Pathogenesis of *Clostridium difficile* and Immune response in health and disease

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Abstract

*Clostridium difficile* is the main cause of antibiotic-associated diarrhoea and the aetiological agent of pseudomembranous colitis (PMC). The recent emergence of a hyper-virulent strain of *C. difficile*, which has caused many deaths worldwide and in the UK, has increased its relevance and interest.

Most patients colonized with *C. difficile* remain asymptomatic. When *C. difficile*-associated disease (CDAD) presents, it ranges from mild diarrhoea to severe diarrhoea that can lead to severe complications which can result in colectomy or even death. It is not clear why some patients remain asymptomatic while others present severe disease but one hypothesis is that patients who develop symptoms have lower levels of specific antibodies to *C. difficile* than those who remain asymptomatic after colonization with *C. difficile*.

The levels of systemic antibodies were analysed in three groups within the elderly hospitalised population of Edinburgh: a) CDAD cases; b) asymptomatic carriers of *C. difficile* and c) non-colonized controls. Specific antibody levels in serum were measured by ELISA (IgG and IgM), cytotoxicity neutralization and western blot. Antigens analysed were the *C. difficile* toxins, the surface proteins and carbohydrate of *C. difficile* and the *E. coli* LPS. Overall, the levels of antibodies to *C. difficile* antigens were highest in the CDAD cases and lowest in the non-colonized controls suggesting that cases are not unable to produce specific antibodies.

CDAD cases were more likely to be co-infected with cytomegalovirus (CMV) than asymptomatic carriers or non-colonized controls. CMV might be an indicator of severe disease or might be contributing towards the worsening of CDAD.
The antibiotics histories of patients were only partially available. However, from the data available, the CDAD cases were more likely to have received β-lactam antibiotics without β-lactamase inhibitors than asymptomatic carriers, and ciprofloxacin was linked to susceptibility to colonization (but not to disease).

The main virulence factors of \textit{C. difficile} are two exotoxins which are encoded in a region of the genome named “pathogenicity locus” (PaLoc) along with three other genes: \textit{tcdC}, a putative negative regulator; \textit{tcdR}, an alternative sigma factor essential in the transcription of the toxins and \textit{tcdE} which is very likely involved in toxin release.

Toxin production can be affected by environmental factors. In this study, it was seen that in \textit{C. difficile} cultures grown in the laboratory toxin is released earlier in the presence of sub-inhibitory concentrations of amoxicillin, clindamycin or vancomycin, but only marginally in the presence of metronidazole. Real-time PCR was used to investigate if antibiotics affected the expression of the PaLoc genes. The presence of clindamycin seemed to induce an increase in the levels of mRNA of the five PaLoc genes while amoxicillin and vancomycin might help toxin release by creating pores in the \textit{C. difficile} wall.

The toxins are the main virulence factors, essential to disease. However, there is no strict correlation between the virulence of a strain and the amount of toxin produced \textit{in vitro}. Therefore, other virulence factors have been considered. In this study it was shown that the addition of surface antigens, innocuous on their own, increased the cytotoxicity of toxin A in Vero and Caco2 cells, particularly the surface carbohydrate.
Publications


1. Introduction

1.1. The microorganism

1.1.1. Description

*Clostridium difficile* is a Gram positive strictly anaerobic rod, with a characteristic motility, characteristic smell and green fluorescence under long-wave UV light (Poxton et al., 1996). It forms sub-terminal spores allowing the microorganism to survive under non ideal circumstances for long periods of time. It is generally found everywhere in hospitals and nursing homes (McFarland et al., 1989). *C. difficile* is the cause of a large proportion of cases of nosocomial (hospital-acquired) antibiotic-associated diarrhoea (Bartlett, 1992) and the aetiological agent of pseudomembranous colitis (PMC). The whole spectrum of disease is referred to as *C. difficile* associated disease (CDAD). *C. difficile* associated disease generates important costs to the health systems of the majority of the Western countries (Wilcox et al., 1996; Kyne et al., 2002).

The sequence of the *C. difficile* genome is now available ([http://www.sanger.ac.uk](http://www.sanger.ac.uk)). The Sanger Institute completed the sequencing of *C. difficile* strain 630, which was isolated from a patient with severe PMC in Zurich, Switzerland in 1982. Strain 630 is a fully virulent, highly transmissible, multi-drug resistant strain. The genome consists of a circular chromosome of 4,290,252 bp with a G+C content of 29.06%, and a circular plasmid of 7,881 bp with a G+C content of 27.9%. 

1
1.1.2. History

*Clostridium difficile* was first described in 1935 as part of the commensal gut flora of neonates (Hall & O’Toole, 1935) and was given the name of *Bacillus difficile* for the difficulties they encountered to isolate it. They described it as a strict anaerobe, actively motile with elongate sub-terminal spores of about the same diameter as the rod. They found that it was highly pathogenic when inoculated into guinea-pigs and rabbits due to the production of a soluble exotoxin. However, *C. difficile* was not ascribed to human disease until the late 1970s.

Pseudomembranous colitis (PMC) was a well described severe gastrointestinal lesion known since the XIX century and characterised by the formation of yellowish plaque-like lesions in the colon, but it was not ascribed to any aetiological agent. In the early 1970s, antibiotics, particularly clindamycin and lincomycin, were related to the appearance of colitis and PMC (Cohen et al., 1973; Tedesco et al., 1974). This was a major turning point at the time since clindamycin was a widely used antibiotic due to its high efficiency against gram-positives without major side effects (Cohen et al., 1973). In some patients, the administration of oral clindamycin led to the appearance of severe diarrhoea, which persisted after withdrawal of clindamycin, and the appearance of PMC plaque lesions from which no pathogen could be isolated (Cohen et al., 1973; Tedesco et al., 1974).

Larson et al. (1977) found that the faecal samples of PMC patients during acute illness produced a cytotoxic effect when tested in cell culture. They discarded viruses since they could not find virus particles and they discarded allergies to the antibiotics.
since these were not seen before or after disease, and the symptoms persisted after the discontinuation of the antibiotic. Rifkin et al. (1977) showed that the cytotoxicity of the PMC stool samples could be neutralized with an anti-toxin to C. sordellii but C. sordellii itself was not isolated from stool samples despite being a relatively easy organism to isolate. Rifkin et al. (1977) also noted that the toxin disappeared from the stool sample after the administration of vancomycin.

In 1978 C. difficile was linked to disease for the first time as the aetiological agent of PMC (Bartlett et al., 1978; Larson et al., 1978; George et al., 1978). This discovery led to an increase in the interest and relevance of this microorganism. When stool flora of PMC patients was more thoroughly examined, they found on those patients an overgrowth of C. difficile which, when inoculated in vitro, was the cause of enterocolitis in hamsters (Larson et al., 1978). The problems isolating C. difficile were solved when George et al. (1979) developed a selective and differential medium for the isolation of C. difficile: CCFA – cycloserine, cefoxitin and fructose agar, which is still used today.

In 1981 it was found that C. difficile produced a second toxin (toxin A) which was different from the previously described cytotoxin (toxin B), and was produced in larger amounts (Taylor et al., 1981). Since then, the importance and relevance of C. difficile has grown. Recently, C. difficile has been the subject of many newspaper headlines due to the appearance of a hyper-virulent strain or “super-bug” that will be discussed later on, that has cost many lives and causes considerable troubles to the NHS.
1.1.3. Typing of *C. difficile*

Typing methods are a tool to the understanding of the epidemiology of *C. difficile* and allow comparisons of isolates worldwide (Brazier, 2001). Over the years many methods have been developed, all of them have advantages and disadvantages.

**Phenotypic typing methods**

The first typing methods were based on phenotypical characteristics. Sell et al. (1983) typed *C. difficile* isolates according to their sensitivity to different phages and bacteriocins. Other phenotypic methods include serogrouping (Delmée et al., 1985) and immunochemical fingerprinting (Poxton et al., 1984; Sharp and Poxton, 1985). Immunochemical fingerprinting was based on the comparison of EDTA extracts of *C. difficile* immunoblotted against antiserum to whole cells of *C. difficile* reference strain NCTC11223. Serogrouping consists on the agglutination of *C. difficile* strains by one or several antisera (Delmée et al., 1985). Serotyping is still the method against which every other typing method is compared (Brazier, 2001).

The polymorphisms of the surface-layer proteins have been used by our laboratory to type strains of *C. difficile* according to the molecular weight of the two subunits (McCoubrey and Poxton, 2001; McCoubrey et al., 2003).

**Restriction Endonuclease Analysis**

Restriction Endonuclease Analysis (REA) consists of the digestion of the total genome with a frequently cutting enzyme followed by the analysis on an agarose gel of the band pattern of the digest which can differ between strains. Devlin et al (1987)
first described this technique on *C. difficile* using *Cfo*I and recognised 12 different patterns. The current method using *Hind*III as the digestive enzyme was first described by Clabots et al. (1993). They recognised 206 different unique patterns. This technique is highly discriminative and reproducible, but it is a highly time consuming technique and the visual assessment can be very difficult (Brazier, 2001).

**Pulsed-Field Gel Electrophoresis**

Another typing method consists of the digestion of the genome with an infrequent restriction endonuclease (e.g. *Smal*) which produces around ten fragments that are visualised by Pulsed-Field Gel Electrophoresis (PFGE). Kristjansson et al. (1994) found that PFGE was affected by partial degradation of the DNA that did not affect REA or Ribotyping. Also, PFGE is a complex and slow procedure and the equipment can be expensive (Brazier, 2001). Some strains have been non-typeable with PFGE (Spigaglia et al., 2001) due to degradation of the DNA but this problem can be solved shortening incubation times and increasing the concentration of proteinase K (Alonso et al., 2005). Better results can be obtained if DNA is extracted from fresh culture containing no spores (Alonso et al., 2005).

**PCR Ribotyping**

PCR ribotyping consists of the amplification of the 16S-23S rRNA intergenic spacer regions. It was developed as a quick simple alternative to the conventional molecular methods involving genome restriction. The intention was to develop a technique that could be used routinely to differentiate between outbreaks and spontaneous fluctuations on the incidence (Cartwright et al., 1995). This technique was preferred
to other PCR methods (such as Arbitrarily-Primed PCR, AP-PCR) that did not have as much discriminatory power (Collier et al., 1996).

O’Neill et al. (1996) adapted this technique to be used by laboratories as a diagnostic routine which Stubbs et al. (1999) used to construct a library of UK ribotypes, isolated from both hospital and community environments, that has since be used as a reference for hospitals in the UK.

Both advantages and disadvantages of this technique have been found. Bidet et al. (2000) found that PCR ribotyping was easier to use than PFGE but more difficult to interpret, but that overall it was preferred to PFGE. Spigaglia et al. (2001) found that ribotyping was less discriminatory than PFGE but ribotyping was more rapid and was not affected by degradation of the DNA as PFGE was.

The lower discriminatory power of ribotyping can be overcome by subtyping the most common ribotypes (e.g. by PFGE or RFLP), since different subtypes can be prevalent in different environments (Fawley et al., 2005, Poilane et al., 2007)

**Toxinotyping**

The toxinotyping method developed by Rupnik et al. (1998) is based on the polymorphisms that Rupnik et al. (1997) found in the genes of toxins A and B. It consists of the PCR amplification of a fragment of each toxin gene followed by the digestion of the fragment with enzymes and restriction fragment length polymorphism (RFLP) analysis. This typing method is less discriminative than PCR-ribotyping (Rupnik et al., 2001). There are at least 24 different toxinotypes.
Surface protein genotyping

The polymorphisms of the surface layer proteins of *C. difficile* (McCoubrey and Poxton, 2001) have been used to type isolates. The *slpA* gene that encodes for the precursor of the surface layer proteins is amplified by PCR followed by digestion with restriction enzymes and RFLP analysis in agarose gels (Karjalainen et al., 2002).

Another alternative is typing by sequencing the *slpA* gene. It has the advantage that it does not require a reference strain and isolates typed in different laboratories can be easily compared (Kato et al., 2005).

Tasteyre et al. (2000) characterised strains through the inter-strain heterogenicity of the *fliC* gene that encodes for the flagellin subunit. They amplified the gene and digested it with four different restriction enzymes and found two different patterns.

1.1.4. The Hyper-virulent strain 027

The interest in *C. difficile* increased recently due the recent discovery of a hypervirulent strain that has been severely affecting hospitals both in Western Europe and North America. Outbreaks were not uncommon before but in the past few years there has been a rise in the number of deaths and morbidity attributed to *C. difficile*.

In late 2002 outbreaks of *C. difficile* started affecting several hospitals in Quebec, Canada, particularly in Montreal and Sherbrooke (Eggerston and Sibbald, 2004; Pepin et al, 2004). The cases of CDAD increased almost five times in two years and
they were more severe, with more complications, more relapses and more deaths than ever seen before in *C. difficile* outbreaks (Loo et al., 2004; Eggerston, 2004). Pepin et al (2004) linked this increase to the increase in the use of quinolones (mainly ciprofloxacin and levofloxacin), and by 2004 it started being apparent that a new more virulent strain of *C. difficile* had emerged.

In the US, the cases of CDAD were also increasing both in numbers and in severity (Dallal et al., 2002). Muto et al (2005) reported an outbreak of *C. difficile* in a teaching hospital in Pittsburgh related to the use of the new generation of fluoroquinolones which were considered low risk antibiotics for CDAD prior to these outbreaks (Wilcox et al., 2000). McEllistrem et al (2005) linked the increase of severe cases of CDAD in a hospital in Pittsburg to a *C. difficile* strain that produced the binary toxin.

The strains of the US and Canada outbreaks were characterised (Loo et al., 2005; McDonald et al., 2005). They belonged to the restriction endonuclease analysis (REA) group BI, North America pulse field gel electrophoresis (PFGE) type 1 (NAP1), toxinotype III, carried the binary toxin and had a 18-kb deletion in the *tcdC* gene. This they had in common with other rare strains that were involved in outbreaks between 1984 and 1993 and that had presented a classic spectrum of disease (McDonald et al., 2005). When the antibiotic susceptibility pattern of the strains of these new outbreaks was compared with that of the strains from outbreaks before 2001 (historical strains), they found that the new strains had acquired a resistance to gatifloxacin and moxifloxacin, and even though the historical strains were already resistant to levofloxacin, the MIC was considerably higher in the new
ones (McDonald et al., 2005). Drudy et al (2007) found in this strain, mutations on the gyrA gene that confers resistance to fluoroquinolones.

The increase of severity of CDAD started also being reported outside North America. In Western Europe, a hyper-virulent strain was first isolated in the southeast of England in November 2003 (Smith, 2005). This outbreak was caused by a strain characterised as ribotype 027 and that was closely related, in fact identical, to the one reported in Canada and the US. It was later reported in outbreaks in the Netherlands (van Steenbergen et al, 2005), Belgium (Joseph et al., 2005) and France (Coignard et al., 2006). Other reports included a British tourist in Austria (Indra et al., 2006), a patient transferred from a hospital in the UK to Ireland (Long et al., 2007), Luxembourg, Poland, Switzerland and in late 2006, in Glasgow, Scotland (Kuijper et al., 2007). The emergence of this strain prompted regular surveillance studies (Kuijper et al., 2007). In Asia, it was first isolated in Japan in March 2005 from a 30 year old Japanese female patient (Kato et al, 2007).

Warny et al (2005) analysed several isolates from outbreaks in Quebec, US and UK and they found that in vitro these isolates produced 16 times more toxin A and 23 times more toxin B than historical isolates. They all carried the binary toxin gene but Warny et al. (2005) did not measure the production of binary toxin in vitro. The role of the binary toxin in pathogenesis is unclear but it seems unable to produce disease on its own (Geric et al., 2006). The mechanism by which this strain emerged and is transmitted remains unknown. The hyper-production of toxin in strain 027 has been attributed to the 18bp deletion in the putative negative regulator gene, tcdC. However, this is not the reason since other strains with the same deletion produce a
functional TcdC (Matamouros et al., 2007). When strain 027 was more closely analysed, it was found that they also had a 1 bp deletion at position 117 in the ATG start codon of tcdC resulting on a frame-shift and a truncated TcdC (Matamouros et al., 2007).

