological changes in intestinal mucosa in experimental models of *Clostridium difficile* infection. Submitted to Journal of Medical Microbiology.

VI Naaber P., Maimets M., Mikelsaar M. *Clostridium difficile* caused diarrhoea: I. Etiology, pathogenesis and clinical picture. Eesti Arst. 1994; 2: 122–124 (in Estonian).

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ABBREVIATIONS

AAD	antibiotic-associated diarrhoea
CCFA	cefoxitin-cycloserine-fructose agar
CD	*Clostridium difficile*
CDCD	*Clostridium difficile* caused diarrhoea
CDI	*Clostridium difficile* infections (includes CD caused diseases ranged from mild diarrhoea to pseudomembranous colitis)
CDT	*Clostridium difficile* latex test
CFU/g	colony forming units per gram
CNA agar	colistin-nalidixic acid agar
CR	colonisation resistance
FAA	fastidious anaerobe agar
FHEL	facultatively heterofermentative lactobacilli
GG or *Lactobacillus* GG	*Lactobacillus rhamnosus* strain GG (ATCC 53103)
GLC	gas-liquid chromatography
ICU	intensive care unit
IMF	indigenous microflora
M/F	male/female ratio
MIC	minimum inhibitory concentration
MLN	mesenteric lymph nodes
MRS medium	de Man-Rogosa-Sharpe medium
NCCLS	The National Committee for Clinical Laboratory Standards (USA)
OHEL	obligately heterofermentative lactobacilli
OHOL	obligately homofermentative lactobacilli
PCR	polymerase chain reaction
PMC	pseudomembranous colitis
PPMO	potentially pathogenic microorganism
QCIM	quantitative composition of intestinal microflora
SCFA	short-chain fatty acid

1. INTRODUCTION

Clostridium difficile is recognised as a major etiological agent of pseudomembranous colitis and antibiotic-associated diarrhoea (Bartlett & Gorbach, 1977; Bartlett, 1994). *C. difficile* infection develops when the stability of intestinal indigenous (normal) microflora has been disrupted and colonisation resistance decreased, mainly due to a recent antimicrobial treatment (Wilson, 1993).

Although *C. difficile* was first isolated more than 60 years ago its real role and importance in medicine was obscure until 80's. During the past decade the number of studies associated with *C. difficile* and microbial ecology has remarkably increased. According to Medline there were published nearly 500 papers about *C. difficile* during the past 5 years. At the same time there could be observed also a high interest in rapid diagnosis and effective therapy of *C. difficile* infection among clinicians. Despite the international attention, *C. difficile* and infection caused by it is quite a new topic in Estonia as microbiological diagnostics of *C. difficile* was not available until the end of 1991. Therefore only little is known about its real epidemiological situation in Estonia so far.

Today *C. difficile* is considered the principal cause of nosocomial diarrhoea, associated with prolonged stay at hospital, increased mortality and additional expenses (Riley, 1996). For example, recent studies in England have shown 4000 pounds (≈ 87 200 EEK) excess cost *per* one *C. difficile* infection case in hospital (Wilcox *et al.*, 1996). *C. difficile* infection is most frequent among elderly antibiotic treated and hospitalised patients. Due to increasing usage of antibiotics and expected demographic changes towards increasing numbers of elderly patients, one can predict also the increase in prevalence of *C. difficile* infection in the future (Riley, 1996). Considering that, there is an urgent need for effective prophylaxis and treatment of *C. difficile* infection.

Vancomycin and metronidazole are still the most widely used and effective medicines for treating of *C. difficile* infection. Unfortunately, this kind of therapy is expensive and frequently associated with relapses (Wilcox & Spencer, 1992). Since antimicrobial agents cause alteration in normal microflora and evoke *C. difficile* infection, the irony of using antibiotics to treat this infection is apparent. Furthermore, recent trends indicating an explosion of antibacterial resistance among common pathogens have raised the concern that we may be approaching the post-antimicrobial era characterised by increased importance of alternative strategies for the treatment of infectious diseases (Mitchell, 1992; Amibile-Cuevas *et al.*, 1995). Therefore, the application of therapy that could

restore host resistance by normalising indigenous microflora and thus eliminating the possibility of *C. difficile* establishing in intestinal tract and cause diarrhoea seems more natural (Borriello, 1990a). Unfortunately, several essential questions remain unsolved: are there particular indigenous microorganisms associated with resistance against *C. difficile*; by what mechanism do they operate; is it possible to influence their population level in the intestinal tract?

Usage of probiotics is one of the best examples of microecological approach in the treatment of *C. difficile* infection. Beneficial effect of lactic acid bacteria on human health and gastrointestinal ecosystem was described by Metchnikoff almost a century ago, and several recent studies have shown their usefulness in case of intestinal disorders, including relapsing *C. difficile* infection (Elmer *et al.*, 1996). However, there still exists an insufficient theoretical basis for this kind of therapy. Due to the extremely complicated nature of intestinal microecosystem it is difficult to understand the effects and operative mechanisms of different biotherapeutical agents. New studies in this field are necessary for assessing the basic mechanisms, as well as for practical application of biotherapeutics.

Evidently, there is an urgent need for a new experimental approach to solve the pathophysiology of microbial ecosystem components and *C. difficile* interactions. The evaluation of human microbial ecosystems in health and disease has been the main goal of investigations in Institute of Microbiology of Tartu University for many years (Mikelsaar, 1992; Sepp, 1994; Mändar 1996). There are old traditions and good experience in the field of examination of lactoflora in Tartu (Uibu, 1972; Lenzner, 1973; Mikelsaar & Mändar, 1993; Karki, 1996). The choice for studying *C. difficile* infection in association with intestinal microecosystem was made thanks to these traditions and experience on the one hand, and the urgent practical needs for introduction of *C. difficile* laboratory diagnostics, on the other hand. So far there have been no data about colonisation by *C. difficile* among hospitalised patients and other persons in Estonia.

In our work special attention has been given to investigation of microecological changes in intestinal tract during *C. difficile* infection. Apart from this, we have also assessed the colonisation by *C. difficile* in some groups of patients and healthy persons, evaluated properties of different strains of *C. difficile* and looked for possible influence of intestinal lactobacilli on the colonisation of *C. difficile*. Our aim was also to elucidate some pathophysiological changes in mucosa and bacterial translocation in experimental *C. difficile* infection and to test some possibilities of preventing *C. difficile* infection.

2. REVIEW OF THE LITERATURE

2.1. *Clostridium difficile*, historical overview

Clostridium difficile (CD) is a gram-positive spore-forming obligate anaerobe (4–8 x 0.5–1 μm, rod-shaped with subterminal or terminal non-bulging oval spores) belonging to genus *Clostridium*. The organism was first isolated by Hall and O'Toole in 1935 from the faeces of infant and designated as *Bacillus difficilis* (Knoop *et al.*, 1993). Although pseudomembranous colitis (PMC) was described more than a century ago, it began emerging in 1960s due to increasing use of new antibiotics and particularly after introduction of clindamycin (Pothoulakis *et al.*, 1993). The link between PMC and CD was made at the end of 1970s when Larson showed the cytotoxic activity of PMC patients' faecal filtrate on cell culture (Larson *et al.*, 1977). Afterwards CD was isolated from intestinal tract of PMC patients and clindamycin treated animals (Bartlett *et al.*, 1977; Bartlett & Gorbach, 1977; George *et al.*, 1978; Larson *et al.*, 1978).

According to the current state of knowledge, suppression of indigenous intestinal microflora due to administration of antibiotics, subsequent colonisation of intestinal tract and production of toxins A and B by CD are the most important factors for development of intestinal infection (Bartlett, 1994). *Clostridium difficile* infection (CDI) is almost always restricted to intestinal tract and only in rare cases CD has been isolated from extraintestinal mixed infections (Lyerly *et al.*, 1988). Spectrum of CD caused intestinal infections ranges from mild diarrhoea to life threatening PMC. The latter situation is rare but associated with severe complications and high mortality (Tabaqchali & Jumaa, 1995). In most cases infection is localised in colon, but also pseudomembranous enteritis has been described (Tsutaoka *et al.*, 1994).

2.2. Epidemiology of *C. difficile*

2.2.1. Carriage rate in population

The carriage rate of CD in intestine of healthy adults varies in different geographical areas from 2% in Sweden to 15% in Japan (Knoop *et al.*, 1993). Unfortunately it is not known if these numbers represent a transient colonisation or is CD a permanent component of the stable flora of these subjects (Bartlett,

1994). The counts of CD in asymptomatic adults are low and the toxins are not detected in their faeces (Bartlett, 1994).

The higher age has been reported a risk factor for colonisation by CD and CDI (Bennett & Greenough, 1993). Some 18.7% of elderly residents have been found colonised by CD in long-term-care facilities (Simor *et al.*, 1993). Also the colonisation of healthy elderly people in population is higher than the average of general population (Nakamura *et al.*, 1981)

CD frequently colonises infants and young children without causing any symptoms of disease. The colonisation rate varies in different studies from 15 to 71% (Al-Jumaili *et al.*, 1984; Lyerly *et al.*, 1988; Tullus *et al.*, 1989; Cherkasskaja *et al.*, 1992; Knoop *et al.*, 1993). Infants become colonised some few days after birth and the high colonisation rate decreases to the adults' level after the age of 2 years (Lyerly *et al.*, 1988; Tullus *et al.*, 1989). From 31 to 94% of CD strains isolated from children were highly toxigenic *in vitro* (Al-Jumaili *et al.*, 1984; Tullus *et al.*, 1989; Pykiel *et al.*, 1990; Knoop *et al.*, 1993). CD toxins have been demonstrated in high titres in faeces of 40% CD positive infants (Knoop *et al.*, 1993).

Some investigators have found a correlation between CD colonisation of infants and their food. According to these studies, breast fed infants are less often colonised than bottle formula fed (Tullus *et al.*, 1989). Also the cytotoxin in faeces is more frequently detectable in formula fed infants (Emeruwa & Oguike, 1990). There are no data whether there may exist geographical differences in CD prevalence in infants, depending on different socio-economic status or different composition of intestinal microflora.

2.2.2. Colonisation of hospitalised patients and *C. difficile* caused diarrhoea

According to different studies, the carriage rate in hospitalised patients varies from 7.1 to 21% (McFarland *et al*, 1989; McFarland *et al.*, 1990; Johnson *et al.*, 1990; Clabots *et al.*, 1992; Merad & Djellout, 1992; Rudensky *et al.*, 1993; Simor *et al.*, 1993; Walker *et al.*, 1993; Samore *et al.*, 1994). During outbreak even more than 50% of patients in the ward may become colonised by CD (Cartmill *et al.*, 1994). Interestingly from 50 up to 85% of these patients may remain asyptomatic while in others there develops *C. difficile* caused diarrhoea (CDCD) (Clabots *et al.*, 1992; Johnson *et al.*, 1990; McFarland *et al.*, 1989; McFarland *et al.*, 1990). It is not known what kind of hidden mechanisms or factors would guarantee resistance to CDI in some patients. At the same time

some 69.9% to 100% of CD strains isolated from hospitalised patients are cytotoxic *in vitro* (Grönroos, 1989; Rotimi & Akindutire, 1989). Some recent studies suggest that the capacity to cause nosocomial outbreaks may be restricted to specific strains of CD that exhibit both increased transmissibility and pathogenicity (Samore *et al.*, 1996).

In case of nosocomial diarrhoeas of adults, CD is considered the most frequent etiologic agent (figure 2.1). Some 21–52% of nosocomial diarrhoeas are caused by CD in industrial countries (McFarland, 1993; Ristola *et al.*, 1993), but only about 10% in developing countries (Niyogi *et al.*, 1991). In community acquired diarrhoea CD is the etiological agent in less than 6% of cases (Riley *et al.*, 1991b; Liesenfeld *et al.*, 1993). In most cases (90–95%) CDI manifests as mild watery diarrhoea (Marino, 1991). PMC is rare manifestation of CDI, but in some surgical departments PMC can affect up to 2.5% of all patients (Sugiwara *et al.*, 1990).

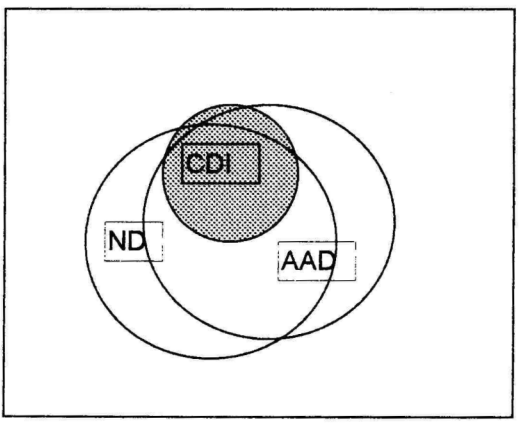


Figure 2.1. Relations between antibiotic associated, nosocomial and CD caused diarrhoeas in adults. The majority of CD caused diarrhoeas are related to previous antibiotic treatment. About 1/3 of AAD's are caused by CD and others are mostly due to dysbiosis of intestinal microflora. At the same time CD is one of the most important causes of nosocomial diarrhoeas (ND) in adults.

CD is the most important causal agent of antibiotic associated diarrhoea (AAD). From 15 to 56% of AAD are caused by CD (De-Barbeyrac *et al.*, 1989; Rotimi & Akindutire, 1989; Drapkin, 1992). Studies evaluating differences in

CD prevalence in nosocomial diarrhoeas of particular profile patients (e.g. surgical, oncological, internal wards) are unfortunately absent.

2.2.3. Other reservoirs of *C. difficile*

In some studies also the vagina has been reported as an important extraintestinal source of CD. Vaginal carriage of CD has been detected in 72% of female patients at a sexually transmitted disease clinic (Hafiz *et al.*, 1975). However, subsequent studies have failed to confirm so high a vaginal carriage rate (Tabaqchali *et al.*, 1984; Al-Jumaili *et al.*, 1984; Knoop *et al.*, 1993; Mändar, 1996).

Some investigators have revealed a high frequency of colonisation of pets by CD: 39.5% of cats and dogs treated in veterinary clinics and 95.3% of neonate dogs appeared to be colonised (Perrin *et al.*, 1993; Riley *et al.*, 1991a). However, using molecular typing methods CD strains isolated frequently from pets usually appeared to be different from human ones (O'Neill *et al.*, 1993).

CD is frequently detected in environmental samples taken from rooms occupied by patients with CDCD: 20–33% of cultures of these samples were CD positive (Drapkin, 1992; Knoop *et al.*, 1993; McFarland, 1993). The microorganism has also been isolated from samples of other environmental sources outside of hospital, including marine sediment, soil, sand, mud, and animal dung (Knoop *et al.*, 1993; Al Saif & Brazier, 1996).

2.3. Pathogenesis of *C. difficile* infection

Factors important in the pathogenesis of *C. difficile* infection (CDI) are the disturbance of intestinal microflora due to exposure to antibiotics, the presence of CD in the patient's intestinal microflora or acquisition of the organism from an environmental source, production of toxin A, and age related susceptibility (Bartlett, 1994). The disruption of colonisation resistance of indigenous microflora to CD is an important prerequisite for the establishment of CD in intestine of humans or experimental animals. The understanding of mechanisms and protective role of indigenous microflora may help to comprehend the preconditions essential for colonisation and development of infection by CD.

2.3.1 Microbial ecology of intestinal tract & its role in the pathogenesis of *C. difficile* infection

2.3.1.1. Indigenous microflora

Gastrointestinal tract of a human being is colonised with more than 400 different species of microorganisms in numbers of more than $10^{11}...10^{12}$ per gram (Simon & Gorbach, 1984). Inhabiting various biotopes (lumen or mucosa of colon, ileum etc.) these microbial populations perform specific microbiocenoses. It has been shown that there are great individual differences in quantitative and qualitative composition of intestinal microflora, but on the other hand, also the stability of microflora in particular individuals has been proven (Meijer-Severs & Santen, 1986; Mikelsaar, 1992).

To describe this individually stable microflora, several such terms as normal, autochthonous, resident or indigenous microflora have been used. Due to absence of general consensus some authors use these as synonyms, but in others these terms designate closely related notions overlapping each other to some extent (Rusch, 1989). According to the modern concepts of microbial ecology, the name "indigenous microflora" has been preferred by several investigators (Savage, 1989). Despite confusions in terminology we proposed to use the term inigenous microflora (IMF) to signify the group of non-pathogenic or potentially pathogenic microbes which are permanently inhabiting a particular biotope of a particular individual and are in symbiotic association with host. The term symbiosis includes different persistent and intimate associations between organisms such as parasitism, neutralism, commensalism and mutualism (Rusch, 1989). Human IMF usually includes anaerobes potentially predominant in the lower intestinal tract such as bacteroids, bifidobacteria, eubacteria and anaerobic cocci (Mikelsaar, 1992).

Apart from IMF, each biotope consists of non-indigenous (transient, allochthonous) microbes originating from environment or from IMF of other biotopes (Rusch, 1989). These pathogenic (e.g. CD) or non-pathogenic microbes can inhabit biotope either for a short time or, in case of perturbation of ecosystem, even for prolonged periods. However, it is not clear yet whether there are any factors which could speed up the restoration of normal microecological relations of IMF.

2.3.1.2. Colonisation resistance

The term microbial colonisation resistance (CR) has been defined as the limiting action of the IMF on colonisation of the bowel by exogenous as well as endogenous potentially pathogenic microorganisms (PPMOs) (Vollaard & Clasener, 1994). Although microbes play the most important role in maintenance of CR, it could also be mediated by anatomical and physiological factors including intact mucosa, salivation, swallowing, secretion of IgA, action of M cells, production of gastric acid, desquamation of cells of the mucus membranes, and normal gastrointestinal motility. It appears, however, that anatomical and physiological CR guaranteeing systems are not capable of keeping the concentration of PPMOs under control if the IMF is absent or disturbed (Vollaard & Clasener, 1994; Salyers & Whitt, 1994; Finlay & Siebers, 1995). Several studies indicate that only anaerobic IMF has the main role in microbial CR, and aerobic PPMOs do not contribute to the normal microbial CR (Borriello, 1989; Vollaard & Clasener, 1994). Also lactobacilli, as part of IMF, may have a particular role in the maintenance of CR (Mikelsaar, 1992; Salminen & Deighton, 1992; Lidbeck & Nord, 1993).

The importance of IMF in maintaining CR to CD can most impressively be demonstrated in hamsters: CD cannot colonise and cause disease in normal hamsters but in antibiotic treated animals even small doses of CD cause lethal infection, and further, previous administration of faecal microflora of normal hamsters to antibiotics treated animals prevents CDI (Borriello, 1990a). Despite clear evidence of the protective role of IMF, the exact mechanisms and bacteria which guarantee CR against CD remain unknown. Various combinations of microorganisms have been used *in vivo* and *in vitro* studies in attempts to inhibit growth and/or toxin production by CD, but in general, attempts to use *in vivo* anything other than the complete flora have been unsuccessful (Borriello, 1989; Borriello, 1990a). Several mechanisms which may be involved in control of CD population in bowel have been described: competition to nutrients and mucosal receptors, inhibitory effect of some short chain fatty acids, and bacteriocin mediated competition (Su *et al.*, 1987; Wilson & Perini, 1988; Borriello, 1990a, Borriello, 1990b; Wilson, 1993). According to recent studies there are some evidences that mucin-microflora interactions (e.g. degradation of mucin by IMF) are linked to CR to CD (Wilson, 1993; Bourlioux, 1996; Karjalainen *et al.*, 1996).

2.3.1.3. Antibiotic associated diarrhoea and *C. difficile* infection

Gastrointestinal symptoms, particularly diarrhoea, are relatively common side effects of antibiotic usage. Antibiotic associated diarrhoea (AAD) designates the diarrhoeas manifesting during or after recent antimicrobial therapy. Although in the majority of cases exact mechanisms of this side effect are not well understood, proposed mechanisms include: direct action of antibiotics on intestinal function; inducing predisposition to infection with enteric pathogen; and factors secondary to disturbance of normal intestinal flora that do not involve infection with a known pathogen (Midtvedt, 1989; Borriello, 1992).

CD is the most frequent enteric pathogen causing AAD (Bartlett, 1992). Nearly all antimicrobial drugs have been reported to induce CDI. The assumption is that modification of faecal flora is an essential feature of these drugs, but a confounding interrelated variable is their activity against CD (Bartlett, 1992). However, according to studies of hamster model and of patients also, the drugs with good activity against CD (including ampicillin and vancomycin) may induce CDI.

Several investigations support the idea that the ability of a particular antimicrobial to induce CDI depends on its spectrum of activity and its pharmacokinetic properties. Antibiotics active against anaerobic bacteria have been found most frequently associated with CDI (McFarland, 1993). According to several studies, the administration of third-generation cefalosporines, broad spectrum penicillins and clindamycin is associated with the highest risk for CDI (Zimmermann, 1991; Bartlett, 1992; McFarland, 1993; Ristola *et al.*, 1993; Hirschhorn *et al.*, 1994; Lumio, 1996). It seems that the frequency and severity of CDI do not appear to be antibiotic dose-related, by contrast in AAD due to dysbiosis (Bartlett, 1992). However, more precise clinical studies are needed to confirm this assumption.

2.3.2. Virulence of *C. difficile* and host factors in *C. difficile* infection

2.3.2.1. Toxins of *C. difficile*

At least five toxic factors of CD have been described, though only two of these, toxin A and B, have been implicated to disease and studied in detail (Borriello *et al.*, 1990). Toxin A and toxin B are polypeptides with molecular mass 308 and 270 kDa. For toxin A four major biological activities were reported: enterotoxic,

cytotoxic, cytotonic and agglutinative capability of rabbit red blood cells, for toxin B only lethal and cytotoxic properties are described (Sullivan *et al.*, 1982; Wolfhagen *et al.*, 1994). The differentiation of toxin A as enterotoxin and toxin B as a cytotoxin is not strictly correct, as both toxins are potent cytotoxins (Tucker *et al.*, 1990). However, toxin B is much more cytotoxic than toxin A for most cell lines (Lyerly *et al.*, 1990). It is thought that toxin A may be more important in the disease process, because all of the known features of ileocaecitis in the hamster model can be reproduced by administration of toxin A alone (Lyerly *et al.*, 1985; Mitchell *et al.*, 1986). Although toxin A has been supposed to be responsible for fluid accumulation in intestine and diarrhoea it has been shown that also toxin B can cause fluid accumulation in small intestine of experimental animals and cause damage to human colonic epithelium (Torres *et al.*, 1990; Riegler *et al.*, 1995).

Toxin A binds to the carbohydrate structures on the enterocyte but the receptors of toxin B are yet unknown (Krivan *et al.*, 1986; Tucker & Wilkins, 1991; Wolfhagen *et al.*, 1994). Although several studies have been carried out to identify factors inducing or suppressing CD virulence *in vivo* there is no confirmed information about these factors yet (Seddon *et al.*, 1991; Kamiya *et al.*, 1992; Barc *et al.*, 1992). One recent study has shown greatly increased CD toxins production in biotin limited conditions (Yamakawa *et al.*, 1996).

Until recent years there was a general agreement that virulent CD strains that can cause disease are always producing both toxin A and B, but avirulent strains did not produce either toxin and could not cause disease. The main role in pathogenesis of diarrhoea was attributed to toxin A (Wilkins *et al.*, 1989; Lyerly *et al.*, 1990; Fluit *et al.*, 1991). The understanding of CDI pathogenesis became more complicated after the discovery of a particular CD strain producing only toxin B and still being capable of inducing diarrhoea in experimental animals (Torres, 1991; Lyerly *et al.*, 1992; Borriello *et al.*, 1992, Depitre *et al.*, 1993). Furthermore, some toxin A and B negative strains have been shown to cause fluid accumulation in experimental studies and also outbreaks of AAD in patients with no other identifiable agent than non-toxigenic CD have been described (Gonzalez-Valenzia *et al.*, 1991; Tabaqchali, 1995). Despite these findings, toxin A and B remain the most important virulence factors in the pathogenesis of most cases of CDI.

2.3.2.2. Other virulence factors

Other virulence factors of CD associated with pathogenesis of CDI include adherence capacity, production of capsule and hydrolytic enzymes (Borriello *et al.*, 1990).

The adherence of CD to intestinal mucosa has been shown in human and animal studies. There appears to be an association between virulence and mucosal adherence of CD: the highly virulent strains attach to mucosa better than poorly virulent or avirulent strains (Borriello, 1990b). Thus, adherence may play a significant role in CDI, caused by nontoxigenic strains (Gonzalez-Valencia *et al.*, 1991). Some cell surface properties such as hydrophobicity and positive cell surface charge may contribute to the adherence of CD to mucus and underlying cells (Krishna *et al.*, 1996). Although some strains of CD possess fimbria, no association between the presence of fimbria and adherence to gut mucosa was demonstrated (Borriello *et al.*, 1990). The role of other CD surface structures e.g. presence of two super-imposed S-layers, in the pathogenesis of CDI is not yet clearly established either (Borriello, 1990b; Mastrantonio *et al.*, 1996).

2.3.2.3. Susceptibility of host

One of the typical characteristics of CDI is the age related susceptibility: neonates and young children are frequently colonised with virulent CD strains without any development of disease. The same phenomenon has been described in neonate hamsters (Rolfe & Iaconis, 1983). The relatively frequent carriage of CD in neonates may be explained on the basis of poor CR, as they have developing and incomplete intestinal flora that does not provide sufficient CR to CD (Borriello, 1990b). However, the mechanism of their resistance to CD toxins remains unclear. A number of hypotheses have been proposed: colostrum contains substances which neutralise toxin A; the infants lack toxin receptors in their intestine; the receptors are in an immature form and do not recognise toxin A; receptors are covered by a thicker layer of mucine (Lyerly *et al.*, 1988). There is no reliable explanation available yet.

Similarly, the role of immune response of the host in the pathogenesis of CDI is not fully understood. Although it has been shown in several experimental studies that anti-toxin immunoglobulins can protect animals against CDI, no correlation between the amount of secretory and systemic anti-toxin A antibodies and clinical course of disease has been demonstrated in patients (Lyerly *et al.*, 1990; Lyerly *et al.*, 1991; Johnson *et al.*, 1992; Viscidi *et al.*, 1983). However, it has been shown that the CD specific serum IgA and IgM levels are higher in asymptomatic carriers than in symptomatic patients (Mulligan *et al.*, 1993). This finding may indicate some protective role of humoral immune response against CDI.

One of the groups most susceptible to CDI are the ICU patients. Beside intensive antibacterial treatment some other factors altering the host resistance may be involved. It is known that lack of enteral nutrition is always associated

with alteration of IMF, overgrowth with PPMOs and septic complications (Bengmark *et al.*, 1995, Bengmark & Gianotti, 1995). However, it is not known if there are associations between ICU patients' state of IMF, colonisation by CD and nosocomial diarrhoeas.

2.3.3. Experimental models for studying colonisation resistance to *C. difficile*

Many experimental studies have been carried out to identify bacteria that are important in CR to CD and may be used for re-colonisation of at-risk patients to restore CR or to treat infected patients. Various combinations of microorganisms have been used in attempts to inhibit growth and/or toxin production by CD both *in vitro* and in *vivo* (Borriello, 1990a).

2.3.3.1. *In vitro* experiments

Many bacteria isolated from faeces have been found to be antagonistic against CD on agar plates. These microorganisms include several anaerobes and lactobacilli but also facultative aerobes (Rolfe *et al.*, 1981; Malamou-Ladas & Tabaqchali, 1982; Barclay & Borriello, 1982). However, these experiments do not correspond to the natural conditions of intestine.

For detection of the inhibitory effect of intestinal content *in vitro* a batch culture method was developed. The inhibitory effect of faeces from healthy humans and untreated hamsters on the growth of CD has been demonstrated by this method (Borriello & Barclay, 1986; Borriello *et al.*, 1988a; Borriello, 1990a).

Recently, bacterial interference between CD and other microbes has been studied in continuous flow cultures. The results of these studies suggest competition for nutrients as an important mechanism in CR (Yamamoto-Osaki *et al.*, 1994; Wilson, 1993)

Beside the studies of interaction of CD and intestinal microflora some investigations have been carried out to establish the mechanisms of adhesion of CD to mucosa using cell culture models (Eveillard *et al.*, 1993; Karjalainen *et al.*, 1994).

2.3.3.2. Animal models

In many studies attempts have been made to reconstitute resistance to CDI in animals using faecal homogenates from healthy normal animals of the same or different species or humans. In most of these studies the administered complete faecal flora provided resistance to CDI in different germfree animals (Wilson *et al.*, 1981; Wilson *et al.*, 1986; Itoh *et al.*, 1987). In contrast to these studies the attempts to use some particular anaerobe or their combination for reconstituting CR against CD have been unsuccessful (Wilson *et al.*, 1986; Borriello, 1990a). Thus, the studies to establish a single microorganism which can provide CR to CD *in vivo* have failed.

2.4. Use of biotherapeutic agents and other strategies for prevention and treatment of *C. difficile* infection

Standard treatment of CDI includes oral administration of vancomycin or metronidazole for 7 to 10 days (Pothoulakis & LaMont, 1993; Tabaqchali &Jumaa, 1995). Although definite improvement is usually noted just 3–4 days after such treatment one or multiple serial relapses of CDI can occur in 10 to 20% of patients (Fekety & Shah, 1993). For this reason new strategies that improve CR have been extensively investigated for treatment and prophylaxis of CDI.

2.4.1. Reparation of indigenous microflora

Preparations that have beneficial effects on human health *via* modulation or reparation of IMF include probiotics, prebiotics and synbiotics. Oral probiotics can be defined as living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition (Fuller, 1991; Fuller, 1994). Prebiotics are substances which serve as nutritional substrates for the beneficial intestinal microorganisms. Synergistic combinations of prebiotics and probiotics are called synbiotics (Schaafsma, 1996).

2.4.1.1. Probiotics

More commonly used probiotics contain lactic acid bacteria (lactobacilli, streptococci) and bifidobacteria. Beside the restoration of CR other important requirements to probiotics include ability to survive transport to the active site;

adhesion to the gut mucosa in active site; ability of reproduction; ability to exist in symbiosis with mucosal biofilm; ability to exist in the presence of factors which disrupt CR (Salminen *et al.*, 1993a; McFarland & Elmer, 1995; Elmer *et al.*, 1996)

There are several reports about treatment of patients with relapsing CDI using different microorganisms, including oral therapy with nontoxigenic CD strain, rectal infusion of normal faeces or mixture of intestinal bacteria (Schwan *et al.*, 1984; Seal *et al.*, 1987; Tvede & Rask-Madsen, 1989). Although some of these attempts were successful there are serious ethical and practical problems with this kind of treatment.