It was also seen that in a human gut model, strain 027 germinates more readily, remains in a vegetative form and produces cytotoxin for substantially longer than ribotype 001 (Freeman et al., 2007).

### 1.2. The disease

*C. difficile* associated disease (CDAD) is a non-invasive colonic infection that ranges from (non-bloody) mild to severe diarrhoea to PMC with toxic megacolon or perforation and ultimately death. *C. difficile* infection is generally acquired in the hospital (Kelly et al., 1994). There is a clear, well-described direct relationship between the disruption of the gut flora by antibiotic treatment and disease.

#### 1.2.1. Incidence and cost

The awareness of CDAD worldwide has been increasing due to the emergence of the hyper-virulent strain 027. It was evidenced that the cases of CDAD had been increasing in number and severity. However, until the introduction of mandatory surveillance it was difficult to establish whether the increase seen was in fact a real increase of the cases of CDAD or if it was a better reporting and an increased awareness among clinicians. In England, Wales and Northern Ireland surveillance indicated that the number of CDAD cases increased a 22% in the first quarter of
2007 when compared to the previous quarter. However, when compared to the same quarter in the previous year, the increase was only 1.6%.

The number of voluntary reports in Scotland had been increasing markedly for the last ten years before surveillance of \( C. \text{difficile} \) became mandatory from 1 September 2006.

![Incidence of CDAD in Scotland](image)

**Figure 1.1** – Number of voluntary reports of CDAD in Scotland between 1996 and 2005. Data obtained from the Health Protection Scotland website (www.hps.scot.nhs.uk)

\( C. \text{difficile} \) infection increases the length of hospital stay (Macgowan et al., 1995). Wilcox et al. (1996) estimated that a case of CDAD increases the cost to the UK health service by approximately £4000, mainly due to the extended stay in hospital. Kyne et al. (2002) estimated that a case of \( C. \text{difficile} \) in the US costs around $3700 due to a 55% increase on the length of stay in hospital, but they thought this figure
might have been an underestimate. They did not include doctors' fees or the cost of any treatment after discharge from hospital. Overall and before the emergence of the hyper-virulent strain, the cost of management of diarrhoea due to C. difficile in the US exceeded $1.1 billion per annum (Kyne et al., 2002). These figures clearly reflect the necessity to control the spread of C. difficile, if for no other reason, to reduce the costs to the health systems.

1.2.2. Ecology of C. difficile

*C. difficile* in the environment

*C. difficile* is an opportunistic pathogen. Spores are commonly found anywhere in hospitals and nursing homes, especially old ones, and although anyone is potentially susceptible, it is traditionally related to old age and long term hospitalisation. It is transmitted faecal - oral human to human. Spores of *C. difficile* can be transmitted by hospital staff as well as hospital equipment such as bedpans (Rogers et al., 1981) and asymptomatic patients can be a reservoir for transmission of *C. difficile* (Johnson et al., 1990b). Al Saif and Brazier (1996) isolated toxigenic *C. difficile* from water samples (rivers, sea water, lake water, inland drainage, swimming pools and tap water), soil samples (public parks, gardens and playgrounds) and raw vegetables suggesting that *C. difficile* is very common in the environment.

All these environment sources can be reservoirs for community acquired CDAD. Johal et al. (2004a) reported that 28% of the CDAD cases they encountered were due to community acquired *C. difficile* following the administration with antibiotics. The isolation rate of *C. difficile* in patients from general practice and community health
centres is approximately 5% (Riley et al., 1986). In a survey of patients with diarrhoea admitted to an infectious diseases unit, *C. difficile* was isolated from 19 patients of which 17 came from their homes (Brettle et al., 1982).

**C. difficile in animals**

Household pets are a potential reservoir for community acquired CDAD (Borriello et al., 1983; Riley et al., 1991). Riley et al. (1991) found that the isolation rate in domestic cats and dogs was around 40%. However half of those isolates were non-toxigenic. O’Neill et al. (1993) isolated toxigenic *C. difficile* from pets and did not find a correlation between those isolates and the isolates predominantly found in human patients and hospital environment. However, even though the pet isolates were rare in human disease, they were toxigenic isolates that could potentially be transmitted to humans and originate disease.

*C. difficile* has also been reported as pathogen in animals producing a very similar disease to that shown in humans. Arroyo et al. (2005) have shown that isolates producing disease in animals (calves, dogs and horses) are very close related to human isolates, sometimes even identical, suggesting a potential interspecies transmission of the disease.

**C. difficile in neonates**

While carriage of *C. difficile* in healthy adults is very low (under 3%), around 50% of healthy infants under 1 year old carry *C. difficile* asymptotically (Kelly and LaMont, 1998). In neonates, *C. difficile* seems to be acquired mainly from
environmental sources rather than transmission from the mother (Bolton et al., 1984). Al Jumaili et al (1984) got vaginal swabs from women in labour and were all negative for *C. difficile* on culture, but almost half of their new born babies were carrying it in stool. *C. difficile* could be isolated from environmental samples of the special care unit. Cooperstock et al. (1982) found that breast-fed babies had a significant lower rate of colonization than babies fed primarily by infant formula. Borriello and Barclay (1986) showed that the gut flora of breast-fed neonates inhibited the growth of *C. difficile* at the same rate as the microbiota of healthy adults. This rate was reduced in bottle-fed neonates, children under 3 years old and geriatric patients.

Many hypotheses have been postulated to explain the tolerance to the toxins in the neonatal bowel such as the lack of toxin receptors in the gut (Borriello, 1995; Kelly and Lamont, 1998). However, several studies (Thomas et al, 1986; Schechter et al., 1999; Parsons et al., 2005) showed that *C. difficile* can cause severe enterocolitis in neonates as a complication of other diseases such as Hirschprung’s disease (severe constipation due to the lack of nerve cell growth in the bowel) or infant botulism, suggesting that they are able to develop disease.
1.2.3. Risk factors

Old age and the administration of antibiotics are the main risk factors linked to CDAD. However, some cases of *C. difficile* diarrhoea have occurred in patients where no antibiotics have been ascribed (Bartlett, 1992) suggesting that many other factors are involved.

Commensal gut flora acts as a protective barrier which can be overcome by most enteric pathogens when these are large in numbers (Borriello, 1995). However, this is not the case with *C. difficile*: without the disruption of the native gut flora, *C. difficile* can not compete for the colonization of the gut (Borriello, 1995). Borriello and Barclay (1986) showed that the growth of *C. difficile* was inhibited when added to faecal emulsions obtained from healthy adult volunteers due to the presence of viable bacteria, and that this inhibition was reduced in patients receiving antibiotics. Caecal emulsions of hamsters treated with clindamycin lost all the inhibitory properties that caecal emulsions of untreated hamsters had on the growth of *C. difficile* (Borriello and Barclay, 1986).

**Antibiotics**

Although clindamycin (Tedesco et al., 1974) and the β-lactams susceptible to β-lactamases (Tedesco, 1975; Starr et al., 2003) are the most common antibiotics associated to CDAD, almost any antibiotic can precipitate disease (Bartlett, 1992), especially if administered orally. It takes approximately 3 weeks to recover the commensal gut flora after the disruption produced by the administration of antibiotics (Bartlett, 1992).
The risk of an antibiotic causing disease is correlated to the frequency of use of such an antibiotic, decreasing the use of a particular antibiotic decreases the risk it presents (Gerding, 2004). A second factor to take into consideration when analysing the risk of an antibiotic, is the level of resistance of *C. difficile* to it (Gerding, 2004). It would seem that the more efficient and more improved the antibiotic, the higher risk it presents. The third generation of cephalosporins were shown to give a higher risk of *C. difficile* infection than first and second generations (Spencer, 1998). The newer generation of fluoroquinolones were at first reported to be low risk to *C. difficile* (Bartlett, 1992; Wilcox et al., 2000) but the acquisition of resistance to fluoroquinolones by the hyper-virulent strain ribotype 027 has made the new generation of fluoroquinolones a major risk (Gaynes et al., 2004; Pepin et al., 2005b).

Baines et al. (2006) studied the effect of tigecycline on the growth and toxin production of *C. difficile*. Tigecycline, which is the first licensed glycylcycline antibiotic to be marketed, has broad-spectrum activity against anaerobes, both obligate and facultative. In the presence of tigecycline, *C. difficile* remained as spores without germinating or producing toxin and therefore was considered a low risk antibiotic for CDAD (Baines et al., 2006). They also considered that the normal flora would be restored by the time the concentration of tigecycline in the gut was reduced to a level where germination of the spores could take place. However, many factors are involved in CDAD and caution should be applied to the use of any broad-spectrum antibiotic, particularly one that fails to eliminate spore-forming bacteria.
K. Sánchez-Hurtado  

Table 1.1 – Antibiotics as risk factors for CDAD – adapted from Mylonakis et al. (2001)

<table>
<thead>
<tr>
<th>High risk</th>
<th>Medium risk</th>
<th>Low risk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clindamycin</strong></td>
<td><strong>Chloramphenicol</strong></td>
<td><strong>Metronidazole</strong></td>
</tr>
<tr>
<td><strong>Ampicillin</strong></td>
<td><strong>Erythromycin and other macrolides</strong></td>
<td><strong>Vancomycin</strong></td>
</tr>
<tr>
<td><strong>Amoxicillin</strong></td>
<td><strong>Other penicillins</strong></td>
<td><strong>Bacitracin</strong></td>
</tr>
<tr>
<td><strong>Cephalosporins</strong></td>
<td><strong>Tetracyclines</strong></td>
<td><strong>Teicoplanin</strong></td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td><strong>Trimethoprim</strong></td>
<td><strong>Rifampin</strong></td>
</tr>
</tbody>
</table>

Other medication and clinical procedures

The disruption of the gut flora can also be caused by other means, such as anti-ulcer medication, cancer chemotherapy (Kelly and Lamont, 1998), diuretics (Raveh et al., 2006), nasogastric tubes (Pierce et al., 1982; Simor et al., 1993) or intestinal surgery (Pierce et al., 1982). Patients who have had recent gastrointestinal endoscopy were four times more likely to develop severe CDAD (Kyne et al., 1999).

The use of proton-pump inhibitors (PPIs) has been related to increase the incidence of CDAD since they decrease gastric acidity which is a barrier against enteropathogens (Dial et al., 2004; Al-Tureihi et al., 2005). Dial et al. (2005) established a relationship between gastric acid suppressive agents, which included H₂-receptor antagonists as well as PPIs, to community acquired CDAD.

Age

CDAD is more frequent among people over 65 years old (Kyne et al., 1999). However, it is not exclusively a disease of the elderly since cases have been reported among younger patients (Cohen et al., 1973; Larson et al., 1977; Rifkin et al., 1977;
Kyne et al., 1999; Kato et al., 2007) and it has been shown that the severity of CDAD is independent of age (Kyne et al., 1999). The elderly are associated with longer stays in hospitals and increased antibiotic exposure which probably explains why CDAD is more frequent in patients over 65 years old (Borriello, 1995). It was also seen that the barrier created by the commensal flora is reduced in the elderly (Borriello and Barclay, 1986).

**Other host factors**

The nature of the underlying disease affecting the patient has also been associated with CDAD (Simor et al., 1993), particularly if the patient suffers from gastrointestinal disease, renal disease or anaemia (Harbarth et al., 2001). *C. difficile* can be found in patients with inflammatory bowel disease (such as ulcerative colitis or Crohn’s disease) that had not been exposed to antibiotics; however, it is more common if antibiotics have been prescribed (Greenfield et al., 1983). The treatment for gastrointestinal diseases implies hospitalization and the use of antibiotics which are high risks for the acquisition of *C. difficile* (Kelly and Lamont, 1998). CDAD has also been reported in patients with leukaemia (Milligan and Kelly, 1979) and HIV infection (Tumbarello et al., 1995).

A correlation between viral gastroenteritis and *C. difficile* infection has also been reported (Wilcox and Fawley, 2007). Rates of *C. difficile* infection are higher in units affected by norovirus gastroenteritis. It is not clear whether this is due to an increase on the risk of transmission or if there is an interaction between noroviruses and *C.
dissemine. Norovirus could be a cause of the alteration of the gut flora which would facilitate *C. difficile* colonization (Wilcox and Fawley, 2007).

Functional and cognitive impairment are normally a reflection of the severity of underlying disease and makes the patient functionally dependent which increases the risk of nosocomial disease including CDAD (Kyne et al., 1999).

Low albumin levels might be an indicator of reduced total protein levels including the levels of serum immunoglobulin that are protective against *C. difficile* toxins (Al-Tureihi et al., 2005). The role of the immune response will be discussed in detail later on (section 1.3)

**Strain**

The level of disease can be determined by the strain colonising the gut. It has been shown that some strains are more virulent than others (Borriello et al., 1987). The emergence of the hyper-virulent strain earlier discussed has caused more deaths and complications than other *C. difficile* strains and therefore patients carrying more virulent strains would be at more risk of developing severe disease.

**Stay in hospitals**

Hospitalized patients are the primary target of *C. difficile* because they receive antibiotic therapy in an environment where spores of *C. difficile* are predominant (Kelly et al., 1994b). *C. difficile* can be isolated from hospital floor, toilets, bedpans, bedding, mops, scales and furniture, especially in rooms where a CDAD patient is being treated or has been recently treated (Kelly et al., 1994b). Health care personnel
carry the bacterium from one patient to another in their hands, stethoscopes, clothing etc. (Kelly et al., 1994b). It was reported that 21% of patients became colonized during their hospital stay (McFarland et al., 1989).

Long stays in hospitals increases the exposure to the organism and generally implies more antibiotic treatments and other high risk clinical procedures (Bignardi, 1998).

1.2.4. Symptoms

Around 50% of the hospitalised population colonized by \textit{C. difficile} remain asymptomatic (Kelly et al., 1994b). The main symptom that presents is one or many episodes of mild to severe diarrhoea, typically mucosal and non-bloody and frequently accompanied by severe abdominal pain and tenderness (Kelly and Lamont, 1998). In a minority of cases, severe complications of CDAD such as toxic megacolon or perforation can present without antecedents of \textit{C. difficile} diarrhoea which can complicate diagnosis (Bartlett, 1992).

Other symptoms that can present are fever, nausea, general malaise, anorexia and very high leukocytosis (up to 40000 WBC/\mu l). Diarrhoea can cause dehydration and inbalanced of electrolytes (Kelly and LaMont, 1998)

Recurrences within a short period of time are not uncommon (Kelly and Lamont, 1998). The patient’s stay in hospital gets extended implying further costs (Wilcox et al., 1996) as well as a negative psychological impact on the patient, and furthermore he/she has now become a source of infection for other patients (McFarland et al., 1989).
In the worst cases, in about 1-3% of patients (Figures are much higher when the hypervirulent strain 027 is involved – Pepin et al., 2005a) it can lead to PMC and the formation of the characteristic yellowish pseudomembranes that can be confined to small patches or cover much larger areas (Kelly and Lamont, 1998). PMC can lead to partial or total colectomy or even death. Other lethal complications include toxic megacolon, peritonitis and perforation of the colon (Kelly and LaMont, 1998).

1.2.5. Diagnosis

Clinical diagnosis

The appearance of diarrhoea after the administration of antibiotics as well as other symptoms, complications, and the consistency and characteristic odour of the stools can be use to diagnose CDAD accurately (Wilcox, 2007). Other indicators of CDAD are high levels of creatinine, high white blood cells (WBC) count (Pepin et al., 2004) or low levels of albumin (Al-Tureihi et al., 2005)

Gastrointestinal endoscopy is used to visualize colitis and the formation of pseudomembranes. However, endoscopes should be heavily disinfected since they can be a source of transmission of C. difficile (Kyne et al., 1999). Flexible sigmoidoscopy at the bedside reduces the risk of transmission associated with endoscopy units (Johal et al, 2004a).

Histologically, PMC lesions are divided in three groups: Type I - patchy epithelial necrosis accompanied by an exudate of fibrin and neutrophils; Type II - “volcanotype” lesions where exudate “erupts” from a focus of epithelial ulceration while the
surrounding mucosa remains intact; Type III – diffuse epithelial necrosis and ulceration, with pseudomembranes consisting of mucin, fibrin, leukocytes and cellular debris (Kelly et al., 1994b).

**Laboratory diagnosis**

The most common way to confirm the CDAD clinical diagnosis is by toxin detection in stool. The first cytotoxicity assays were based on the neutralization of the cytopathic effect (CPE) produced by *C. difficile* with *C. sordellii* antitoxin (Chang et al., 1979; Bartlett, 1981). However it takes between 1-3 days to get results and requires the maintenance of a cell line.

Many commercial kits are available which provide results within the hour. They detect either toxin A or both A and B toxins by enzyme-immuno assay (EIA). Toxin A was believed to be the main toxin but the appearance of A"B+ strains (Alfa et al. 2000) made it necessary to include toxin B in diagnosis.

CDAD can be diagnosed by culture of faecal samples in selective media, such as the earlier mentioned CCFA medium developed by George et al. (1979) or CCEY which contains cycloserine, cefoxitin, egg yolk, lysed blood and sodium cholate for the germination of spores (Brazier, 1993). Colonies can be identified by odour, colony morphology (see Figure 1.2) and fluorescence under U.V. light (Brazier, 1993). Culture is recommended to avoid missed cases of CDAD (Delmée et al., 2005). The cost of toxigenic culture of every faecal specimen was not as expensive as had been suggested especially when compared to the cost of an outbreak due to the spread of
spores by an untreated patient (Delmée et al., 2005). However it takes between 1-3 days to get results and requires facilities for anaerobic growth.

Further identification of C. difficile colonies can be carried out by gas liquid chromatography, but this is not available in most diagnostic laboratories (Bowman et al., 1986).

![Typical colonies of C. difficile on a CCEY plate](image)

**Figure 1.2 – Typical colonies of C. difficile on a CCEY plate**

Latex particle agglutination is a very sensitive test, simple to perform and at a very low cost, however, since it reacts to glutamate dehydrogenase which is present in other bacteria, cross reactions are common (Bowman and Riley, 1986a)

Molecular methods have also been developed. A non-commercial PCR detects toxigenic strains directly from stool samples by amplification of the toxin B gene (Gumerlock et al., 1993). A multiplex PCR targeting three genes: the triose phosphate isomerase gene (tpi), a housekeeping gene, and the two toxins genes, tcdA and tcdB, was developed to combine a quick diagnosis and immediate identification of strains of C. difficile, mainly by detecting partial deletions on the tcdA gene or differentiating between toxigenic and non-toxigenic strains (Lemee et al., 2004).
Real time PCR for the detection of toxin B is a rapid screening method with a high concordance with toxigenic culture (Van den Berg et al., 2007).

1.2.6. Treatment

First episode

The first step in treatment of CDAD is when possible to stop the current antibiotic treatment, or, if necessary, it could be substituted with a low-risk antibiotic (Bartlett, 1992). If there is no remission, it should be followed by the oral administration of metronidazole or vancomycin, with metronidazole being the first choice due to the high cost of vancomycin (Bartlett, 1992; Kelly and Lamont, 1998) and the need to use vancomycin for the treatment of MRSA (methicillin resistant *Staphylococcus aureus*) infections. In cases of extreme diarrhoea, the antibiotic treatment is supported with rehydration and electrolyte replacement.

Bartlett (1992) recommended following the standard treatment of metronidazole/vancomycin with a 3-week treatment to help restablish the normal gut flora and avoid relapses.

Anti-peristaltics normally used to stop diarrhoea are not recommended in the case of *C. difficile* diarrhoea since they can delay recovery. A normal intestinal peristaltic activity is necessary for the clearance of the organism (Kelly and Lamont, 1998).

So far, there is no evidence of any significant difference in efficiency between metronidazole and vancomycin (McFarland, 2005). Vancomycin is also a selective antibiotic for vancomycin resistant enterococci (VRE) that can cause infections.
(Garbutt et al., 1999). For this reason, vancomycin is saved for those cases where administration of metronidazole is not possible (allergies and incompatibilities). The main problem with metronidazole is the appearance of resistance and reduced susceptibility to it (Brazier et al., 2001; Pelaez et al., 2002), and more rarely, neurotoxic complications (Beloosesky et al., 2000).

In a human gut model, the reduced susceptibility to metronidazole was attributed to a reduction of the gut concentration of metronidazole to sub-inhibitory concentrations possibly due to degradation by other gut bacteria. This phenomenon could be an explanation for metronidazole failure in treatment (Freeman et al., 2007).

**Recurrences**

Most patients will respond very favourably to treatment and CDAD will be resolved, but in 25% of the cases (Kyne and Kelly, 2001) the patient relapses (spores that have not been killed in the first treatment germinate) or gets reinfected (by spores in the environment). Recurrent CDAD is a difficult treatment problem, once a patient has one recurrence of the disease it is most likely that further recurrences will occur (Kelly et al., 1994b), and when that is the case, it can take up to many months or even years to recover.

The recommended treatment of recurrences is to retreat the patient with metronidazole or vancomycin: (McFarland et al., 1994; Gerding et al, 1995; McFarland et al., 2002). Switching from metronidazole to vancomycin is not necessarily going to make a difference since the development of resistance is not usually the cause of relapse (Kelly et al., 1994b; Sanchez et al., 1999).
There has been a debate whether recurrences are due to re-infections after treatment (during the period of time when the host is still susceptible to colonization by spores transmitted by the hands of hospital personnel or contaminated surfaces) or relapses due to the germination of spores that treatment failed to kill (due to discontinuation of the treatment or administration of low dose). It seems that half of the recurrences are re-infections and the other half relapses (Johnson et al., 1989; Wilcox et al., 1998). However, reports that multiple strains can be colonizing the patient at the same time make epidemiological studies difficult since it can be difficult to establish whether the new episode of diarrhoea is due to a re-infection from environmental sources or whether is a relapse (van den Berg et al., 2005a).

Recurrences represent an enormous financial cost since the patient has to extend its stay in hospital, but above all, is a cost to the patient’s quality of life. Consequently, other therapies are being proposed and will be discussed in the next section.

1.2.7. Alternative therapies

Alternative therapies to metronidazole or vancomycin are being proposed, especially for the treatment of recurrences. The increase of dosage of vancomycin gave better results than the standard vancomycin dose (McFarland et al., 2002), however increasing dosage is not recommended since it can lead to the appearance of resistance. Vancomycin tapering (decreasing) dosage and pulsed dosage of vancomycin (every 2-3 days over 3 weeks) also gave better results (McFarland et al., 2002). With pulsed dosage the idea is to allow spores to germinate so that they can be killed.
Other antibiotics

Other antibiotics have been suggested with good results but not better results than those of metronidazole and vancomycin: bacitracin (Young et al., 1985; Dudley et al., 1986), rifampicin (Langrotteria et al., 2006), fusidic acid (Wenisch et al., 1996; Ackermann et al., 2005) and teicoplanin (Wenisch et al., 1996)

Probiotics

Since the disruption of the gut flora is probably the main conditioning for the development of disease, one of the first alternative therapies to reduce the incidence of CDAD in hospitalised patients is to re-establish the altered gut flora and prevent the overgrowth of *C. difficile*. Biotherapy or probiotic therapy is the administration of live non-pathogenic bacteria that can colonize the host’s intestine without detriment.

Surawicz et al. (1989) used *Saccharomyces boulardii* as prophylaxis to prevent antibiotic-associated diarrhoea (AAD). The group receiving the probiotic had a significantly lower incidence of diarrhoea than the group receiving a placebo. *Lactobacillus rhamonosus* GG has also been very efficient at preventing antibiotic-associated diarrhoea (Surawicz, 2003).

*S. boulardii* in conjunction with antibiotics, has also been successfully used to treat recurrent CDAD (McFarland et al., 1994; Surawicz et al., 2000). *S. boulardii* inhibits the binding of toxin A to its gut receptor and produces a protease that hydrolyzes toxin A (Castagliuolo et al., 1996). Qamar et al (2001) showed that *S. boulardii*
stimulates the production of mucosal anti-toxin A IgA when co-administered with a toxoid, suggesting that it acts as a mucosal adjuvant.

Plummer et al. (2004) tested a probiotic treatment based on *Lactobacillus* and *Bifidobacterium* in conjunction with antibiotics and showed a considerable reduction in toxins levels in patients’ samples.

Lactinex (*L. acidophilus* and *L. bulgaricus*) and Bioflorin (*Enterococcus faecium*) are commercially available probiotics for the prevention of AAD.

**Faecal transplants**

One step further in the reconstitution of the commensal flora is the use of “faecal transplants”. This consists either in the irrigation of the bowel with enemas prepared from healthy stools, or feeding through nasogastric tube. It was first described by Bowden et al. (1981) and Schwan et al (1984) as a treatment of punctual cases of recurrent severe diarrhoea that did not respond to traditional treatment. Borriello and Barclay (1986) showed that the viable bacteria in the stools of healthy adults inhibited the growth of *C. difficile*.

Over a period of eight years, Aas et al. (2003) treated 18 cases of severe recurrent CDAD with donor stools which were administered via nasogastric tube. The success rate was 94%. Despite it not being very aesthetic, it is a patient driven technique. The main risk that this technique presents, apart from those that nasogastric feeding already entail, is the presence of infectious agents, so the stools should be screened before administration.
Non-toxigenic strains

The use of non-toxigenic strains of *C. difficile* to prevent CDAD has also been considered. Patients that asymptomatic carry *C. difficile* are at a lower risk of developing CDAD during their hospital stay (Shim et al., 1998).

Borriello and Barclay (1985) prevented the growth of toxigenic *C. difficile* by administering non-toxigenic strains that competed for the same niche. Sambol et al. (2002) protected hamsters by prompting the colonization of the gut with non-toxigenic resistant strains of *C. difficile*. They administered clindamycin to the hamsters to kill the commensal flora before inoculating them with a non-toxigenic strain and once colonization of the non-toxigenic *C. difficile* was confirmed, they challenged the hamsters with a toxigenic strain of *C. difficile* and monitored them for at least two months. Control hamsters were not inoculated with the non-toxigenic strains and died within 48 hours after challenge with the toxigenic strain. All the hamsters that failed to be colonised with the non-toxigenic strain died within 48 hours after challenge with the toxigenic strain. Colonisation with the non-toxigenic strain prevented co-colonisation with the toxigenic strain in the majority of the hamsters (74 – 100%; mean: 90%). The rate of protection against CDAD in the hamsters colonised with the non-toxigenic strain ranged from 93 to 100% (mean: 98%) and the protection against death from 96 to 100% (mean: 99%).
Inmobilisation of toxins

Another strategy is based on the immobilisation of toxins released in the colon rather than killing or preventing growth of *C. difficile*. Toxins are necessary for the pathogenesis of *C. difficile*. The idea is to sequester the toxin permanently before it can enter and damage the enterocyte. Resins such as cholestyramine and colestipol (Ariano et al., 1990) and non-absorbed polymers such as tolevamer (Barker et al., 2006) have been tested for this purpose. The main problem with cholestyramine is that it also binds to vancomycin (Kelly and LaMont, 1998).

Genzyme® is currently in phase III clinical trials in the development of a tolevamer therapy that binds irreversibly to toxins A and B. However, recent data showed that tolevamer therapy was not better at resolving CDAD permanently than the standard prescribed oral dose of vancomycin (http://www.genzyme.com.).

SYNSORB Cd® is an oligosaccharide bound to an inert polymer matrix that acts as a decoy toxin receptor. However, the efficacy of SYNSORB remains to be fully studied (McFarland, 2005).

Immunotherapy

Therapies to boost the immune system have also been explored. The immune response plays an important role in CDAD, particularly when it comes to recurrences (this subject will be treated in more detailed in section 1.3).

The intravenous administration of human pooled immunoglobulin has proved successful in treating severe colitis cases (Salcedo et al., 1997; Beales, 2002; Wilcox,
2004). However, its current costs make it unavailable for widespread use being cost effective only in intractable cases (Wilcox, 2004).

It was also seen that cows immunized with a *C. difficile* toxoid produced toxin-neutralizing immunoglobulin in their milk (Kelly et al., 1996). High titres of antibodies against *C. difficile* were maintained in the immunised cows. The whey fraction of milk was isolated, concentrated and tested for neutralizing activity. This polyclonal enriched whey was administered to patients (both recurrent and first episodes) after the diarrhoea episode and no recurrence was observed in any of the patients during the follow-up after completion of treatment (Van Dissel et al., 2005).

**Vaccines**

Approaches to vaccines have also been made. Corthier et al (1991) protected mice passively using anti *C. difficile* toxin A monoclonal antibodies. A toxoid vaccine induced a marked systemic antibody response in healthy volunteers when compared to non-vaccinated controls (Kotloff et al., 2001; Aboudola et al., 2003). Sougioulitzis et al. (2005) tested a toxoid vaccine in three multi-recurrent cases and showed an increase of anti-toxins (both A and B) IgG, as well as neutralizing activity, in two of them. All three subjects were able to stop their long-term vancomycin treatment after vaccination and had no further relapses. Currently, Acambis is in the process of developing a toxoid vaccine based on both toxins A and B (www.acambis.co.uk).

Other antigens have been considered as vaccine candidates. The surface proteins are highly immunogenic and can induce the production of cytokines in human monocytes and dendritic cells (Ausiello et al., 2006). O’Brien et al. (2005) protected
hamsters using an anti-SLPs serum and Péchiné et al (2007) protected mice from *C. difficile* colonization by rectal administration of different combinations of surface antigens.

**Blocking the immune response**

Another strategy tried is to keep the immune response at bay. Since intestinal inflammation is mediated by the infiltration of neutrophils into the lumen as a host response, a strategy for the reduction and reverse of inflammation could be the blocking of the migration of leucocytes to the intestinal lumen. Kelly et al. (1994a) showed that after blocking the recruitment of neutrophils in the rabbit ileum with a monoclonal antibody to CD18, the levels of toxin A enterotoxicity and mucosal damage were dramatically reduced.

1.2.8. **Control and Prevention**

*C. difficile* forms heat-resistant spores that can persist in the environment for months or even years (McFarland et al., 1989; Kelly et al., 1994b). Wards should be exhaustively disinfected using chlorine-based solutions since it was seen that they were more effective against *C. difficile* than ammonium-based solutions (Mayfield et al., 2000) but there was no major differences between the use of hypochlorite or neutral liquid detergent (Wilcox et al., 2003).

Since *C. difficile* is a spore-forming bacterium, one single patient is a contamination risk for other patients and the ward environment and therefore CDAD patients should be isolated and their faeces carefully disposed of (Larson et al., 1978). McFarland et
al. (1989) found that patients sharing a room with a patient colonized by *C. difficile* were at higher risk to acquire *C. difficile* themselves.

Asymptomatic patients could be a reservoir of infection; however, treatment of asymptomatic patients did not reduce the risk of transmission since 67% reacquired it and is not recommended (Johnson et al., 1992). Asymptomatic patients have a lower risk of developing CDAD (Shim et al., 1998).

*C. difficile* infection is a nosocomial disease transmitted from patient to patient, often thorough the hands of hospital staff (McFarland et al., 1989) and hospital equipment and furniture such as bedpans, floors and toilet seats (Kim et al., 1981).