More profound experimental and clinical studies have been carried out with two promising probiotic strains: *Lactobacillus* GG and *Saccharomyces boulardii*. *Lactobacillus* GG is resistant to bile and low pH, it can adhere to intestinal mucosa and produce antimicrobial substances (Silva *et al.*, 1987; Saxelin, 1995; Goldin & Gorbach, 1996). *Lactobacillus* GG has been successfully used for treatment of relapsing CDI (Gorbach *et al.*, 1987; Gorbach, 1990; Biller *et al.*, 1995). Administration of *Lactobacillus GG* has also shown beneficial effects in prevention or treatment of rotavirus diarrhoea, AAD, traveller diarrhoea, milk hypersensitivity and in normalisation of intestinal microflora (Oksanen *et al.*, 1990; Siitonen *et al.*, 1990; Isolauri *et al.*, 1991; Saxelin, 1995; Goldin & Gorbach, 1996; Benno *et al.*, 1996; Pelto *et al.*, 1996; Elmer *et al.*, 1996).

The prevention of CDI using *Saccharomyces boulardii* has been shown in several experimental, as well as in clinical trials (Toothaker & Elmer, 1984; Surawicz *et al.*, 1989a; Surawicz *et al.*, 1989b; Elmer & Corthier, 1991; McFarland & Bernasconi, 1993; McFarland *et al.*, 1994). The protective effect of *S. boulardii* seems to be due to inactivation of toxin A or inhibition of its binding to receptors (Lucas & Corthier, 1990; Pothoulakis *et al.*, 1993).

2.4.2. Other strategies

The other important new strategies under investigation are the use of prebiotics and passive immunisation for the treatment of CDI. Experimental studies have shown some beneficial effects of administration of soy fibres and fructo-oligosaccharides: normalisation of intestinal microflora, suppression of growth of CD and the decrease of toxin A amounts, amelioration of mucosal lesions (Frankel *et al.*, 1994; May *et al.*, 1995; Gaskins *et al.*, 1996). Also clinical studies have revealed that intake of poorly absorbed disaccharides can modify IMF, decrease pH in colon and suppress the growth of CD (Ito *et al.*, 1997).

Bovine colostrum of immunised cows has been shown to neutralise CD toxins *in vitro* and protect hamsters against CDI (Lyerly *et al.*, 1991; Kelly *et al.*, 1996; Kelly *et al.*, 1997). In recent years more attention has been paid to antibodies treatment against bacterial toxins (Casadevall, 1996).

3. OBJECTIVES OF INVESTIGATION

The general aim of present investigation was to study intestinal microecology during CD colonisation and infection with two perspectives:

(1) better understanding of the basic nature and mechanisms of colonisation resistance to CD and

(2) more successful application of microecological approach in prophylaxis and therapy of CDI. The practical needs to introduce laboratory diagnostics of CD in Estonia and lack of data about local epidemiological situation increased the actuality of this topic.

Accordingly, the present study had the following aims:

* Determination of occurrence of CD in:

(1) patients with nosocomial diarrhoea — as a syndrome frequently associated with CD;
(2) hospitalised patients of ICU — as examples of persons with high risk for CD colonisation;
(3) infants — for detection of possible geographical differences of early colonisation;
(4) pregnant women — as possible source in infant colonisation;

* Evaluation of quantitative composition of intestinal microflora in persons frequently colonised by CD (infants and hospitalised patients) for assessment of possible differences in CD positive and negative persons;

* Evaluation of some characteristics (counts in intestinal content, toxigenicity, antimicrobial susceptibility) of CD strains of hospitalised patients;

* Elaboration of experimental models for revealing associations between the state of intestinal microflora, mucosal morphology and bacterial translocation during development of CDI;

* Assessment of potential strategies for the prevention of CDI in experimental models:

Inhibition of adhesion of CD

Correction of microflora by prebiotics and probiotics

4. MATERIAL AND METHODS

4.1. Hospitalised patients and nonhospitalised persons

Altogether 241 hospitalised and 150 nonhospitalised subjects (infants and pregnant women) were included in the study. 308 analyses of faecal samples from hospitalised and 150 from nonhospitalised subjects were performed. These persons were divided between different studies as follows (table 4.1).

4.1.1 Study groups

Group I: Screening for CD positivity among hospitalised patients with diarrhoea. Between December 1991 and January 1994 Laboratory of Clinical Microbiology of Maarjamõisa Hospital received faecal samples of 203 patients for detection the etiologic agent of nosocomial diarrhoea. These patients were hospitalised in various departments (intensive care units (ICU), 78; surgical, 49; internal, 79 patients) of Tartu University Hospitals. For detection of CD latex agglutination test (CDT) was applied.

Group II: Evaluation of quantitative composition of intestinal microflora (QCIM) of CD positive patients. This group consists of 18 patients (from Group I) whose faecal samples were CD positive and met requirements for quantitative analysis.

Group III: Screening of hospitalised patients for colonisation by CD. This group consists of 38 patients hospitalised in neurological ICU of Neurological Hospital of Tartu University. Patients who stayed in the ICU for at least 2 days within the observation periods and who passed the stools were included. During the first two-months period (February–March 1994) from 25 patients (Group III A) we succeeded to get 40 faecal samples and in the second two-months period (March–April 1995) 27 samples from 13 patients (Group III B) were collected.

Group IV: QCIM studies of hospitalised patients. This study group includes all group III B patients.

Group V and VI: Quantitative estimation of lactobacilli and CD in 1 year old infants. 27 Estonian (Group V) and 29 Swedish (Group VI) 12 months old healthy infants born in 1994 were included. There were no significant differences in previous antimicrobial treatment and feeding (breast or formula) between Estonian and Swedish infants.

Table 4.1.

Data of investigated persons

Group	Study	No. of persons	Sex M/F	Age range/ median (y)	Hospital.* range/median	AB** range median	No. of analyses	Original paper
I	CDT of hospitalised patients with diarrhoea	203	ND	ND	ND	ND	203	II
II	QCIM of CDT + patients♦	(18)	14/4	6–78 54	4d-2.5 m 23d	1–6 4	18	II
IIIA	CD colonisation of ICU patients	25	12/13	8–71 50	1d–5 m 8d	0–9 3	40	III
IIIB	CD colonisation of ICU patients	13	12/1	14–77 55	1d–45d 13d	2–7 4	27	III
IV	QCIM of ICU patients⇧	(13)	12/1	14–77 55	1d–45d 13d	2–7 4	20	III
V	QCIM of 1 year old Estonian infants	27	12/15	1	– 0	0–3 0	27	III
VI	QCIM of 1 year old Swedish infants	29	14/15	1	– 0	0–3 0	29	III
VII	QCIM of 2 year old Estonian infants	36	18/18	2	– 0	ND	36	PS
VIII	QCIM of 2 year old Swedish infants	36	19/17	2	– 0	ND	36	PS
IX	CD colonisation of pregnant women	22	0/22	ND	– 0	ND	22	Ref. 141

* = Duration of hospitalisation before testing; ** = number of different antibiotics administrated before testing; ♦ = randomised patients from Group I, ⇧ = randomised samples from Group IIIB patients; ND = not determined; PS = present study

Group VII and VIII: Quantitative estimation of lactobacilli and CD in 2 year old infants. 36 Estonian (Group VII) and 36 Swedish (Group VIII) 2 year old healthy infants born in 1994–95 were included.

Group IX: Screening of nonhospitalised pregnant women for colonisation by CD. We studied the intestinal carriage of CD in women in the third trimester of pregnancy. Some 22 consecutive women who visited Tartu Maternity Clinic during June 1994 for routine control were included.

4.1.2. Collection of specimens

Faecal samples of group I patients were collected by hospitals personnel and sent to the Laboratory of clinical microbiology of Maarjamõisa Hospital as routine samples for detection of CD. Approximately 2–5 g of faeces was collected in sterile container and processed within 2 hours or stored at +4° C until CDT testing (up to 2 days). Out of CDT positive analyses stored no longer than 2 hours 18, were randomly selected for detection of QCIM (Group II).

Faecal samples from Neurological ICU patients (Group III) were collected by ICU personnel. From all patients who stayed in ICU at the beginning of the study or were admitted during the observation period faecal samples were collected once and repeated weekly. The samples were stored at +4° C until cultivation (no longer than 24 hours). Approximately 2 g of the faeces out of each sample was frozen and stored at –20° C for further testing (Group IV).

From infants (Groups V–VIII) approximately 1 g of faeces was collected into sterile containers and stored for no more than 2 hours in refrigerator at +4°C and then frozen at –20° C until testing. Samples of Estonian infants were collected from October to December 1995 (Group V) and from March 1996 to January 1997 (Group VII) by K. Julge and M. Vasar (Department of Paediatrics, University of Tartu). Samples of Swedish infants were collected from March to June, from October to December 1995 (Group VI) and from January to June 1996 (Group VIII) in Linköping according to the collaboration study with B. Björksten (Department of Paediatrics, University of Linköping, Sweden).

Samples from pregnant women (Group IX) were collected by R. Mändar (Institute of Microbiology). Rectal swabs were put immediately into Stuart anaerobic transport medium and cultivated within 2 hours (Naaber *et al.*, 1996).

4.2. Experimental animals

Altogether 29 mice and 22 hamsters were included in our experimental study of CDI (table 4.2). During experiments the animals were held in separate cages disinfected before study. Cages were disinfected, and also bedding and drinking water were replaced every day. Animals were fed with standard animal chow *ad libitum*. The hamster experiments were made in Tartu in co-operation with Seppo Salminen (University of Turku, Finland) and were approved by ethical committee of Turku.

4.2.1 Study design

4.2.1.1. Mice study

Adult BALB/c mice (n=29) weighing approximately 25 g were distributed into 4 groups (figure 4.1):

Group 1 served as control (7 mice);

Group 2 received cefoxitin (Mefoxitin, MSD Sharp & Dohme GmbH), daily dose 100 mg/kg (administered intragastrically with a stainless steel feeding tube) for 5 days (8 mice);

Group 3 received cefoxitin (similarly to Group 2) and were inoculated with virulent strain of CD (ME 82, culture collection of University of Tampere) 24 h after the last antibiotic dose (7 mice).

Group 4 received CD (7 mice)

Mice were sacrificed using chloroform anaesthesia (Group 2: 24 h after the last antibiotic dose; Groups 3 and 4: 5 days after challenge with CD).

4.2.1.2. Hamster study

Adult Syrian hamsters (*Mesocricetus auratus*) weighing 70–100 g were divided into 4 groups (figure 4.2):

All hamsters received ampicillin (3 mg intragastrically) and after 24 h, were challenged with CD (toxic strain VPI 10463).

Group 1 (6 hamsters) served as control.

Table 4.2.

Experimental animals and material of investigation

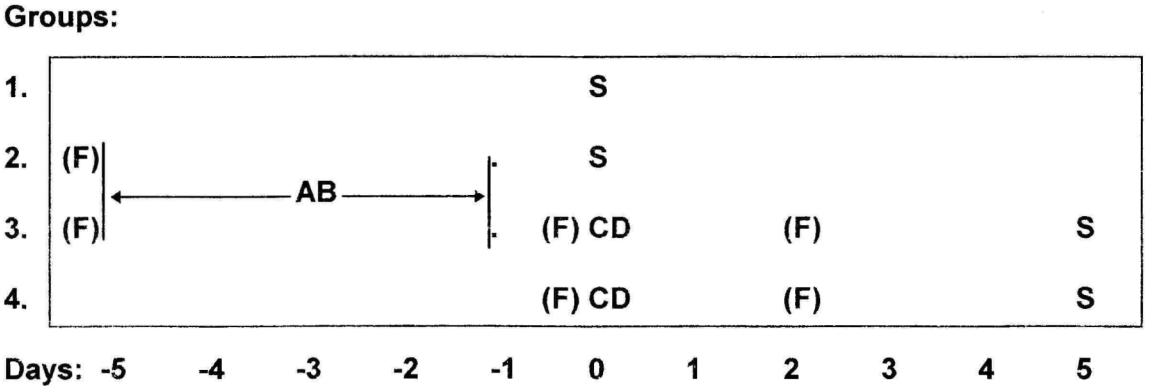
Animals	Group	No of animals	No of analyses				Original paper
			QCIM*	Mucosal histology**	Bacterial translocation ***	Toxin detection	
Mice	1.	7	28	14	28	–	IV; V
"	2.	7	28	14	28	–	IV; V
"	3.	8	32	16	32	–	IV; V
"	4.	7	28	–	–	–	IV
Hamsters	1.	6	24	12	16	6	V
"	2.	5	30	15	20	–	V
"	3.	6	–	–	–	–	PS
"	4.	6	–	–	–	6	PS

* Mice: luminal and mucosal samples of ileum and colon;
hamsters: luminal and mucosal samples of ileum, caecum and colon

** Mice: mucosal samples of ileum and colon;
hamsters: mucosal samples of ileum, caecum and colon

*** Samples of blood, liver, spleen and mesenteric lymph nodes

PS Present study



AB = administration of antibiotic (cefoxitin); CD = challenge with CD; (F) = collection of faecal samples; S = sacrificing and microbiological & histological analyses

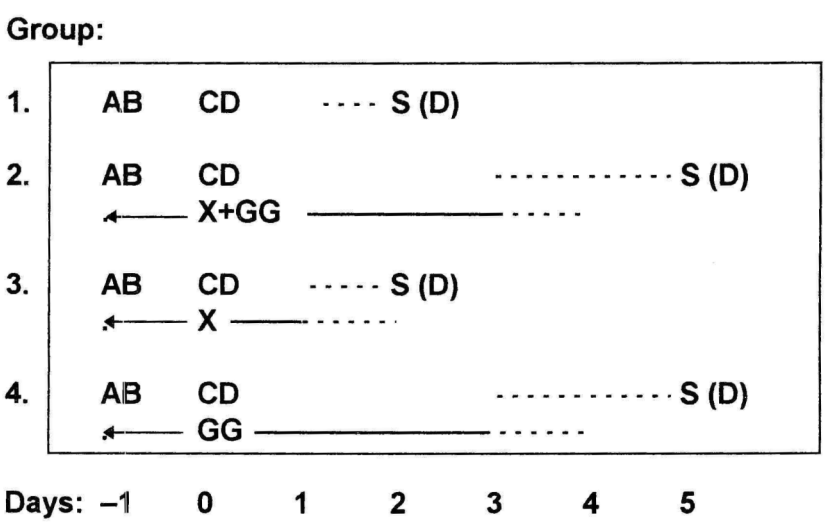
Figure 4.1. Study design of experiments with mice

Group 2 (5 hamsters) received additionally once a day 0.5 ml broth culture of *Lactobacillus* GG (approximately 10^8 cells/ml) and xylitol (1 ml of 20% solution) starting 20 h before administration of CD.

Group 3 (5 hamsters) received additionally solution of xylitol once a day (as Group 2).

Group 4 (6 hamsters) received additionally Lactobacillus GG (as Group 2).

Asymptomatic hamsters were sacrificed 5 days after challenge with CD. Also the moribund hamsters were all sacrificed.



AB = administration of antibiotic (ampicillin); CD = challenge with CD; GG = administration of Lactobacillus GG; S (D) = sacrificing (or death) and microbiological & histological analyses; X = administration of xylitol

Figure 4.2. Study design of experiments with hamsters

4.2.2. Samples

For collection of samples from viscera the autopsy of recently dead or sacrificed animals was performed.

4.2.2.1. Samples for detection of quantitative composition of intestinal microflora

Faecal samples were taken from mice before any experiments (all mice), immediately before administration of CD (Group 3 mice) and 2 days after administration of CD (Groups 3 and 4 mice).

Samples of intestinal content and mucosa were taken in autopsy of animals. From mice these samples were taken from ileum (1–2 cm from ileo-caecal valve) and colon (2–3 cm from anus), and from hamsters from ileum (approximately 2 cm from ileo-caecal valve), caecum and colon (from approximately 2 cm from anus) (Mikelsaar et al., 1987).

All these samples were processed immediately after collection.

4.2.2.2. Samples for detection of bacterial translocation

Blood from heart and samples from liver, spleen and mesenteric lymphatic nodes were aseptically removed during autopsy and processed immediately. The samples from liver, spleen and mesenteric lymphatic nodes were homogenised in saline before culturing for revealing the translocated microorganisms (Mikelsaar & Türi, 1990).

4.2.2.3. Samples for histological studies

Samples from mice were taken from terminal ileum (1–2 cm from ileo-caecal valve) and colon (2–3 cm from anus), and from hamsters from ileum (approximately 2 cm from ileo-caecal valve), caecum and colon (from approximately 2 cm from anus) during autopsy and stored in 4% neutral formalin until processing.

4.2.2.4. Samples for detection of toxin A

For detection of toxin A in intestinal tract of hamsters samples, from caecal content of Group 1 and Group 4 animals were taken and processed immediately.

4.3. Investigation adhesion of adherence of *C. difficile* to Caco-2 cells

For investigation of different possibilities to inhibit the adhesion of CD we used a Caco-2 cell culture model. This study was carried out at Viable Bioproducts Ltd. and Centre for Biotechnology, Turku, Finland.

4.3.1. Caco-2 cell culture

Enterocyte-like Caco-2 cells (ATCC HTB 37) were grown in Dulbecco's modified Eagle's minimal essential medium at 37° C in an atmosphere of 10% CO_2/90% air and were used for experiments at post-confluence after 14...16 days of culturing (Pinto *et al.*, 1983; Elo *et al.*, 1991).

4.3.2. Tested solutions

For inhibition of CD adhesion we used: (1) cell culture medium without supplements for control experiments; cell culture medium supplemented with: (2) 1%, 5% and 10% xylitol (Sigma Chemical Co.); (3) 1%, 5% and 10% bovine colostrum whey (Bioenervi™, Viable Bioproducts Ltd., Turku, Finland); (4) 5% colostrum whey without preservatives (sodium benzoate and lemon flavour); (5) 5% milk whey and (6) 20% supernatant of *Lactobacillus* GG culture in MRS broth.

4.3.3. Bacterial strains

A highly virulent CD strain VPI 10463 was used. Some 5 ml of 24 hours old culture of CD was centrifuged, cells were washed once and then re-suspended in 5 ml cell culture medium or cell culture medium supplemented with xylitol or colostrum. *Lactobacillus* GG was incubated in MRS broth (Oxoid) for 24 h and centrifuged. Supernatant was filtered through 0.45 μm filter (Millipore) before using in inhibition test.

4.3.4. Adherence inhibition tests

Before adherence tests both post-confluent Caco-2 cell cultures and CD cells were pre-incubated with test solutions. After pre-incubation CD cells were heated 10 min at 60° C and then seeded in to test solution to Caco-2 cells and incubated for 1.5 hours at 37° C in air.

After incubation unbound bacteria were washed for five times, cell culture was dried in air, fixed with methanol, stained by Gram and examined microscopically at the magnification of x1000. From at least three different glass coverslips of cell monolayer the numbers of adherent bacteria were counted in 26 microscopic fields selected at random. The adhesion index was calculated as the average number of adhering bacteria per microscopic field from at least three different assays (Eveillard *et al.*, 1993).

4.4. Microbiological methods

4.4.1. Detection of *C. difficile* in faecal samples

For detection of CD the CDT latex test and the cultivation on selective medium were used.

4.4.1.1. Latex test

For detection of CD antigens in faecal samples of Group I patients Culturette CDT latex agglutination test (BBL) was applied. Preparation of specimen and interpretation of results was carried out according to manufacturer's instructions. This test detects an antigen closely related to CD that has been identified as glutamate dehydrogenase (Lyerly *et al.*, 1991)

4.4.1.2. Cultivation of *C. difficile*

For detection of CD in Group III A and III B patients, their faecal samples were pre-treated with ethanol as described by Marler *et al.* (1992) for destroying non-spore-forming bacteria. Then the samples were seeded into modified Cefoxitin Cycloserine Fructose Agar (CCFA, Buggy *et al.*, 1985; Kamiya *et al.*, 1989; Bartley *et al.*, 1991; Niyogi & Pal, 1992). CCFA Base (Oxoid) supplemented with Cefoxitin Cycloserine Selective Supplement (Oxoid), 0.1% Sodium

Taurocholate (Sigma) 0.003% neutral red (Sigma) and 5% Egg Yolk Emulsion (Oxoid). Media were incubated in anaerobic environment (AnaeroGen, Oxoid) for 48 hours. In the other investigated groups CD was cultivated without ethanol pre-treatment as described in 4.4.2.

4.4.2. Determination of quantitative composition of intestinal microflora

4.4.2.1. Preparation of samples and cultivation

The weighed samples of faeces and intestinal content were serially diluted $10^{-2}...10^{-9}$ ($10^{-1}...10^{-9}$ in Group II) under a stream of CO_2 in pre-reduced phosphate buffer (pH 7.2). The samples from mucosa were washed with 5 ml saline, weight, homogenised aseptically and then serially diluted $10^{-2}...10^{-5}$. The bacteria were quantified by seeding of serial dilutions (0.05 ml) on different freshly prepared media (table 4.3.) i.e. Fastidious Anaerobe Agar (FAA; LabM) for total anaerobes, Columbia CNA agar (BBL) for grampositive anaerobes, Schaedler agar (BBL) with GN Selective Supplement (Oxoid) for gramnegative anaerobes, modified CCFA for CD, yeast-extract agar for total aerobes, yeast-extract agar with 6.5% sodium chloride for staphylococci, Endo agar for coliforms, Enterococcel Agar (BBL) for enterococci MRS agar (LabM or Oxoid) for lactobacilli and streptococci, Sabouraud dextrose agar supplemented with penicillin (50000 U/l) and streptomycin (40000 U/l) for yeasts and fungi were used (Mikelsaar & Siigur, 1992). The total counts of clostridia were estimated on FAA after ethanol treatment (Marler *et al.*, 1992).

FAA, Columbia CNA, Schaedler agar and CCFA medium were incubated 5 days in anaerobic atmosphere (GasPak Plus, BBL or AnaeroGen, Oxoid). MRS medium was incubated in 10% CO_2 atmosphere for 72 h. Other media were incubated in air for 24–48 h.

4.4.2.2. Identification and quantification of microorganisms

The colony counts of different dilutions were recorded and all morphologically distinct colonies from the highest dilutions with growth were Gram stained and subjected to microscopy. The microbial counts (CFU/g) and relative amount of every group of microorganisms in total count (%) were calculated employing special programme "BioQuant" (Mändar *et al.*, 1992). Detection level of intestinal bacteria was ≥2.3 (Group II) or ≥3.3 log CFU/g (in other Groups).

Table 4.3.

Media and incubation

Group	Micro-organisms	Media	Incubation
Anaerobes			
Persons of Groups II; IV-VIII Mice, hamsters	Total count	FAA	37° C, 5 days in anaerobic atmosphere: AnaeroGen or GasPak Plus
	Total clostridia	FAA after ethanol treatment	
Persons of Groups II-IX	CD	Modified CCFA	
Persons of Groups IV-VIII Mice, hamsters	Gram-negative	Schaedler + GN	
	Gram-positive	Columbia CNA	
Aerobes, facultative aerobes, microaerophiles			
Persons of Groups II, IV-VIII Mice, hamsters	Lactobacilli Streptococci	MRS Agar	37° 3 days in 10% CO_2
	Total aerobes	Yeast-extract agar	37° in air, 48 h
	Coliforms	Endo agar	37° in air, 24 h
	Yeasts	Sabouraud agar	37° in air, 72 h
	Staphylococci	Yeast-extract agar + NaCl	37° in air, 24 h
Hamsters	Enterococci	Enterococcsel Agar	

The total counts of indigenous anaerobes were calculated as total counts of anaerobes, excluding the counts of CD. To characterise mucosal microflora in experimental animals we determined predominant microbes. A microbial group was considered as predominant if its proportion in the total count of mucosal microflora was more than 30% at least in two animals of the same study group.

The microorganisms were identified mostly on genus level, according to their growth on selective media, colonial and cellular morphology. For anaerobes the inability to grow in aerobic and microaerophilic environment was detected.

4.4.3. Identification and determination of properties of isolated *C. difficile* strains

4.4.3.1. *C. difficile* strains

Altogether 24 different CD strains were tested: 21 strains were collected from hospitalised patients with AAD (including 11 strains isolated from Group II and 6 from Group IIIB patients); one wild strain was isolated from mice before experiments (FE 4) and two strains were applied in animal experiments (VPI 10463 and ME 82).

4.4.3.2. Identification

CD was identified according to its ability to grow on special selective medium for CD (CCFA) by forming typical colonies (large, irregular, yellow, due to fructose fermentation reaction, without lipase and lecitinase reaction) with typical smell; gram reaction and cellular morphology (gram-positive slender regular rods); and absence of growth in aerobic and microaerophilic environment (Murray *et al.*, 1995). In some problematical cases, the fermentation pattern was determined by RapidANA II (Innovative Diagnostic Systems Inc.) for confirmation of CD.

For confirmation of the preliminary identification of strains of CD, the detection of SCFA by GLC was used. Strains were cultivated in peptone yeast glucose broth for 24 h in an anaerobic chamber with (Sheldon Manufacturing Inc. with gas mixture: 5% CO_2; 5% H_2; 90% N_2) and esterificated as described elsewhere (Siders, 1995). The end products of strains were detected using GLC (M3700) with capillary column (SE-30, 25 m) and SCFA standards: acetic, isobutyric, butyric, isovaleric, valeric and isocapronic acid (Sigma). SCFA

profiles of isolated strains were compared with those of control strain (VPI 10463) and with reference data (Morton, 1995).

4.4.3.3. Antimicrobial susceptibility testing

By disc-diffusion method the antimicrobial susceptibility test was performed for all isolated 24 CD strains. Susceptibility for 12 antimicrobial agents (penicillin G, piperacillin, cefomandole, cefoxitin, cefotaxime, imipenem, chloramphenicol, tetracyclin, erythromycin, clindamycin, rifampicin, vancomycin) was tested. For this purpose Isosensitest Agar (Oxoid) supplemented with 5% sheep blood, Kirby-Bauer level antibiotic discs (Oxoid) and CD suspension in saline with density equal to 0.5 MacFarland standard were used (Barry *et al.*, 1990). After 24 h incubation in an anaerobic chamber growth inhibition zones were estimated according to NCCLS standards for gram positive bacteria (NCCLS M100-S5, 1994).

The minimal inhibitory concentrations (MIC) were determined for 12 strains isolated from patients by E-test (AB BIODISK). The E-test is a special quantitative technique for susceptibility testing of bacteria. The test consists of a plastic carrier coated with exponential gradient of antibiotic. During incubation on inoculated agar plate the antibiotic diffuses and inhibits bacterial growth seen as ellipse-like inhibition zones (Citron *et al.*, 1991; Olsson-Liljequist & Nord, 1994).

Additionally antimicrobial susceptibility of 22 strains (20 isolated from patients, ME 82 and VPI 10643) were tested also using the broth break-point test (ATB ANA, bioMérieux) as a reference method. This test includes one or two break-point dilutions of 25 different antimicrobial agents in a microwell plate. The susceptibility tests were performed according to manufacturer instructions and incubated at 37° C in anaerobic atmosphere of 5% CO_2, 5% H_2 and 90% N_2 in an anaerobic incubator house in an anaerobic chamber (Sheldon Manufacturing Inc.).

4.4.3.4. Toxin production of *C. difficile* strains

For testing *in vitro* production of toxin A, some 24 strains were inoculated into Brain Heart Infusion broth (Oxoid) supplemented with 1% Yeast Extract (Oxoid) and 0.1% L-cysteine hydrochloride (Oxoid, 1996) and incubated for 4 days in anaerobic chamber (Sheldon Manufacturing Inc.). Toxin A was detected in culture filtrates using sandwich immunoassay *C. difficile* Toxin A Test (Oxoid)(Borriello, 1996). According to the intensity and rapidity of positive

reaction the results were evaluated semiquantitatively (+, ++ or +++). The same test was applied for detection of toxin A produced *in vivo* from caecal content of hamsters.

4.4.4. Detection of bacterial translocation

The samples from sterile body sites (blood, liver, spleen, mesenteric lymph nodes) were seeded into blood agar and FAA. The blood agar plates were incubated in 10% CO_2 and FAA. in an anaerobic atmosphere (anaerobic chamber or jars) for 5 days. Bacteria were identified to *genus* level as described previously.

4.4.5. Properties of lactobacilli of experimental animals

In 34 lactobacilli strains isolated from mice and 30 strains from hamsters some particular properties (grouping according to their biochemical activity, susceptibility to some antibiotics, antagonistic activity against CD) were estimated.

4.4.5.1. Grouping of strains

All these strains were classified as OHOL, FHEL or OHEL using a special identification system as described by Lenzner *et al.* (1984) which estimates gas production from glucose, ability to grow at 15°C, fermentation of sorbitole and tagatose.

4.4.5.2. Antimicrobial susceptibility

Antimicrobial susceptibility of 10 strains isolated from mice and 22 from hamsters (strains of all 3 groups of lactobacilli were included) was tested by disc-diffusion method. Sensitivity was estimated for ampicillin and cefoxitin, using Kirby-Bauer level antibiotic discs (Oxoid) and Isosensitest agar (Oxoid) supplemented with 5% horse blood. The plates were incubated for 48 h in 10% CO_2 at 37° C (Mändar, 1996).

4.4.5.3. Detection of *in vitro* antagonistic activity of isolated lactobacilli against *C. difficile*

For detection of *in vitro* antagonistic activity of lactobacilli against CD (strain VPI 10463) some 10 hamster strains of different groups of lactobacilli were included. Antagonism was estimated as inhibition of growth of CD by lactobacilli on Wilkins-Chalgren agar (Oxoid) (Tvede & Rask-Madsen, 1989).

For one hamster strain showing the most potent antagonistic activity on agar also the antagonistic activity against CD in broth culture was detected. Schaedler (BBL) broth tubes were inoculated with CD, CD together with lactobacilli and separately with lactobacilli. The broth cultures were incubated anaerobically and samples for quantitative cultures were taken at different intervals after inoculation (0; 6; 12; 24 and 36 h), using calibrated loops (1 and 10μl). The number of colonies of lactobacilli were counted on MRS agar and that of CD on FAA, and the counts of microbes (CFU/ml) were calculated.