Hospital staff should wash their hands with soap and water before and after contact with patients. Alcohol rubs are not recommended since they do not inactivate spores (Surawicz, 2006), in fact, ethanol shock (1 hour with 100% ethanol) can be use to select for sporulating bacteria (Bartlett, 1981).

The use of vinyl gloves has been shown to reduce the number of CDAD cases (Johnson et al., 1990a). Johnson et al. (1990a) found that easy accessibility to the gloves was a key factor for the effective use of gloves.

Doctors should be aware of the consequences of prescribing antibiotics and proton-pump inhibitors and should not prescribe them lightly (Wilcox, 2000). Establishing restriction policies on the antibiotic(s) associated to a particular CDAD outbreak has been a successful control measure to stop such outbreaks (Ho et al., 1996; Quale et
al., 1996; Jones et al., 1997; McNulty et al., 1997; Climo et al., 1998; Thomas et al., 2002; Carling et al., 2003; Khan and Cheesbrough, 2003; Kuijper et al., 2006).

It is important to have proper surveillance to detect outbreaks and initiate protocols of disinfection and antibiotic restriction policies as soon as possible (McDonald et al., 2007). In all, efficient control is the result of a combination of measures. Apisarnthanarak et al. (2004) initiated an environmental-infection intervention to control the transmission of *C. difficile* in two units of a hospital. Infection control measures included contact isolation, disposable thermometers, the use of gloves and gowns and posting hand-washing signs. Environmental control programmes included daily cleaning and disinfection of patient rooms and staff work and lounge areas with unbuffered 1:10 hypochlorite solution. They managed to reduce the incidence of CDAD by 50% without any change on antibiotic use. The reduced rates persisted for a year after the intervention.
1.3. **Immune response to C. difficile**

Around 60% of healthy adults have antibodies against *C. difficile* toxins (Kelly et al., 1992). Asymptomatic carriers had higher levels of anti-toxin A IgG and anti-nontoxin antigens IgM than those who developed symptoms (Kyne et al., 2000). The levels of antibodies against several surface proteins were also lower in CDAD patients than in healthy controls (Pechiné et al., 2005b).

1.3.1. **Cases and controls**

Kyne et al (2000) prospectively studied the immune response to *C. difficile* colonization of 47 patients that were being administered antibiotics and that were colonized with *C. difficile* during their stay in the hospital (if they had acquired *C. difficile* prior hospitalization, they were discarded from the study). Of those 47 patients, 28 (60%) developed CDAD and 19 (40%) remained asymptomatic (4 of them were colonized with non-toxigenic strains) during their whole stay in the hospital and during the 30-day follow-up after discharge. The levels of IgG antibodies in serum against toxin A were significantly higher in the asymptomatic group but the levels of anti-toxin B IgG were not significantly different. The levels of IgM in serum against “nontoxin antigens” (product of the sonication of non toxigenic strains of *C. difficile*) were also significantly higher in the asymptomatic group. Serum levels of anti-toxins IgM and IgA were not significantly different. Levels of faecal IgG and IgM against every antigen tested were also not significantly different.

Kyne et al (2000) in their study also noted that asymptomatic carriers were on average suffering less severe diseases than the ones that developed CDAD
symptoms. They suggested toxin A as a protective antigen and a good candidate for vaccines.

Drudy et al (2004) studied the antibodies levels to surface layer proteins (SLPs) in a population of 146 hospitalised patients. They divided them into cases, carriers and controls according to their colonisation status, but they did not find differences in the levels of anti-SLP IgG, IgM or IgA. Pechine et al. (2005a) showed that 33 CDAD patients had significant lower levels of antibodies (anti-human polyvalent immunoglobulin) against several adhesins than 40 healthy controls (women and children): the N terminal domain of Cwp66, the flagella (FliC and FliD) and a fibronecting-binding protein, Fbp68, but not against toxins A or B, suggesting that these proteins have an important role in pathogenesis.

Johal et al. (2004b) found no differences in levels of systemic antibodies (IgG, IgM and IgA) between CDAD patients, patients with severe PMC and controls. However, they found differences in the levels of mucosal antibodies. They analysed a total of 28 biopsies of patients with and without CDAD and different grades of severity, and found that patients with CDAD had lower numbers of mucosal macrophages than controls (non-C. difficile self-contained diarrhoea, with normal sigmoidoscopy and normal histology). No differences were found in lamina propria T-cells but B/plasma cells were lower in CDAD patients and decreased with severity of CDAD. IgA producing cells were reduced in CDAD patients and numbers decreased with severity. No differences were found in IgM producing cells except in the most severe PMC group where they were significantly reduced. By contrast, IgG producing cells were higher in CDAD than controls except in the most severe group.
1.3.2. Immune response in recurrences

Kyne et al (2001) monitored 44 patients that developed *C. difficile* diarrhoea: 22 (50%) had a single episode of diarrhoea that remitted after treatment and 22 (50%) had recurred after standard treatment with metronidazole. The levels of anti-toxin A IgG and IgM in serum were significantly higher in the group that had single episodes enforcing the idea of the protective role of anti-toxin A antibodies. They also found other factors to be correlated to recurrences. Mean age in recurrent group was 10 years higher with 14 out of the 22 patients with single episodes (64%) being under 65 years old. The disease that led to hospitalisation was considered severe or extremely severe in 68% of the patients in the group that recur while 68% of the patients in the single episode group were considered to have mild or moderate levels of disease. Finally, 65% of patients that recurred had other antibiotics during the 60-day follow-up period versus 27% of the patients that had a single episode of diarrhoea.

Kyne et al (2001) showed that the levels of IgA in serum against “nontoxin antigens” (product of the sonication of non toxigenic strains of *C. difficile*) were significantly higher in the group of recurrent episodes, a situation they could not explain. However, Drudy et al (2004) analysed the antibodies levels in the serum of 55 CDAD patients and observed that those that had a single episode of diarrhoea had significantly higher levels of IgM against surface layer proteins (SLPs) than those who recurred. They suggested that SLPs antibodies conferred protection against recurrence to *C. difficile*. No differences in IgG or IgA levels were found.
When the mucosal antibodies were analysed, it was observed that patients with severe PMC that recurred had fewer B plasma cells, IgA and IgG producing cells than patients with severe PMC which did not recur (Johal et al. 2004b).

1.4. PaLoc

1.4.1. Description

![Diagram of PaLoc](image)

Figure 1.3 – Schematic representation of the PaLoc

The pathogenicity locus or PaLoc is a 19.6 kb region that encodes for five genes: toxin A gene (tcdA), toxin B gene (tcdB), tcdC, tcdR and tcdE. A pathogenic strain of C. difficile is defined by the ability to produce at least one of the two main toxins A and B. The total absence of the PaLoc leads to the non-toxigenicity of the strain, and therefore to its avirulence (Cohen et al., 2000). Delmée et al. (2005) reported that over 2% of the isolates obtained by routine culture of faecal samples were non-toxigenic strains.

The PaLoc is highly stable and conserved. Although the total absence of it results in the non-virulence of the strain, partial deletions are compatible with toxin production (Cohen et al., 2000). The existence of strains lacking toxin A but able to produce toxin B (A'B-) have been reported and these are as virulent as strains producing both toxins (Alfa et al., 2000). This contradicted the earlier belief that toxin A was the
most important of the two toxins (Borriello et al., 1987). Partial deletions in the \textit{tcdC} gene have also been reported (Spigaglia and Mastrantonio, 2002).

Most of the A"B+ strains are toxinotype VIII, characterized by a 1.8 kbp deletion in \textit{tcdA} (Rupnik et al., 1998). Other toxinotypes include toxinotype X (Rupnik et al., 1998), XVI and XVII (Rupnik et al., 2003). The analysis of the isolates from an outbreak associated to fluoroquinolone resistant A"B+ strains (belonging to toxinotype VIII and PCR ribotype 017), showed that they had a substitution of Asp426 for Val in \textit{gyrB} which was associated with a high level of resistance to all the fluoroquinolones tested (ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin). The substitution for valine in position 426 results in a loss of a negative charge and quite possibly a shape alteration of the drug-binding pocket (Drudy et al., 2006).

Strains producing toxin A but lacking toxin B (A'+B- strains) have not yet been reported, which might imply that toxin B is more important in the pathogenicity of \textit{C. difficile}. Rupnik et al. (2001) described a non-toxigenic strain that completely lacked the \textit{tcdB} gene but that had 2/3 of the \textit{tcdA} gene but produced neither toxin A or B. This strain, which was called A'+B-, belonged to toxinotype XI. Despite the fact that this A'+B- do not produce toxin A at detectable levels by ELISA, since they have most of \textit{tcdA} gene they might still have a role in pathogenesis (Cohen et al., 2000).

The PaLoc is always integrated into the same one site of the chromosome; none of the genes of the PaLoc exists at any other site of the genome (Braun et al., 1996). The border sequences of the PaLoc are perfectly conserved among all toxinogenic
strains suggesting that the PaLoc defines a distinct genetic element (Braun et al., 1996). However, the PaLoc is not by itself an independent mobile genetic element. No transposon, plasmid, phage-like elements or any other characteristic structures of mobile genetic elements was found in the border region of the PaLoc (Braun et al., 1996).

1.4.2. Toxins: mechanism of action

Toxins A and B are part of a group of toxins known as “Large Clostridial Toxins” (LCTs), which are the largest bacterial protein toxins known (Schirmer and Aktories, 2004). They are the two main virulence factors, although it has been shown that there is no strict correlation between the amount of toxin produced in vitro and the severity of disease caused (Borriello et al., 1987). Therefore, other virulence factors that might not be essential, but may still be important to understand fully the pathogenicity of C. difficile, have been considered and will be referred to later in section 1.6.

Both toxins are secreted by C. difficile (exotoxins) and internalized by the gut cells. They both are toxic in cell culture but toxin B produces between 100 to 1000 times more damage to cell monolayers than toxin A and therefore toxin B is referred to as the cytotoxin (Schirmer and Aktories, 2004). Toxin A was labelled the enterotoxin under the belief that it was more responsible for the diarrhoea symptoms than toxin B and did not produce any cellular damage to enterocytes. However, it has since been shown to damage enterocytes in the absence of toxin A (Alfa et al., 2000).
Both toxins are highly similar in molecular weight, sequence and mode of action (Voth and Ballard, 2005). They can be divided in three regions: the N-terminal region is the catalytic domain, the C-terminal region is the domain that binds to the toxin receptor, and in the middle there is a hydrophobic section which is involved in the translocation of the toxin into the cytosol (see Figure 1.4). The uptake of the toxins and their translocation into the cytosol are essential for the toxins to reach their substrate (Schirmer and Aktories, 2004).

![Diagram of toxin structure](image)

**Fig 1.4** – The structure of toxin B – from Schirmer and Aktories (2004). The uptake and action of the toxins are summarised in Figure 1.5.

**Binding to the receptor**

The receptor-binding domain of the toxins, which is located in the carboxyl terminal, is composed of repetitive oligopeptide elements (known as CROPs) commonly accepted as motifs for binding to host cell surface sugar structures (Just and Gerhard, 2004). In toxin A this domain covers a third of the whole molecule and contains 38 of these CROPs (Just and Gerhard, 2004). The CROPs found in toxin B are more divergent and less frequent than those found in toxin A (Voth and Ballard, 2005).
Toxin A has been shown to be able to bind to several structurally related carbohydrates structures (Just and Gerhard, 2004). Toxin A binds to the human carbohydrates I, X and Y (Tucker and Wilkins, 1991) and to bovine thyroglobulin (Krivan and Wilkins, 1987) among others.

Receptors for toxin B remain unknown but it is believed to be ubiquitous (Voth and Ballard, 2005). The binding of the toxin(s) to cell surface structures induces receptor-mediated endocytosis into endosomal structures within the cell cytoplasm (Just and Gerhard, 2004).

**Translocation into the cytosol**

The translocation into the cytosol is pH-dependent as evidenced by studies that inhibited cytotoxicity by blocking the endosomal H⁺ pumps. However, if the medium was acidified, toxin was taken up despite the blockage (Barth et al., 2001). The central region of the toxins is highly hydrophobic. The low pH in the endosomes induces a structural change of the toxins exposing its hydrophobic region (Qa’Dan et al., 2000). The increased hydrophobicity allows toxin B to interact with the endosomal membrane to form pH-dependent channels which mediate translocation into the cytosol (Barth et al. 2001). The pH-dependent channel-formation activity of toxin A has been reported to be also cholesterol-dependent (Giesemann et al., 2006).

Immunoblot analysis showed that only the enzyme domain of the toxin B is transported into the cytosol while the rest of the toxin probably remains in the endosome (Pfeifer et al., 2003). Toxins are processed by autocatalytic cleavage dependent on inositol-hexaphosphate (Reineke et al., 2007). The autocatalytic
cleavage does not happen inside the endosomes since it can be inhibited by the addition of proteases (Pfeifer et al., 2003). This cleavage might occur either at the cell surface before internalization or after translocation through the membrane of the endosome by a cytosolic enzyme (Schirmer and Aktories, 2004).

![Diagram of toxin entry and translocation](image_url)

**Fig. 1.5** – Representation of the toxins entry in the cell and translocation into the cytoplasm - from Aktories (2007).

**Enzymatic activity**

Once in the cytosol, the enzymatic domain of the toxins (N-terminal) acts on its target: small GTPases of the Rho and Ras subfamilies present in the cytoplasm including Rho, Rac and Cdc4 (Just and Gerhard, 2004). The substrate is recognised
by the C-terminal section of the enzymatic domain (Just and Gerhard, 2004), particularly 5 aminoacids were identified in toxin B to be essential in substrate recognition (Jank et al., 2007).

In their inactive form, the Rho proteins are bound to a molecule of GDP. Activation is carried out by guanine exchange factors (GEFs) which transfer a molecule of phosphorus from a GTP to the GDP bound to the Rho protein. Once activated, the GTPase binds to its effector. Inactivation is carried out by GTPase activating proteins (GAPs) which hydrolyse the GTP back to GDP (Schirmer and Aktories, 2004).

![Diagram](image-url)

**Fig 1.5: Glucosylation of GTPases by toxins A and B** – from Schirmer and Aktories (2004). The glucosylation of the Rho-GTPases proteins by the toxins (LCC) blocks the coupling of Rho-GTPases with effectors (1), inhibits the nucleotide exchange induced by GEFs (2), blocks the Rho/GDI interaction (3) and inhibits GTP hydrolysis stimulated by GAPs (4).
The toxin transfers a molecule of glucose from an UDP-glucose to a conserved threonine in position 35/37 of the GTPase, which is irreversible. Glucosylation of the inactive form prevents the activation of the GTPase and the glucosylation of the active form prevents both inactivation and the coupling with the effector (see Figure 1.5). In all, glucosylation leads to the biological inactivity of the GTPase (Schirmer and Aktories, 2004).

The glucosyltransferase activity probably lies within a highly conserved DXD motif (aspartate–X–aspartate) in the enzymatic domain of the toxin which is present in many families of glucosyltransferases (Wiggins and Munro, 1998). The exchange of either aspartate to alanine or asparagine leads to an important loss of enzyme activity (Busch et al., 1998). Other aminoacids in the catalytic domain have been recognised as essential for enzyme activity (Jank et al., 2007).

**Consequences of glucosylation of GTPases**

The irreversible glucosylation of the Rho protein results in the disruption of different processes regulated by small GTPases of the Rho and Ras families. It leads among other things to the depolymerization of the actin cytoskeleton which is lethal for the cell and results in the typical rounded phenotype observed and know as CPE (cytopathic effect). Rounded cells detach from the matrix.

Another process regulated by the small GTPases is the maintenance of tight junctions of epithelial barriers. Inactivation of the GTPases by toxins A and B results in the opening of the tight junctions, increasing colonic permeability and leading to the main symptom of CDAD: diarrhoea (Voth and Ballard, 2005). Neutrophil release
through the opened tight-junctions and the initiation of the inflammatory response is another contribution to pathogenesis. An ulcer forms and the release of mucus and inflammatory cells result in the formation of a pseudomembrane (Thelestam and Chaves-Olarte, 2000).