4.5. Histological methods

For histological examination, the slices of intestinal mucosa were fixed in neutral formalin. The paraffin embedded sections were stained with hematoxylin and eosin, acridine orange and by modified Gram method (Brown & Hopps, 1973). Different inflammatory changes (PMN cells, infiltration, hyperaemia, mitotic activity, haemorrhages, erosions, pseudomembranes) in mucosa were evaluated. The histopathological evaluation was done in a blinded manner on coded slides, using scale from 0 to 5, by experienced pathologists (Endel Türi, M. D. and Raik-Hiio Mikelsaar, M. D.). The changes were given a numerical score: no changes = 0; mild changes = 1; moderate changes = 2; marked changes = 3, 4 and severe changes = 5 (May *et al.*, 1995; Axelsson *et al.*, 1996).

4.6. Statistics

For statistical analyses the following tests were used: Mann-Whitney rank sum test for unpaired data (data of children, patients and experimental animals); Kruskall-Wallis' test (hospitalised patients; children); Wilcoxon rank test for paired data (experimental animals); 95% confidence interval and t–test (adhesion study); χ^2 or Fisher's exact test (children; hospitalised patients, experimental studies).

5. RESULTS AND DISCUSSION

5.1. Studies of hospitalised and nonhospitalised persons

5.1.1. Colonisation with *C. difficile* of subjects under investigation

5.1.1.1. Detection of *C. difficile* in patients with nosocomial diarrhoea

In the total 203 patients under investigation the CDT test was positive in 74 cases (37%). In different departments of Tartu University Hospital the prevalence of positive CDT varied: intensive care units — 49% (38 positive tests out of 78); surgical departments — 46% (21 out of 46); internal departments — 19% (15 out of 79). The prevalence of positive results was significantly lower in internal departments as compared with ICU (χ^2=15.5; $p<0.05$) and also with surgical departments (χ^2=10.1; $p<0.05$).

In all of the 18 patients with positive CDT test included to Group II the culture for CD was also positive. The counts of CD in their faeces varied between 2.3 and 8.4 log CFU/g (figure 5.1), forming <0.001 to 81 % (median 0.02%) of their total microflora.

5.1.1.2. Colonisation of patients in the neurological ICU

In the first observation period none of the 25 patients was colonised by CD, although CD was detectable in an environmental sample from ward.

In the second period CD was isolated from 7 patients (in 10 samples) out of 13. In 3 of these patients CD was negative in the initial sample but they became colonised during one or two weeks of staying in hospital. In one patient CD was present in the first sample but was absent in the second and third one.

The counts of CD in positive faecal samples varied within the ranges of 4.3 to 8.1 log CFU/g (median 7) forming <0.001 to 35% (median 5.9%) of their total microflora.

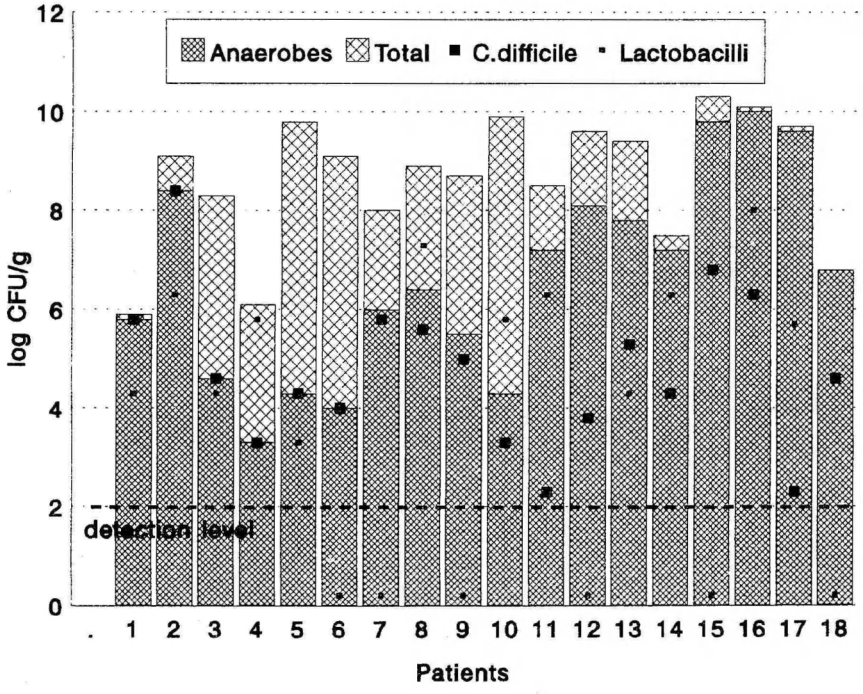


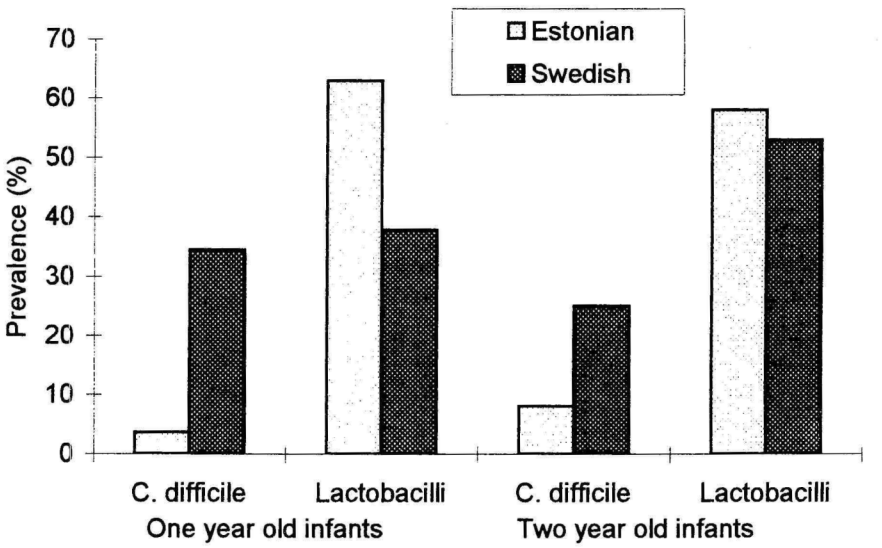
Figure 5.1. Individually different counts of CD, anaerobes, total microbes and lactobacilli in faecal samples of Group II patients

5.1.1.3. Colonisation of Estonian and Swedish infants

One year old infants. We found that the intestinal tract of 10 Swedish infants out of 29 (35%) and only one Estonian infant out of 27 (4%) was colonised by CD (χ^2=8.39; p<0.05; figure 5.2). Also the counts of CD were higher in Swedish infants (0...7.5 log CFU/g, median 0) than in Estonian ones (0...4.0 log CFU/g, median 0; p<0.05).

Two year old infants. Like to one year old group we also found higher prevalence of CD in Swedish two year old infants as compared with Estonian ones (χ^2=4.98; p<0.05). Some 9 out of 36 Swedish infants and 3 out of 36 Estonian ones were colonised by CD. The counts of CD varied from 0 to 7.6 log CFU/g (median 0) in Estonian and from 0 to 7.9 log CFU/ (median 0) in Swedish infants (p=0.08).

The relative amount of CD in total microflora of CD positive infants from both groups was from 0.007 to 22.7% (median 0.13) in one year old infants and from <0.0001 to 0.9% (median 0.02%) in two year old ones.



$p < 0.05$ Estonian *vs.* Swedish infants: prevalence of CD in one and two year olds; lactobacilli in one year olds

Figure 5.2. Prevalence of CD and lactobacilli in faecal samples of Estonian and Swedish infants

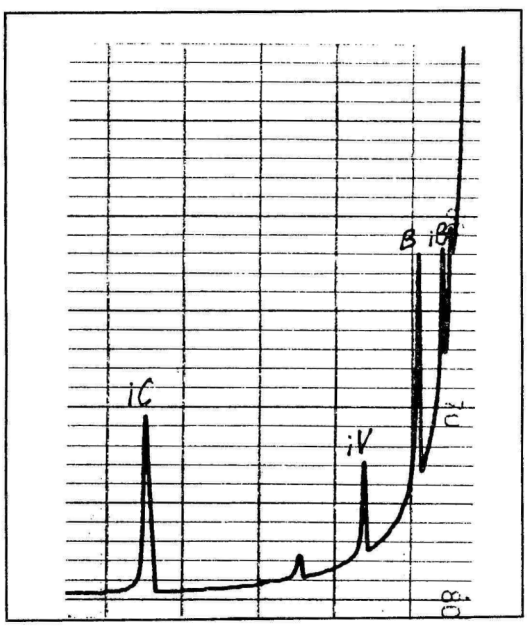
5.1.1.4. Colonisation of nonhospitalised pregnant women

Among the 22 pregnant women investigated, we could find CD only in one faecal sample (5%).

5.1.2. Properties of isolated *C. difficile* strains

5.1.2.1. Production of short-chain fatty acids

The end products profile of isolated strains was identical to CD strain from culture collection (VPI 10463). All strains produced SCFAs typical of CD: isobutyric, butyric, isovaleric and isocapronic acids (Figure 5.3)



iB = isobutyric; B = butyric; iV = isovaleric; iC = isocapronic

Figure 5.3. Typical SCFA profile of CD (strain no. 12)

5.1.2.2. Toxin production

Of all the 21 CD strains isolated from patients with nosocomial diarrhoea only one was unable to produce toxin A *in vitro*. The other 20 strains appeared slightly (6 strains), moderately (3) or highly (11) toxigenic. The CD strains VPI 10463 and ME 82 were highly (+++) and the mouse wild strain FE 4 slightly (+) toxigenic. There was no noticeable association between toxin production *in vitro*, CD counts *in vivo* and in the presence of diarrhoea of these patients (data not shown).

5.1.2.3. Antimicrobial susceptibility

Susceptibility data of CD strains of patients (n=20) to antimicrobial agents by broth break-point method are shown in figure 5.4. All strains were susceptible to most of tested beta-lactame antibiotics (excl. cefoxitin and ceftizoxime), metronidazole and vancomycin. Susceptibility to chloramphenicol, tetracycline, clindamycin, erythromycin and rifampicin varied. For most antimicrobial agents there was revealed a good correlation with disc-diffusion method (data not shown). Few strains with intermediate sensitivity to tetracycline or erythromycin by disc-diffusion method were either sensitive or resistant by break-point method. Three strains were sensitive to chloramphenicol by break-point but resistant by disc-diffusion method. Remarkable discrepancies between these two methods appeared in case of cefotaxime: all strains were resistant by disc-diffusion but only 2 strains by break-point method.

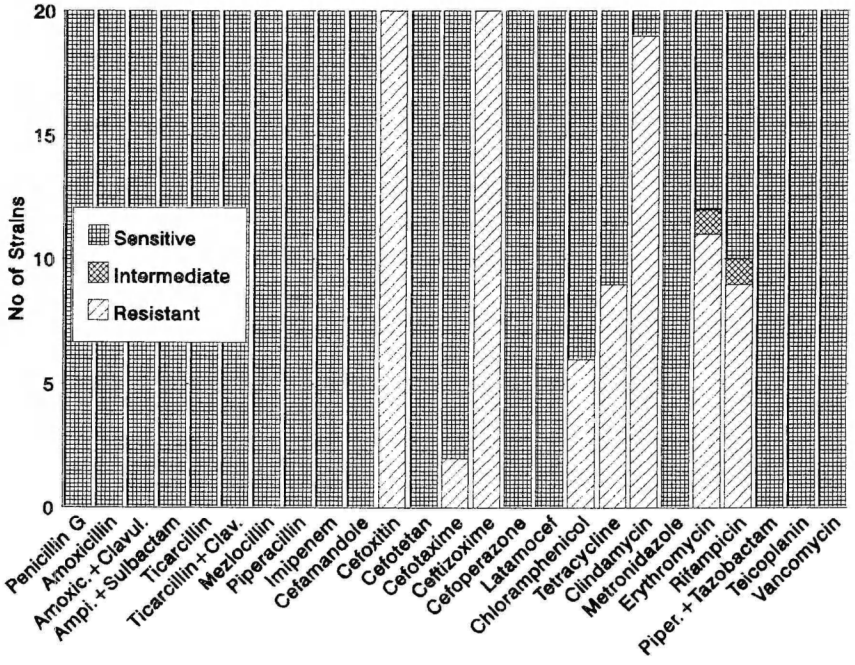


Figure 5.4. Antimicrobial susceptibility of CD strains (n=20) isolated from patients with nosocomial diarrhoea (break-point method).

According to MIC values in E-test all the 12 tested strains appeared to be sensitive to ampicillin (MIC: 0.25...3.0 μg/ml) and resistant to ciprofloxacin (MIC: 12...>32 μg/ml, table 5.1).

Table 5.1

Minimum Inhibitory Concentrations of ampicillin and ciprofloxacin against 12 CD strains(E-test)

	MIC (μg/ml) / no of strains							
	0.25	0.38	0.5	1.0	1.5	3.0	12	>32
Ampicillin	1	1	3	5	1	1	0	0
Ciprofloxacin	0	0	0	0	0	0	1	11

Comparing the sensitivity of isolated strains to antibiotics with varying activity against CD strains (chloramphenicol, tetracycline, erythromycin and rifampicin) and toxigenicity of strains by Fisher's exact test we found that strains with higher toxigenicity (++ or +++) were usually resistant to three or four of these agents. The strains with lower toxigenicity (– or +) were sensitive to two or more of these antibiotics ($p=0.01$, table 5.2). Comparing the resistance of strains for particular agents we found similar association with toxigenicity in case of chloramphenicol ($p<0.01$).

Table 5.2.

Resistance (to chloramphenicol, tetracycline, erythromycin, rifampicin) and toxigenicity of 20 CD strains isolated from patients with nosocomial diarrhoea

Toxigenicity *in vitro*	n	Resistance (number of strains)	
		Low resistance	High resistance
		Resistant to 0 1 2 agents	Resistant to 3 4 agents
Low –	1	0 1 0	0 0
toxigenicity +	6	4 1 1	0 0
High ++	3	2 0 0	0 1
toxigenicity +++	10	2 0 1	0 7
Total	20	8 3 2	0 8

5.1.3. Quantitative and qualitative composition of intestinal microflora in investigated persons

5.1.3.1. Comparison of intestinal lactoflora of Estonian and Swedish infants

One year old infants. We found that the prevalence of lactobacilli was higher in Estonian infants (63%) as compared with Swedish ones (38%; figure 5.2). Also the counts of lactobacilli were significantly higher in Estonian infants' (0...10 log CFU/g, median 4.2) intestinal microflora than in that of Swedish ones (0...4.0 log CFU/g, median 0, $p<0.01$). There was no association between the prevalence of CD and previous antimicrobial therapy.

Two year old infants. There were no significant differences between Estonian and Swedish infants in prevalence (21/36 and 19/36) and counts (ranges 0...8.3 and 0...7.1; medians 3.95 and 3.3 log CFU/g) of intestinal lactobacilli. Comparing CD positive and negative infants we found that prevalence and also the counts of lactobacilli were significantly higher in CD negative infants (table 5.3).

Table 5.3.

Comparison of counts and prevalence of lactobacilli of CD positive and negative two year old infants

	CD positive	CD negative	statistical difference
Prevalence of lactobacilli	3/12	37/60	$\chi^2=5.44$ $p<0.05$
Counts of lactobacilli Range Median (log CFU/g)	 0–5.3 0	 0–8.3 4.2	 $p<0.05$

5.1.3.2. Intestinal microflora and clinical data of hospitalised patients

Group II. In patients of Group II total counts of intestinal microbes varied from 6.1 to 10.3 log CFU/g (median 10.0), the counts of anaerobes from 3.3 to 10.0 log CFU/g (median 6.6) and the counts of lactobacilli from 0 to 8.0 log CFU/g (median 4.3). The proportions of these microbial counts were individually different (figure 5.2). Out of 18 patients in 13 cases the counts of aerobes outnumbered those of anaerobes. In 7 cases CD was the predominant anaerobe.

Comparing these data with healthy adults' faecal microflora counts (Mikelsaar, 1992) we found that both the counts of anaerobes and lactobacilli were lower in CD positive patients (medians 6.0 *vs.* 10.3 and 4.3 *vs.* 7.0 respectively, $p<0.05$).

Group IV. Comparing the counts of lactobacilli and anaerobes in faecal samples of Group IV patients we found that the counts of lactobacilli were significantly higher ($p<0.05$) in CD negative samples (0...8.8 CFU/g, median 5.3 log) than in CD positive ones (0...7.1 log CFU/g, median 0, table 5.4.). However, there were remarkable individual differences in counts of lactobacilli in both groups. There were no significant differences in the counts of anaerobes between the CD positive and negative samples. The counts of both anaerobes and lactobacilli were lower in samples of hospitalised patients as compared with healthy adults. In most faecal samples counts of aerobes outnumbered those of anaerobes, indicating the imbalance of intestinal microflora. Only in 2 CD negative samples the proportions of aerobes and anaerobes were similar to those of healthy persons.

T a b l e 5.4.

Comparison of clinical data of Group IV CD positive & negative patients and samples

	CD positive	CD negative
Patients (n=13)		
Number of different antimicrobial agents administered (range/ median)	2–7/ 5*	2–4/ 3
Age (range/ median)	14–55/ 54	40–77/ 54
Prevalence of diarrhoea (at least one episode)	6/7	5/6
Samples (n=20)		
Prevalence of diarrhoea during sampling	5/10	10/17
Staying (days) at ICU before sampling (range/ median)	4–59/ 30.5	1–85/ 11
Counts of lactobacilli (range/median; log CFU/g)	0–7.1/ 0*	0–8/ 5.3
Prevalence of lactobacilli	3/9*	9/11

* Statistically significant differences ($p<0.05$): number of administered antimicrobial agents — CD positive *vs.* negative patients; counts and prevalence of lactobacilli — CD positive *vs.* negative samples.

All of these patients had received multiple different antimicrobial agents (III generation cefalosporins, penicillins, aminoglycosides, quinolones, sulphonamides). Comparing the CD positive and negative patients we found that CD positive ones had received more different agents (2 to 7, median 5) than

negative ones (2 to 4, median 3). In 11 patients out of 13 numerous episodes of diarrhoea had been recorded by personnel of ICU with similar frequency in both CD positive and CD negative patients. Commonly CD positive patients have been kept in hospital longer than CD negative ones.

5.1.4. Discussion

Detection of CD. For detection of prevalence of CD in faecal samples of Group 1 patients we used Culturette Brand CDT latex agglutination test (Becton Dickinson). This test was initially devised for detection of toxin A but later investigations revealed that the latex-reactive protein was not the toxin (Lyerly *et al.*, 1991). Compared with other CD detection methods the sensitivity of CDT test has been reported from 67 to 92% and specificity 95...100% (Bennett *et al.*, 1989; Johnston & Bloy, 1994; Staneck *et al.*, 1996). Despite these discrepancies it is one of the simplest and quickest methods for detection of CD. According to manufacturer the storage of samples up to 3 days at 4° C does not influence the sensitivity of test.

For detection of CD in other patients and experimental animals the cultivation on modified CCFA medium was used. Fresh or frozen samples were used for cultivation. Although it has been shown that CD can be cultivated from positive faecal samples stored 4 days at 5° or 25° C (Bowman & Riley, 1986) it is not known how storage and freezing can influence the counts of CD in faecal samples. To avoid the influence of different storage conditions on the counts of CD we compared only the data of those groups whose samples had been similarly treated.

In some previous studies the sensitivity level of cultivation of faeces for CD has been about 10^3 bacteria in one gram of faeces on selective CCFA medium (Lyerly *et al.*, 1988). Media containing sodium taurocholate can recover significantly more CD than ordinary CCFA formula due to better outgrowth of spores (Kamiya *et al.*, 1989; Buggy *et al.*, 1985). One recent study has shown good detection rate of CD in faecal samples using taurocholate supplemented CCFA as compared with PCR (Arzese *et al.*, 1995). Because of that we supplemented our CCFA with sodium taurocholate. In case of hospitalised patients we used the ethanol pretreatment of faecal samples since the ethanol or heat shock had been shown to increase the recovery of CD (Bartley & Dowell, 1991; Marler *et al.*, 1992; Hanff *et al.*, 1993).

In all faecal samples (Group IIIB) that were CD positive by initial cultivation after ethanol shock, CD was also detectable after freezing and thawing for quantitative study in counts > 10^4 CFU/g. Thus it seems that if CD colonises an individual, the numbers of microorganisms reach relatively high numbers.

Colonisation of nonhospitalised persons with CD. Our results of intestinal colonisation by CD of pregnant women were not different from data of healthy nonhospitalised adults in Europe (Knoop *et al.*, 1993). However, it is not known if there are any differences in intestinal CD carriage rate between pregnant women and the whole population or not. The failure to isolate CD even from hospitalised patients during our first observation period (Group III A) indicates that carriage in the whole of Estonian population is probably quite low. Further studies are needed for confirmation of this expectation. If intestinal carriage of CD in healthy adults has been found quite similar in different studies, there are remarkable differences in data about vaginal carriage rate. Previous studies have shown low vaginal CD carriage in Estonian women (Mändar, 1996). The factors influencing vaginal colonisation by CD are not known.

The colonisation by CD of Estonian infants has been found relatively low as compared with other studies. The possible explanations of this finding are discussed below.

Patients with nosocomial diarrhoea. According to our study the mean frequency of CD as a causative agent of nosocomial diarrhoea in Tartu University Hospitals was similar to the data of other investigators. Although there are no data in the literature about the percentage of CD causing diarrhoea in different departments of one hospital the results of studies carried out in different hospitals were largely variable. We have found that in departments with different profiles the proportion of CDI was different. The higher frequency of CD in surgical and ICU patients was corresponded to our expectations, since patients undergoing surgery or in intensive care have usually more risk factors (administration of more antibiotics, severe underlying disease, parenteral or nasogastral feeding etc.) associated with CDI (McFarland *et al.*, 1990; McFarland, 1993).

ICU patients. Studying the colonisation of patients by CD in the particular department we discovered remarkable differences during different time periods (5.1.2.). It seems that periods of high colonisation and infection rate alternate with periods without colonisation by CD. This has been noted also in case of other microbial agents of hospital infections (Emori & Gaynes, 1993). In case of outbreaks more than half of the patients in the ward could be involved in contrast to relatively low baseline of colonisation rate (Cartmill *et al.*, 1994). It has been shown in several studies that spores of CD can survive in environment for several months and may be transmitted from patient to patient mainly by hands of medical personnel (Araujo *et al.*, 1991). In the first observation period we also sampled the ICU environment and found CD in one sample. While comparing ICU patients colonised and uncolonised by CD we particularly focused on factors connected directly with intestinal colonisation resistance (prior antimicrobial therapy, quantitative composition of indigenous microflora) and

ignored factors that could affect patients' general resistance (underlying disease, immune status etc.). We found that CD positive patients had received larger quantities different antimicrobial agents than negative ones. Most of CD positive patients had received third-generation cefalosporins, penicillins, aminoglycosides and antifungal agents. Although it is not known which microbial groups are responsible for maintenance of colonisation resistance to CD, empirical data indicate that agents active to anaerobic and/or grampositive microflora are associated with higher risk to CDI (McFarland, 1993). This was also true in our case.

Antimicrobial agents and CDI. The confounding fact that most CD strains are sensitive to the same antibiotics has been described previously (Lyerly *et al.*, 1988; Gorbach, 1992; Dzink & Bartlett, 1980). Testing the CD strain isolated from hospitalised patients we also found that all strains were sensitive to all penicillins and to most cefalosporines used for treatment of these patients. Several speculations could be set up to explain this discrepancy: (1) The antibiotic with good activity against CD *in vitro* did not achieve inhibitory level in terminal colon against CD but the concentration was high enough to alter the micro-organisms antagonistic to CD. (2) Although initial concentration of antibiotic in the colon was sufficient to destroy vegetative cells of CD, after some decrease in the concentration the overgrowth by CD from surviving spores or an acquired new strain is possible, since recovery of colonisation resistance takes more time. (3) It is known that quantitative and also the qualitative composition of luminal and mucosal flora is different (Mikelsaar *et al.*, 1987; Poxton *et al.*, 1997). Administrated antibiotic could achieve inhibitory concentration only in intestinal mucosa and therefore alter only indigenous microflora intimately associated with mucosa by direct bactericidal effect or inhibition of adhesion. It is impossible to decide in our study which antimicrobial was associated with a higher risk for CD colonisation and which one might protect against it, since all patients had received several antimicrobial agents simultaneously and the therapy was also changed several times before and during testing.

It is known that antimicrobial treatment may modify both luminal as well as mucosal microflora, causing decrease in anaerobes and increase in facultative organisms (Mikelsaar *et al.*, 1987; Mikelsaar & Siigur, 1992). Comparing the quantitative composition of intestinal microflora of patients undergoing antimicrobial therapy with data of healthy subjects (Mikelsaar, 1992) we found a significant decrease in the counts of total anaerobes and lactobacilli. In different individuals these changes were different: in some cases we did not find indigenous anaerobes in detectable counts at all and the only detectable anaerobe was CD, in some other cases the proportions of total counts of aerobes and indigenous anaerobes were similar to those of healthy subjects.

IMF and CDI. Although several experimental studies have been made to identify microbial groups that are important in maintenance of colonisation resistance against CD, no attention has been given to possible differences in quantitative and qualitative composition of intestinal microflora of patients who are and who are not colonised by CD. Comparing quantitatively the microflora of CD positive and negative patients we revealed significantly higher counts of lactobacilli in CD negative ones. It has been described previously that lactobacilli have protective role against some intestinal pathogens (Salminen & Deighton, 1992). Some strains of lactobacilli have antagonistic activity against CD *in vitro* and some probiotics can terminate relapsing colitis caused by CD (Silva *et al.*, 1987; Goldin & Gorbach, 1996). However, the main role in maintenance of CR against CD has been usually attributed to anaerobic microflora. Although we found imbalance of intestinal microflora (absolute and relative reduction of counts of anaerobes) in patients colonised with CD, there were no significant differences in the counts of intestinal anaerobes between CD positive and CD negative ICU patients.

Toxigenicity. All isolated strains, except one, were toxigenic *in vitro*. This finding is in good correlation with the data of the literature: in most of the other studies more than 90% of strains isolated from patients have appeared to be toxigenic (Grönroos, 1989). We found that the capability of producing toxin A *in vitro* varied in different strains. We have also found that in most cases higher toxigenicity was related to higher resistance to antimicrobial agents, particularly to chloramphenicol. The determinant of resistance to chloramphenicol, *catD,* was shown to be cromosomal and non-transferable (Sebald, 1994). Whether this or other related genes have any role in expression of virulence of CD needs further investigation. It is not known if strains highly toxigenic *in vitro* are more virulent *in vivo* and associated with a more severe disease. Toxins level produced by CD in intestinal tract depends on several factors, such as population level of CD in intestine (CFU/g), capability of luminal content (e.g. fibres) and antibodies to neutralise the toxins and probably some other unknown factors inducing or inhibiting the production of toxins *in vivo*. It has been shown in animal experiments that strains with similar toxigenicity *in vitro* may produce different amounts of toxins *in vivo* (Borriello, 1990b). The problem of correlation of toxigenicity and infection became more complicated due to several recent reports describing the cases of AAD caused by strains unable to produce toxins A and B (Tabaqchali, 1995). We have also found one atoxigenic strain in patient with AAD. One can speculate that some other virulence factors beside toxins may also been implicated in the pathogenesis of CD associated diarrhoea in such cases.

Susceptibility testing. Sensitivity testing of isolated CD strains is not routine procedure in clinical laboratory, since sensitivity testing of anaerobes is time-consuming and expensive. According to literature 99% of CD strains are

sensitive to metronidazole and vancomycin — the agents used for treatment of CDI (Gorbach, 1992; Dzink & Bartlett, 1980). We have also found that all our strains were sensitive to these agents. Comparing our results with those of other studies we saw that sensitivity of CD strains to most antibiotics was similar. Discrepancies appeared in case of rifampicin, imipenem and cefotaxime. Rifampicin has been previously reported to be highly active against CD and recommended for treatment of CDI by some authors (Gorbach, 1992). But only less than half of our strains appeared to be sensitive to this antibiotic.

In previous studies 100% of CD strains has been found resistant to imipenem with unknown mechanism (Sanders *et al.*, 1992). Both methods used by us showed high activity of imipenem against all investigated CD strains. However, using heavy inoculum in disc-diffusion method few colonies with intermediate resistance appeared inside the inhibitory zone. After isolation and re-testing of these colonies different subpopulations with high and intermediate sensitivity to imipenem appeared. It seems that MIC of imipenem to CD strains is slightly below 8 μg/ml, the concentration used in our break-point method test panel and also referred as intermediate MIC of imipenem for anaerobes by NCCLS. These discrepancies with published data are caused by the use of different breakpoints for interpretation of the sensitivity of imipenem in other studies. In case of cefotaxime the results of the two methods were contrariwise. It seems that in these cases the NCCLS interpretation criteria of disc-diffusion method for aerobes are not applicable to anaerobic bacteria. Although the disc-diffusion method is not recommended for susceptibility testing of anaerobes, some studies have shown its convenience for testing rapidly growing anaerobic bacteria (Barry *et al.*, 1990). Our study revealed that for the testing of CD, the disc-diffusion method gives reliable results in case of most of the studied antimicrobial agents.

IMF of infants. Comparing Estonian and Swedish infants we found several differences in the quantitative composition of their intestinal microflora. In one year old infants the prevalence of lactobacilli was significantly higher in Estonian ones, but the counts and prevalence of CD were higher in Swedish. There were also some differences in other microbial groups (Sepp *et al.*, in press). The prevalence of lactobacilli in two year old Swedish infants has increased as compared to one year olds and has reached the same level as in Estonian infants. Although the prevalence of CD in Swedish infants has decreased during the development of intestinal microflora (2 years *vs.* 1 year old) there were still significant differences present in comparison with the same age Estonian ones. At the same time there were no significant differences in the prevalence of lactobacilli and CD between one and two year old Estonian infants.

The differences in intestinal microflora of people from different geographical areas have been noted by several investigators (Salminen *et al.*, 1995). Most of these differences have been associated with dietary variations (Benno &

Mitsuoka, 1991; Peltonen *et al.*, 1992). The differences in maternal microflora and cleaning procedures during delivery can also influence formation of indigenous microflora of neonates (Mändar, 1996). It is known that infants up to 2 years are frequently colonised by CD (Tullus *et al.*, 1989). However, the data of infants' colonisation rate varies highly in different studies. These variations have been explained mostly by different degrees of environmental exposure to CD in maternity clinics, rather than by different maternal colonisation (Al-Jumaili *et al.*, 1984). However, low CD vaginal and intestinal carriage rate in Estonian pregnant women may also be one cofactor supporting the low colonisation of Estonian neonates by CD. It is not known if the higher colonisation of infants by CD has any impact on physiological or pathological developments. However, there are some indications that in neonates CD may play some role in the regulation of intestinal motility also under physiological conditions (Midtvedt, 1989).

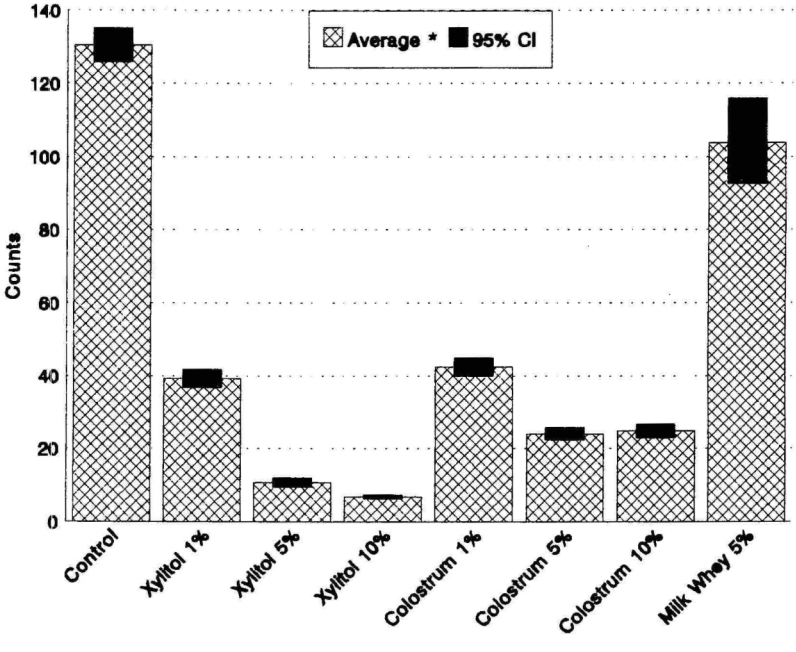
There are no data in the literature about possible differences in the quantitative composition of intestinal microflora of infants depending on their high or low colonisation by CD. Our findings suggest that differences in counts of some groups of indigenous microbes (e.g. lactobacilli) may be one possible explanation of such remarkable differences in colonisation rate with CD in infants. It seems that development of intestinal lactoflora is probably important in eradication of CD from intestinal tract of infants serving as CR controlling factor. Similarly to hospitalised patients, the association between colonisation by CD and lower counts of intestinal lactobacilli was evidently demonstrated in two year old infants. In case of one year old infants there was higher variability in counts of lactobacilli in CD positive as well as in negative infants. This could be explained by the developing stage of intestinal microflora of one year old infants whereas intestinal microflora of two year olds has been further developed, its composition resembling that of adults.

5.2. Experimental models for pathogenesis of *C. difficile* infection

5.2.1. Inhibition of adhesion of *C. difficile* to cell cultures

Our study revealed that both xylitol and bovine colostrum inhibited the adhesion of CD to Caco-2 cells (figure 5.5). The inhibition of adhesion by xylitol was dose dependent. There were statistically significant differences in adherence of CD between control and 1% xylitol ($p<0.01$); 1% and 5% xylitol ($p<0.01$) and 5% and 10% xylitol ($p<0.01$). We found that inhibiting effect of colostrum was partially dose dependent. The adherence was statistically different between

control and 1% colostrum ($p<0.01$), as well as 1% and 5% colostrum ($p<0.01$) but 5% and 10% colostrum had similar inhibition effect on adherence of CD. There were no differences in inhibition by 5% colostrum with and without its preservatives (sodium benzoate and lemon flavour). We found that also 5% milk whey had some influence on adhesion of CD ($p<0.01$) but it was about four and a half times lower than in 5% colostrum whey. Supernatant of *Lactobacillus* GG culture had no influence on adhesion of CD to Caco-2 cells.



Counts = counts of adherent bacteria per microscopic field

Significant differences ($p<0.01$): xylitol 1–10% *vs.* control; xylitol 1% *vs.* xylitol 5 & 10%; xylitol 5 % *vs.* xylitol 10%; colostrum 1–10% *vs.* control; colostrum 1% *vs.* colostrum 5 & 10 %; milk whey 5 % *vs.* control

Figure 5.5. Inhibition of adherence of CD to Caco-2 cells.

5.2.2. Mice model

During our experiments all mice remained healthy and no macroscopic lesions were revealed in necropsies.

5.2.2.1. Changes in intestinal microflora

One day after the administration of the last dose of cefoxitin (Group 2) an absolute, as well as a relative decrease in the total of anaerobes was observed as compared with control (figure 5.6). However, this reduction appeared statistically significant only in the ileal and colonic luminal microflora ($p<0.001$). In all mice of Group 2, the counts of anaerobes were below detection level (<3.3 log CFU/g). Six days after the last administration of cefoxitin and challenge with CD (Group 3) the counts of anaerobes were somewhat lower than those in controls but the difference was statistically insignificant. Thus the counts of anaerobes were almost restored in a week after administration of cefoxitin stopped.

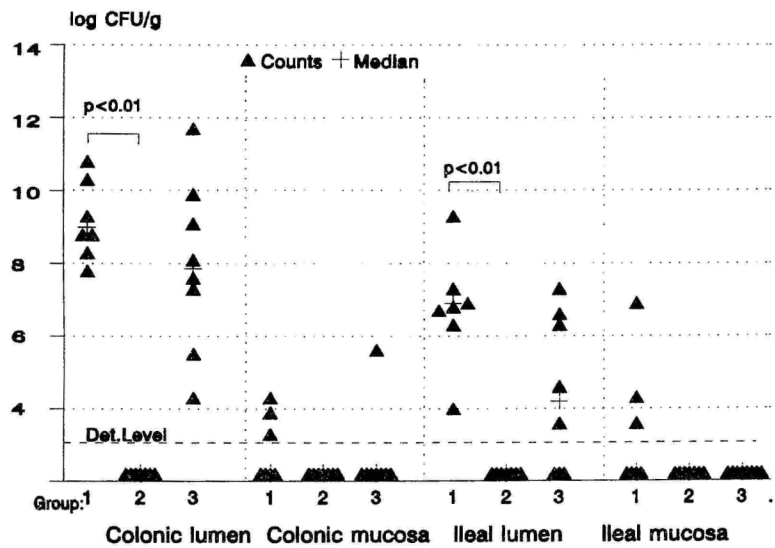


Figure 5.6. Counts of anaerobes in different loci of intestine of mice Group 1(control), Group 2 (received cefoxitin) and Group 3 (received cefoxitin and CD)

The counts of most of the aerobes (coliforms, staphylococci, streptococci) also decreased after the administration of cefoxitin. Enterococci were the only

group of aerobes that increased after antibiotic treatment: their counts increased in Group 2, as compared with the control group in the jejunal luminal and mucosal microflora ($p=0.01$). In the jejunal lumen these counts remained on a higher level even until the 6th day of discontinuation of the antibiotic administration and challenge with CD ($p<0.05$). The administration of cefoxitin had no significant effect on intestinal lactobacilli. The counts of yeasts were increased in Group 2 but the significant difference from control occurred only in jejunal lumen ($p<0.05$). There were no significant differences in the counts of aerobes and anaerobes between Group 4 (received only CD) and the controls (Group 1).

5.2.2.2. Colonisation of animals by *C. difficile*

The faecal samples collected before our experiments from all animals showed that only one mouse from Group 2 was colonised by wild strain of CD (5.3 log CFU/g). After administration of cefoxitin, CD did not occur in its faeces in detectable counts. No CD was found in the intestine of any of control animal.

48 h after the administration of CD all cefoxitin compromised mice (Group 3) appeared to be colonised. The counts of CD varied in their faeces between 4.3 and 9.6 log CFU/g (median 7.1; figure 5.7). Of 7 mice untreated with cefoxitin (Group 4) 5 were colonised 48 h after the challenge with CD (median 5.3 log CFU/g). Five days after administration of CD 7 mice out of 8 in Group 3 were colonised. After the same time only 2 mice out of 7 in Group 4 had remained colonised. CD occurred in detectable amounts only in colonic lumen. The counts varied between 0 and 8.1 (median 4.6) in Group 3 and between 0 and 6.3 log CFU/g (median 0) in Group 4 mice ($p<0.05$). The counts of CD decreased significantly from day 2 to day 5 in both Group 3 and Group 4 ($p<0.05$).

5.2.2.3. Morphological changes in intestinal mucosa

In histological studies of mice we found more clearly expressed changes in colonic mucosa than in ileal one. In the sections of colon we discovered higher inflammatory activity in both, mice Group 2 and mice Group 3, as compared with control mice of Group 1 (table 5.5). In colonic mucosa of these animals more solid lymphoid follicles, diffuse infiltrate and polymorphonuclear (PMN) cells were found. In some animals hyperaemia was present and mitotic activity was enhanced. In mice of group 2 these changes were mild, but of Group 3, moderately expressed. No bacteria were seen in mucosa of Gram-stained samples, although they were visible in the lumen of gut.

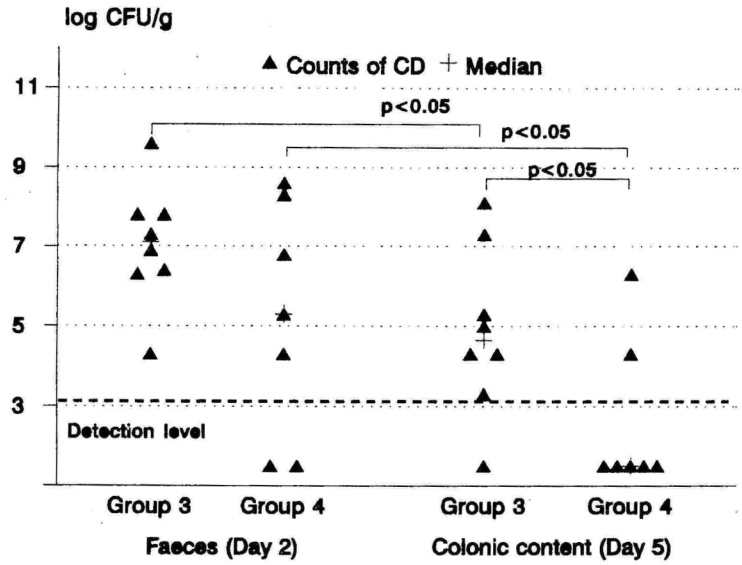


Figure 5.7. Counts of CD in faeces (day 2) and colonic content (day 5) of mice of Group 3 (received cefoxitin and CD) and Group 4 (received only CD)

Table 5.5.

The results of histological examination of colonic mucosa of Groups 1; 2 and 3 mice

Group		Ranges and medians of score values				
		Lymphoid follicles	Lymphoid infiltrate	PMN	Hyper-aemia	Mitotic activity
Group 1 (n=7)	Range	0–1	0	0	0–1	0
	Median	0	0	0	0	0
Group 2 (n=8)	Range	0–2	0–2	0–2	0–2	0
	Median	1	1	1	0	0
Group 3 (n=7)	Range	1–4	0–3	1–2	0–2	0–1
	Median	2	1	2	1	1

Statistically significant difference between groups ($p<0.01$):
In lymphoid follicles: group 1 *vs.* 2 &3 and 2 *vs.* 3; lymphoid infiltrate: group 1 *vs.* 2 & 3; PMN: group 1 *vs.* 2 & 3; hyperaemia: group 1 *vs.* 2 & 3 and 2 *vs.* 3; mitotic activity: group 3 *vs.* 1&2.

5.2.2.4. Bacterial translocation

Bacterial translocation was most frequent (10 cases) in Group 2 i. e. in mice whose intestinal microflora was most deeply disturbed due to recent antibiotic administration. In Group 3 mice whose intestinal flora had recovered we found only one case of translocation, despite of more pronounced inflammatory activity in colonic mucosa. In control group (Group 1) there were no cases of translocation.

The most frequently translocating bacteria were lactobacilli and enterococci, and sometimes the lactobacilli were found even in blood cultures (table 5.6). The translocating microorganisms were usually also predominant in different locations of intestinal mucosa of particular animals.

Table 5.6.

Bacterial translocation (number of cases) and mucosal predominance of microorganisms in Groups 1; 2 and 3 mice

Bacteria	Group 1		Group 2		Group 3	
	Translo-cation	Predomi-nance	Translo-cation	Predomi-nance	Translo-cation	Predomi-nance
Bacteroids	–	Ileum Colon				Colon
Lactobacilli	–	Ileum Colon	3 MLN 1 Spleen 1 Liver 2 Blood	Ileum	1 MLN	Ileum Colon
Enterococci	–		1 MLN 1 Liver	Ileum Colon		
Yeasts	–		1 MLN	Ileum		Ileum

5.2.3. Hamster model

5.2.3.1. Protection of hamsters by xylitol and *Lactobacillus* GG

In our experiments all Group 1 and also Group 3 (received additionally xylitol) hamsters became moribund or died within 1.5 and 2.5 days after challenge with CD. Of 6 hamsters who received additionally *Lactobacillus* GG (Group 4) 3 died within three days and 3 remained healthy and were sacrificed five days after challenge with CD. Among the hamsters who received additionally *Lactobacillus*

GG and xylitol (Group 2) 4 animals out of 5 remained healthy and were sacrificed. One hamster died 3 days after challenge with CD. In Groups 1 and 4 of hamsters also toxin A was detected in caecal content after necropsy: the toxin was present in all animals who acquired CDI but absent in those who remained healthy.

In four animals of Group 1 and all Group 2 hamsters, QCIM and mucosal status were evaluated. After further analysis of the data we grouped these hamsters according to their pathological findings into two groups: hamsters with (n=5) and without (n=4) enterocolitis.

5.2.3.2. Intestinal microflora of hamsters with and without enterocolitis

The most important difference in the intestinal microflora between hamsters with and without enterocolitis was revealed in the counts of indigenous anaerobes (i.e. others than CD). In some hamsters with enterocolitis indigenous anaerobes were absent from all mucosal samples, nor were they any more the predominant microbes of luminal flora (figure 5.8). CD was present in high counts (6.0....11.3 log CFU/g) in all tested luminal and in most mucosal (up to 7.0 log CFU/g) samples of these animals.

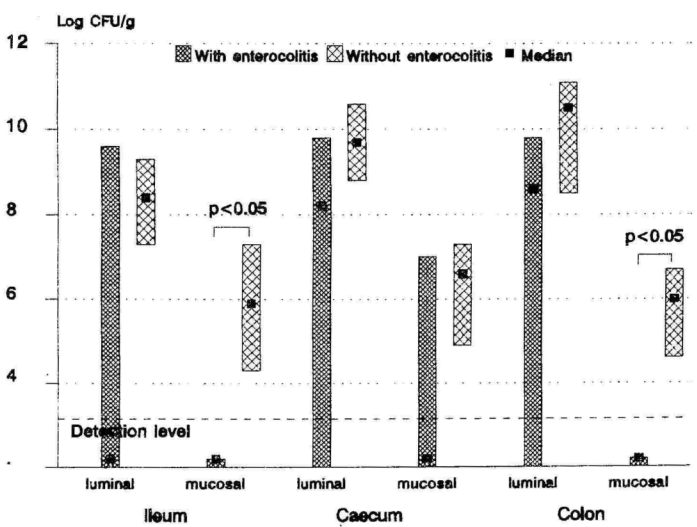


Figure 5.8. Counts of indigenous anaerobes in different intestinal loci of hamsters with and without enterocolitis

In hamsters without enterocolitis these anaerobes were predominant microbes in all sampled intestinal loci in both lumen and mucosa. CD was detectable in low counts in two of these animals: in ileal and caecal lumen in one hamster (3.3 log CFU/g) and in colonic lumen in the other one (4.3 log CFU/g).

5.2.3.3. Morphological changes in intestinal mucosa and bacterial translocation

In all hamsters of Group 1 and in one hamster of Group 2 there were signs of serious enterocolitis: infiltration with lymphocytes and PMN cells, erosions of epithelial surface, haemorrhages in mucosa, formation of pseudomembranes (table 5.7). These changes were equally observable in terminal ileum and caeum but to a lesser degree in colon. In gram-stained sections we detected gram-positive rods adhering to the mucosa. In some cases also different morphotypes of bacteria (gram-positive and negative) were detectable within mucosa in samples stained by Gram or with acridine orange. No enterocolitis was observed in 4 hamsters out of group 2 and only mild inflammatory changes were detected in their intestinal mucosa.

Table 5.7.

The results (ranges and median of score values) of histological examination of ileal mucosa of C. difficile infected hamsters with and without enterocolitis

Inflammatory changes	Hamsters with enterocolitis (n=5)		Hamsters without enterocolitis (n=4)	
	Range	Median	Range	Median
Infiltration*	4–5	5	1–2	2
PMN*	3–5	4	1–2	2
Hyperaemia	2–4	3	0–3	1
Haemorrhages*	1–3	2	–	0
Destruction*	1–3	2	–	0
Pseudomembranes*	1–2	2	–	0

* $p < 0.05$ hamsters with *vs.* without enterocolitis

Bacterial translocation was more frequent in animals with pseudomembranous enterocolitis. In these hamsters translocated bacteria were microorganism dominating in mucosal flora: coliforms, enterococci and lactobacilli and in two

animals also CD. In hamsters without enterocolitis, translocation was rarer and microbes were mostly indigenous anaerobes (table 5.8).

Table 5.8.

Bacterial translocation (number of cases) and mucosal predominance of microorganisms in hamsters with and without enterocolitis

Bacteria	With enterocolitis		Without enterocolitis	
	Trans-location	Predominance	Trans-location	Predominance
Anaerobic G+ cocci	–	–	1 MLN	Ileum Colon
Bacteroides	–	–	1 MLN 1 Spleen 1 Liver	Ileum Caecum Colon
C. difficile	1 MLN 1 Spleen	Ileum Caecum Colon	–	–
Lactobacilli	1 MLN 1 Spleen 2 Liver	Ileum Caecum	1 MLN 1 Spleen 1 Liver	–
Enterococci	2 MLN	Ileum Caecum Colon	–	–
Coliforms	3 MLN 4 Spleen 4 Liver	Ileum Caecum Colon	–	–

5.2.4. Properties of intestinal lactobacilli

5.2.4.1. Grouping and susceptibility

Some 34 strains (20 before and 14 after antibiotic treatment) of lactobacilli isolated from Group 2 mice faecal samples belonged to different groups as follows: OHOL — 26 strains (76%), OHEL — 6 strains (18%) and FHEL — 2 strains (6%). There were no differences in proportions of different groups between strains isolated before and after administration of cefoxitin. All 10 strains randomly selected for antimicrobial susceptibility testing appeared to be resistant to cefoxitin and sensitive to ampicillin.

From faecal samples of Group 2 hamsters 24 different strains were isolated before administration of ampicillin. After administration of antibiotic only 6 strains could be isolated. Lactobacilli belonging to OHOL group were

prevalent in samples taken before (19 strains) but totally absent in samples taken after administration of ampicillin. Out of 22 randomly selected strains 20 appeared sensitive and 2 with intermediate sensitivity to ampicillin *in vitro*. To cefoxitin 10 strains were sensitive, the other 10 resistant and 2 strains displayed intermediate sensitivity.

5.2.4.2. Antagonistic activity against *C. difficile in vitro*

Three strains of lactobacilli (2 OHOL; 1 FHEL) out of 10 isolated from hamsters showed some antagonistic activity against CD on agar plate with inhibitory zones from 4 to 10 mm of CD growth. In co-cultivation with most antagonistic lactobacilli strain (FHEL) CD lost its viability during 6 h (figure 5.9). The counts of lactobacilli did not differ in cultures with and without CD (data not shown).

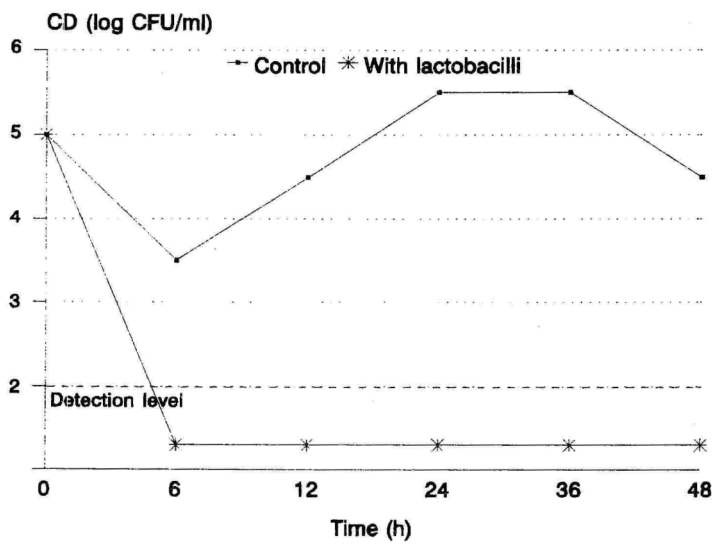


Figure 5.9. Counts of CD cultivated in broth with and without (control) lactobacilli

5.2.5. Discussion

Adhesion of CD. Our studies revealed that both xylitol and colostrum can significantly reduce the adhesion of CD to Caco-2 cells. Adhesion of intestinal pathogens e.g. CD to the mucosa of gut is the initial step essential for

colonisation, expression of virulence and subsequent development of infection (Borriello *et al.*, 1988b; McLlane, 1995). Hence, inhibition of adhesion could be one possible strategy for prevention of colonisation and infection. Prevention of oral colonisation with cariogenic *Streptococcus mutans* by application of xylitol is one example of feasibility of this approach (Mäkinen, 1978).

Cell cultures have been used as experimental models for studying the adherence of intestinal pathogens including CD (Bernet *et al.*, 1993; Finlay & Falkow, 1990; Nishikawa *et al.*, 1994; Eveillard *et al.*, 1993). In earlier studies it has been shown that some carbohydrates, such as glucose and galactose can inhibit the adhesion of CD to cell culture (Karjalainen *et al.*, 1994). Unfortunately, these substances are quickly absorbed or degraded in the gastrointestinal tract and their concentration cannot reach the necessary level in colon after *per os* administration. Since xylitol is absorbed more slowly from gastrointestinal tract than most other common carbohydrates, it may, under circumstances of high dietary intake, achieve considerable concentrations in the large intestine and therefore influence bacterial adhesion (Salminen *et al.*, 1985).

Several studies have shown that administration of normal or hyperimmunised colostrum has positive results in treatment of different gastrointestinal infections (Stephan *et al.*, 1990; Tacket *et al.*, 1988). Bovine colostrum contains a very high level of several bioactive compounds, such as antibodies and growth factors (Korhonen *et al.*, 1978). It has been shown that also colostrum of nonimmunised cows contains a low level of anti-CD IgG. Blocking of receptors by antibodies or other bioactive substances is the putative operating mechanism of colostrum in our experiments. According to several reports the immunoglobulines of bovine colostrum resist digestion in human intestinal tract and hence could be used to influence pathological processes in different parts of intestinal tract (Kelly *et al.*, 1997). Milk whey has shown similar but much weaker effect than colostrum, since cow's milk contains approximately ten times less immunoglobulines than colostrum (Kelly *et al.*, 1996). The supernatant of Lactobacillus GG culture has no influence on adhesion of CD.

Experimental models. In our experimental studies we used two different animal models for CDI: ampicillin compromised hamsters and cefoxitin compromised mice. In the first model we got lethal infection in all CD challenged controls but none in mice model.

Antibiotic treated hamsters are the most frequently used animal models of CDI. In hamster model, untreated animals resisted challenges with very large doses of CD, whereas antibiotic treated animals were susceptible to very low doses of microorganisms. Earlier experiments have shown that all hamsters remain susceptible to CDI for 2 days after administration of 3 mg ampicillin *per os* (Larson & Borriello, 1990). It has also been shown that in studies into

protection of hamsters with various agents, susceptibility or resistance is absolute, partial susceptibility manifesting itself in terms of mild illness or prolonged diarrhoea has never been described in hamsters (Larson & Welch, 1993). We could observe the same phenomenon in our experiments: hamsters either died within 3 days or remained completely healthy. Thus, despite some differences in localisation and course of CDI between hamsters and humans the hamster model seems suitable for screening for efficacy of different agents in case of fatal infection.

As compared with hamsters, mice are relatively resistant to CD: infection is not so fulminate and mortality is low (Wilson *et al.*, 1986). Some previous studies have shown that CDI could be induced in cefoxitin treated BALB/c mice (Wilson *et al.*, 1986; May *et al.*, 1995). Although all cefoxitin treated mice became colonised in our experiment, no evident symptoms of disease developed. Possible reasons for this are discussed below. Since CDI in mice is usually mild and also the pathological changes resemble those of humans, the mice model seems to be suitable for studying changes in intestinal microflora and reaction in mucosa during CDI.

Intestinal microecology in experimental CDI. Comparing the intestinal microflora of cefoxitin treated mice with controls we revealed the most remarkable changes in numbers of anaerobes: the total count, as well as the counts of different groups of anaerobes decreased in all intestinal loci due to antimicrobial treatment. At the same time the counts of enterococci increased. These changes are typical of decreased CR in intestinal tract resulting from antibiotic usage (Nord & Edlund, 1991). This imbalance of intestinal microflora was rapidly recovered and 6 days after discontinuation of administration of cefoxitin the IMF was almost restored. Although there were significant differences in CD population levels between pre-treated and untreated mice, CD did not become predominant in the intestinal ecosystem even in cefoxitin treated mice. Also the counts of CD in 5 days after its administration were significantly lower in colonic content in both groups as compared with initial colonisation (2 days after CD challenge). One possible explanation for this barely moderate weakening of CR against CD may be the stability of lactoflora during cefoxitin treatment. The total counts of lactobacilli remained stable in all intestinal loci during the treatment. Also all of the isolated strains of lactobacilli were resistant to cefoxitin and there were no differences in lactoflora group composition isolated before or after administration of cefoxitin.

Comparing the intestinal microflora of hamsters with and without enterocolitis we found remarkable changes in their counts of anaerobes: in hamsters with enterocolitis these counts were lower in all intestinal loci. However, we did not find significant differences in counts of lactobacilli in these hamsters. Although hamsters without enterocolitis (Group 2) were fed with

lactobacilli these microbes did not become predominant in their mucosal flora. The finding that the used probiotic strain did not become dominant in intestinal microflora correlates with previous studies with infants (Sepp *et al.*, 1993). Some other studies have shown that administration of a probiotic strain of lactobacilli can increase the counts of the other members of IMF such as bacteroids and bifidobacteria (Orrhage *et al.*, 1994; Benno *et al.*, 1996).

Although we did not compare the counts of lactobacilli before and after administration of ampicillin we can draw some conclusions about the strength of influence of lactobacilli. Studying the strains of lactobacilli isolated before and after antibiotic administration we revealed that OHOL group that was predominant before was totally absent after administration of antibiotics. Also most of the strains were sensitive to ampicillin. This finding indicates that antibacterial treatment can influence group composition of intestinal lactoflora. Similar findings that OHOL group of lactobacilli is the most sensitive to external influences has also been described before (Karki, 1996).

Influence of histological and microbial shifts on bacterial translocation in CDI. In our mice study bacterial translocation was most frequent in animals whose intestinal microflora was more deeply disturbed due to recent administration of antibiotics. The translocating bacteria were lactobacilli and enterococci i.e. bacteria predominating in mucosal microflora. In CD challenged mice whose indigenous intestinal microflora had been partly recovered the translocation was quite rare regardless of even more pronounced inflammation in their intestinal mucosa. Thus, it seems that in cases of mild CDI the extent of disturbance of intestinal microflora is more important for promoting the bacterial translocation than the degree of inflammatory activity in mucosa. However, we have found some inflammatory changes in intestinal mucosa of mice who had received antibiotic but not CD. This inflammatory reaction could be caused by an imbalance of microecosystem and subsequent changes in metabolic endproducts of microorganisms or associated directly with antibiotic. It is possible that these changes of mucosa are also one of the cofactors supporting translocation.

In case of serious enterocolitis with profound intestinal mucosal damage in hamsters, bacterial translocation was detected in all animals. Translocating bacteria (coliforms, lactobacilli, enterococci) were also predominant in intestinal mucosa. In hamsters who did not develop enterocolitis the translocation was rare and usually caused by anaerobic bacteria normally predominating in intestinal mucosa and content. Translocation of CD was infrequent despite of its high counts in mucosa of infected hamsters. Also in clinical studies CD has been rarely found in blood and other extraintestinal sites (Feldman *et al.*, 1995; Qualman *et al.*, 1990).

Our finding that changes in intestinal microecology *i.e.* reduction of colonisation resistance and overgrowth by some subordinate bacteria can promote bacterial translocation from intestinal tract correlates with previous data (Berg, 1992; Van Leeuwen *et al.*, 1994). In our experiments the translocating bacteria were also in all cases the predominant microbes in the intestinal mucosa. This finding correlates with previous experiments (Mikelsaar & Türi, 1990).

It has been also shown in clinical studies that in most cases of bacteremia due to bacterial translocation from the intestinal tract the causing agent is the same facultative gram-negative organism that was simultaneously predominating in faeces (Wells *et al.*, 1988). It has been shown that CDCD is a predisposing factor for gram-positive especially *Enterococcus spp.* bacteremia (Borek *et al.*, 1996). Enterococci are typically resistant to many antimicrobial agents and therefore may survive the antimicrobial treatment inducing CDCD and overgrowth after reduction of colonisation resistance. In our experiments we have also seen translocation of enterococci, however, some other microorganisms were frequently found in MLN and other organs. Although it is known that some microbes are more capable of translocation than others it seems that in case of AAD the composition of individual intestinal microflora and the spectrum of activity of the applied antibiotic are the most important factors for selecting the microorganism that can potentially translocate and cause bacteremia.