Another consequence of the glucosylation of GTPases by toxins A and B is cell apoptosis (Calderon et al., 1998; Zhao et al., 2003).

1.4.3. \textit{tcdC}

When the transcription of the PaLoc genes was analysed (Hundsberger et al., 1997), it was seen that TcdC was produced at high levels in early exponential phase while the rest of the genes remained at low levels, and that this situation reversed in stationary phase. It seemed that TcdC had a negative influence on the transcription of the other genes of the PaLoc, and consequently TcdC was proposed as the negative regulator. The absence of a functional \textit{tcdC} gene in the hyper-virulent strain is believed to be the cause of the hyper production of toxin (Warny et al., 2005), supporting the evidence earlier given by Hundsberger et al. (1997).

Matamouros et al. (2007) showed that the presence of TcdC, which can produce dimers, has a negative effect on the transcription of \textit{tcdA} and \textit{tcdB}. TcdC does not bind to the promoters of \textit{tcdA} or \textit{tcdB}. It inhibits transcription by by destabilizing the complex that TcdR forms with the RNA polymerase, most likely by competing with the enzyme for TcdR. As explained in the next section, the formation of the TcdR-RNA polymerase complex is essential for the transcription of the toxin genes.
1.4.4. *tcdR*

Hundsberger et al (1997) proposed TcdR as the positive regulator. Mani and Dupuy (2001) gave evidence that TcdR acted as an alternative sigma factor for the RNA polymerase. They showed that TcdR was indispensible for the transcription of toxins. Using shift motility assays they showed that TcdR does not bind to the toxins promoters on its own, it binds to the RNA polymerase even in the absence of DNA and this complex (TcdR-RNA polymerase) is the one able to interact with the toxin promoters.

1.4.5. *tcdE*

The *tcdE* gene is located in between the toxins A and B genes. It encodes for a highly hydrophobic protein that is transcribed alongside the toxins and has high homology to phage holin proteins, proteins used by phages to lyse the bacterial cell at a programmed moment (Tan et al., 2001).

TcdE is very likely involved in the release of the toxins which otherwise would accumulate in the bacterial cytoplasm (Tan et al., 2001). Goh et al (2005) infected *C. difficile* with phages that contained *tcdE* but no *tcdA* or *tcdB* and showed an increase of the production of toxin B. Phages did not convert non-toxigenic strains to toxin production. Transcription of *tcdB* was not altered by phage infection but the production of toxin B was increased after phage infection, which evidenced the effect of *tcdE* on toxin release.
Hundsberger et al. (1997) showed that the transcription peak of tcdE occurred at stationary phase and Tan et al. (2001) showed clear evidence of the protein’s involvement in bacterial lysis. Tan et al. (2001) showed that the presence of tcdE in the medium prompted a reduction of turbidity (OD 600nm) of E. coli cultures that could not be explained by other environmental factors (temperature, nutrients etc). They suggested that C. difficile releases the toxins by creating membrane lesions. They did not determine whether these lesions resulted in cell death for C. difficile or the evolutionary significance of dying cells releasing the toxins, but they did point out that sporulation could be key to the understanding of this phenomenon. Kamiya et al. (1992) added acridine orange to inhibit sporulation and saw a reduction of the cytotoxicity even though there were no changes in the number of vegetative cells.

1.5. Expression of the PaLoc

Hammond et al. (1997) analyzed the transcription of the PaLoc by in-gel hybridizations of the extracted RNA with probes for the five genes of the PaLoc. The tcdC probe did not bind with the RNA. The other four genes all bound to a 17.5kb transcript, and tcdB, tcdR and tcdE also bound to a second processing intermediate of 8.1 kb of length. All four genes also bound to individual transcripts of 8.4 kb (tcdA), 7.4 kb (tcdB), 1 kb (tcdE) and 700 bp (tcdR). The model they proposed was that the transcript for tcdA was immediately processed from the full length transcript while the transcript for tcdB was part of a second processing intermediate which consisted of tcdB linked with either tcdR or tcdE. Each of the individual transcripts is ultimately derived from the processing intermediates. However, they detected that individual tcdA and tcdB transcripts were present at much greater relative amounts
than the 17.5 kb and 8.1 kb processing intermediate transcripts and suggested a
model of transcription which combined the process of the full length transcript into
individual transcripts as well as the direct transcription of single genes.

The PaLoc is regulated at the transcription level (Hammond et al., 1997). Regulation
is very probably complex. The regulation of the transcription of the PaLoc by
quorum sensing has been postulated after the identification of a $luxS^{cd}$ gene in C.
difficile 630 which is primarily transcribed in late exponential growth and encodes
for an AI-2 synthase (Carter et al., 2005). The $luxS^{cd}$ gene is conserved in both
pathogenic and non-pathogenic strains (Lee and Song, 2005). The autoinducer-2 (AI-
2) is a chemical signalling molecule used to communicate information between cells
about the external environment and cell density population (Lee and Song, 2005).
The $luxS^{cd}$ gene is regulated by a pair of genes, $rolA$ and $rolB$. RolA is a negative
regulator of $luxS^{cd}$ (Carter et al., 2005).

1.5.1. Environmental regulation of the PaLoc

It was seen that toxin production could be regulated by environmental factors. The
alteration of redox potential or the elevation of temperature dramatically increased
the levels of toxin in the medium (Onderdonk et al., 1979).

Toxin production is highly dependent on temperature, with maximal yields at 37°C
and low production at 22°C and 42°C (Karlsson et al., 2003). The presence of $tcdR$
regulates toxin production but only at 37°C. At 22°C or 42°C, there is very little
expression regardless of the presence of $tcdR$. 
Karlsson et al. (2003) showed that the increase of toxin seen at 37°C was accompanied by a rise in the production of butyric acid. The addition of butyric acid increased toxin production at 22°C but not at 42°C, and in contrast, the addition of butanol decreased toxin production at 37°C. Growth was not affected by the addition of either butyric acid or butanol. Butyric acid might be acting as a metabolic stress indicator. The absence of glucose leads to the fermentation of certain amino-acids which results in the formation of butyric acid (Karlsson et al., 2003). Adding glucose to the growth medium suppressed the transcription of tcdA, tcdB and tcdR (Karlsson et al., 2003).

The transcription of tcdA and tcdB declined as the concentration of glucose in the medium was increased, achieving complete repression in the presence of 1% glucose (Dupuy and Sonenshein, 1998). This could be an indication that the evolutionary sense of toxin production is to obtain nutrients by killing other cells (Dupuy and Sonenshein, 1998).

The reduction in the concentrations of biotin in the medium, resulted in a reduction of growth and the increase of toxin production (Yamakawa et al., 1996; Karlsson et al., 2003). By contrast, excess of biotin in the medium enhances growth and inhibits the production of toxin (Yamakawa et al., 1998). The disruption of the gut flora would result in a decrease of the biotin concentration in the gut that would precipitate the production of toxins (Yamakawa et al., 1996).

Yamakawa et al. (1998) added different amino-acids to cultures growing at biotin-limited conditions and found that toxin production was inhibited in the presence of
lysine, asparagine, glutamic acid and glutamine. Under biotin-limited conditions, growth was enhanced by asparagine, glutamic acid and glutamine, but was inhibited by lysine.

1.5.2. Antibiotics and the expression of the PaLoc

Antibiotics have been linked to CDAD since *C. difficile* was first ascribed to disease. As has been explained before, oral ingestion of antibiotics leads to the disruption of the commensal gut flora which makes the patient susceptible to colonization of the gut by *C. difficile* spores present in the environment. However, the presence of antibiotics in the medium could be a source of stress equivalent to the lack of nutrients and might lead to the upregulation of toxin production. Antibiotics have been demonstrated to increase cell adherence (Waligora et al., 1999; Hennequin et al., 2001b).

Onderdonk et al. (1979) saw that the presence of sub-inhibitory concentrations of both vancomycin and penicillin in the medium produced a 1000-fold increase of toxin within 4h. Drummond et al. (2003b) showed that sub-MIC concentrations of antibiotics, induced an earlier production of toxin, particularly with metronidazole, amoxicillin and clindamycin.
1.6. Other virulence factors

1.6.1. Binary toxin

Popoff et al (1988) found a strain of *C. difficile* that produced a third toxin, the binary toxin. This binary toxin (CDT) is an actin-specific ADP-ribosyltransferase unrelated to toxins A and B but with similarities to other binary toxins of other clostridia. CDT does not produce the typical cytopathic effect produced by toxins A and B.

The frequency of the binary toxin is very low, ranging from 2% (Rupnik et al., 2003) to 25% (Spigaglia & Mastrantonio, 2002), although this latter Figure was obtained in a collection of just 32 strains. Barth et al (2004) estimated that the average frequency of the CDT in hospital and patient’s isolates of *C. difficile* was 6% in the UK and 16% in the US. These Figures are much higher in outbreaks where the strain responsible carries the binary toxin. McEllistrem et al. (2005) reported that over 65% of the isolates from an outbreak in a hospital in Pittsburgh, US, between 2001 and 2002, carried CDT, accounting for almost 90% of the severe cases. That strain was later reported to be the hyper-virulent strain 027.

CDT consists of two subunits: CDTa and CDTb. CDTa as the enzymatic subunit, ADP-ribosylates monomeric actin leading to the disorganization of the cytoskeleton. CDTb is the binding subunit and is responsible for the translocation of CDTa into the cell cytoplasm. (Barth et al., 2004)
The role of the binary toxin in disease is uncertain. Due to its low frequency, it is understood that it is not essential in pathogenesis, however it has been theorised that the presence of the binary toxin increases the severity of diarrhoea (Barbut et al., 2005). In some cases of diarrhoea in hospitalised patients, it has been reported the presence of strains of *C. difficile* that lacked both toxins A and B but carry CDT (Geric et al., 2003). However, it could not be determined whether these strains were the cause of diarrhoea. Geric et al. (2006) challenged hamsters that were previously treated with clindamycin, with four strains of *C. difficile* that lacked both toxins A and B but had the binary toxin (A′B′CDT+). The result of this challenge was positive colonization of *C. difficile* but there was no diarrhoea or signs of other CDAD symptoms. However, the binary toxin preparations did have enterotoxic activity producing non-haemorrhagic (as opposed to toxins A and B) fluid responses in ileal loop segments from rabbits. They determined that the role of the binary toxin was most probably adjunctive to toxins A and B since it was unable to cause disease on its own in hamsters.

### 1.6.2. Surface proteins

Borriello et al. (1987) compared high-virulence strains (caused death in hamsters) with low-virulent strains and showed that there was no straight correlation between the amount of toxin produced *in vitro* and the damage these strains produced *in vivo*. They suggested that virulence was related to a rapid production toxin which would probably depend on the efficiency of colonization. Borriello et al. (1987) theorised that the ability to associate with the gut mucosa was more important to determine the virulence than the amount of toxin that the strain is able to produce.
The S-layer is a protective surface which helps maintain the shape and envelope rigidity of some bacterial cells. It is not a cell membrane (Sara and Slater, 2000). The S-layer of *C. difficile* consists of two subunits. The molecular mass of each subunit can vary from strain to strain (McCoubrey and Poxton, 2001). Both subunits derive from a common precursor which is encoded by the *slpA* gene (Calabi et al., 2001). The S-layer proteins are involved in the adherence to the gut epithelial cells (Calabi et al., 2002).

Several other proteins of the surface of *C. difficile* have been identified as adhesins, including Cwp66, GroEL, Fbp68 and the flagella.

Cwp66 (“clostridial wall protein 66 KDa”) is a heat-shock protein of the surface of *C. difficile* with a two-domain structure (Waligora et al., 2001). Analysis of its amino-acid sequence and its homology to other known proteins showed that the N-terminal domain of Cwp66 is probably involved in adhesion. Cell attachment properties of this protein after heat-shock were confirmed by competitive inhibition with polyclonal antibodies (Waligora et al., 2001).

Another protein likely to be involved in adherence is a heat-shock protein (Hsp60), GroEL (Hennequin et al., 2001a). Different types of stress (heat, lack of iron, high osmolarity, acid and antibiotics) increase adherence and the expression of GroEL, suggesting that GroEL mediates adherence in response to stress. Excess of iron or low osmolarity inhibit the production of GroEL (Hennequin et al., 2001b). Hennequin et al. (2003) also identified a fibronectin-binding protein (Fbp68) that binds fibronectin both soluble and immobilised.
Both components of the flagella, flagellin filament (FliC) and the flagellin cap protein (FliD), have also been identified as adhesins (Tasteyre et al., 2001)


1.6.3. Others

Other virulence factors have been considered. *C. difficile* has a capsule which might be involved in evasion of the immune system (Davies and Borriello, 1990); Borriello et al. (1988) found fimbriae in some strains of *C. difficile*, but they found no correlation to virulence and suggested they had no role in disease.

The presence of hydrolytic enzymes that degrade tissue might contribute to the tissue damage produced by *C. difficile* (Steffen and Hentges, 1981; Seddon et al., 1990).
1.7. AIMS of the thesis

This thesis consists of three studies:

1) A comparative study of the immune response in the hospitalised elderly population in the Edinburgh area to *Clostridium difficile* (toxins and cellular antigens) and also to an indicator of their general immunity. The indicator chosen was *E. coli* LPS which is highly immunogenic and antibodies to it are almost always present in general population. The hospitalised elderly population was divided into three groups according to their *C. difficile* colonization status: cases (symptomatically colonized), carriers (asymptomatically colonized) and controls (non-colonized). It has been hypothesized that those colonized by *C. difficile* that do not present symptoms have higher levels of specific IgG and IgM in serum than those who develop symptoms.

The main aim of this study was to test this hypothesis by comparing the levels of serum antibodies in the three groups by ELISA, neutralization of the cytotoxicity and Western blot.

2) Previous work from the group analysed the effect of the presence of sub-inhibitory concentrations of antibiotics on the production of toxins. Antibiotics have a major role in the pathogenesis of this organism disrupting the commensal gut flora and allowing the colonization of *C. difficile*. The hypothesis of this study is that on top of that, the presence of antibiotics stimulates the production of toxins (more quantity and/or earlier production), and that this stimulation is done at the regulation
level. Both the knock-out of the putative negative regulator \((tcdC)\) and the over-stimulation of the positive regulator \((tcdR)\) as well as that of \(tcdE\) (believed to be involved in release of toxin), would lead to the expected result: increased toxin production.

The main aim of this study was to compare by Real-time PCR the expression of the five genes encoded in the PaLoc in the presence and in the absence of antibiotics.

3) The two toxins are essential in pathogenesis; however, they are not the only virulence factor associated to disease. A third study was carried out to determine if the surface antigens of \(C.\) \(difficile\) and \(E.\) \(coli\) LPS have a role as enhancers of the cytotoxicity of \(C.\) \(difficile\) toxins \textit{in vitro}. Monolayers of Vero and Caco2 cells were challenged with purified toxin A with and without surface antigens to test if the addition of the cellular antigens further decreased cell viability.
2. Materials and Methods

2.1. Bacterial strains and clinical specimens

2.1.1. Strains used

Two strains were used throughout this work. Reference strain 630 (PCR ribotype 012; Sebaihia et al., 2006) is the first strain of *C. difficile* to be fully sequenced and was a natural choice for the RNA work. Strain MPRL338a (PCR ribotype 001, toxinotype 0, S-type 5236) is a local strain found during a previous epidemiological study (McCoubrey, 2002) and was chosen as the strain from which antigens were extracted due to its proximity to other local strains. Both strains were stored in cooked meat broth at room temperature.

2.1.2. Collection of samples and consenting forms

Blood and stool samples were collected in accordance with the Local Research Ethics Committee from every major hospital in Edinburgh, but the main target was the Royal Victoria Hospital since it is the main geriatric assessment and rehabilitation hospital for the north of Edinburgh.