Effect of Lactobacillus GG and xylitol. We have found that both GG alone and in combination with xylitol had partial protective effect against CDI in hamsters. Since the groups were quite small, the protective effect could be statistically shown only in case of combination of GG and xylitol. Considering the experience that control hamsters (compromised with antibiotic and challenged with CD) always died, also the protective effect of GG then administered alone, becomes more obvious.

The beneficial effect of GG in case of diarrhoeal disease has been reviewed above (2.4.). Despite the success in clinical studies the effect of GG against CDI has not been proven in experimental models before. The operative mechanisms of GG in this protection remains unclear.

In the case of xylitol we expected putative protection by two mechanisms. The first possible action could be the inhibition of adhesion of CD according to our results with cell cultures. The other effect of xylitol is the modification of intestinal flora reported previously (Salminen *et al.*, 1985; Salminen *et al.*, 1993b). Since the modification of IMF takes usually more time, the other mechanism is unlikely to be important in such a fulminate course of infection, which is typical of hamsters. Although we did not discover any effect of xylitol alone in hamsters studies, the other animal models with milder CDI may be useful for detection its possible impact on IMF. Thus, whether co-administration

of xylitol plus GG has any effect in CDI as compared with GG alone needs further studies.

6. GENERAL DISCUSSION

The understanding of natural colonisation resistance to CD is a crucial problem in comprehension of pathogenesis of CDI and a prerequisite for more successful application of microecological approach in its prophylaxis and treatment.

For studying CD and IMF interactions several models could be used. The simplest one is the detection *in vitro* of antagonistic activity of members of IMF against CD. Several isolated strains have been shown to own antagonistic activity against CD in previous experiments and in our study as well. However, the value of these results is problematical since production of inhibitory substrates may only figure as one putative regulatory mechanism out of several, functioning *in vivo*.

Use of animal models has several advantages over clinical studies in investigation of pathogenesis of CDI. These models permit to study microbiological and histological changes at different biotopes, bacterial translocation to extraintestinal sites and other parameters in different stages of disease. However, it is not clear to what extent we can use experimental studies as model of CDI in humans since there are significant differences in intestinal microflora, infection sites and course between humans and experimental animals.

For studying the possible impact of different microbial groups of IMF on CR to CD in intestinal tract of humans we were particularly interested of applicability of two approaches: (1) comparison of IMF of persons commonly colonised and with similar exposure to pathogen (e.g. patients of the same ICU) and (2) following the changes of intestinal microflora and eradication of CD from intestine during the development of IMF of infants. Our primary results indicate the usefulness of these approaches and encourage us to continue the studies in this field.

Despite extensive studies in intestinal microecology during CDI attempts to isolate particular organisms or to find a single mechanism responsible for CR to CD have been unsuccessful. Obligate anaerobes of IMF are microorganisms most commonly associated by several investigators with intestinal CR. It has been shown in many studies that antimicrobial treatment suppresses the intestinal anaerobic microflora. Also our experimental and clinical studies have shown that significant decrease in counts and percentage of anaerobes in different intestinal loci is the most common effect of antimicrobial therapy on intestinal microflora. Despite the decrease in total counts of anaerobes in patients under antibacterial therapy not all of them become colonised by CD and we could not reveal any difference in total counts of anaerobes between CD positive and negative patients. Also in our mice study only moderate weakening of CR could be observed after suppression of anaerobic microflora.

At the same time we found that colonisation of infants and also hospitalised patients by CD was associated with lower counts of intestinal lactobacilli. However, despite of this common trend there were noticeable individual variations. Also our experimental studies indicate that intestinal lactobacilli may probably have an important role in maintenance of CR to CD.

There are also some theoretical considerations that make questionable the monopolistic role of anaerobes in maintenance of CR. For example, despite very high activity of metronidazole against most obligate anaerobes its administration is not associated with development of CDI. Some investigators try to solve this discrepancy by explanation with pharmacokinetics of metronidazole: the drug is completely absorbed, so that its concentrations in the colon are virtually nil (Bartlett, 1994). On the other hand, then it is difficult to explain how metronidazole can be one of the most effective drugs for treatment of CDI localised in colon. Furthermore, despite it supposedly low concentrations in colonic lumen, metronidazole can significantly suppress mucosal indigenous anaerobes in experimental model (Mikelsaar & Siigur, 1992). At the same time lactobacilli are relatively resistant to metronidazole and most intestinal lactobacilli are also resistant to vancomycin. But they are sensitive to penicillins, cefalosporines and clindamycin i.e. antibiotics most frequently associated with CDI (Lenzner *et al.*, 1980; Miller & Shah, 1994; Mändar, unpublished data). These data fit in with our supposition that lactobacilli may play an important role in guaranteeing resistance to CD.

Despite the attractiveness of the theory that one microbial group (*genus, species*) is responsible for maintenance of CR to CD, inconsistency of abundant clinical and experimental data indicate that there is no single microbial group that controls establishment of CD in intestinal tract by one mechanism. It is more likely that several diverse microbes may be involved by different mechanisms, forming functional groups with similar action: competition for nutrients or adhesion site; production of metabolites with inhibitory activity to growth, adhesion or toxin production; neutralisation of toxins; blockade of toxin receptors; activation of host responses etc. This approach is in accordance with previous experimental data that reconstitution of CR to CD with complete faecal flora of different animals or humans was almost always successful but failed if several particular isolates or their combinations were used.

As IMF varies from person to person different microbes may have a leading role in one functional group and also the role of particular functional group in maintenance of CR to CD may vary in different individuals. Every functional group may make its individual contribution to maintenance of CR, and if this (total) pool decrease under certain level the establishment of CD in intestinal microecosystem becomes possible. We have also found marked individual differences in counts of anaerobes, lactobacilli and other members of IMF in

healthy, as well as in CD positive patients. It seems that not only the total count of lactobacilli, but also the group and species composition of intestinal lactoflora may be important in CR. Thus, the total count of intestinal lactobacilli may be an indirect indicator of CR so far as its shifts reflect similar trends in particular groups or species of lactobacilli highly antagonistic to CD in particular individual.

The individual differences in intestinal microflora with a leading role in the maintenance of CR explain why one antimicrobial agent can cause CDCD in some patients but not in others. This approach can also help to understand the large variation of disease from asymptomatic colonisation to PMC e.g. switching off one protective mechanism allows the CD to colonise intestinal tract but the full effect of virulence could be inhibited by another one. Thus, on the one hand, alteration of all functional groups for development of CDI is not necessary, and on the other hand, suppression of one functional group may not lead to CDI.

For restoring CR several approaches have been used including substitution of colonic flora by enemas of faeces of healthy persons, and supporting recovery of normal flora by administration of prebiotics or probiotics. Administration of certain microorganisms (probiotic) in high doses may restore CR to CD and/or prevent CDI by mechanism different from that was prevalent in maintenance of CR by indigenous microflora. It seems that for complete manifestation of protective effect, probiotic may need co-operation with some functional groups of IMF and if these groups are absent, the effect is weaker. It is known that some probiotics (similarly to prebiotics) can increase the counts of some microbial groups of IMF. For example, *Saccharomyces boulardii* enhances somewhat the counts of intestinal lactobacilli and this could be one of the mechanisms by which it restores CR to CD (Karki *et al.*, 1996). Necessity of some groups of IMF for successful action of probiotic is one possible explanation why administration of GG does not provide protection in all experimental animals (and probably not in all patients either). Beside other important factors the efficacy of a probiotic depends on how many different antagonistic mechanisms against CD it owns. An illustration of this is the effective use of nontoxigenic strains of CD: toxigenic and nontoxigenic strains compete for the same nutrients and the same mucosal receptors.

Thus, we have found some degree of association between the presence of intestinal lactobacilli and CR to CD. Further studies are needed to clarify the role of particular groups and species of lactobacilli in the maintenance of CR.

7. CONCLUSIONS

1. The occurrence of *Clostridium difficile* in patients with nosocomial diarrhoea has been investigated in Tartu University Hospitals. Nearly one third of studied patients (36.5%) were colonised with *C. difficile*. The occurrence of *C. difficile* was higher in intensive care units and surgical departments as compared with internal ones.

2. The frequency of colonisation by *C. difficile* in the intensive care unit in different periods of observation was widely varying. The occurrence of *C. difficile* was higher in patients treated with a larger number of antibiotics.

3. Studying the carriage of *C. difficile* in Estonian nonhospitalised persons we found a low carriage rate (4...8%) among infants as compared with investigations in other countries. The colonisation of pregnant women (5%) was not different from data of healthy nonhospitalised adults in Europe.

4. In quantitative composition of intestinal microflora of patients colonised with *C. difficile* the imbalance (reduction of anaerobes and increase of aerobes) was present in most cases. The counts of *C. difficile* were individually different in these patients, forming from <0.001 to 80.7% of total faecal microflora. The counts of intestinal lactobacilli were higher in *C. difficile* negative patients as compared with positive ones from the same department.

5. *C. difficile* strains isolated from hospitalised patients were mostly (95%) toxigenic. All these strains were susceptible to metronidazole and vancomycin, antibiotics widely used for treatment of *C. difficile* infection. Only half of the strains were susceptible to rifampicin, antimicrobial agent previously reported as highly active against *C. difficile* and also recommended for treatment of *C. difficile* infection.

6. There were considerable differences in *C. difficile* colonisation between Estonian and Swedish infants. Swedish one and two year old infants were more frequently colonised with *C. difficile* than Estonian ones. The colonisation of infants with *C. difficile* was associated with lower counts of intestinal lactobacilli. Thus, the geographical differences in colonisation by *C. difficile* in infants may be caused by differences in the composition of their intestinal microflora.

7. For studying the pathogenesis of *C. difficile* infection two different experimental animal models were developed by which the quantitative composition of intestinal microflora, mucosal injury and translocation of microorganisms were compared. Administration of cefoxitin caused dramatic decrease in counts of intestinal anaerobes in mice but did not affect lactoflora. These changes supported the colonisation by *C. difficile*, accompanied by

mild inflammation of mucosa but no development of obvious symptoms of infection. In ampicillin challenged hamsters *C. difficile* caused fatal pseudomembranous enterocolitis. This model of severe infection provided a possibility to assess the protective effect of different interventions.

8. In case of mild experimental *C. difficile* infection bacterial translocation was more closely associated with antibiotic introduced disturbance of intestinal microflora than with the degree of inflammatory activity in mucosa after challenge with *C. difficile*. Severe *C. difficile* infection with destructive damage of mucosa led to massive bacterial translocation from intestine. Translocating microbes in both experimental models were mostly facultative anaerobes, predominant in intestinal mucosa. The translocation of *C. difficile* was rare, occurring in some cases of fatal enterocolitis.

9. Studying the strategies for prevention of *C. difficile* infection we revealed that *Lactobacillus* GG administered *per os* showed partial protective effect against lethal *C. difficile* infection in hamster model. Using cell culture model we found that bovine colostrum and xylitol can inhibit the adhesion of *C. difficile*. Thus, pre- and probiotics could be possible strategies for influencing intestinal microecology to prevent colonisation by *C. difficile*.

10. Our clinical and experimental studies indicate that both indigenous intestinal and introduced lactobacilli may play a significant role in maintaining colonisation resistance to *C. difficile*. However, remarkable individual differences could be seen in the counts and relative amounts of lactobacilli in the total intestinal flora of persons colonised by *C. difficile*. Thus, these differences could be one cofactor influencing the development of *C. difficile* infection.

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CLOSTRIDIUM DIFFICILE INFEKTSIOON JA SEEDETRAKTI MIKROOBIÖKOLOOGIA

Kokkuvõte

C. difficile (*CD*) on peamine antibakteriaalse raviga seotud diarröa tekitaja ning samuti olulisim täiskasvanute haiglasisese diarröa põhjustaja. *CD*-infektsiooni eeldusteks peetakse seedetrakti mikroobiökoloogia muutusi: *CD*-vastast kolonisatsiooniresistentsust tagava indigeense mikrofloora tasakaalu häirumine. Siiani ei ole aga täpselt teada, millistel mikroobigruppidel on oluline osa selle resistentsuse tagamisel ning milliste mehhanismidega see toimub.

CD-ga sageli koloniseeritud gruppideks on hospitaliseeritud patsiendid ja alla kaheaastased lapsed. Viimaste koloniseerituse sagedus on eri uuringute põhjal erisugune. Uurides faktoreid, mis soodustavad *CD*-ga koloniseerumist on peamiselt keskendutud välistele teguritele (antibiootikumide kasutamine, ekspositsioon jne.), kuid vähem on uuritud erinevusi seedetrakti mikroökosüsteemis.

CD-kandluse ja -infektsiooni sageduse kohta Eestis andmed siiani puudusid, sest kuni 1991 aastani puudus *CD* mikrobioloogilise diagnostika võimalus. Käesoleva töö teema valiku üheks ajendiks oligi *CD* mikrobioloogilise diagnostika juurutamise vajadus haiglas, millest kasvas välja põhjalikum seedetrakti mikroobiökoloogia uurimine.

Uurimistöö eesmärk

Töö peamiseks eesmärgiks oli *CD*-kolonisatsiooni ja *CD*-infektsiooniga seotud seedetrakti mikroökosüsteemi muutuste selgitamine. Konkreetsed eesmärgid:

(1) *CD* esinemise selgitamine erinevates inimgruppides (hospitaliseeritud patsiendid, lapsed, rasedad);

(2) *CD*-positiivsete ja -negatiivsete patsientide ning laste seedetrakti mikrofloora hulgalise koostise võrdlemine;

(3) patsientidelt isoleeritud *CD*-tüvede mõningate omaduste uurimine (hulk seedetraktis, resistentsus, toksigeensus);

(4) mõnede *CD*-infektsiooni patogeneesiga seotud faktorite (seedetrakti mikrofloora ja limaskesta muutused, bakterite translokatsioon seedetraktist) vaheliste seoste uurimine eksperimentaalsetel mudelitel;

(5) mõningate *CD*-infektsiooni profülaktika võimaluste (adhesiooni pärssimine, probiootikumi tüve kasutamine) hindamine.

Uuritavad ja meetodid

Inimesed

203 nosokomiaalse diarröaga patsiendil (grupp I), kes olid hospitaliseeritud Tartu kliinikutes määrati latekstestiga *CD* esinemine roojas, nendest 18 *CD*-positiivsel (grupp II) teostati rooja kvantitatiivne uuring. Kahe kahekuulise perioodi vältel uuriti Tartu Ülikooli Närvikliiniku intensiivravi-patsientide koloniseeritust *CD*-ga (rooja külv): esimesel perioodil 25 patsienti (grupp IIIA, 40 analüüsi) ja teisel perioodil 13 patsienti (Grupp IIIB, 27 analüüsi). Viimase grupi patsientide 20 analüüsis (grupp IV) määrati mikrofloora kvantitatiivne koostis. Võrreldi laktobatsillide ja *CD* esinemist rootsi ja eesti ühe- (29+27 last) ja kaheaastaste (36+36) laste roojas (grupid V...VIII). Uuriti 22 raseda naise seedetrakti koloniseeritust *CD*-ga.

Eksperimentaalsed uuringud

Tsefoksitiini ja/või *CD*-ga mõjutatud hiirtel (kokku 29) hinnati seedetrakti mikrofloorat ja limaskesta muutusi erinevates lokalisatsioonides ning bakteriaalset translokatsiooni. Ampitsilliini ja *CD*-ga ning ksülitooli ja/või *Lactobacillus GG*-ga mõjutatud hamstritel (kokku 23) hinnati infektsiooni tekkimist, seedetrakti mikrofloorat ja limaskesta muutusi ning bakteriaalset translokatsiooni.

Caco-2 koekultuuril uuriti *CD* adhesiooni mõjutamist ksülitooli, kolostrumi- ja piimavadaku ning laktobatsillide kultuuri supernatandiga.

Patsientidelt isoleeritud *CD*-tüvedel (20) määrati toksiin A produktsioonivõime ning erinevate meetoditega antibiootikumi tundlikkus. Uuriti ka hamstritelt isoleeritud laktobatsillide mõningaid omadusi (grupiline kuuluvus, tundlikkus, antagonism *CD* suhtes).

Uurimistöö peamised tulemused

Leiti, et *CD*-d oli umbes kolmandikul (36,5%) nosokomiaalse diarröaga patsientidest. *CD* esinemissagedus oli oluliselt kõrgem ($p<0,05$) intensiivravi- (48,7%) ja kirurgia- (45,7%) osakondades, võrreldes sisehaiguste osakondadega

(19%). *CD* hulk patsientidel indiviiditi erines (2,3...8,4 log PMÜ/g), moodustades <0,001 kuni 80,7% mikroobide üldhulgast. Märkimisväärselt erines ka anaeroobide, aeroobide ja laktobatsillide üldhulk.

Intensiivravipatsientide uurimisel leidsime, et esimesel perioodil ei olnud ükski patsient *CD*-ga koloniseeritud, teisel aga seitse patsienti 13-st. *CD*-ga koloniseeritud patsiendid olid saanud rohkem mitmesuguseid antibiootikume kui *CD*-negatiivsed (mediaan 5 *vs.* 3; p<0,05). Samuti oli *CD*-positiivsetel patsientidel roojas harvem laktobatsille (3/9 *vs.* 9/11; p<0,05).

Patsientidelt isoleeritud *CD*-tüved olid enamasti toksilised (19/20). Kõik tüved olid tundlikud beeta-laktaam antibiootikumide (välja arvatud tsefoksitiin ja tseftisoksiim), metronidasooli ja vankomütsiini suhtes. Tundlikkus Klooramfenikool-, tetratsükliin-, klindamütsiin-, erütromütsiin ja rifampitsiintundlikkus varieerus.

Võrreldes rootsi lastega oli eesti üheaastastel lastel roojas sagedamini laktobatsille (17/27 vs. 11/29; p<0,05) ja harvem *CD*-d (1/27 vs. 10/29; p<0,05). *CD* osas oli sarnane seos ka kaheaastastel lastel (3/36 vs. 9/36; p<0,05). Laktobatsillide esinemissageduses kaheaastastel eesti ja rootsi lastel olulisi erinevusi ei olnud. Võrreldes kõiki *CD*-positiivseid ja -negatiivseid kaheaastaseid lapsi oli esimestel laktobatsille harvem (3/12 vs. 37/60; p<0,05).

Hiirte mõjutamine tsefoksitiiniga põhjustas anaeroobide hulga olulist vähenemist kõigis soole lokalisatsioonides, kuid laktobatsillide hulk ei muutunud. Sellised mikrofloora muutused soodustasid küll hiirte koloniseerumist *CD*-ga, kuid infektsiooni sümptoome ei tekkinud, ilmnes vaid jämesoolelimaskesta mõõdukas põletikureaktsioon. Bakteriaalne translokatsioon oli sagedasem vahetult pärast antibiootikumi manustamist kui viis päeva pärast *CD*-ga koloniseerumist, mil normaalne mikrofloora oli juba osaliselt taastunud. Translotseerusid peamiselt soolelimaskestal domineerivad fakultatiivsed anaeroobid. Seega, kerge *CD*-infektsiooni mudelil oli translokatsiooni soodustavaks faktoriks pigem seedetrakti mikrofloora tasakaalustamatus kui limaskesta põletikulised muutused.

Ampitsilliini ja *CD*-ga mõjutatud hamstritel kujunes kolme päeva jooksul fataalne enterokoliit. Täiendavalt *Lactobacillus GG*-d ja ksülitooli saanud hamstritest haigestus viiest üks ning ainult *Lactobacillus GG*-d saanutest kuuest kolm. Ksülitool üksinda hamstreid *CD*-infektsiooni eest ei kaitsnud. Enterokoliidiga loomadel olid sooles ulatuslikud destruktiivsed muutused ning massiline bakteriaalne translokatsioon. Translotseerusid peamiselt limaskestas domineerivad enterobakterid ning üksikutel juhtudel ka *CD*.

Kasutades koekultuurimudelit, leidsime, et nii ksülitool kui ka kolostrumi-preparaat inhibeerivad *CD* adhesiooni. Ksülitooli adhesiooni pärssiv toime oli

sõltus doosist ning kolostrumil osaliselt doosist. *Laktobacillus GG* supernatandil inhibeerivad omadused puudusid.

Järeldused

(1) *CD*-d oli umbes 1/3-l nosokomiaalse diarröaga patsientidest. Sagedus varieerus osakonniti, olles kõrgem intensiivravi- ja kirurgia osakondades.

(2) Intensiivraviosakonna patsientide koloniseeritus *CD*-ga varieerus perioodiliti ning oli seotud suurema hulga mitmesuguste antibiootikumide kasutamisega.

(3) Eesti laste koloniseeritus *CD*-ga oli suhteliselt madal, rasedatel aga sarnane kirjanduse andmetega.

(4) Enamikul *CD*-ga patsientidel oli düsbioos. *CD* hulk seedetraktis varieerus indiviiditi. Lactobatsille oli rohkem *CD*-negatiivsetel patsientidel kui *CD*-positiivsetel.

(5) Kõik patsientidelt isoleeritud *CD*-tüved olid metronidasool- ja vankomütsiintundlikud ning enamik ka toksigeensed

(6) Võrreldes eesti lastega oli rootsi lastel *CD*-d sagedamini, laktobatsille aga harvem.

(7) Tsefoksitiiniga mõjutatud hiirtel jäi laktobatsillide hulk sooles muutumatuks ning infektsiooni välja ei kujunenud. Ampitsilliiniga mõjutatud hamstritel tekkis fataalne pseudomembranoosne enterokoliit.

(8) Kerge infektsiooni korral oli bakteriaalne translokatsioon seotud pigem seedetrakti mikrofloora muutustega, kui soole limaskesta põletikuga.

(9) Uurides *CD*-infektsiooni profülaktika võimalusi, leidsime et *Lactobacillus GG* kaitses osaliselt hamstreid letaalse infektsiooni eest. Ksülitool ja kolostrum pärssisid *CD* adhesiooni koekultuuril.

(10) Seega viitavad meie nii kliiniliste kui ka eksperimentaalsete uuringute tulemused laktobatsillide olulisusele *CD*-vastase kolonisatsiooniresistentsuse tagamisel.

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FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Inhibition of adhesion of *Clostridium difficile* to Caco-2 cells

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Abstract

For many microorganisms, including *Clostridium difficile*, mucosal association is an important factor influencing intestinal colonisation and subsequent infection. Inhibition of adhesion of *C. difficile* to intestinal mucosa could be a new promising strategy for prevention and treatment of antibiotic-associated diarrhoea. We investigated the possibilities of influencing the adhesion of *C. difficile* by xylitol and bovine colostrum whey. Caco-2 cells and *C. difficile* cells were incubated with 1%, 5% and 10% solutions of xylitol and colostrum. Our study revealed that both xylitol and colostrum inhibited the adhesion of *C. difficile* to Caco-2 cells. Inhibition by xylitol was dose-dependent. When compared to the control, the count of adherent *C. difficile* decreased 3.4 times when treated with 1% xylitol, 12 times when 5% xylitol was applied, and 18.7 times when treated with 10% xylitol. The inhibition of adherence by colostrum was partially dose-dependent: 3.1 times in the case of 1%, and 5.5 times in the cases of 5% and 10% colostrum. Further experimental and clinical studies are needed for the application of xylitol and colostrum in the treatment and prophylaxis of pseudomembraneous colitis.

Keywords: *Clostridium difficile*; Colostrum; Xylitol; Mucosal adhesion; Caco-2 cell

1. Introduction

Clostridium difficile is recognized as a major etiological agent of pseudomembraneous colitis and antibiotic-associated diarrhoea. *C. difficile* infection develops when the stability of the indigenous intestinal flora has been disrupted and colonisation resistance decreased [16]. The major known virulence factors of *C. difficile* are the toxins A and B [2,8].

However, the ability of *C. difficile* and other bacteria to adhere to gastrointestinal cell surfaces is becoming recognised increasingly as a prerequisite for colonisation of the gut, expression of virulence and development of infection [3,9]. It is clearly established that *C. difficile* can associate with the intestinal mucosa in humans and hamsters [3]. There appears to be an association between virulence and mucosal adherence of *C. difficile*: the highly virulent strains attach to the mucosa better than poorly virulent or avirulent strains [3]. Hence, inhibition of mucosal adhesion of *C. difficile* could be one new promising strategy for the prevention of colonisation of the intestinal tract with *C. difficile* and subsequent infection. For inhibition of adhesion several blocking substances, such as soluble receptor analogues or anti-receptor antibodies, could be used.

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During recent years the polarized human intestinal epithelial cell line Caco-2 has been used to study the intestinal attachment of *C. difficile* and other pathogens. It has been shown that adherence of *C. difficile* to Caco-2 cells is greatly enhanced after heat-shock and could be blocked by anti-*C. difficile* antibodies and partially by glucose and galactose [4,5]. These data suggest the possibility of using Caco-2 cells as a model for studying different *C. difficile* adhesion blockers.

The aim of our study was to determine the influence of bovine colostrum whey and xylitol to adhesion of *C. difficile* to Caco-2 cells.

2. Materials and methods

2.1. Caco-2 cell culture

Enterocyte-like Caco-2 cells (ATCC HTB 37) were grown in Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, MO, or Gibco Life Technologies Ltd., Paisley, UK) supplemented with 30 mg/l human transferrin (Sigma Chemical Co.), 2 mM L-glutamine (Sigma Chemical Co.), 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (Biological Industries) at 37°C in the atmosphere of 10% CO_2/90% air. Cells were trypsinized and split every 7 days. Monolayers of cells were prepared on glass coverslips which were placed in 24-well tissue culture plates (Greiner GMBH, Nürtingen, Germany). Cells were seeded at a concentration of 500 000 cells/ml to obtain confluence. The culture medium was changed every other day, and one day before bacterial adhesion experiments the culture medium was replaced by antibiotic-free medium. Cells were analysed for growth and confluence using bright field microscopy. Cell cultures were used at post-confluence after 14–16 days of culture.

2.2. Bacterial strains

A highly virulent *C. difficile* strain VPI 10463 was grown in Schaedler broth (BBL Microbiology Systems, Cockeysville, MI) for 24 h in anaerobic jars with gas generating envelopes (Oxoid, Basingstoke, Hampshire, UK) at 37°C. After incubation *C. difficile* broth cultures were mixed and divided into 5 ml aliquots, centrifuged at 1500 × *g* for 10 min, cells were washed once with 5 ml PBS and resuspended in 5 ml cell culture medium or cell culture medium supplemented with xylitol or colostrum.

2.3. Tested solutions

Cell culture medium without supplements for control experiments; cell culture medium supplemented with 1%, 5% and 10% xylitol (Sigma Chemical Co.);

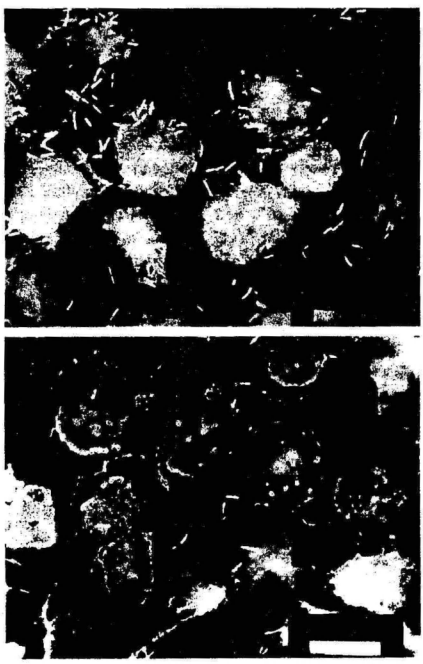

Fig. 1. *C. difficile* adhesion to Caco-2 monolayer. Stained with acridine orange (×10000). Top panel: Control experiment. *C. difficile* binds to Caco-2 cells unevenly forming clusters or as single cells. More bacteria adhered preferentially to the edges of cells. Bottom panel: Inhibition of adhesion of *C. difficile* after influence with 1% xylitol. Only single bacteria could be seen and the majority of enterocytes examined was completely devoid of bacteria.

1%, 5% and 10% bovine colostrum whey (Bioenervi™, Viable Bioproducts Ltd., Turku, Finland); 5% colostrum whey without preservatives (sodium benzoate and lemon flavour) and 5% milk whey.

2.4. Inhibition of adherence

Before adherence tests both post-confluent Caco-2 cell cultures and *C. difficile* cells were preincubated with test solutions. Caco-2 cells were preincubated for 40 min at 37°C in 5% CO_2 atmosphere and washed *C. difficile* cells for 20 min at 37°C in air. After preincubation *C. difficile* cells were heated 10 min at 60°C and then 1 ml of *C. difficile* suspension in test solution was seeded to each well with preincubated Caco-2 cells. Cell cultures with *C. difficile* were incubated for 1.5 h at 37°C in air. After incubation, unbound bacteria were washed five times with 1 ml of PBS, cell culture was dried in air, fixed with methanol, stained by Gram and examined microscopically at a magnification of ×1000. From at least three different glass coverslips of cell monolayers the numbers of adherent bacteria were counted in 26 microscopic fields selected at random. The adhesion index was calculated as the average number of adhering bacteria per microscopic field from at least three different assays.

2.5. Statistics

Student's *t*-test was performed to compare the inhibition of *C. difficile* adhesion with different tested solutions.

3. Results

3.1. Influence of xylitol on adhesion of C. difficile

In control experiments *C. difficile* cells were bound unevenly to Caco-2 monolayer, forming bacterial clusters (Fig. 1, top panel). Our study revealed that xylitol remarkably inhibited the adhesion of *C. difficile* to Caco-2 cells (Fig. 1, bottom panel). The inhibition of adhesion by xylitol was dose-dependent (Fig. 2). There were statistically significant differences in adherence of *C. difficile* between control and 1% xylitol ($P < 0.01$); 1% and 5% xylitol ($P < 0.01$) and 5% and 10% xylitol ($P < 0.01$).

3.2. Influence of colostrum and milk whey on adhesion of C. difficile

We found that inhibiting effect of colostrum was partially dose-dependent. The adherence was statistically different between control and 1% colostrum

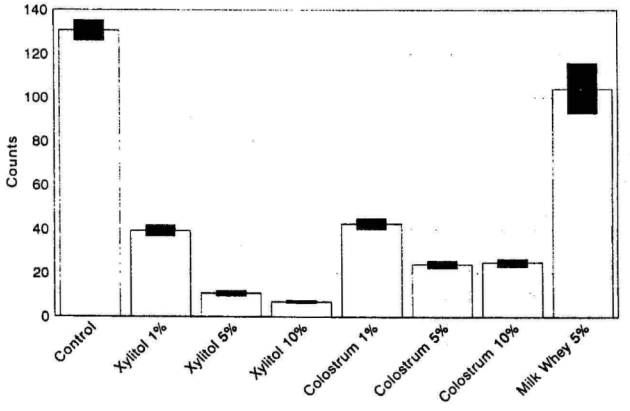

Fig. 2. Inhibition of adherence of *C. difficile* to Caco-2 cells. Counts = Counts of adherent bacteria per microscopic field; CI = Confidence interval. * Average from 3 different Caco-2 assays in each counted 26 fields in 3 different coverslips.

($P < 0.01$), as well as 1% and 5% colostrum ($P < 0.01$) but 5% and 10% colostrum had similar inhibition effect to adherence of *C. difficile* ($P > 0.05$). There were no differences in inhibition by 5% colostrum with and without its preservatives (sodium benzoate and lemon flavour).

We revealed also that 5% milk whey had some influence on adhesion of *C. difficile* ($P < 0.01$) but it was about four and a half times lower than in 5% colostrum whey.

4. Discussion

Our study shows that both xylitol and colostrum whey can significantly reduce the adhesion of *C. difficile* to Caco-2 cells. The post-confluent Caco-2 cell culture served as a suitable model for in vitro studies of inhibition of *C. difficile* adhesion.

The polarized human intestinal epithelial cell line Caco-2 has been used to study the adherence or invasion of many different enteropathogenic microbes. This cell line spontaneously differentiates in culture and undergoes morphological and functional differentiation similar to mature enterocytes [4,11]. In our experiments we preincubated both *C. difficile* and cell culture with tested solutions before performing the adherence assays since these conditions seemed to be more similar to the in vivo situation. In previous experiments also some other cell lines, such as mucus secreting HT29-MTX cell line has been used [4,5]. Since the opinions about the role of mucus in pathogenesis of *C. difficile* infection are contradictory and mucus itself can interfere in adhesion of *C. difficile* we preferred to use Caco-2 cells. Although in control experiments *C. difficile* tends to adhere unevenly, forming clusters on Caco-2 cells, the number of bacteria was still countable.

We have found that xylitol inhibits significantly the adhesion of *C. difficile*. The application of soluble receptor blockers, such as carbohydrates, can desorb attached bacteria or prevent attachment. The feasibility of this approach has been established in principle in models of urinary tract infections. Installation of alpha-methyl mannoside with *E. coli* strains processing type 1 fimbriae into the urinary tract of mice prevented the development of bacteriuria [1]. Xylitol has also been previously reported to prevent colonisation of oral streptococci, especially *Streptococcus mutans* [10].

The exact mechanism of xylitol influence on adhesion of *C. difficile* and other bacteria is not clear. In earlier studies it has been shown that carbohydrates, such as glycose and galactose and also gelatin can partially inhibit *C. difficile* adhesion to Caco-2 cells [15]. Unfortunately these substances are quickly absorbed or degraded in the gastrointestinal tract and their concentration cannot reach the necessary level in colon after per os administration. Xylitol is a five-carbon sugar alcohol. Since xylitol is absorbed more slowly from gastrointestinal tract than most other common carbohydrates, it may, under circumstances of high dietary intake, achieve considerable concentrations in the large intestine [13]. It can be supposed that in the intestine xylitol might interfere with adhesion of *C. difficile* similarly to that shown by our in vitro studies.

We found that application of bovine colostrum to Caco-2 cells reduces significantly the attachment of *C. difficile*. There are several examples of antibodies of normal or hyperimmunized colostrum having given positive results in the treatment of different gastrointestinal infections [14,15]. Bovine colostrum contains a very high level of several bioactive components, such as antibodies and growth factors [7]. Its ability to inhibit adhesion of *C. difficile* may be influenced by some receptor blocking substances (e.g. antibodies). In some studies it has been shown that also colostrum of non-immunised cows contains a low level of anti-*C. difficile* IgG [6]. Several reports suggest that bovine colostral immunoglobulins resists digestion in human intestinal tract and hence could be used to influence pathological processes in different parts of the intestinal tract [6]. The milk whey had shown similar but much weaker effect since cow's milk contains approximately ten times less immunoglobulin than colostrum. It is also known that human milk can inhibit *C. difficile* toxin A-receptor binding. This mechanism could be important in protecting infants against *C. difficile*-associated intestinal disease [12].

Further experiments are needed to study the influence of xylitol and bovine colostrum on mucosal adhesion and further colonisation of *C. difficile* in vivo, and also the possibilities of their application in treatment and prophylaxis of *C. difficile* infection.

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II

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Clostridium difficile põhjustatud diarröa. Mikrobioloogilise diagnoosimise esmane kogemus Tartus

Paul Naaber

Clostridium difficile, **mikrobioloogiline diagnoosimine, kolonisatsioon, ravimitundlikkus**

Clostridium difficile on üks sagedamaid haiglasiseseid diarröa tekitajaid täiskasvanuil. Peaaegu alati on *C. difficile* põhjustatud diarröale eelnenud antibakteriaalne või kasvajavastane ravi. Diarröa ja koliidi patogeneesis on oluline soole limaskesta kahjustus, mille on põhjustanud *C. difficile* produtseeritud toksiinid (10).

Samuti on teada, et *C. difficile* on võimeline koloniseerima soolt vaid siis, kui soole mikrofloora barjäärfunktsioon, mis tagab kolonisatsiooniresistentsuse patogeensete mikroobide vastu, on kahjustatud antibakteriaalse raviga. Tervel inimesel on seedekulgla erinevate osade mikroökosüsteemides üksikute mikroobirühmade vahel püsivad kvantitatiivsed suhted. Sellises tasakaalustatud süsteemis on osa mikroobirühmi domineerivad ja teised alluvad, kusjuures jämesooles ületab domineerivate anaeroobide hulk aeroobide hulga kuni tuhat korda (8). Missugune on inimese mikrofloorasse kuuluvate erinevate mikroobide osa kolonisatsiooniresistentsuse tagamisel, ei ole siiani täpselt teada.

C. difficile põhjustatud diarröa mikrobioloogilist diagnoosimist alustati Eestis 1991. aastal Tartus Maarjamõisa Kliiniku mikrobioloogialaboris. Käesoleva töö eesmärgiks oli välja selgitada *C. difficile* hulk ja tähtsus diarröaga patsientide rooja mikroflooras, *C. difficile* põhjustatud diar-

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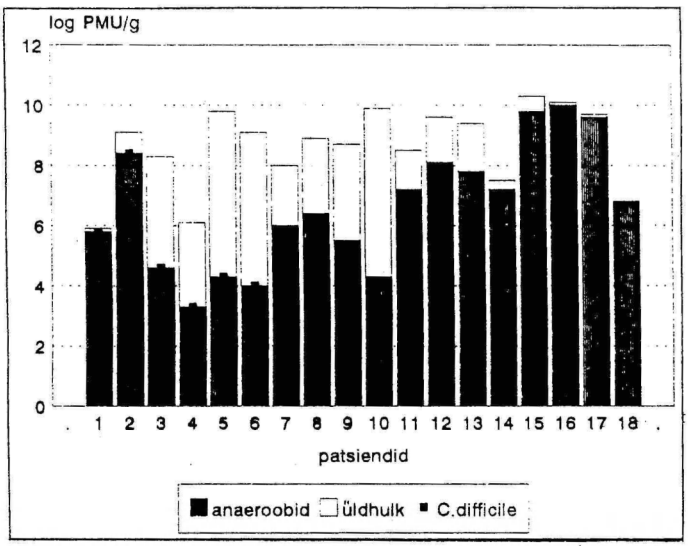


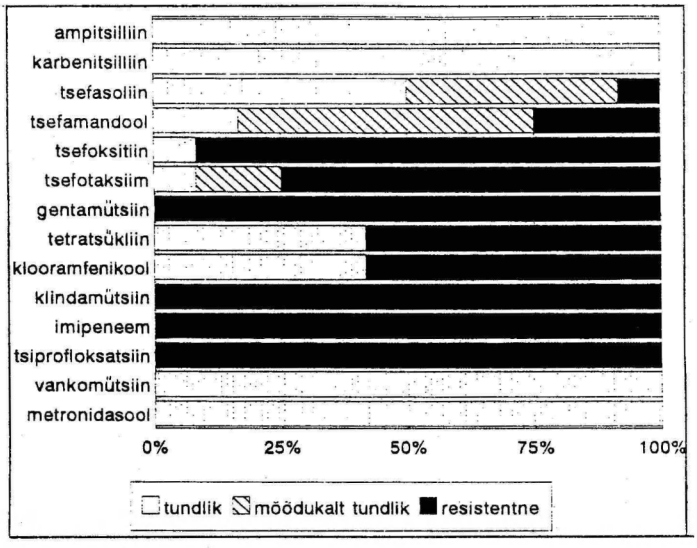
Joonis 1. *C. difficile*, **anaeroobide ja mikroobide üldhulk.**

röa esinemissagedus Tartu kliinikute erinevates osakondades ning meie isoleeritud *C. difficile* tüvede ravimitundlikkus.

Uurimismaterjal ja -metoodika. 1991. aasta detsembrist kuni 1994. aasta jaanuarini uuriti Tartu Maarjamõisa Kliiniku mikrobioloogialaboris 203 patsiendi analüüsi, kellel oli tekkinud antibiootikumide manustamise tulemusena diarröa. Nad olid hospitaliseeritud Tartu kliinikute erinevates osakondades järgmiselt: intensiivraviosakondades 78 patsienti, kirurgiaosakondades 46 ja sisehaiguste osakondades 79 patsienti. *C. difficile* määramiseks roojast kasutati sõeltestina *Culturette Brand'i* CDT–lateks–aglutinatsiooni testi *(Becton Dickinson).*

Lisaks nimetatule määrati 18 juhuslikult valitud patsiendil, kelle CDT–latekstest oli positiivne, fekaalse mikrofloora kvantitatiivne koostis. Selleks valmistati eelnevalt kaalutud roojatükist CO_2 keskkonnas lahjendused redutseeritud fosfaatpuhvris. Mikroobide hulga määramiseks kasutati järgmisi söötmeid: *Fastidious Anaerobe Agar* (F.A.A., LAB M) — anaeroobide üldhulga määramiseks; pärmiekstrakti sisaldav põhisööde — aeroobide üldhulga määramiseks; *Cycloserine Cefoxitin Fructose Agar* (CCFA, *Oxoid*) — *C. difficile* määramiseks (7, 12). F.A.A. söödet inkubeeriti 5 päeva ja CCFA–d 48 tundi temperatuuril 37°C anaeroobses keskkonnas, seejuures kasutati anaerostaati *Anaerobic Systeme* 100 ja anaeroobset keskkonda genereerivaid pakendeid *GasPak Plus* (mõlemad *Becton Dickinson).* Põhisöödet inkubeeriti 48 tundi temperatuuril 37°C aeroobses keskkonnas. Väikseim selle metoodikaga määratav mikroobide hulk oli > 2 log PMÜ/g roojas. Mikroobide hulga ja osatähtsuse arvutamiseks kasutati arvutiprogrammi *BioQuant* (9). *C. difficile* põhjustatud diarröaga patsientidelt isoleeritud 12 *C. difficile* tüvel määrati ravimitundlikkus 14 antibiootikumi suhtes agardifusioonimeetodil, kasutades antibiootikumidiske *(bioMerieux)* ja F.A.A. söödet (LAB M). Ampitsilliini minimaalse inhibeeriva kontsentratsiooni (MIK) määramiseks kasutati E–testi (AB *Biodisk)* (2).

Uurimistulemused ja arutelu. 203 uuritust oli CDT–test positiivne 74 juhul (36,5%–l). Osakonniti oli positiivsete tulemuste osatähtsus erinev: intensiivraviosakondades — 48,7%–l (38 positiivset 78–st), kirurgiaosakondades 45,7%–l (21 positiivset 46–st) ja sisehaiguste osakondades 19%–l (15 positiivset 79–st).



Joonis 2. *C. difficile* **tüvede ravimitundlikkus.**

C. difficile hulk patsientide roojas varieerus 2,3—8,4 log PMÜ/g, anaeroobide hulk 3,3—10,0 log PMÜ/g ja mikroobide üldhulk 6,1—10,3 log PMÜ/g. Vaadeldes neid arve iga patsiendi puhul eraldi, leidsime neis suuri erinevusi (vt. joonis 1). Aeroobide üldhulk ületas anaeroobide üldhulga 13 patsiendi analüüsis. *C. difficile* osatähtsus mikroobide üldhulgast oli väga erinev, ulatudes 0,000001—80,7%-ni. *C. difficile* oli domineerivaks anaeroobiks seitsmel juhul ning moodustas kuuel juhul ligikaudu 100% anaeroobide üldhulgast.

C. difficile tüvede ravimitundlikkus on esitatud joonisel 2.

Kõik uuritud tüved olid tundlikud ampitsilliini, karbenitsilliini, metronidasooli ja vankomütsiini suhtes. Klooramfenikooli, tetratsükliini ja tsefalosporiinide suhtes oli tundlikkus erinev, kusjuures I põlvkonna tsefalosporiinide (tsefasoliin) suhtes oli tundlikke tüvesid rohkem, kuid II ja III põlvkonna ravimite suhtes olid enamik resistentsed. Kõik uuritud tüved olid resistentsed klindamütsiini, gentamütsiini ja tsiprofloksatsiini suhtes. Kuigi imipeneem pidurdas kõigi uuritud tüvede kasvu, leidus suhteliselt palju resistentseid mutante, mistõttu tuleb nimetatud tüvesid pidada resistentseks imipeneemi suhtes. Ampitsilliini minimaalne inhibeeriv kontsentratsioon uuritud *C. difficile* tüvede suhtes oli järgmine: 0,25 μg/ml — ühel tüvel; 0,38 μg/ml — ühel tüvel; 0,5 μg/ml — kolmel tüvel; 1,0 μg/ml — viiel tüvel; 1,5 μg/ml — ühel tüvel ja 3,0 μg/ml — ühel tüvel. Seega olid kõigi tüvede MIK-d alla 4 μg/ml, mida peetakse ampitsilliinil tundlikkuse piiriks (1).

C. difficile põhjustatud diarröa diagnoosimiseks kasutatakse mitmeid meetodeid, mis põhinevad kas mikroobi või tema toksiinide määramisel roojas. Meil kasutatava CDT-latekstesti tundlikkuse ja spetsiifilisuse kohta esineb kirjanduses vastukäivaid andmeid (12). Selle meetodi eeliseks on kiirus ja suhteliselt madal hind. *C. difficile* toksiinide määramise meetodid ei ole meil praegu igapäevases diagnoosimises kasutatavad, seda nii vastavate rakukultuuride puudumise kui ka kõrge hinna tõttu. Väljakasvatamine CCFA-söötmel on laialt kasutatav, kuid

suhteliselt aeganõudev meetod. Rooja kvantitatiivse koostise ja *C. difficile* hulga määramist roojast on siiani veel vähe kasutatud.

C. difficile on antibakteriaalse raviga seotud diarröa põhjustajaks kirjanduse andmeil keskmiselt 25%–l juhtudest (4). Mõningal määral suurem esinemissagedus meie uurituil (36,5%) võis olla tingitud sellest, et *C. difficile* määramise algusjärgul pöörduti meie labori poole vaid kliiniliselt raskekujulisema diarröa korral. Intensiivravi– ja kirurgiaosakondades oli *C. difficile* suurem esinemissagedus diarröa tekitajana ootuspärane, sest seal viibivatel haigetel on resistentsus enamasti vähenenud ning neile on pikka aega manustatud mitmesuguseid antibakteriaalseid preparaate suhteliselt suurtes annustes.

C. difficile põhjustatud diarröaga patsientide rooja mikrofloora kvantitatiivne uurimine näitas soole mikroökosüsteemi tasakaalustamatust. Enamikul juhtudel olid domineerivaks aeroobsed mikroobid ning nende hulk ületas anaeroobsete hulga kuni 10000 korda. Mitmel patsiendil oli *C. difficile* ainuke määratavas koguses esinev anaeroob *faeces*'es. Ma ei ole kirjandusest leidnud andmeid, mis kirjeldaksid selliseid märkimisväärseid muutusi seedetrakti mikroflooras *C. difficile* põhjustatud diarröa korral. Kuigi ei ole teada, missugused mikroobid tagavad kolonisatsiooniresistentsuse *C. difficile* suhtes, on mitmed autorid üksmeelel, et sellest protsessist võtab osa palju erinevaid mikroobirühmi (5). Samuti viitavad mitmed *in vitro* ja *in vivo* tehtud eksperimendid sellele, et nendeks võiksid olla tervetel inimestel domineerivad anaeroobsed mikroobid (3). Meie uurimistulemused, mis näitavad anaeroobsete mikroobide hulga vähenemist *C. difficile* põhjustatud diarröaga patsientidel, kinnitavad nimetatud seisukohta. Siiski ei esinenud aeroobide ülekaalu kõigil uurituil, mis näitab, et mikrofloora seisund pärast antibakteriaalset ravi võib patsientidel olla erinev. Peab muidugi arvestama, et haiguse patogeneesi seisukohalt ei ole oluline mitte ainult *C. difficile* hulk sooles, vaid ka koloniseeriva tüve mitmed virulentsusfaktorid, nagu kinnistumine limaskestale ja toksiinide produtseerimisvõime. Ei tohi unustada ka asjaolu, et rooja mikrofloora peegeldab vaid osaliselt soole eri osade limaskestale adhereerunud mikroobide omavahelisi suhteid (8).

Kuigi meie uuritud *C. difficile* tüvedest olid kõik *in vitro* tundlikud ampitsilliini, karbenitsilliini ning enamik tundlikud või mõõdukalt tundlikud tsefasoliini suhtes, ei ole β–laktaamide kasutamine *C. difficile* infektsiooni raviks näidustatud. Ravi efekti puudumine *in vivo* nimetatud preparaatide kasutamise korral võib ühelt poolt tingitud olla nende farmakokineetilistest omadustest, näiteks antibiootikum ei saavuta *C. difficile* suhtes vajalikku püsivat bakteritsiidset kontsentratsiooni soole limaskestas. Teiselt poolt on ilmselt oluline ka nende preparaatide pärssiv toime kolonisatsiooniresistentsust tagavatesse mikroobidesse, mistõttu antibiootikumi toimiva kontsentratsiooni vähenemisel soole limaskestas on võimalik uus *C. difficile* ülekasv kas soole allesjäänud eostest või uuest väliskeskkonnast pärit nakatumisest.

Empiiriliselt on *C. difficile* põhjustatud diarröa juhtudel efektiivseks osutunud ravi vankomütsiini või metronidasooliga. Meie täheldatud 100%–line tundlikkus vankomütsiini ja metronidasooli suhtes on vastavuses kirjanduse andmetega, mille põhjal ligikaudu kõik *C. difficile* tüved on *in vitro* tundlikud nende preparaatide suhtes (6). Nimetatud ravi edukust võiks seletada ka asjaoluga, et neil ravimitel on teistest väiksem toime kolonisatsiooniresitentsust tagavasse indigeensesse mikrofloorasse. Näiteks metronidasool ei toimi laktobatsillidesse ja bifidobakteritesse ning neil on madal aktiivsus eoseid mittemoodustavatesse grampositiivsetesse anaeroobidesse (13). Siiski esineb ka nen-

de preparaatide kasutamise korral küllalt sageli diarröa retsidiive, on isegi kirjeldatud juhtumeid, kus pärast vankomütsiini ja metronidasooli manustamist on tekkinud *C. difficile* infektsioon. Need faktid kinnitavad seisukohta, et kolonisatsiooniresistentsust tagav indigeenne mikrofloora on individuaalselt erinev. Üks võimalikke kolonisatsiooniresistentsuse tõstmise ja ravitulemuste parandamise viise oleks sobiva probiootikumi manustamine antibakteriaalse ravi ajal või pärast seda (11).

Kokkuvõte. Ajavahemikul 1991. aasta detsembrist kuni 1994. aasta jaanuarini uuriti Tartu kliinikutes hospitaliseeritud ja antibakteriaalse raviga seotud 203 diarröaga patsiendi rooja analüüsi *C. difficile* suhtes. Neist 74-l (36,5%-l) oli CDT-latekstest positiivne. 18 patsiendil, kellel määrati kvantitatiivselt rooja mikrofloora koostis, esines *C. difficile* hulgas 2,3—8,4 log PMÜ/g. Neist 13-l ületas aeroobide hulk anaeroobide hulga, mis näitab soole mikrofloora tasakaalustamatust, ning 7-l juhul oli *C. difficile* domineerivaks anaeroobiks. 12 uuritud *C. difficile* tüvest olid kõik tundlikud vankomütsiini ja metronidasooli suhtes.

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Summary

Clostridium difficile Caused Diarrhea: Primary Experience of Microbiological Diagnostics in Tartu. Between Dec, 1991 and Jan, 1994, 203 consecutive hospitalized patients with antibiotic associated diarrhea were investigated for the presence of *C. difficile*. In 74 (36.5%) cases CDT-test appeared to be positive. In 18 random patients with positive CDT-test the quantitative composition of the fecal microflora was determined. The counts of *C. difficile* varied between 2.3 and 8.4 log CFU/g. In 13 samples the counts of aerobes exceeded those of anaerobes which indicates the imbalance of the intestinale microflora. In 7 cases *C. difficile* was the predominant anaerobe. All 12 investigated strains of *C. difficile* occurred susceptible to vancomycine and metronidazole.

III

Clinical Infectious Diseases, 1997; 25 (Suppl 2)

Colonization of Infants and Hospitalized Patients with *Clostridium difficile* and Lactobacilli. Paul Naaber, Karin Klaus, Epp Sepp, Bengt Björksten, and Marika Mikelsaar. From the Institute of Microbiology, University of Tartu, Tartu, Estonia; and the Department of Paediatrics, University Hospital, Linköping, Sweden

Clostridium difficile is the most frequent agent of nosocomial diarrhea in industrialized countries. Approximately 10%–25% of hospitalized patients become colonized by *C. difficile* [1]. Among nonhospitalized adults, the rate of carriage of *C. difficile* in the intestinal tract varies in different countries, ranging from 2% in Sweden to 15% in Japan [2]. Asymptomatic carriage of *C. difficile* is more common among infants and young children, ranging from 15% to 63% [2].

It is generally agreed that the disruption of the indigenous intestinal microflora is an essential prerequisite for colonization by *C. difficile*. The presence of lactobacilli as a component of the indigenous intestinal microflora has traditionally been associated with resistance to colonization with intestinal pathogens [3, 4]. However, it is not clear if the counts of lactobacilli differ significantly between persons who are colonized by *C. difficile* and those who are not. The aim of our study was to compare the prevalence and counts of *C. difficile* and intestinal lactobacilli in Estonian and Swedish children and to compare the counts of lactobacilli in hospitalized Estonian patients in relation to the presence of *C. difficile*.

We investigated the fecal microflora of 27 healthy Estonian infants and 29 healthy Swedish infants (all 1 year of age) and 34 consecutive patients (66 fecal samples) in the neurological intensive care unit of Tartu University Hospital (Tartu, Estonia). The patients had been hospitalized because of neurological surgery or brain trauma for periods ranging from 2 days to 5 months (median duration, 11 days) before sampling. These patients had been treated with up to nine different antimicrobial agents (median number, four; some had not received any agents). The study was done during two 2-month periods separated by a 10-month interval. To detect *C. difficile* in these hospitalized patients, the fecal samples were seeded after alcohol shock into cefoxitin-cycloserine-fructose agar and incubated anaerobically for 4 days. In addition, the counts of *C. difficile* and lactobacilli in all samples from the infants and in 20 randomized samples from the hospitalized patients that were collected in the second period were determined as described previously [5].

Ten (35%) of 29 Swedish infants and only one (4%) of 27 Estonian infants were colonized with *C. difficile* ($P < .05$; figure 1A). Furthermore, counts of *C. difficile* in feces were significantly higher in Swedish infants than in Estonian infants ($P < .05$; figure 1B). However, both the prevalence and counts of lactobacilli were significantly higher among the Estonian infants than among the Swedish infants ($P < .005$).

None of the 21 hospitalized patients was colonized by *C. difficile* during the first 2-month observation period, whereas seven of 13 patients were found to be colonized during the second period. The counts of *C. difficile* varied from 4.3 log cfu/g to 8.1 log cfu/g (median count, 7.0 log cfu/g). The counts of lactobacilli were significantly higher ($P < .05$) in *C. difficile*-negative samples (0–8.8 log cfu/g; median count, 5.3 log cfu/g) than in *C. difficile*-positive samples (0–7.1 log cfu/g; median count, 0 log cfu/g).

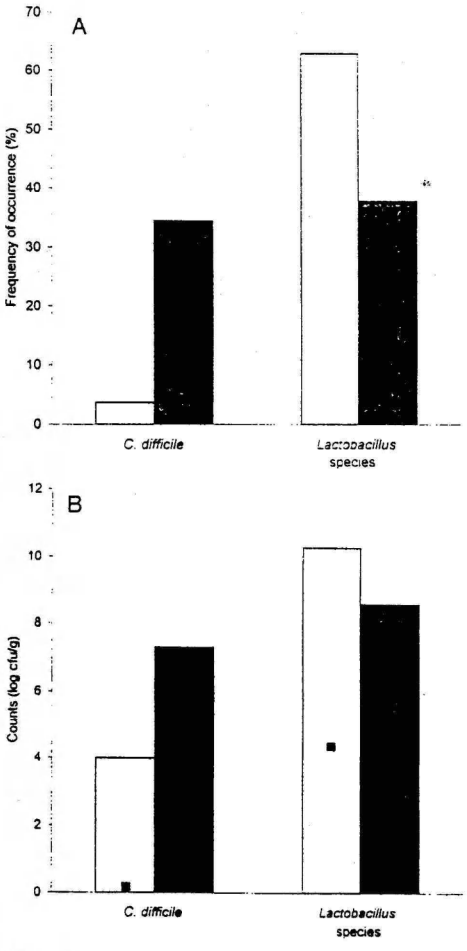

Figure 1. *A*, Frequency of the occurrence of *Clostridium difficile* and lactobacilli in Estonian (□) and Swedish (■) infants. *B*, Ranges and medians (■) of counts of *C. difficile* and lactobacilli in Estonian (□) and Swedish (■) infants.

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Thus, we found that the intestinal carriage rate for *C. difficile* was relatively low among Estonian infants as compared with that among Swedish infants. At the same time, Estonian infants were more often colonized with lactobacilli, and the counts were higher. It is known that some lactobacilli are antagonistic to *C. difficile* in vitro and in vivo [3, 4]. Therefore, we speculate that high counts of lactobacilli in the intestines of Estonian infants are protective against colonization by *C. difficile*. The differences in the prevalence of lactobacilli and some other intestinal microbes among Estonian and Swedish infants could be due to different diets and/or other conditions under which the sources for normal intestinal microflora are established (authors' unpublished data). The maternal intestinal microflora and the hospital environment are the most likely determinants of acquisition of *C. difficile* by neonates. Thus, the low carriage rate for *C. difficile* among Estonian pregnant women (authors' unpublished data) could also explain the low prevalence of *C. difficile*.

The counts of lactobacilli were significantly lower in the *C. difficile*-positive hospitalized patients. Furthermore, the patients in whom the lactobacilli remained relatively intact after antimicrobial treatment were less likely to be colonized by *C. difficile*. Whether lactobacilli play a significant role in the maintenance of resistance to *C. difficile* colonization in various populations and hospitalized patients needs further study.

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IV

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ANTIBIOTIC - COMPROMISED MURINE MODEL OF *CLOSTRIDIUM DIFFICILE* INFECTION

PAUL NAABER, and MARIKA MIKELSAAR

SUMMARY

Cefoxitin compromised murine model was used for the quantitative evaluation of the changes of microflora in different intestinal biotypes during *C. difficile* infection. After the administration of cefoxitin the counts of anaerobes and most groups of aerobes decreased, the counts of enterococci increased and those of lactobacilli did not change significantly. In spite of the *C. difficile* colonisation on the mouse colon, no macroscopic pathological lesions of the viscera could be revealed and the normal intestinal microflora recovered rapidly. Our study showed that not only the anaerobes/aerobes ratio but also some other microorganisms, e.g. the amount of intestinal lactobacilli, seem to be important in guaranteeing the colonisation resistance against *C. difficile*.

INTRODUCTION AND AIM

Clostridium difficile has been firmly ascertained as a cause of pseudomembranous colitis and of many cases of antibiotic-associated diarrhoea. The normal indigenous flora of the gastrointestinal tract provides an important protective barrier against colonisation by *C. difficile*. Disruption of the stability of the gut flora, particularly that resulting from the use of antibiotics, facilitates the establishment of *C. difficile* (*Wilson*, 1993). Unfortunately attempts to isolate and identify these particular organisms which provide resistance against *C. difficile* colonisation have been unsuccessful. Experimental antibiotic-associated colitis has been induced in several animal species. The mice model resembles the disease in humans more precisely than the hamster model since mice are relatively resistant to *C. difficile* whereas pseudomembranes are quite commonly seen in their caeca (*Wilson* et al., 1986). Unfortunately, experimental studies do not report any comparative data on the changes of microflora in the intestinal content and the mucosa during *C. difficile* infection.

The aim of the present study was to examine the quantitative relations between the major microbial groups of different intestinal locations in experimental *C. difficile* infection in mice.

MATERIAL AND METHODS

Animals

Twenty-nine adult BALB/c mice (approx. 25 g) were distributed into 4 groups and kept in individual cages: group 1 was the control group (7 mice); group 2 (7 mice) received cefoxitin; group 3 (8 mice) received cefoxitin and *C. difficile*; group 4 (7 mice) received *C. difficile*.

Administration of cefoxitin

Cefoxitin (Mefoxitin, Merck Sharp & Dohme GmbH) was administered once a day with a daily dose of 100 mg/kg intragastrically for 5 days.

Administration of *C. difficile*

We used a toxin producing strain of *C. difficile* (ME-82) isolated from a patient with *C. difficile* associated colitis*. 0.5 ml of 24 h old *C. difficile* culture in Columbia Broth (Becton Dickinson) was administered intragastrically to group 3 mice 24 h after the last administration of cefoxitin, and to group 4 mice 24 h after their separation to individual cages.