Patients aimed for were over 65, in long stay hospitalisation and under antibiotic treatment. Patients with dementia were not considered since they could not consent for themselves.
2.1.3. Toxin assay on fresh stool samples

Toxins assays were performed on fresh stools (under 24 h from collection) using a commercial kit for the detection of \textit{C. difficile} toxins A+B (TechLab) and following the instructions of the manufacturer.

2.1.4. Culture of stool samples and identification of \textit{C. difficile}

Stools were cultured on Brazier’s CCEY agar and incubated at 37°C in anaerobic conditions (10% H$_2$, 10% CO$_2$ and 80% N$_2$) for 24 to 48 h. Positive results were seen within that period, if negative, the plates were then incubated for another 48 h to confirm negative results.

Preliminary identification of \textit{C. difficile} was done by colony morphology, visualization of motility in light microscopy and green fluorescence under long wave U.V. light (Wood’s Lamp). Further identification included Gram film and toxin assay.

2.1.5. Growth of \textit{C. difficile}

\textit{C. difficile} was grown at 37°C and anaerobic conditions (10% H$_2$, 10% CO$_2$ and 80% N$_2$) in a DWS Mark III workstation (Shipley, UK). PPY medium (pH=7.4) contains 20 g/L Proteose peptone (Oxoid L85); 10 g/L Yeast extract (Difco 0127-017); 5g/L NaCl; 0.75 g/L Cysteine-HCl and 0.4 g/L Na$_2$CO$_3$; Anaerobic Identification Medium (AIM) (pH=7.1) contains 20 g/L Proteose peptone (Oxoid L85); 5 g/L Yeast extract (Oxoid L21); 5g/L Trypticase (BBL 11921); 5g/L NaCl; 0.75 g/L Cysteine-HCl and 0.4 g/L Na$_2$CO$_3$. 

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2.1.6. Detection of toxin production in vitro

*C. difficile* was grown at 37°C and anaerobic conditions for five to six days in PPY medium. Alternatively, AIM medium can be used with same results. Toxin was detected using the Techlab kit as instructed by the manufacturer.

2.1.7. Ribotyping

Ribotyping was done following the method described by O’Neill et al. (1996). Specific oligonucleotide primers complementary to the 3’ end of the 16S rRNA gene (positions 1445-1466) and the 5’ end of the 23S rRNA gene (positions 1-20) were used to amplify the variable-length intergenic spacer region. Primers were obtained from MWG. The sequences of the primers were:

16S  5’ CTG GGG TGA AGT CGT AAC AAG G 3’

23S  5’ GCG CCC TTT GTA GCT TGA CC 3’

The total volume of the PCR reactions was 100μL containing PCR buffer (20mM Tris HCl, 50mM KCl; Invitrogen), 2.25mM MgCl₂ (Invitrogen), 2U of Taq polymerase (Invitrogen), 200μM each dNTP (Amersham Pharmacia Biotech), 50 pmol of each primer and 10μL of template DNA. Initial denaturation was carried out at 94°C for 3 min. Then the mixtures were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. A final elongation step was included at 72°C for 5 min.
The amplification products were concentrated to a final volume of 12-15μL by heating at 75°C for 100 min and were run in 3% Metaphor agarose gels (ABgene) at 80 volts for 2.5 h. As a size marker a 100bp DNA ladder (ABgene) was used.

Patterns were identified by comparison with those already included in the laboratory database. The concentrated PCR products of those that were different were sent to the Anaerobe Reference Unit in Cardiff (UK) to be identified.

2.1.8. Toxinotyping

Toxinotyping was done following the method described by Rupnik et al. (1997) and Rupnik et al (1998). The first 3kb of tcdB (PCR fragment B1) and the 3kb repetitive region of tcdA (PCR fragment A3) were detected and characterized by RFLP. Primers were obtained from MWG. The sequences of the primers were as follow:

**tcdA** (product: 3100 bp)

A3C 5' TAT TGA TAG CAC CTG ATT TAT ATA CAA G 3'

A4N 5' TTA TCA AAC ATA TAT TTT AGC CAT ATA TC 3'

**tcdB** (product: 3100 bp)

B1C 5' AGA AAA TTT TAT GAG TTT AGT TAA TAG AAA

B2N 5' CAG ATA ATG TAG GAA GTA AGT CTA TAG

PCRs were performed in a final volume of 50μL with a reaction mixture containing PCR buffer (20mM Tris HCl, 50mM KCl; Invitrogen), 3mM MgCl₂ (Invitrogen), 1U of Taq polymerase (Invitrogen), 200μM each dNTP (Amersham Pharmacia Biotech),
15 pmol of each primer and 5 μL of template DNA. For amplification of A3 fragments it was also added 1% W-1 (Invitrogen), TMA (tetramethylammonium chloride, Sigma) to a final concentration of $10^{-4}$M and BSA (Bovine serum albumin; Promega) to a final concentration of 0.1 mg/mL. After initial denaturation at 93°C for 3 min, B1 products were amplified for 30 cycles and A3 products for 35 cycles of annealing and extension at 47°C for 8 min and denaturation at 93°C for 4 sec. Final extension was performed at 47°C for 10 min.

Amplified fragments were visualized on 1% agarose and subjected to restriction enzyme digestions using the restriction enzymes AccI and HincII for B1 and EcoRI for A3. Restriction enzymes were obtained from New England BioLabs. Incubation with 1 μl (final volume: 20 μl) of the enzymes occurred at 37°C for 2 h. In the restriction of B1 with HincII, 10% BSA (Promega) was also added.

After electrophoresis of the digestion products in 1% agarose gels, toxinotypes of all tested isolates were identified as described by Rupnik et al. (1997) and Rupnik et al (1998).

2.1.9. **S-layer typing**

S-layer proteins were extracted as described later on in this Chapter (2.2.4). Dialysis was found to be essential to avoid interference when running in the gel. Samples were run in SDS-PAGE as described below.

S-types were determined by comparison with known S-types since it was noticed that small changes in procedure can result in different results. The method of SDS-PAGE
described below is preferred to that of "mini-gels" described later on in this Chapter because it detects smaller differences in bands size.

2.1.10. SDS-PAGE and Coomassie blue stain

Samples were mixed in equal volumes of double strength buffer, boiled for 3-5 min and run in a 10% acrylamide separating gels, with 4% stacking gels using the buffer system of Laemmli (1971). An initial voltage of 60 V was used to pull the proteins through the stacking gel. The voltage was increased to 150 V and run until the dye front had moved 7.5 cm through the separating gel. The separated proteins were stained with Coomassie blue as described by Hancock and Poxton (1988).

2.2. Extraction of antigens

2.2.1. Dialysis culture filtrate

An overnight culture (1ml) of a toxigenic strain of C. difficile (strain MPRL338a) was used to inoculate a sterilised, pre-reduced dialysis bag containing before sterilisation 50 mL of 0.85% NaCl suspended in a 1L of PPY medium in a stoppered, vented conical flask. After four days of anaerobic growth at 37°C, the contents of the dialysis bag were harvested (10000g, 15 min) and the supernate dialysed overnight against TBS buffer (0.05M Tris-HCl, 0.15M NaCl; pH 7.0) at 4°C. It was then filter-sterilised and kept at 4°C.

This filtrate which contains toxins A and B together with other extracellular products but none of the medium components, will be referred to as "non-purified toxin".
2.2.2. Purification of toxin A

The method is based on those followed by Krivan and Wilkins (1987) and Kamiya et al. (1989) and utilises the property that Toxin A binds to bovine thyroglobulin and can be eluted at 37°C.

CNBR-activated Sepharose gel (4.4 g: Amersham Biosciences) was washed thoroughly in 1mM HCl to get rid of the additives present. It was then allowed to react overnight (4°C) with 60 ml of a 5g/L thyroglobulin in 0.1M MOPS buffer (pH 7.0) that was previously centrifuged (8000g, 10 min) and filtered (0.22 μm) to remove insoluble particles.

The mix was blocked with 40 ml of 0.1M ethanolamine for 30 min (4°C) and then washed in 0.1M MOPS buffer (pH 7.0).

The coupled beads were packed into a column (C10 Amersham Biosciences) and washed at 37°C with 120 ml of prewarmed basic buffer (0.1M glycine-NaOH, 0.5M NaCl; pH 10.0) and 120 ml of prewarmed acidic buffer (0.1M glycine-HCl, 0.5M NaCl; pH 2.0). The column was then equilibrated at 4°C with 120 ml of TBS (0.05M Tris-HCl, 0.15M NaCl; pH 7.0).

C. difficile culture filtrate from section 2.2.1 (100 mL at 4°C) was added and eluted with 25 ml of TBS at 4°C followed by 50 ml of TBS at 37°C. Fractions were collected, filter sterilised and kept at 4°C.

To reuse the column, it was washed with 60 ml of acidic buffer at room temperature and equilibrated with 60 ml of TBS at 4°C.
The fractions were monitored for absorbance at $A_{280}$, run on 1D SDS-PAGE as described earlier to check purity and tested for toxin activity with the Techlab kit for *C. difficile* toxin A/B detection. Cytotoxicity assays were also carried out in both Vero and Caco2 cells.

### 2.2.3. EDTA extraction of surface proteins

An overnight culture of *C. difficile* in PPY medium (or AIM medium) was used as a starter to inoculate (1% w/w) 20 mL of pre-reduced PPY. It was incubated at 37°C in anaerobic conditions overnight or until enough turbidity was achieved ($OD_{600}=0.6$).

Cells were harvested at room temperature (5000g, 20 min) and washed twice in PBS. The pellet was then drained and re-suspended in 1.5 ml of 10mM EDTA buffer and incubated at 45°C for 90 min. The mixture was sonicated in a sonic bath for 1 min followed by a 10 sec mix in a vortex mixer. Bacteria were removed by centrifugation twice (13000g, 2 min). The supernatant fluids containing the extracts were stored at -20°C.

Dialysis to remove EDTA was carried out when needed in 1L 10mM Tris-HCl pH 7.4, overnight at 4°C in a magnetic stirrer. The buffer was changed and the dialysis continued for 4 more hours. The dialysed samples were stored at -20°C.
2.2.4. Extraction of Surface layer proteins (SLPs)

S-layer proteins from whole cells were extracted using 5M guanidine hydrochloride.

A 1% inoculum of a *C. difficile* overnight culture (in AIM or PPY) was inoculated to 4 ml of PPY and incubated anaerobically at 37°C overnight. Purity checks of both starter and final culture were carried out by phase contrast microscopy, Gram stain and subculture to blood agar both aerobically and anaerobically. The overnight culture was then harvested (3000g, 20 min), and washed twice in the same volume of PBS. The pellet was then drained and re-suspended in 0.3 ml of a 5M GHCl solution, and transferred into a screw-capped Eppendorf tube. The samples were shaken in the red blood cell shaker at room temperature for 2 hours and then centrifuged twice at 13000g for 2 min to remove remaining bacteria. The final supernate was stored at -20°C.

Dialysis was carried out to remove guanidine-HCl in 1L 6.25 mM Tris-HCl buffer, pH 6.8 overnight at 4°C in a magnetic stirrer. The buffer was changed and dialysis continued for 4 more hours. The dialysed extracts were stored at -20°C.

2.2.5. Surface lipocarbohydrate (LC)

This method was based on those described by Poxton and Cartmill (1982) and Sharp and Poxton (1986).

6L of pre-reduced PPY were inoculated with an overnight *C. difficile* culture and grown anaerobically overnight at 37°C. Then they were harvested (10000g, 10 min, 4°C) and washed in PBS. The cells were then suspended in PBS and broken by
sonication (5 x 1 min) at 10 μm (MSE Soniprobe). The cell walls were removed by centrifugation (35000 g, 30 min) and the supernate collected into a weighed container and lyophilised.

The lyophilized supernate was defatted with 2 x 200 ml of chloroform/methanol (2:1) over 24 h. The dry defatted membrane was resuspended in distilled water to 10% (w/v) and mixed with an equal volume of 80% phenol solution. The mixture was stirred at room temperature for 30 min and then centrifuged at 2500 g, 20 min (4°C). The upper layer was collected and dialysed against tap water to remove phenol.

The dialysed material was then mixed with an equal volume of 0.2M acetic acid buffer, pH 5.0 containing 0.02M MgCl₂ and ribonuclease (Sigma) and deoxyribonuclease (Sigma). The mixture was incubated overnight at 37°C in the presence of toluene.

The phenol extraction was repeated and after dialysis of the upper layer, lyophilized in a pre weighed container. This was labelled crude membrane antigen and stored at room temperature. The antigen was run in SDS-PAGE as described earlier in this Chapter and silver stained.
2.2.6. Silver stain of PAGE gels

The gel was left on a fixative solution (25% propan-2-ol, 7% acetic acid) overnight. The gel was then incubated for 15 min in a fresh oxidizer solution (containing 0.7% periodic acid in dilute fixative) followed by four 1-hour washes with distilled water.

The gel was then stained in ammoniacal silver nitrate solution freshly made for 15 min. It was washed four times (10 min each wash) in distilled water and developed in freshly prepared developer (0.005% citric acid in 0.019% formaldehyde solution) until the required intensity was reached and then washed again in distilled water several times to stop the development.

2.3. Immunoassays

2.3.1. ELISA

Polysorp plates (Nunc) were coated with 100 µl of the appropriate concentration (as stated in the Results) of the chosen antigen in antigen buffer (0.05M sodium carbonate buffer pH 9.6 + 0.02% sodium azide) and incubated overnight in darkness at room temperature.

Plates were washed in PBS (pH=7.4; Oxoid tablets) four times and blocked with 100 µl of TG buffer (0.05M phosphate buffer pH 7.4 containing 2% teleostean gelatin (TG) and 0.1% Tween 20) 90 min.

After four washes in PBS, the plates were incubated for 90 minutes with 100 µl of the chosen serum diluted in antiserum buffer (0.05M sodium phosphate buffer, pH
7.4 containing 0.085% NaCl, 0.05% Tween 20 and 0.02% sodium azide), and then washed four times as previously.

It was then incubated in a 1 in 10000 dilution in antiserum buffer of the anti-human (IgG or IgM) AP-conjugate (Sigma) for 90 min and developed with alkaline phosphatase substrate (Sigma tablets) in APS buffer (0.05M sodium carbonate pH 9.8 containing 1mM MgCl₂).

The A₄₀₅ was measured at 15 minutes intervals for 1 h using an ELISA plate reader (Anthos reader 2001) with the reference filter at 620nm.

Three kind of negative controls (just buffers, antigen and no serum, serum and no antigen) were included as well as a positive control (sera from young (under 65) healthy (non-hospitalised) volunteers) that also ensured reproducibility of the assay.

2.3.2. Cytomegalovirus IgG ELISA

The levels of IgG against CMV were measured by a commercial ELISA kit (IBL Hamburg) as specified in the manufacturer’s instructions.

2.3.3. Immunoblots

Surface proteins extracted with EDTA were prepared as described earlier in this Chapter (2.2.3). Samples were mixed in equal volumes of double strength buffer, placed in a 100°C waterbath for 3-5 min and then run in 10% bisacrylamide-Tris PAGE gels (Invitrogen) following the manufacturer’s instructions and using the MOPS SDS running buffer supplied by the manufacturer. These gels were referred to
as "mini-gels" to differentiate them from the SDS-PAGE gels described earlier. Gels were run at 150V until the front reached the bottom of the gel (approx. 30-45 min).

The products were transferred from the gel into a nitrocellulose membrane at 150V for 100 min at 4°C. Then the membrane was blocked with 8% skimmed powder milk (Marvel) in PBS overnight at 4°C.

After three ten-minutes washes in PBS at room temperature, the membrane was incubated (rocking) with a 1 in 1000 human serum dilution in 1% Marvel in PBS + 0.05% Tween 20 for 3 hours at room temperature.

The membrane was then washed three times (ten minutes each, room temperature) with 1% Marvel in PBS + 0.05% Tween 20, and then incubated with the second antibody (anti-human IgG HRP-conjugate) at 1 in 10000 dilution in 1% Marvel and 0.05% Tween 20 for 90 min.

After three ten-minutes washes in 1% Marvel in PBS + 0.05% Tween 20 at room temperature, the membrane was incubated with ECL (enhanced chemoluminescence) solution (containing 250 mM luminol and 90 mM p-coumaric acid in DMSO) for 5-10 min and developed by autoradiography using Hyperfilm ECL (Amersham Biosciences) and an X-Ray Film Developer (Protec).
2.4. **Cell culture**

2.4.1. Cell lines

The Vero cell line is a well established cell line obtained from the kidney epithelia of the African green monkey.

The Caco2 lineage was obtained from carcinogenic human colon and is used as a model for the human intestine. This lineage spontaneously differentiates after reaching confluency.

2.4.2. Growth of cells

Vero and Caco2 cells from the Medical Microbiology frozen stock were grown at 37°C, 5%CO₂ in 75cm² flasks in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma), 100 units/ml of penicillin G sodium (Gibco), 100 μg/ml of streptomycin sulphate (Gibco), 292 μg/ml L-glutamine (Gibco) and 1% non essential aminoacids (Sigma), and incubated at 37°C, 5% CO₂.

Proliferating cells were split when they reached 90% confluency with 0.05% trypsin in 0.53mM EDTA and spun down at 1000 g, 10 min. A 10% inoculum (10⁶ cells/ml) was used to inoculate new flasks.

Caco2 cells started differentiating spontaneously after reaching confluency.
2.4.3. Neutralization of the cytotoxicity.

Microtitre plates were inoculated with 100 µl of a \(10^6\) cells/ml +FBS solution and incubated at 37°C, 5% CO₂ overnight until confluence.

Blanks were inoculated with 20 µl of DMEM medium, toxin controls were inoculated with 10 µl of toxin dilution in DMEM medium and 10 µl of DMEM medium, and serum controls were inoculated with 10 µl of undiluted serum and 10 µl of DMEM medium. Tests were inoculated with 10 µl of toxin dilution and 10 µl of human serum dilution. There were at least two replicates of each test.

Plates were incubated at 37°C, 5% CO₂ and viability measured by an MTT assay that is described later (2.4.5).

2.4.4. Antigen enhancement of the cytotoxicity

Microtitre plates were set up as described earlier in this Chapter. Blanks were inoculated with 20 µl of DMEM medium, toxin controls were inoculated with 10 µl of a dilution of toxin in DMEM medium and 10 µl of +FBS medium, and tests were inoculated with 10 µl of the same toxin dilution and 10 µl of an appropriate concentration of the antigen. There were at least two replicates of each test.

Plates were incubated at 37°C, 5% CO₂ and viability measured by an MTT assay that is described later (2.4.5).
2.4.5. MTT assay

This assay is a cell viability test. Viable cells are able to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which is yellow into formazan which is purple, and this reduction (which is being carried out by a mitochondrial reductase) can be easily measured by a spectrophotometer.

MTT solution (20 µl of a 5 mg/L solution in sterile PBS) was added to each well. Plates were incubated at 37°C, 5% CO₂ for 4 hours. The formazan produced is solubilised in DMSO (100 µl per well) and colour measured at A₅₇₀.

2.5. Antibiotics and expression

2.5.1. Growth in the presence of sub-MIC concentrations of antibiotics

An overnight culture of C. difficile in PPY medium (or AIM medium) was used as starter to inoculate (1% w/w) pre-reduced PPY containing 4x MIC, 2x MIC, MIC, ½ MIC, ¼ MIC, 1/8 MIC or 1/16 MIC concentrations of antibiotic or the same volume of pyrogen free water in controls. It was incubated at 37°C in anaerobic conditions for different times up to 24 h.

Antibiotics chosen for the study were vancomycin (V2002; Sigma) and metronidazole (M1547; Sigma), the two main antibiotics used for the treatment of CDAD; and two others that are associated with precipitation of the disease: amoxicillin (A8523; Sigma) and clindamycin (C5269; Sigma). MICs were determined by Drummond et al. (2003a) and confirmed by broth macrodilution.
2.5.2. RNA extraction and purification

RNA was extracted using a commercial kit (RiboPure Bacteria, Ambion) with a few modifications. *C. difficile* was grown anaerobically in PPY medium at 37°C. 15 mL were centrifuged at 5000 g for 20 min and the supernatant was discarded. The pellet was resuspended in the reagent (containing phenol) provided by the kit as instructed.

Cells were transferred to a tube containing Zirconia beads as instructed but then they were lysed by homogenization in a bead beater for 3 mins. The rest of the steps were performed as instructed in the handbook.

Elution of the purified RNA was done twice using 40 μl of elution buffer each time. The final DNase treatment considered optional in the handbook was also applied but 8 Units of Turbo DNase (Ambion) were used instead of 8 Units of DNase I for better results. Incubation was increased to 1 h. Inactivation of the enzyme was performed as instructed.

The purified RNA was stored at -70°C.

2.5.3. Quantification of RNA/DNA

The RNA concentration of the purified extracts was measured using the Nanodrop ND-1000 Spectrophotometer (Labtech).
2.5.4. Reverse transcription

The reverse transcription was carried out using a commercial kit (Retroscript, Ambion) as instructed by the manufacturer, using the two-step protocol with heat denaturation of the RNA. Random decamers were preferred to Oligo(dT) primers as the handbook stated that better yields were obtained with random primers.

The reverse transcription was performed at twice the volumes indicated in the handbook to ensure that there was enough cDNA for all the PCRs needed. Total RNA (3 μg) in a final volume of 40 μL were retro-transcribed each time.

2.5.5. Real time PCR

Real time PCR was performed using a Stratagene thermocycler mx3000P. The experiment type selected was SYBR Green (with Dissociation curve).

To the cDNA obtained (5 μl in a final volume of 50 μL), SYBR green Jumpstart Taq Ready Mix for Quantitative PCR (25 μl: Sigma) containing SYBR green I, 20mM Tris-HCl (pH 8.3), 100mM KCl, 7mM MgCl₂, 0.4 mM of each dNTP and 0.05 units/μl of Taq DNA Polymerase was added. Primers were added in the concentrations required and the total volume was achieved by adding nuclease free water. Primers were obtained from MWG. Sequences of the primers can be found in table 2.1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>tcdA</td>
<td>TA1</td>
<td>5' ATG ATA AGG CA A CTT CAG TGG 3'</td>
<td>624</td>
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<td></td>
<td>TA2</td>
<td>5' TAA GTT CCT CTT GCT CCA TCA A 3'</td>
<td></td>
<td>Spigaglia &amp; Mastrantonio, 2002</td>
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<tr>
<td>tcdB</td>
<td>TB1</td>
<td>5' GAG CTG CTT CAA TTG GAG AGA 3'</td>
<td>412</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB2</td>
<td>5' GTA ACC TAC TTT CAT AAC ACC AG 3'</td>
<td></td>
<td>Spigaglia &amp; Mastrantonio, 2002</td>
</tr>
<tr>
<td>tcdC</td>
<td>Tim2</td>
<td>5' GCA CCT CAT CAC CAT CTT CAA 3'</td>
<td>345</td>
<td>Cohen et al., 2000</td>
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<tr>
<td></td>
<td>Struppi2</td>
<td>5' TGA AGA CCA TGA GGA GGT CAT 3'</td>
<td></td>
<td></td>
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<tr>
<td>tcdR</td>
<td>Tim3</td>
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<tr>
<td></td>
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<tr>
<td>tcdE</td>
<td>Tim1</td>
<td>5' GTT TAA GTG CAA TAA AAA GTC GTA 3'</td>
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<td>Cohen et al., 2000</td>
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<td>Struppi1</td>
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<td>tpi-F</td>
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<td></td>
<td>tpi-R</td>
<td>5' CAT AAT ATT GGG TCT ATT CCT AC 3'</td>
<td></td>
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</tbody>
</table>

Table 2.1 Sequence of primers used in Real time PCR

This table shows the sequence as well as the size of the product of the primers used in Real time PCR.

The PCR program and the primer sequences for tcdA and tcdB were based on Spigaglia and Mastrantonio (2002). For tcdA, 100 pmoles of each primer was added to each 50 μl reaction and for tcdB, 25 pmoles of each primer was needed. An initial denaturation at 94°C for 2 min was followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C.

For tcdC, tcdR and tcdE, sequence of the primers and PCR program were based on Cohen et al (2000). PCR was not done in multiplex as described by Cohen et al (2000). To every 50 μl reaction, 50 pmoles of each primer of the chosen gene was added. An initial denaturation at 95°C for 2 min was followed by 40 cycles of 1 min at 95°C, 1 min at 52°C and 1 min at 72°C.

For the triose phosphate isomerase (tpi) gene that was used as a housekeeping gene, the program and the primer sequences were based on Lemee et al (2004). Each 50 μL
reaction contained 25 pmoles of each primer. After an initial denaturation at 95°C for 3 min, followed 40 cycles of 30 sec at 95°C, annealing for 30 sec at temperatures decreasing from 65°C to 55°C (1°C decrement per cycle for the first 11 cycles and the rest fixed on 55°C) and extension at 72°C for 30 sec.

Every PCR finished with a dissociation cycle which consisted of 30 sec at 95°C, 1 min at 60°C and 30 sec at 95°C.

The negative controls consisted of a blank which contained every reagent but no cDNA, and a non-RT control which instead of cDNA contained 5 μL of the purified RNA prior to the reverse transcription step to guarantee the non contamination with genomic DNA.

Standard curves (which also served as positive controls) were performed with eight known concentrations of genomic DNA from reference strain 630. Genomic DNA was extracted with a commercial kit (Nucleospin Tissue; Machery-Nagel) as instructed by the manufacturer. The DNA concentration was measured with the Nanodrop as described earlier in this Chapter (2.5.3). The first concentration in the standard curve was 5 μL of the undiluted DNA extract and the following ones were obtained in 10-fold dilutions.

PCR products were stored at 4°C and then run in 1% agarose gels to check the size of the products.
2.6. Statistics

All statistics were done using the GraphPad Prism 4 package. Data was checked for normality and then compared to each other. If the distribution of the values was Gaussian, unpaired t-test was performed. If the distribution was not normal, the Mann-Whitney test was used instead.
3. Immune response in the hospitalised elderly population.

3.1. Introduction

This chapter is a comparative study of the levels of antibodies to *C. difficile* in three groups within the hospitalised elderly population in Edinburgh:

- **Cases:** diagnosed cases (toxin A/B positive), symptomatically colonized with *C. difficile*.

- **Carriers:** asymptomatic for *C. difficile* associated disease (CDAD) but at least toxin A/B or culture positive.

- **Controls:** asymptomatic for *C. difficile* and both toxin (A/B) and culture negative.

As mentioned earlier in Chapter 1, the spectrum of disease is very wide, ranging from no symptoms or mild ones to severe disease with difficult to treat complications. The ability of the host to produce specific antibodies against toxins and/or cellular antigens has been postulated as a main key factor to explain this variability, offering protection against the negative effects of *C. difficile*. Several authors (Kyne et al., 2000; Kyne et al., 2001; Drudy et al., 2004; Johal et al., 2004) have found evidence of the importance of the antibody-mediated immune response.

The aim of this study was to test the hypothesis that patients with lower levels of antibodies in serum (less effective immune system) are more susceptible to disease.
3.1.1. The use of the core of E. coli LPS

When the main hypothesis was postulated at the start of this project (that the inability or the weakening of the ability to produce a systemic antibody response is a main factor in the development of disease), the question of whether this hypothetical inability/weakening was a general drop in the host’s systemic immunity or if it was a specific lack of response to C. difficile or its antigens arose. Therefore, it was concluded that what was needed was an immunogenic antigen to which the vast majority of the target population is exposed. It was not known what vaccines these individuals had as infants and there did not seem to be a practical way of finding out. Flu vaccine was suggested since it is common practice to vaccinate the elderly before the winter months, but that again raised practicality issues.

Finally the core of Escherichia coli LPS was suggested. This laboratory has extensively worked and still is working with LPS and its immunogenicity and consequently both knowledge of the antibody levels in the normal population and the LPS itself were readily available.

LPS can be divided in three regions: 1) lipid A which is the region responsible for the endotoxic activity of the LPS; 2) the core oligosaccharide and 3) the repeating units of O-polysaccharide and major target to the host antibody response. While the O-antigen polysaccharide of bacterial endotoxins is highly variable, the polysaccharide structures of the core are more limited and conserved. There are over 170 different O-antigens serotypes known for E. coli but only 5 core types (R1, R2, R3, R4 and K12). The antibody response to whole LPS is the result of the responses
to both the core and the O-antigen. The specific response to the O-antigen is protective but is also very specific to that particular serotype, while the specific response to the core is thought to offer protection to several serotypes. A previous study from the group (Gibbs et al., 2004) showed that the *E. coli* R1 core was the most frequent core type of the *E. coli* isolated from the faeces of healthy humans, accounting for 63% of the total. R2 and R4 came second accounting for 15% and 14% respectively. R3 and K12 accounted only for 4% each. Gibbs et al (2004) also screened for specific antibodies against the five different core types in sera of healthy humans. They found that the levels of anti-R4 core antibodies were significantly higher than the rest and that the levels of anti-K12 were significantly the lowest. The levels for R1, R2 and R3 were found to be intermediate and without significant differences between them.

For the experiments carried out for this thesis, both the cores of an R1 LPS (the most frequent in humans) and an R4 LPS (the most immunogenic in humans) were used.

### 3.1.2. *C. difficile* and Cytomegalovirus

Cytomegalovirus (CMV) is a type of herpes virus which can cause infection, although in most of the cases infection is harmless and asymptomatic. According to the Health Protection Agency (2007), between 60 and 90% of the world population has been in contact with the virus at one point in their lives. Once the person is infected, the virus lies dormant until the immune system is weakened but causes no problems or very minor problems except in vulnerable groups of people such as babies, pregnant women or people with weakened immune systems (transplants,
chemotherapy, HIV ...). In these groups of people, infection can severely affect organs such as the eye, the liver, the gastrointestinal system (causing colitis) or the nervous system.

CMV was chosen for this study for several reasons. Firstly, CMV was chosen as an indicator of immunodeficiency. In patients that are infected with CMV and whose immune system is compromised, the virus leaves its dormant stage and affects the host with symptoms that can easily be obscured in the hospital environment: mild fever, sweats, rash, anaemia, fatigue etc (Health Protection Agency, 2007). The presence of CMV antibodies in CDAD patients would help support the idea of an affected immune system that will explain the loss in the ability of producing specific antibodies against *C. difficile*.

Secondly, CMV infection increases the risk of mortality from other diseases. Looney et al (1998) related changes on the T-cell population of volunteers to CMV infection, changes that were independent to age, while age was a risk factor for CMV infection. These changes can be permanent due to the nature of CMV infection, latently infecting macrophage precursors and becoming activated during the differentiation of the mononuclear phagocytes. Looney et al (1998) suggested that these changes may be affecting the immune function, and that they could be a reason for the decreased response to other infections such as influenza in the elderly population. In this study, we looked at the relationship between CMV antibodies and *C. difficile* antibodies, suggesting the over-bearing response to CMV as one of the reasons for the cases not to be developing a protective immune response against *C. difficile*. 

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Finally, CMV has also been reported as a cause of pseudomembranous colitis (PMC) in cases of severely immunocompromised patients (such as AIDS, leukaemia and transplant patients), both on its own (Olonfilade and Chiang, 2001; Cadena et al., 2007) and in co-infection with *C. difficile* (Ives and Smith, 1996; Riva et al., 2005). Hutin et al. (1997) studied the prevalence of *C. difficile* in patients admitted to an AIDS ward and showed no correlation between infection with HIV and *C. difficile* carriage. However they showed a significant association between *C. difficile* carriage and history of CMV infection. The co-infection with CMV in patients carrying *C. difficile* might help to precipitate the symptoms associated to CDAD, and therefore patients carrying both CMV and *C. difficile* would be at a higher risk to develop symptoms.