Samples

Faecal samples were collected before the administration of cefoxitin, and before and 48 h after the administration of *C. difficile*. Using chloroform anaesthesia the mice were sacrificed: group 1 mice 24 h after their separation to individual cages; group 2 mice 24 h after the last administration of cefoxitin; group 3 and 4 mice 5 days after the administration of *C. difficile*. Samples of the luminal contents and slices of the mucosa were obtained from the ileum (1-2 cm from the ileo-caecal valve) and from the colon (2-3 cm from the anus). To remove the microbes not attached to the mucosa, the slices were vigorously washed for 1 min in 2 ml of prereduced phosphate buffer. The weighed slices of mucosa were then homogenised with sterile glass powder and serial decimal dilutions under a stream of CO_2 were prepared in prereduced phosphate buffer. Likewise, the samples of the luminal contents were weighed and serially diluted in prereduced phosphate buffer.

Bacteriological studies

The concentrations of microbes were determined by serial dilution and seeding on different freshly prepared media: Fastidious Anaerobe Agar (F.A.A., LAB M) for total anaerobes; Columbia CNA Agar (Becton Dickinson) for Gram-positive anaerobes; kanamycin-vancomycin agar - F.A.A. supplemented with kanamycin 100 mg/l and vancomycin (Lilly) 7.5 mg/l for Gram-negative anaerobes; Cycloserine Cefoxitin Fructose Agar - CCFA Agar Base (Oxoid) supplemented with cefoxitin (Mefoxitin, Merck Sharp & Dohme GmbH) 16 mg/l, cycloserine (Sigma) 250 mg/l, sodium taurocholate (Sigma) 1 g/l, neutral red indicator (Merck) 30 mg/l and 20 % egg-yolk suspension for *C. difficile*. F.A.A., Columbia CNA Agar, kanamycin-vancomycin agar and CCFA were incubated at 37°C for 5 days in an anaerobic atmosphere (GasPak 100 Anaerobic System, Becton Dickinson). For the detection of clostridia alcohol shock was used: equal amounts of each dilution and absolute ethanol were mixed for 30 min. and then seeded on F.A.A. medium (*Marler* et al., 1992). *C. difficile* was identified by its ability to grow on

* This strain was kindly presented to us by prof. P.Grönroos (Tampere, Finland).

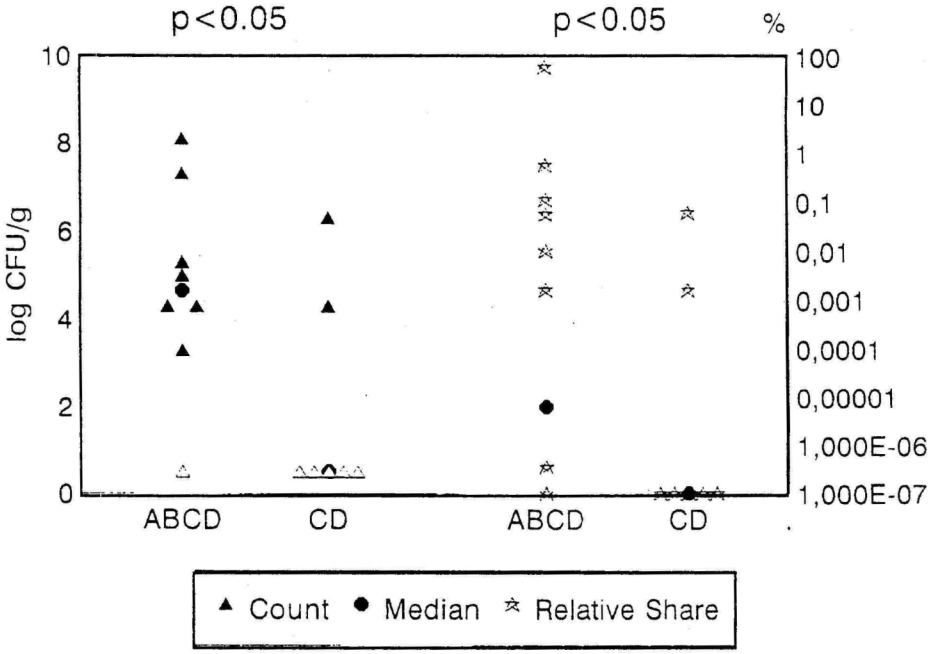

Figure 1: Counts and relative proportion of *C. difficile* in colonic luminal microflora.
ABCD : Received cefoxitin and *C. difficile*.
CD : Received *C. difficile*.

CCFA, colonial and cellular morphology, specific smell and with RapID-ANA II anaerobic identification system (Innovative Diagnostic Systems). All other microbial groups were isolated and identified as described previously (*Mikelsaar* and *Siigur*, 1992). The detection level of our method was 3 log CFU/g. For microorganisms counting the computer program "BioQuant" was used (*Mändar* et al., 1992).

RESULTS

After the administration of cefoxitin (group 2) both an absolute as well as a relative decrease of the total anaerobes and their different groups occurred. However, this reduction appeared statistically significant only in the luminal microflora ($p<0.001$). Six days after the last administration of cefoxitin and challenge with *C. difficile* (group 3) the counts of anaerobes were somewhat lower than those in the controls. After the administration of cefoxitin the counts of most of the aerobes also decreased (coliforms, staphylococci, streptococci). Enterococci were the only group of aerobes that increased after antibiotic treatment: their counts went up in group 2, as compared to the control group, in the jejunal, luminal and mucosal microflora ($p=0.01$). In the jejunal lumen these counts remained on a higher level even until day 6 after the interruption of the antibiotic and challenge with *C. difficile* ($p<0.05$). The

administration of cefoxitin had no effect on intestinal lactobacilli. There were no significant differences in the counts of aerobes and anaerobes between the group 4 and the controls.

Colonisation by *C. difficile*

The faecal samples collected before our experiments showed one mouse to be colonised by *C. difficile* (5.3 log CFU/g). After the administration of cefoxitin *C. difficile* did not occur in its faeces in detectable counts. Forty-eight hours after the administration of *C. difficile*, all cefoxitin compromised mice (group 3) and 5 from the 7 untreated mice (group 4) were found colonised. Five days after the administration of *C. difficile* in group 3 seven mice out of 8 and in group 2 two mice out of 7 were colonised. *C. difficile* occurred in detectable amounts only in the colonic lumen. The counts and the relative proportion of *C. difficile* in the total counts were individually different (Figure 1). During the study all mice remained asymptomatic and no macroscopic pathological lesions of the viscera could be revealed in necropsies.

DISCUSSION

Although there were significant differences in the *C. difficile* population levels between the pretreated and nontreated mice, all animals remained asymptomatic. *C. difficile* does not usually become predominant in the intestinal microbial ecosystem even in cefoxitin-pretreated mice. Our study shows that in mice cefoxitin causes only a moderate weakening of *C. difficile* colonisation resistance. There is general agreement that anaerobes play an important role in the maintenance of the stability and balance of the intestinal microecosystem and that might be true also about *C. difficile* colonisation resistance. In our study the total counts of anaerobes decreased more than 1,000,000 times due to cefoxitin treatment and so it indicates that anaerobes which persist in high numbers in the intestinal tract are not the only important microbial group providing colonisation resistance against *C. difficile*. There were no significant changes in the counts of intestinal lactobacilli after the administration of cefoxitin. The relative stability of lactobacillary microflora could also be a possible factor helping to maintain colonisation resistance in mice.

CONCLUSION

Our study shows that not only the anaerobe/aerobe ratio but also the amount of some other microorganisms, e.g. intestinal lactobacilli, seem to be important in guaranteeing the colonisation resistance against *C. difficile*.

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Bacterial Translocation, Intestinal Microflora and Morphological Changes of Intestinal Mucosa in Experimental Models of *Clostridium difficile* Infection

Paul Naaber, Seppo Salminen, Marika Mikelsaar

Abstract

Bacteremia and subsequent septic process is one possible complication of *C. difficile* infection. The aim of our study was to correlate bacterial translocation with morphological changes of intestinal mucosa and shifts of intestinal microflora in experimental models of *C. difficile* infection. We used mice model to study postantibiotic shifts and mild *C. difficile* infection and hamsters as model of fatal enterocolitis. We also studied the influence of pro- and prebiotics (lactobacilli and xylitol) in hamster model. Quantitative composition of luminal and mucosal microflora was evaluated in different intestinal loci, inflammatory changes of mucosa were estimated in histological sections and bacterial translocation was detected in samples from blood, liver, spleen and mesenteric lymph nodes. We found that in case of mild *C. difficile* infection the extent of disturbance of intestinal microflora appears to be a more important promoting factor of translocation than inflammatory activity in mucosa. In fatal enterocolitis translocation is frequent with facultative anaerobes predominating in intestinal mucosa and in some cases also with *C. difficile*. Combination of lactobacilli and xylitol have some protective effect against *C. difficile* infection.

Introduction

Clostridium difficile is the established cause of antibiotic associated diarrhoea. Depending on its virulence and the susceptibility of host this pathogen produces a wide spectrum of disease, ranging from self-limited watery diarrhoea to fatal pseudomembranous colitis (PMC). Disruption of the stability of the host's indigenous microflora is a prerequisite for colonisation by *C. difficile* and subsequent infection. This alteration of colonisation resistance occurs mainly after antibiotic therapy in hospitalised patients. In cases of complicated PMC colonic perforation, peritonitis and secondary infections, mainly due to bacteremia, may result [1]. In spite of emergency surgical intervention and heavy antimicrobial treatment these severe conditions are associated with high mortality [2].

In some patients with severe underlying disease bacterial translocation from mucosal membranes, particularly of the intestinal tract, is an important cause of bacteremia and subsequent septic processes [3]. Intestinal bacterial translocation is the passage of viable indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes, spleen, liver, peritoneal cavity and bloodstream [4]. The most frequent translocating organisms include *E. coli* and other members of *Enterobacteriaceae,* but rarely anaerobes [3]. The exact microorganisms that translocate through *C. difficile* damaged intestinal mucosa have not been identified. An experimental model for such studies is urgently needed.

To study the pathogenesis of *C. difficile* induced infections and its virulence factors several experimental animals have been used, including Guinea pigs, rats, rabbits,

mice and hamsters [5]. The most frequently used animal model for studying severe *C. difficile* enterocolitis is the antibiotic compromised hamster model, since hamsters are extremely sensitive to *C. difficile* toxins.

C. difficile diarrhoea could be induced also in cefoxitin treated conventional BALB/c mice [5]. Mice are relatively resistant to *C. difficile* toxins and the morphological findings in caecum during infection are similar to changes in human intestine [5]. The mouse model of *C. difficile* infection has been successfully used for investigation of histological changes in intestinal mucosa and shifts in intestinal microflora in animals undergoing different diets [6].

Although experimental studies have shown that administration of antimicrobial agents and physical alteration of mucosal barrier can enhance bacterial translocation from the intestinal tract it is not known whether translocation correlates with histological changes in intestinal mucosa and with shifts in intestinal mucosal and luminal microflora after antibiotic therapy or during development of *C .difficile* infection. Nor is it known if probiotic administration can influence the spectrum of translocating organisms in pseudomembranous colitis.

Aim

Our aim was to study if it is possible to predict the type of bacterial translocation knowing the degree of inflammatory changes in intestinal mucosa and the state of intestinal microflora in experimental *C. difficile* infection.

Material and methods

Study design.

To study the influence of possible alterations in intestinal microbial ecology important for the pathogenesis of antibiotic associated diarrhoea we used several experimental animal models: (1) antibiotic compromised mice as a model of post-antibiotic shifts in intestinal tract; (2) antibiotic compromised and *C. difficile* challenged mice as a model of mild *C. difficile* infection; (3) antibiotic compromised and *C. difficile* infected hamsters as a model of severe enterocolitis and (4) antibiotic compromised and *C. difficile* challenged hamsters receiving xylitol and lactobacilli as a model for putative influence of probiotics on *C. difficile* infection.

Experimental animals.

Adult BALB/c mice (n=22) were used in experiments. The mice were housed individually in stainless steel cages. The cages were sterilised before using and the bedding was changed daily. The mice were distributed into 3 groups: group 1 served as control (7 mice); group 2 received cefoxitin (Mefoxitin, MSD Sharp & Dohme GmbH), daily dose 100 mg/kg (administrated intragastrically with a stainless steel feeding tube) for 5 days (8 mice); group 3 received cefoxitin (similarly to group 2) and was inoculated with virulent strain of *C. difficile* ME 82 (7 mice) 24 h after the last antibiotic dose. Mice were sacrificed using chloroform anaesthesia (group 2: 24 h after the last antibiotic dose; group 3: 5 days after challenge with *C. difficile*) and samples were taken from ileum and colon for histological and bacteriological studies.

Nine adult Syrian hamsters (*Mesocricetus auratus*) were divided into 2 groups and held in the same conditions as mice. The group 1 hamsters (n=4) received ampicillin (3 mg intragastrically) and after 24 h, were challenged with *C. difficile* (toxic strain VPI 10463). Group 2 (n=5) received additionally *Lactobacillus* GG and xylitol (1 ml of 20% solution) once a day starting 20 h before administration of *C. difficile*. Asymptomatic hamsters were sacrificed 5 days after challenge with *C. difficile*. Also moribund hamsters were sacrificed. Samples of sacrificed or freshly deceased hamsters were taken from ileum, caecum and colon for bacteriological and histological studies. Samples were also taken from all mice and hamsters of blood, mesenteric lymph nodes (MLN), spleen and liver for detection of bacterial translocation.

Bacterial strains

A toxigenic strain of *C. difficile* ME 82 was isolated from a patient with PMC in Tampere University Hospital, Finland. The highly virulent strain VPI 10463 was kindly given to us by Microbiology Department at University of Turku. The bacterial inoculum for use in the animal model was prepared by incubating the strains of *C. difficile* anaerobically at 37° C 24 h in Columbia Broth (BBL). The culture was centrifuged (3000 r/min for 10 min) and resuspended in an equivalent volume of saline (approx. 10^8/ml) and 0.5 ml of microbial suspension was given to animals intragastrically.

Lactobacillus rhamnosus strain GG (ATCC 53103) was kindly provided by Valio Ltd., Finland. Some 0.5 ml of 24 h old culture in MRS Broth (Oxoid) was administered to hamsters intragastrically.

Histological samples

Histological samples from different locations of intestinal mucosa were fixed with formalin. Paraffin sections were cut and stained with hematoxylin-eosin, by the modified method of Gram [7] and also with acridine orange for better detection of bacteria. In hematoxylin-eosin stained samples inflammatory changes in mucosa were evaluated (score 0...5): the number of solid lymphatic follicles, diffuse lymphatic infiltrate, infiltration by polymorphonuclears (PMN), hyperaemia, mitotic activity. For diagnosis of PMC several signs were taken into consideration: formation of pseudomembranes, haemorrhages, erosions of surface epithelium and inflammation in *lamina propia* of intestinal mucosa.

Bacteriological samples

Quantitative composition of luminal and mucosal microflora was estimated as described previously [8]. The counts (log CFU/g) of principal groups of intestinal microbes (coliforms, enterococci, staphylococci, lactobacilli, peptostreptococci, clostridia, bacteroids, yeasts) and the total counts of aerobes and anaerobes were estimated. To characterise mucosal microflora we determined the predominant microbes. A microbial group was considered as predominant if its proportion in the total count of mucosal microflora was more than 30 % at least in two animals of the same study group [9].

For bacteriological detection of bacterial translocation the homogenised samples from spleen, liver, mesenteric lymph nodes and heart blood were seeded to blood agar (Blood Agar Base No 2, Oxoid) and Fastidious Anaerobe Agar (F.A.A., LabM). Blood agar was incubated for 48 h in aerobic, F.A.A. in anaerobic atmosphere (AnaeroGen & AnaeroJar, Oxoid) for 5 days.

Statistics

For detection of significant differences in score values of pathological changes and in the counts of microbes the data were analysed with Mann-Whitney rank sum test using program "Statgraphics".

Results

Development of *C. difficile* infection

In our first study all mice of control group (group 1) and cefoxitin influenced group (group 2) survived and remained healthy. No symptoms of infection were discovered in animals challenged with *C. difficile* (group 3), although the intestine of all mice became colonised by *C. difficile*.

In our second experiment with hamsters who received ampicillin and *C. difficile* (group 1) the pseudomembranous enterocolitis developed in all animals. Affected caeca became distended with watery stool, congested and haemorrhagic. Lesions occurred in the caecum and terminal ileum. Colons of affected animals contained no formed stool. Hamsters died or became moribund during 1.5 to 2.5 days. Among hamsters who received additionally lactobacilli and xylitol only one animal out of 5 developed enterocolitis during 3 days. No symptoms of enterocolitis were revealed in other four hamsters of this group.

Histological studies of intestinal mucosa

In histological studies of mice we found more clearly expressed changes in colonic mucosa than in ileal one. In the sections of colon we discovered higher inflammatory activity in both, mice group 2 and mice group 3, compared with control mice of group 1 (table 1). In colonic mucosa of these animals more solid lymphoid follicles, diffuse infiltrate and polymorphonuclear (PMN) cells were found. In some animals hyperaemia was present and mitotic activity was enhanced. In mice of group 2 these changes were mild, but of group 3, moderately expressed. No bacteria within mucosa in Gram-stained samples were seen, although they were visible in the lumen of gut.

In all hamsters of group 1 and in one hamster of group 2 the signs of serious enterocolitis were present: infiltration with lymphocytes and PMN cells, erosions of epithelial surface, haemorrhages in mucosa, formation of pseudomembranes (table 2.). These changes were equally expressed in terminal ileum and caeum but in a lesser degree in colon (figure 1b). In Gram-stained sections we detected gram positive rods adhering to the mucosa (figure 1c, d). In some cases also different morphotypes of bacteria (Gram negative and negative) were detectable within mucosa in samples stained by Gram or with acridine orange (figure 1e). In 4 hamsters out of group 2 no

enterocolitis was observed and only mild inflammatory changes were detected in their intestinal mucosa (figure 1a).

Bacteriological studies of intestinal microflora

Details in the changes of luminal and mucosal intestinal microflora of these experimental mice have been described in our previous paper [8]. After administration of cefoxitin (mice group 2) both an absolute and a relative decrease of total anaerobes and their different subgroups occurred in ileum and colon. Counts of enterococci and yeasts were increased in the intestinal mucosa and lumen of group 2 mice. These changes in intestinal microflora had been partially recovered in group 3 mice since they were sacrificed 6 days after administration of last cefoxitin dose. All animals of this group were colonised by *C. difficile*.

We further grouped the hamsters according to their pathological findings into two groups: with and without enterocolitis. The most important difference in the intestinal microflora between hamsters with and without enterocolitis was revealed in the counts of indigenous anaerobes (i.e. others than *C. difficile*). In hamsters with enterocolitis indigenous anaerobes were absent from all mucosal samples, nor were they any more the predominant microbes of luminal flora (figure 2). *C. difficile* was present in high counts (6.0....11.3 log CFU/g) in all tested luminal and in most mucosal (up to 7.0 log CFU/g) samples of these animals.

In hamsters without enterocolitis these anaerobes were predominant microbes in all sampled intestinal loci in both lumen and mucosa. *C. difficile* was detectable in low counts in two of these animals: in ileal and caecal lumen in one animal (3.3 log CFU/g) and in colonic lumen in the other hamster (4.3 log CFU/g).

Bacterial translocation

In mice bacterial translocation was most frequent in antibiotic compromised mice of group 2 and no translocation was detected in control group (group 1). The most frequently translocating bacteria were lactobacilli and enterococci, and sometimes the lactobacilli were found even in blood cultures (table 3). Lactobacilli were also the predominant microbes in the intestinal mucosa of all groups of mice.

In hamsters bacterial translocation was more frequent in animals with pseudomembranous enterocolitis (all animals from group 1 and one animal from group 2, table 4). In most cases the translocated bacteria were coliforms but in some cases also enterococci and lactobacilli. In two animals also *C. difficile* was translocating to MLN and spleen. In hamsters without enterocolitis translocation occurred more rarely and in most cases with indigenous anaerobes (i.e. other anaerobes than *C. difficile*).

Discussion

We report that both, changes in intestinal microflora, and mucosal status can influence bacterial translocation. In our mice study bacterial translocation was most frequent in animals whose intestinal microflora was more deeply disturbed due to recent administration of antibiotics. The translocating bacteria were lactobacilli and enterococci i.e. bacteria predominating in mucosal microflora. In *C. difficile*

challenged mice whose indigenous intestinal microflora had been partly recovered the translocation was rarer regardless of even more pronounced inflammation in their intestinal mucosa. Thus, it seems that in cases of mild *C. difficile* infection the extent of disturbance in intestinal microflora is more important for promoting the bacterial translocation than the degree of inflammatory activity in mucosa. However, we have found some inflammatory changes in intestinal mucosa of mice who had received antibiotic but not *C. difficile*. This inflammatory reaction could be caused by an imbalance of microecosystem and subsequent changes in metabolic endproducts of microorganisms or associated directly with antibiotic. It is possible that these changes of mucosa are also one of the cofactors supporting translocation.

In case of serious enterocolitis with profound intestinal mucosal damage in hamsters, bacterial translocation was detected in all animals. Translocating bacteria (coliforms, lactobacilli, enterococci and *C. difficile*) were also predominant in intestinal mucosa. In hamsters who did not develop enterocolitis the translocation was rare and usually caused by anaerobic bacteria normally predominating in intestinal mucosa and content. Although these hamsters were fed with lactobacilli these microbes did not become predominant in their mucosal flora. The finding that used probiotic strain did not become predominant in intestinal microflora correlates with previous clinical studies [10].

Our findings that changes in intestinal microecology *i.e.* reduction of colonisation resistance and overgrowth by some subordinate bacteria can promote bacterial translocation from intestinal tract correlates with previous data [4; 11]. In our experiments the translocating bacteria were in all cases also the predominant microbes in the intestinal mucosa. This finding correlates with previous experiments [9]. It has been also shown in clinical studies that in most cases of bacteremia due to bacterial translocation from the intestinal tract the causing agent is the same facultative gram-negative organism that was also found to be predominant in faeces [3]. It has been shown that *C. difficile* associated diarrhoea is a predisposing factor for Gram-positive esp. *Enterococcus spp.* bacteremia [12]. Enterococci are typically resistant to many antimicrobial agents and therefore may survive the antimicrobial treatment inducing *C. difficile* diarrhoea and overgrowth after reduction of colonisation resistance. Although it is known that some microbes are more capable of translocation than others it seems that in case of antibiotic associated diarrhoea the composition of individual intestinal microflora and the spectrum of activity of the used antibiotic are the most important factors for selecting microorganism that can potentially translocate and cause bacteremia. Despite of predominance in mucosal flora of hamsters with enterocolitis *C. difficile* was infrequent translocating organism. Also in clinical studies *C. difficile* have been find rarely in blood and other extraintestinal sites [13].

Our study revealed that combination of lactobacilli and xylitol have some protective effect against *C. difficile* infection. Four hamsters out of five receiving probiotics remained healthy. Previously published experiments have shown that the susceptibility or resistance of hamsters to *C. difficile* is absolute [14]. Partial susceptibility, manifesting itself in terms of mild illness or prolonged diarrhoea, has not been observed earlier. Thus clear-cut end-points can be obtained without using large numbers of animals, for instance in a previous study four or five out of five animals in

each experimental group either remained completely healthy or developed typical symptoms of enterocolitis within 3-5 days [15]. These data are in good agreement with our experiments.

It has been shown that administration of *Lactobacillus* GG could be used for treatment of recurrent *C. difficile* colitis [16; 17]. Xylitol as nonabsorbable sugar appears to be a stable substrate for intestinal lactic acid bacteria [18]. On the other hand, we have shown in our previous experiments that xylitol itself can also inhibit the adhesion of *C. difficile* to Caco-2 cells [19]. Further experiments are needed to study the possibilities of application of such probiotics combined with substrate for treatment and prophylaxis of *C. difficile* infection.

From our results we can conclude that in case of the mild *C. difficile* diarrhoea alteration of mucosal indigenous microflora and overgrowth by some resistant facultative aerobes is more important for promoting translocation than inflammation in mucosa. Serious destruction of the intestinal mucosa due to pseudomembranous enterocolitis leads to massive polymicrobial translocation by predominating mucosal microflora. Thus, in patients with a serious underlying disease whose intestinal microflora has been disturbed owing to antimicrobial therapy, detection and sensitivity testing of overgrowing facultative flora could be important for predicting possible cause of sepsis and selecting appropriate empirical antimicrobial therapy. Application of probiotics with suitable substrates to restore indigenous intestinal microflora may also be useful for prevention of bacterial translocation from intestinal tract in these patients.

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Table 1. The results of histological examination of colonic mucosa of antibiotic compromised and *C. difficile* challenged mice

Group of animals		Ranges and medians of score values				
		Lymphoid follicles	Lymphoid infiltrate	PMN	Hyper-aemia	Mitotic activity
Group 1 (n=7)	Range	0 - 1	0	0	0 - 1	0
	Median	0	0	0	0	0
Group 2 (n=8)	Range	0 - 2	0 - 2	0 - 2	0 - 2	0
	Median	1	1	1	0	0
Group 3 (n=7)	Range	1 - 4	0 - 3	1 - 2	0 - 2	0 - 1
	Median	2	1	2	1	1

Statistically significant difference between groups ($p<0.01$):
In lymphoid follicles - group 1 vs. 2 &3 and 2 vs. 3; lymphoid infiltrate - group 1 vs. 2 & 3; PMN group 1 vs. 2 & 3; hyperaemia group 1 vs. 2 & 3 and 2 vs. 3; mitotic activity 3 vs. 1&2.

Table 2. The results (ranges and median of score values) of histological examination of ileal mucosa of C. difficile infected hamsters with and without enterocolitis

Inflammatory changes	Hamsters with enterocolitis (n=5)		Hamsters without enterocolitis (n=4)	
	Range	Median	Range	Median
Infiltration*	4 - 5	5	1 - 2	2
PMN*	3 - 5	4	1 - 2	2
Hyperaemia	2 - 4	3	0 - 3	1
Haemorrhages*	1 - 3	2	-	0
Destruction*	1 - 3	2	-	0
Pseudomembranes*	1 - 2	2	-	0

* $p< 0.05$ hamsters with vs. without enterocolitis

Table 3. Number of cases of translocation and mucosal predominance of intestinal microbes in mice group 1, 2 and 3

Bacteria	Group 1		Group 2		Group 3	
	Trans-location	Predomi-nance	Trans-location	Predom inance	Trans-location	Predomi nance
Bacteroids	-	Ileum Colon				Colon
Lactobacilli	-	Ileum Colon	3 MLN* 1 Spleen 1 Liver 2 Blood	Ileum	1 MLN	Ileum Colon
Enterococci	-		1 MLN 1 Liver	Ileum Colon		
Yeasts	-		1 MLN	Ileum		Ileum

*MLN- Mesenteric lymph nodes

Table 4. Number of cases of translocation and mucosal predominance of intestinal microbes in hamsters with (n=5) and without (n=4) enterocolitis

Bacteria	With enterocolitis		Without enterocolitis	
	Trans-location	Predominance	Trans-location	Predominance
Anaerobic G+ cocci	-	-	1 MLN*	Ileum Colon
Bacteroides	-	-	1 MLN 1 Spleen 1 Liver	Ileum Caecum Colon
C. difficile	1 MLN 1 Spleen	Ileum Caecum Colon	-	-
Lactobacilli	1 MLN 1 Spleen 2 Liver	Ileum Caecum	1 MLN 1 Spleen 1 Liver	-
Enterococci	2 MLN	Ileum Caecum Colon	-	-
Coliforms	3 MLN 4 Spleen 4 Liver	Ileum Caecum Colon	-	-

*MLN- Mesenteric Lymph Nodes

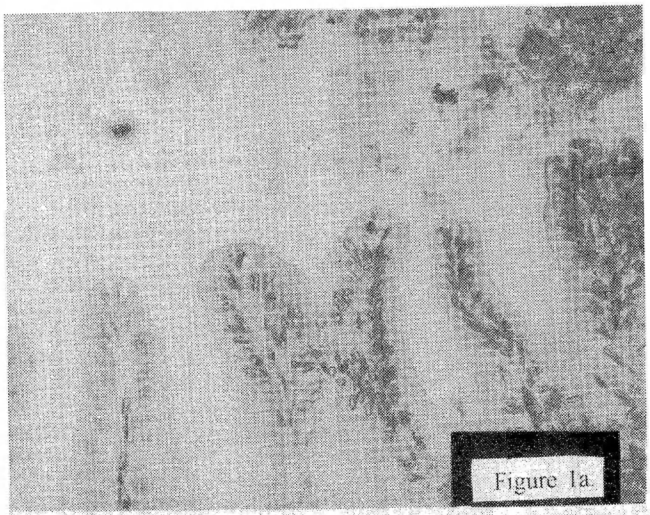

Figure 1a.

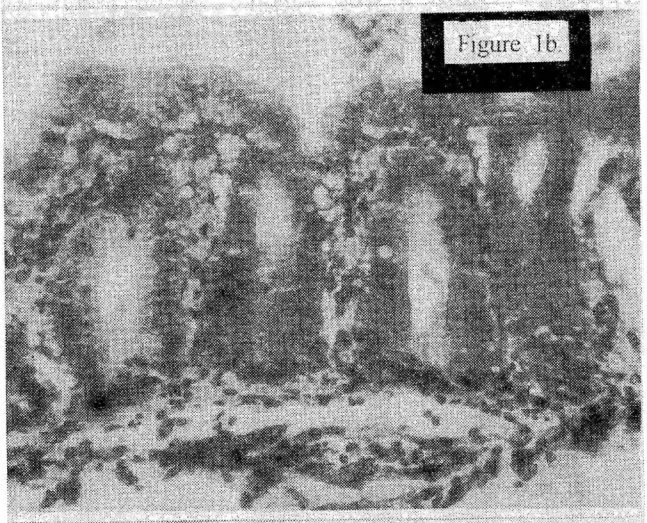

Figure 1b.

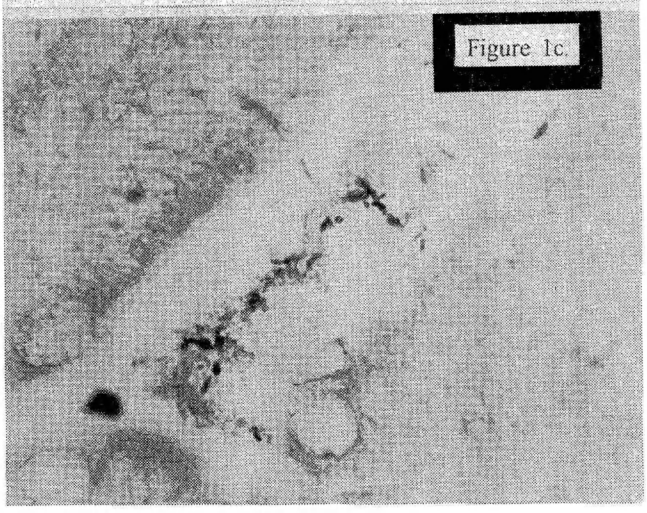

Figure 1c.

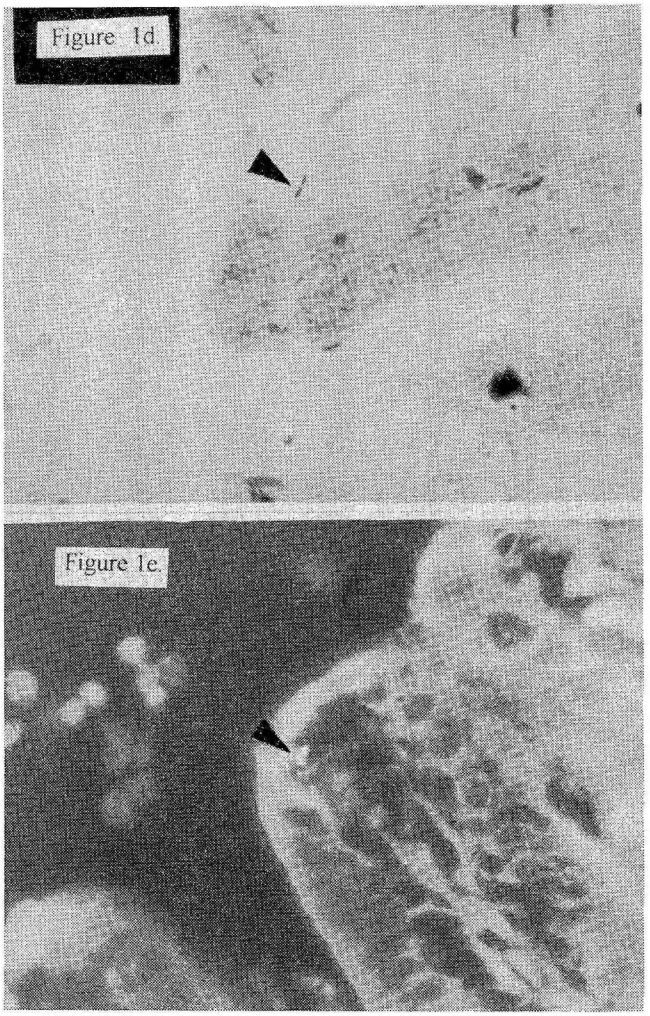
Figure 1d.
Figure 1e.

Figure 1a. Caecal mucosa of hamsters without enterocolitis (hamsters treated with lactobacilli and xylitol) demonstrating regular short crypts and smooth epithelial cell surface. In some animals mild inflammatory changes (hyperaemia and infiltration) could be seen (hematoxylin-eosin X400).

Figure 1b. Caecal mucosa of hamsters with enterocolitis. The lamina propria is inflamed, the surface epithelium is focally eroded. Adherent to the mucosal surface is an obvious inflammatory pseudomembrane. The areas of haemorrhage are not shown (hematoxylin-eosin X400).

Figure 1c. Caecal mucosa of hamsters with enterocolitis. Gram positive rods and other morphotypes of bacteria in the crypt base (modification of Gram staining X1000)

Figure 1d. Caecal mucosa of hamsters with enterocolitis. Gram-positive rods adherent to mucosal surface (modification of Gram staining X1000).

Figure 1e. Some few bacteria within the intestinal mucosa of hamsters with enterocolitis (stained with acridine orange, UV microscope X1000).

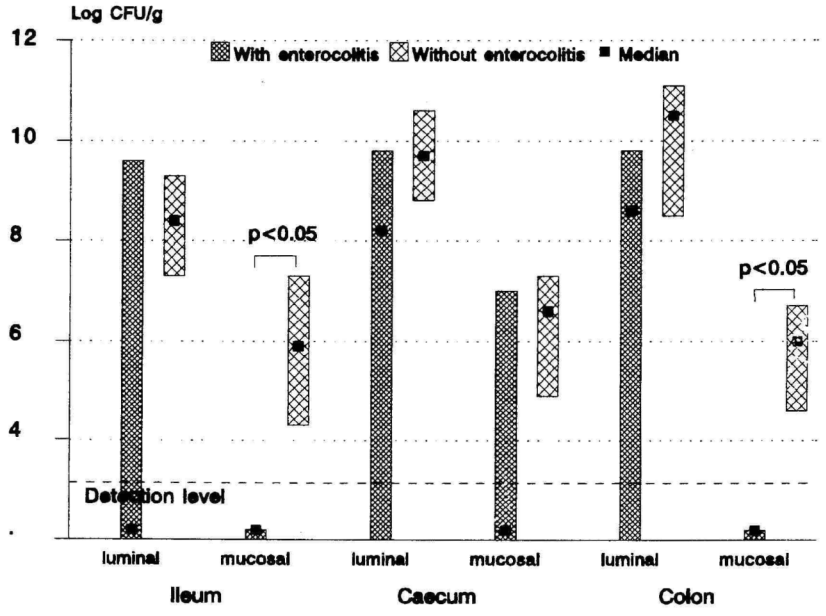

Figure 2. Counts (ranges and medians, log CFU/g) of indigenous anaerobes in different loci of hamsters with (n=5) and without (n=4) enterocolitis.

VI

Eesti Arst 1994; 2: 122–124

Eesti Arst, 1994, 2, 122—124

Clostridium difficile põhjustatud diarröa. I. Etioloogia, patogenees ja kliiniline pilt

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***Clostridium difficile*, pseudomembranoosne koliit, diarröa, antibakteriaalne ravi**

Igapäevapraktikas on küllalt sageli ette tulnud juhtumeid, kus antibakteriaalse ravi ajal või järel tekib haigel diarröa. Antibakteriaalse raviga seotud diarröa (*antibiotic-associated diarrhea*, AAD) üks sagedamaid tekitajaid on anaeroobne mikroorganism — *Clostridium difficile*. Enamasti möödub *C.difficile* põhjustatud diarröa (*C.difficile-associated diarrhea*, CDAD) pärast antibakteriaalse ravi lõpetamist, kuid mõningatel juhtudel kujuneb välja raske kliiniline sündroom — pseudomembranoosne koliit.

Üks varajasemaid pseudomembranoosse koliidi kirjeldusi pärineb 1893. aastast, kui Finney kirjeldas pärast operatsiooni noorel naisel surmaga lõppenud raskekujulist verist diarröad. Lahangul leiti maost ja sooltest plaatjaid membraane, ning Finney andis sellele sündroomile nimeks difteriitiline koliit. Pärast antibiootikumravi kasutuselevõttu pseudomembranoosse koliidi juhud sagenesid ning kirjanduses ilmus palju töid erinevate oletustega selle fataalse haiguse etioloogia kohta (10, 11).

Etioloogia. 1960-ndatel aastatel domineerinud seisukoha järgi peeti tekitajaks *S. aureus*'t, kuigi katseloomade nakatamisel stafülokokkidega koliiti tekitada ei õnnestunud. *C.difficile*, mille olid juba 1935. aastal isoleerinud Hall ja O'Toole, seos pseudomembranoosse koliidiga hakkas selguma alles 1970-ndate aastate lõpul. Nimelt leiti, et pseudomembranoosset koliiti põdejate *faeces*'e filtraadil on tugev tsütotoksiline toime, mida on võimalik neutraliseerida *Clostridium sordellii* antitoksilise seerumiga. Ühtlasi leiti nende sooles hulgaliselt *C.difficile*'t (2, 10, 11, 12).

Patogenees. *C.difficile* põhjustatud diarröa ja koliidi tekke eeldusteks on olulised mitmed nii haigest kui ka haigusetekitajast sõltuvad faktorid: 1) patsiendi seedetrakti koloniseerumine virulentse *C.difficile* mikroobitüvega; 2) soole normaalse mikrofloora kaitsevõime vähenemine antibakteriaalse ravi tulemusena; 3) haige vanusest sõltuv vastuvõtlikkus (9, 12).

***C. difficile* virulentsusfaktorid.** *C.difficile* olulisemateks virulentsusfaktoriteks on A- ja B-toksiin, mida toksilised tüved produtseerivad alati üheaegselt. Atoksilised tüved seevastu ei produtseeri kumbagi toksiini ning nad ei ole võimelised tekitama koliiti (8). Mõlemad toksiinid on suure molekulmassiga proteiinid, kuid nende toime on erinev.

A-toksiin ehk enterotoksiin põhjustab soole limaskesta histotoksilise kahjustuse kaudu sooleepiteeli permeaabluse suurenemist ja sellega hemorraagilise ning albumiinirikka vedeliku kogunemist sooleväendikku.

B-toksiin ehk tsütotoksiin kutsub enamikus imetajate rakkudes esile mittespetsiifilisi muutusi, nagu DNA, RNA ja valkude sünteesi languse. Tsütotoksilisuse testis nähtav rakkude ümardumine on ilmselt seotud mikrofilamentide süsteemi kahjustusega ning on neutraliseeriv *C. sordellii* antitoksilise seerumiga (3, 5, 11). Tegelikult on ka A-toksiinil tsütotoksiline aktiivsus, kuid see on 100 kuni 1000 korda B-toksiini omast nõrgem.

Haiguse patogeneesis peetakse tähtsamaks A-toksiini toimet, kuna loomkatsetes B-toksiin üksinda soolde viiduna haigust ei tekita, kuid A-toksiin põhjustab vedeliku kogunemist ja katseloomade surma. Arvatavasti vajab B-toksiin oma toi-

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me täielikuks avaldumiseks soole limaskesta eelnevat kahjustamist A-toksiini poolt, seega toimivad mõlemad toksiinid sünergistlikult (3, 5, 10, 11).

Erinevate *C.difficile* toksiliste tüvede produtseeritav toksiini kogus võib olla väga erinev. Sellega on ka osaliselt seletatav haiguse kliinilise raskuse suur varieeruvus (11). Toksilise tüve *in vitro* produtseeritav toksiini kogus aga ei ole alati vastavuses *in vivo* moodustuva toksiini kogusega (3). See, missugused faktorid mõjutavad toksiinide produktsiooni *in vivo*, ei ole veel selge. Varem avaldatud arvamus, et toksiinide produktsiooni mõjutab antibiootikumide kontsentratsioon sooles, ei ole hiljem kinnitust leidnud (1, 11).

C.difficile tüvede virulentsus on seotud ka nende adhesioonivõime, kihnu olemasolu ja mitmesuguste hüdrolüütiliste ensüümide produktsiooniga. On leitud, et kõrge virulentsusega tüvede adhesioon soole limaskestale on tugevam kui madala virulentsusega tüvedel. Resistentsust polümorfonukleaarsete leukotsüütide fagotsütoosi suhtes seostatakse kihnu olemasoluga. *C.difficile* kõrge virulentsusega tüvedel on kollagenaasi aktiivsus suurem kui mittevirulentsetel. Need ensüümid on vajalikud mikroobile oluliste toitainete kättesaamiseks peremeesorganismi kudedest (3, 5, 10).

Kolonisatsiooniresistentsus. Kui *C.difficile* ei koloniseeri soolt või esineb seda seal väikestes kogustes, siis ta haigust ei põhjusta. *C.difficile* mikroobirakkude arvukus allub aga soole indigeense mikrofloora kontrollile (kolonisatsiooniresistentsus). See tähendab, et tervetel inimestel esinevad seedetrakti eri osade mikroflooras tasakaalustatud ja püsivad suhted mikroobigruppide vahel (14). Erinevate soolemikroobide tähtsus kolonisatsiooniresistentsuse tagamisel ei ole veel lõplikult selge. Tänaseks on leitud, et *C.difficile* kasvu pärssimisel on oluline osa mutsiini lõhustavatel bakteritel, seevastu fakultatiivsed gramnegatiivsed mikroobid *C.difficile* kolonisatsiooni ei mõjuta. Kontrollmehhanismis on ilmselt kõige olulisem mikroobide võitlus erinevate toitainete pärast ja erinevate mikroobide paljunemise kiirus (3, 4, 14).

Antibakteriaalse ravi ajal pärsitakse paratamatult ka normaalset soole mikrofloorat, mis põhjustab kolonisatsiooniresistentsuse vähenemist. Järelikult on selge, miks antibiootikumide kasutamine on üks olulisi *C.difficile* infektsiooni riskitegureid. Diarröad võivad esile kutsuda väga paljud antibiootikumid, kuigi erinevatel preparaatidel on see võime erinev, sõltudes nii toimespektrist kui ka farmakokineetilistest omadustest (2, 12). Sagedamini on *C.difficile* põhjustatud diarröa tingitud tsefalosporiinide, ampitsilliini ja klindamütsiini, väga harva aga aminoglükosiidide, sulfoonamiidide, metronidasooli ja tetratsükliini kasutamisest (2, 9). Mõnikord on kirjeldatud *C.difficile* põhjustatud diarröa teket ka pärast tsütostaatikumravi (1).

Patsiendi vastuvõtlikkus infektsiooni suhtes ja nakkusallikas. *C. difficile* kandlus tervetel täiskasvanutel varieerub erinevates uuringutes ja erinevates geograafilistes piirkondades, moodustades enamasti 2...3 % populatsioonist. Antibakteriaalset ravi saanute hulgas on asümptomaatilisi mikroobikandjaid 5...15 % (9). Oluline *C.difficile* allikas on haigla keskkond: mikroobikandlus sageneb vastavalt haiglas viibitud ajale. Nii esineb 10...25 %-l hospitaliseerituist *C.difficile* ning nendest umbes ühel kolmandikul võib tekkida diarröa (7, 9, 11).

C.difficile'ga nakatumise risk ja kliinilise diarröa raskusaste on suurem kõrges vanuses patsientidel. Üheks selle seletuseks on fakt, et vanemaealistel (>65 a.) on noortega võrreldes suurenenud lümfotsüütide kemotaktiline vastus A-toksiinile, mis väljendub tugevama soole limaskesta põletikuna (10).

C.difficile unikaalseks omaduseks on tema sage sümptoomideta esinemine vastsündinutel ja imikutel. Vastsündinute kolonisatsiooni esinemissagedus on erinevatel andmetel 10...90 % (9). Andmete lahknevust seletatakse pigem erinevustega sünnitusmajade keskkonnas kui ema-

de erineva vaginaalse mikroobikandlusega (10). Sagedamini esineb kandlust esimesel 8 elukuul, pärast teist eluaastat on see langenud tervete täiskasvanute tasemele (9, 10).

Et vastsündinutel ja väikelastel tekib pseudomembranoosset koliiti väga harva, vaatamata *C.difficile* ning ka toksiinide suurele hulgale sooles, siis peab neil olema ka väga efektiivne kaitsemehhanism. Nii on leitud, et rinnapiim sisaldab mitmeid toksiine neutraliseerivaid substantse, ilmselt sekretoorseid antikehi (11). Ometi on probleem komplitseeritum, sest ka kunstlikul toidul olevad imikud ei haigestu. Üks võimalikke seletusi on asjaolu, et loote intestinaalrakud on toksiinide suhtes vähem tundlikud. A-toksiini retseptorid laste soole limaskesta rakkudel ühe hüpoteesi järgi kas puuduvad või on need kaetud paksema limakihiga kui täiskasvanutel (10, 11). Kaitsemehhanism toksiinide toime vastu on olemas ka vastsündinud hamstritel (3).

Kliiniline pilt. *C. difficile* põhjustatud diarröa tekib sagedamini 4...9. päeval pärast antibakteriaalse ravi alustamist, kuid ta võib avalduda ka pärast ühekordset antibiootikumi annust või alles nädalaid pärast antibakteriaalse ravi lõpetamist.

Kliiniline pilt võib varieeruda kergest diarröast kuni eluohtliku pseudomembranoosse koliidini. Erinevate sümptoomide esinemissagedus on toodud tabelis.

Tabel. *C.difficile* põhjustatud diarröa nähtude esinemissagedus (protsentides)

Sümptoom	Esinemissagedus
Vesine diarröa	90...95
Verine diarröa	5...10
Palavik üle 38°C	kuni 66
Leukotsütoos üle 15000 raku mm^3-s	40...50
Kõhuvalu	80...90

Pseudomembranoosse koliidi tüsistusena võivad tekkida toksiline megakoolon, hüpovoleemiline šokk, kooloni perforatsioon, sekundaarne sepsis ja verejooks (10, 13).

Pseudomembranoosse koliidi juhtudel on endoskoopiliselt sooles nähtavad plaatjad kollakasvalged 2...20 mm suurused pseudomembraanid. Need koosnevad põletikulisele koele kleepunud fibriinist, limast, nekrootilistest epiteelirakkudest ja leukotsüütidest. Pseudomembraanid võivad vahelduda hüpereemiliste ja normaalse limaskestaga aladega. Kuigi patoloogilisi muutusi esineb sagedamini pärasooles ja sigmasooles, võib põletikust olla haaratud kogu seedetrakt, söögitoru ja magu kaasa arvatud (9, 10, 12).

Lisaks seedetrakti infektsioonidele võib *C.difficile* harvadel juhtudel osaleda ka muudes sellistes lokaalsetes ja süsteemsetes infektsioonides nagu abstsessid, haavanakkused, osteomüeliit, pleuriit, peritoniit, sepsis ja urogenitaaltrakti infektsioonid (11). On kirjeldatud ka *C.difficile* toksiinide indutseeritud reaktiivset artriiti (6).

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Summary

***Clostridium difficile* Caused Diarrhea: I. Ethiology, Pathogenesis and Clinical Picture.** This article gives a review on the antibiotic-associated diarrhea caused by *Clostridium difficile*. In the first part of the article the historical background, pathological factors and clinical manifestations of this disease are discussed.

VII

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Eesti Arst, 1994, 3, 210—212

Clostridium difficile põhjustatud diarröa. II. Diagnoosimine, ravi ja profülaktika

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Clostridium difficile, **pseudomembranoosne koliit, diarröa, antibakteriaalne ravi**

Kirjutise esimeses osas käsitlesime *C. difficile* põhjustatud diarröa etiopatogeneesi ja kliinilist sümptomaatikat (Eesti Arst, 1994, 2). Järgnevalt peatume selle haiguse diagnoosimisel ja ravil.

Diagnoosimine. Kliinilised kriteeriumid. Oluline kliiniline kriteerium on kõhulahtisus. Diarröa põhjustatud diagnoos põhineb vähemalt kahel vedela väljaheitega defekatsioonil päevas, kusjuures kõhulahtisus kestab vähemalt kaks päeva.

Teiseks oluliseks kriteeriumiks on antibakteriaalne või tsütostaatiline ravi mitte üle kuue nädala tagasi.

Kõhuvalu ja palavikku esineb ainult osal patsientidel. Diagnoosi kinnitab allumine metronidasool- või vankomütsiinravile (5).

Endoskoopia. Pseudomembranoosse koliidi diagnoosi kinnitab pseudomembraanide olemasolu sooles. Painduva fiiberendoskoobiga, millega on nähtav umbes 60 cm soolt, õnnestub pseudomembranoosset koliiti diagnoosida kuni pooltel juhtudel. Väike endoskoopia efektiivsus *C. difficile* infektsiooni diagnoosimisel on tingitud sellest, et ei ole võimalik vaadelda tervet soolt ning et pseudomembraanid võivad kergetel juhtudel ja haiguse varajases staadiumis puududa. Seega ei välista negatiivne endoskoopiline leid pseudomembranoosse koliidi või *C. difficile* põhjustatud diarröa võimalust (5).

Laboratoorne diagnoosimine. Laboratoorsed meetodid põhinevad kas *C. difficile* enda või tema **toksiinide** määramisel väljaheitest. Leukotsüütide leidumine väljaheites, mida varem peeti sagedaseks ja iseloomulikuks haiguse tunnuseks, on tegelikult olulise diagnostilise väärtuseta (14).

C. difficile **kultiveerimiseks** kasutatakse selektiivset CCFA-söödet, milles leiduvad tsükloseriin ja tsefoksitiin pärsivad teiste soolemikroobide kasvu. *C. difficile* esinemist sooles on võimalik CCFA-l kindlaks teha, kui ühes grammis väljaheites leidub vähemalt 100 *C. difficile* rakku. Diarröa korral on see hulk tavaliselt tunduvalt suurem — 10^4...10^9/g (12). 48-tunnise inkubatsiooni järel anaeroobsetes tingimustes on CCFA-l nähtavad iseloomuliku morfoloogiaga ebakorrapärased 4...8 mm-se läbimõõduga kollased pesad, mis helenduvad rohekalt ultraviolettvalguses. Kultiveerimine ei võimalda eristada toksilisi tüvesid atoksilistest (5, 10, 12).

Laialt kasutatavaks toksiini määramise meetodiks on *faeces*'e filtraadi **tsütotoksilisuse kindlakstegemine koekultuuril.** *C. difficile* tsütotoksiin (B-toksiin) põhjustab koekultuuris tsütopaatilist efekti, mis väljendub eeskätt rakkude ümardumisena. Seda efekti pärsib *C. sordellii* antitoksiline seerum. Selle meetodiga on võimalik avastada tsütotoksiini, mitte aga haiguse patogeneesi seisukohalt olulisemat enterotoksiini (A-toksiini), mille tsütotoksiline aktiivsus on tunduvalt väiksem. Kuigi toksilised tüved produtseerivad mõlemat toksiini, võib nende kogus *in vivo* varieeruda suures ulatuses. On leitud ka üksikuid *C. difficile* tüvesid, mis produtseerivad küll hulgaliselt tsütotoksiini, kuid mitte määratavas koguses enterotoksiini. Sellele vaatamata on korrelatsioon tsütotoksilise efekti ja haiguse kliinilise raskuse vahel enamikul juhtudel hea ning *C. difficile* põhjustatud diar-

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röaga haigetel on test positiivne *faeces*'e lahjendustes 10^{-3}...10^{-5}. Meetodi puudusteks on see, et lõplik vastus saadakse alles 24 tunni pärast, samuti vajadus koekultuuride järele. Meetodi eeliseks aga on väga kõrge tundlikkus — tsütopaatilise efekti esilekutsumiseks piisab 1pg B-toksiinist (3, 5, 11, 12).

Suhteliselt kiire ja lihtne on **lateksaglutinatsioonitest.** Hilisemad uuringud on näidanud, et lateksreaktiivne proteiin ei ole mitte toksiin, vaid *C. difficile* poolt produtseeritav ensüüm — glutamaadi dehüdrogenaas. Test reageerib nii toksilistele kui ka atoksilistele *C. difficile* tüvedele ning *Peptostreptococcus anaerobius*'ele ja *Porphyromonas asaccharolytica*'le. Arvamused latekstesti sensitiivsuse ja selektiivsuse kohta on küllalt lahknevad, kuid üksmeelel ollakse selles, et oma kiiruse tõttu on see sobiv sõeluuringutestina, kuid positiivsed tulemused vajavad hilisemat kinnitamist teiste meetoditega (10).

Viimase kümne aasta jooksul on nii A-toksiini kui ka korraga mõlema toksiini määramiseks kasutatud paljude firmade poolt väljatöötatud ELISA-teste. Need testid nõuavad vähe aega (2,5...3,5 tundi), nende sensitiivsus ja spetsiifilisus on hea, kuid siiski madalam kui koekultuurimeetodil (1, 3, 10).

Kiire, spetsiifiline ja tundlik **polümeraasi ahela reaktsioon** (PCR) on võimeline kindlaks tegema isegi 10 *C. difficile* rakku 10^{10}...10^{11} bakteri hulgast ühes grammis väljaheites ning sobivate praimerite (ribonukleotiidsondide) valiku korral eristama ka toksilisi tüvesid atoksilistest (5, 8, 10).

Ravi. Paljud haiged paranevad spontaanselt pärast diarröad esilekutsunud antibiootikumi manustamise lõpetamist. *C. difficile* põhjustatud diarröa vajab spetsiifilist ravi siis, kui põhihaiguse antibakteriaalset ravi ei ole võimalik katkestada; patsient on raskelt haige ja esinevad süsteemsed tüsistused või patsiendil diarröa püsib, seda esilekutsunud antibakteriaalse ravi lõpetamisele vaatamata (3).

C. difficile põhjustatud diarröa ravis kasutatakse **vankomütsiini,** mis on peaaegu alati efektiivne. Tüüpilise raviskeemi järgi määratakse sissevõtmiseks 125 mg vankomütsiini neli korda päevas 7...14 päeva vältel. Ravi tulemusena langeb palavik enamasti 24 tunni jooksul ja diarröa kaob 4...5 päeva jooksul (13).

Alternatiivse preparaadina kasutatakse **metronidasooli,** mille eeliseks on tunduvalt madalam hind, kuid efektiivsuselt ei jää see vankomütsiinile alla. Metronidasooli tavaliseks annuseks suu kaudu võetuna on 500...750 mg kolm korda päevas või 250 mg neli korda päevas. Efektiivne on ka **batsitratsiinravi** (25 000 ühikut neli korda päevas), kuid sellise ravi edukuse kohta on seni andmeid avaldatud veel vähe ning mõned *C. difficile* tüved on batsitratsiini suhtes osutunud resistentseks (3, 6, 11, 12).

Intravenoosse ravi edukuses on kirjanduse andmed vastukäivad ning seda tuleks kasutada üksnes siis, kui suu kaudu manustamine ei ole võimalik. Sellistel juhtudel soovitatakse manustada 500 mg metronidasooli iga 6...8 tunni järel (10, 13).

Juba enne pseudomembranoosse koliidi etioloogia selgumist kasutati selle haiguse ravimisel kolestüramiini, mis seob *C. difficile* toksiinid A ja B. Tavaliseks annuseks on 4 g kolestüramiini kolm kuni neli korda päevas. Sellise ravi efektiivsust on hinnatud erinevalt: osa autorite järgi annab see peaaegu alati tulemusi, teiste järgi puudub ravitulemus kuni pooltel haigetel ning üldiselt soovitatakse kolestüramiini vaid kergematel haigusjuhtudel. Et kolestüramiin võib siduda ka vankomütsiini, tuleks hoiduda neid üheaegselt manustamast (6, 12).

Et *C. difficile* põhjustatud diarröa tekke eelduseks on soole mikrofloora düsbioos, püütakse leida sobivaid normaalsest soole mikrofloorast pärinevaid mikroobitüvesid (probiootikume), mis oleksid võimelised soole normaalset kolonisatsiooniresistentsust taastama. **Probiootikumidest** on edukalt kasutatud atoksilisi *C. difficile* tüvesid ja *Lactobacillus casei* tüve GG (4, 7, 12). Tulemusi on

andnud ka pärmseen *Saccharomyces boulardii*, mis soolt küll ei koloniseeri, kuid mis neutraliseerib toksiinid või pärsib nende produktsiooni (17).

C. difficile põhjustatud diarröa antibakteriaalse raviga kaasnevad 10...40%-l haigetest retsidiivid. Üks retsidiivide tekke põhjusi on mikroobieoste säilimine sooles, millest pärast ravi lõpetamist võivad areneda vegetatiivsed vormid (6, 11). Võimalik on ka uus infitseerumine väliskeskkonnast. Retsidiivide korral on üle pooltel juhtudel nakkuse tekitajaks olnud uus *C. difficile* tüvi ning ülejäänutel sama tüvi, mis võis pärineda kas väliskeskkonnast või organismis säilinud eostest (15). Retsidiivide vältimiseks soovitatakse pärast metronidasool- või vankomütsiinravi lõppemist jätkata ravi kolme nädala jooksul kas kolestüramiini (3 g neli korda päevas), vankomütsiini (125 mg ülepäeviti) või laktobatsillaarsete probiootikumidega (1 g või enam päevas) (6).

Profülaktika. *C. difficile* põhjustatud diarröa profülaktika seisukohalt on esiteks oluline vältida mittevajalikku antibakteriaalset ravi. Et *C. difficile* infektsioon tekib enamasti haiglas, on selle ärahoidmiseks tähtis nakkuse haiglasisese leviku tõkestamine. Seetõttu soovitatakse isoleerida diarröaga patsiendid ja pärast diagnoosi selgumist alustada spetsiifilist ravi. Kliiniliste sümptoomideta *C. difficile* kandjate ravimist vankomütsiini või metronidasooliga ei soovitata, sest see annab kas lühiaegse efekti või ei anna efekti üldse (9). Taolistel juhtudel võib mikrofloora normaliseerimiseks kasutada probiootikume.

Haiglasisene levik toimub peamiselt *C. difficile* eoste kaudu, mis võivad säilida väliskeskkonnas eluvõimelistena mitmeid kuid. Eosed kanduvad edasi personali käte ja endoskoopide, klistiiriotsikute, siibrite või muude meditsiiniinstrumentide kaudu (2, 10).

Profülaktikameetmeteks, mis võivad *C. difficile* põhjustatud diarröa esinemissagedust oluliselt vähendada, on sage käte pesemine ning ühekordselt kasutatavate kinnaste kandmine (2, 10). Desinfektsioonivahenditest kasutatagu selliseid, mis eoseid hävitavad, nagu naatriumhüpokloriidilahust, mis sisaldab vähemalt 15% vaba kloori, või 2%-list aluselist glutaaraldehüüdilahust (12, 16).

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Summary

***Clostridium difficile* Caused Diarrhea. II. Diagnosis and Therapy.** This article gives a review of the antibiotic-associated diarrhea caused by *Clostridium difficile*. In the second half of the article the diagnosis prophylaxis and therapy are discussed.

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Society for Microbial Ecology and Therapy
Anaerobe Society of Americas

Teadustöö
Peamiseks uurimisvaldkonnaks on *Clostridium difficile* ja seedetrakti mikroflooraga seotud probleemid. Ilmunud 19 teaduspublikatsiooni.

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