### 3.2. Materials and Methods

Antibodies levels in serum were measured by ELISA (IgG and IgM), neutralization of the cytotoxicity in cell monolayers and Western blot against surface proteins.

Antigens (non-purified toxin, purified toxin A, EDTA extractions, guanidine-HCl (GHCl) extractions, and lipocarbohydrate: LC) were obtained as described in Chapter 2. Already extracted LPS from *Escherichia coli* core R1 and *E. coli* core R4 were obtained from the laboratory (MPRL) frozen stocks. For the experiments carried out for this thesis, both the cores of an R1 LPS (the most frequent in humans) and an R4 LPS (the most immunogenic in humans) were used. These LPS were already extracted, lyophilised and stored at minus 20°C. Core-PolymyxinB conjugates were produced as described in Gibbs et al. (2004) and provided from the
laboratory’s stocks. The level of IgG against Cytomegalovirus was measured by a commercial ELISA kit.

This study was done in collaboration with Dr. John M. Starr, consultant geriatrician in the Royal Victoria Hospital. In total, 69 patients consented for this study. Blood and stool samples were collected mostly by Dr. María Corretge (54 samples) and Dr. Rowan McIlhagger (11 samples) that were at the time SHOs based at the Royal Victoria Hospital. The rest of the samples (4) were collected by Dr. Lucia Pareja-Cebrián and Dr. Tahir Nazir. There was no interaction between me and any of the patients selected for this study.

The isolates from cases and carriers were characterised to ensure that the appearance of symptoms after colonization with *C. difficile* was not dependant on the strain with which the host was colonised. The hyper-virulent strain ribotype 027 was not found in this population.

The antibiotic history of the patients of this study was partially obtained retrospectively to try to analyse differences in treatment that could explain why cases developed disease while carriers remained asymptomatic. Information on the administration of proton-pump inhibitors was also provided since they have also been related to the increase of CDAD among hospitalised patients (Dial et al., 2004).
3.2.1. Young volunteers

The serum of healthy young volunteers was used to develop the assays and subsequently as a control to ensure the reproducibility of the assays and as a positive control. This collection of serum was not meant to be representative of the healthy population as a whole.

3.2.2. Samples and Characterization of Isolates

The Ethical approval request was written and submitted by Prof. John M. Starr, a consultant in geriatrics in the Royal Victoria Hospital of Edinburgh, a geriatric hospital. Ethical approval was restricted to the region of the Lothians that encompasses Edinburgh and to non-demented patients that were able of understanding the consent form and consent for themselves. Once consent was granted, samples were collected mainly from patients in the Royal Victoria Hospital and mainly by Dr. Maria Corretge and Dr. Rowan McIlhagger with the help of the nursing staff.

In total 69 patients were consented, 58 patients came from the Royal Victoria Hospital, 6 from the Astley Ainslie Hospital, 4 from the Royal Infirmary of Edinburgh and 1 from the Western General Hospital. Symptomatic patients were enrolled as soon as possible after the diagnosis of CDAD.

All the patients were elderly patients. Age ranged from 61 to 95 years old, with just one patient being under 66. That patient was accepted in the study because despite being 61 her medical condition was such that she was considered an elderly patient.
and was hospitalised in a geriatric unit. The mean age for whole group was 81.9 years old. The mean age for each of the groups was 82.9 in cases, 82.8 in carriers and 80.5 in controls. These differences were not significant.

In the study population, 65% were females (45 females, 22 males, 2 not available). In cases that percentage decreased to 63% (12 females, 7 males). The female percentage in carriers was 67% (14 females, 7 males) and controls had the higher percentage of females, 70% (19 females, 8 males).

The 69 patients were consented and sampled for blood. Out of these, 47 were asymptomatic for CDAD. One patient had CDAD-like symptoms but was toxin A+B negative so was discarded from the study. The remaining 21 were diagnosed cases of CDAD. Stool samples were obtained from all the asymptomatic patients within the 24 h prior to blood sampling. Of the 21 symptomatic patients, three were only sampled for blood, eight were sampled for stool within the 24 h prior to blood sampling and for 10 patients stool samples were obtained from the diagnostic laboratory. All of the stool samples were tested for toxins A+B as described and cultured in CCEY agar. Serum was obtained from the clotted blood samples and stored at -20°C.

Out of the 18 symptomatic cases from which a stool sample was available, 13 were culture positive for *C. difficile*. Three of the five culture negative stools were considered too old since they came from the frozen store of the diagnostic laboratories. The other two were fresh samples that tested positive for toxin A+B, but
the patients had probably been given metronidazole to treat for *C. difficile* at the time of sampling.

Out of the 47 stool samples from the asymptomatic patients, 11 were both culture and toxin A+B positive, 8 were culture positive but toxin A+B negative and 2 were culture negative but toxin A+B positive. These 21 patients were grouped as asymptomatic carriers of *C. difficile*. The remaining 26 (culture negative, toxin A+B negative) were grouped as controls.

<table>
<thead>
<tr>
<th>cases (n=21) carriers (n=21) controls (n=26)</th>
<th>symptoms</th>
<th>stool available</th>
<th>toxin (A+B) positive culture positive</th>
<th>toxin (A+B) positive culture negative</th>
<th>toxin (A+B) negative culture positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 (all)</td>
<td>18</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>21 (all)</td>
<td>11</td>
<td>2</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>26 (all)</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Collection of samples – summary table
This table shows the number of stool samples from each group that were toxin or culture positive.

From the culture positive stool, 32 isolates (13 cases and 19 carriers) were obtained and then ribotyped, toxinotyped, S-typed and tested for production of toxin. All but one isolate produced toxin in vitro. The one isolate that didn’t produce toxin, was a non-toxigenic strain, ribotype 010 and S-type 5438. The isolates were mainly ribotype 001 (72%) followed by ribotype 106 (12.5%), the two main types found in South-East Scotland. They were mostly toxinotype 0 (91%) and S-types 5236 (69%) and 5242 (21%), which were found to be the two most common isolates in Edinburgh in a previous epidemiological study (McCoubrey, 2002).

The majority of isolates in this study were considered typical of the Edinburgh area as established by previous work from our group. Mutlu et al (2007) reported that 75.8% of the isolates of *C. difficile* in the region of the Lothians were ribotype 001,
the second most common being ribotype 106, with 96.6% of the isolates of *C. difficile* in Edinburgh and the Lothians being toxinotype 0.

The cases were selected under the requirement that they were toxin positive in stool to avoid misdiagnosis of the symptoms. If the patient had diarrhoea or was suspected to have CDAD but was toxin negative in stool, the patient was not considered for the study independently of their colonisation status. Therefore, all the isolates from cases were toxin positive in both stool and in vitro.

### 3.3. Results

#### 3.3.1. Toxin production

All the stools from the 47 asymptomatic patients were tested for *C. difficile* toxins and plated in CCEY agar medium. Nineteen were culture positive out of which 11 were toxin positive while the remaining 8 were toxin negative. Two samples were toxin positive but no typical *C. difficile* colonies could be isolated, they were considered carriers but with reservations.

Out of the 19 isolates, 18 produced toxin when incubated in vitro. The one that did not produce toxin after a week of incubation was later on confirmed as non-toxigenic strain and ribotype 010 mentioned above.

#### 3.3.2. PCR Ribotyping

Dr. Esvet Mutlu ribotyped 25 of the total 32 isolates. Of these 32 isolates, 23 were ribotype 001 (9 cases and 14 carriers) and 4 were ribotype 106 (1 case and 3
carriers). The remaining five were the non-toxigenic isolate which was ribotype 010, a ribotype 023, a ribotype 046, a ribotype 070 and a ribotype 139.

Of the 23 isolates that were ribotype 001, all but one were toxinotype 0, the last one (case) being toxinotype I. The four ribotype 106 isolates, the ribotype 046 isolate, the ribotype 070 isolate and the ribotype 139 isolate were also all toxinotype 0. The last one (ribotype 023) was a toxinotype IV.

3.3.3. Toxinotyping

Dr Esvet Mutlu toxinotyped 27 of the total 32 isolates. Of these 32 isolates, 29 were toxinotype 0 (11 cases and 18 carriers). Of those, 22 were ribotype 001, 4 were ribotype 106, 1 was ribotype 046, 1 was ribotype 070 and 1 was ribotype 139. The other 3 isolates were a toxinotype I (ribotype 0, a case), a toxinotype IV (ribotype 023, a case) and the non-toxigenic isolate.

3.3.4. S-typing

The surface layer proteins (SLPs) were extracted with 5M GHCl, following the protocol and the nomenclature described by McCoubrey (2002).

McCoubrey (2002) reported that 77% of the isolates of C. difficile in the Royal Victoria Hospital of Edinburgh were type 5236, followed by S-type 5242 which represented 13% of the isolates, S-type 5046 which were 3%, S-types 5438 and 5140 that represented 2% each and others who were less than 0.5% each.
In this study, 22 isolates were S-type 5236 (9 cases, 12 carriers), 6 were 5242 (1 case, 5 carriers), 2 were 5438 (cases), one was undetermined and the last one (non-toxigenic, ribotype 010) was type 5046.

All of the 22 isolates S-type 5236, were ribotype 001. Of the 6 isolates S-type 5242, 4 were ribotype 106, 1 was ribotype 001 and the other one was a ribotype 139. Of the two S-type 5438, 1 was ribotype 046 and the other one was a ribotype 070.

The six S-type 5242, the two S-types 5438 and 19 out of the 20 S-type 5236, were all toxinotype 0. The last of the S-type 5236 was a toxinotype I.

<table>
<thead>
<tr>
<th>Ribotype (number)</th>
<th>Toxinotype for each ribotype</th>
<th>S-type for each ribotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>001 (23)</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>106 (4)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>010 (1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>023 (1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>046 (1)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>070 (1)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>139 (1)</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2: Characterization of isolates – summary table.

This table shows the number of isolates belonging to each ribotype, and the toxinotype and S-type these belong to.
3.3.5. Antibody levels by ELISA - IgG

Levels of IgG in serum against six antigens of *C. difficile* (obtained as previously described) as well as the core of *E. coli* LPS were measured by ELISA. Plates were coated overnight as described in Chapter 2 with the following concentrations of antigens:

- Non-purified toxin: 3.1 mg of protein /L.
- Purified toxin A: 0.42 mg protein /L.
- EDTA extractions of surface proteins of strain 338a: 30 mg of protein /L.
- EDTA extraction of surface proteins of the patient’s own isolate: 30 mg of protein /L.
- GHCl extractions of SLPs: 30 mg of protein /L.
- *C. difficile* surface lipocarbohydrate (LC): 40 mg/L.
- LPS core (*E. coli* core type R1 (70%) and core type R4 (30%)): 30 mg/L.

By definition, the group of controls did not have isolates of their own so no data is available for EDTA against their own isolate in this group. The mean and standard error of the mean (SEM) were calculated for each of the three groups (carriers, cases and controls) and are compared in Figure 3.1.
Figure 3.1: ELISA IgG

This Figure shows the mean levels of specific systemic IgG of cases, carriers and controls against seven different antigens as measured by ELISA (A\textsubscript{405/620nm}): Non-purified toxin (\textit{C. difficile} dialysis culture filtrate); Toxin A purified by affinity chromatography to bovine thyroglobulin; EDTA extraction of the surface proteins of strain 338a; EDTA extraction of their own isolate; GHCI extraction of SLPs of strain 338a; \textit{C. difficile} surface lipocarbohydrate (LC) extracted with aqueous phenol; mix of the cores of two \textit{E. coli} LPS: core type R1 (70%) and core type R4 (30%) in polymyxin B.

Error bars represent the standard error of the mean (SEM). * indicate significant difference (*p=0.05).

As it can be seen in Figure 3.1 for all seven antigens, cases do not have lower levels than carriers or controls as predicted, in fact, for some antigens the levels of antibodies is higher in cases. The antibodies levels against non-purified toxin are highest in the cases. When the means were statistically analysed, they were found to be statistically different between cases and controls (p=0.042) but not between cases and carriers. However, when antibodies against purified toxin A were measured, no differences were found between the groups.
The antibodies levels against the EDTA extraction of the whole surface proteins of a standard strain, were not significant between the three groups. However, when the antibodies levels against the guanidine-HCl extraction (which specifically extracts the two surface layer proteins – SLPs) of the same standard strain were analysed, differences were found. As with non-purified toxin, levels were the highest in the cases. Means were analysed statistically and differences were significant between cases and controls \((p=0.042)\), but again, not between cases and carriers.

Levels of antibodies against the EDTA extraction of their own isolate as opposed to a standard strain were measured as well. The control group was not included since by definition they had no isolates of their own. There were no differences found.

The levels of antibodies against the surface lipocarbohydrate of \(C.\)\(\text{difficile}\), seemed higher in the controls group, but that difference was not statistically significant. No differences between cases and controls were seen.

Antibodies against the core of the \(E.\)\(\text{coli}\) LPS were measured. The aim was to determine if the differences between the groups were specific to \(C.\)\(\text{difficile}\) or were a general state of the immune system. Antibodies levels were predicted to be lower in the cases, but for every \(C.\)\(\text{difficile}\) antigen tested, when there were differences they were higher in the cases group. Antibodies against the core of the \(E.\)\(\text{coli}\) LPS were highest in the cases although this difference was not significant.

The levels of IgG against Cytomegalovirus were measured by a commercial ELISA kit as described by the manufacturer. Due to serum availability at this point, only 64
out of the original 68 patients could be tested in duplicate (18 cases, 20 carriers and 26 controls). Results are shown in Figure 3.2.

Figure 3.2: CMV infections

This graph shows the percentage of patients with CMV infections versus the patients with no CMV infection in the total population, in cases, carriers and controls. n=number of patients analysed.

Out of the 64 patients, 44 (69%) tested positive for CMV infection, with OD values beyond the maximum detectable by the spectrophotometer (OD>2.999), 14 were cases, 13 were carriers and 17 were controls. One, a carrier, was in the grey area between positives and negatives and the remaining 19 (4 cases, 6 carriers and 9 controls) were negatives.

In percentages, there was a 13% difference between CMV in cases (78%) and both carriers (65%) and controls (65%), suggesting that cases are more likely to have a
CMV infection than carriers or controls. When analysed statistically, it was seen that those differences were not quite significant (p=0.06).

**Antigen correlations**

The correlation between different antigens was analysed in order to determine if there was a linear covariance between antigens, this is, to see if a patient with high levels of antibodies for one antigen had high levels for the others. The square of the correlation coefficient ($R^2$) represents the proportion of values of the total variation that can be explained by the relationship between the variable. Two totally independent variables would have a coefficient of 0, and the closer this coefficient is to 1, the stronger the linear relationship is between the variables.
Table 3.2 (A,B,C,D): R² values tables

These tables show the correlation between the different antigens tested. Table A shows the R² values of the population as a whole. Table B shows the R² values of cases. Table C shows the R² values of carriers. Table D shows the R² values of controls.

Toxin A: purified toxin A; np: Non purified toxin; edta: whole surface proteins extracted with EDTA; ghcl: SLPs extracted with guanidine-HCl; lc: surface lipocarbohydrate of C. difficile; lps: the core of E. coli LPS.
Figures 3.3 (A, B, C): Correlation graphs

Three examples of correlation graphs. Figure A shows toxin A vs non-purified toxin. Figure B shows non-purified toxin vs GHCL extract and Figure C shows EDTA extract vs GHCL extract. ♦ for cases; ▒ for carriers and ▲ for controls.
Toxin A has some correlation with every antigen except the core of *E. coli* LPS, particularly and not surprisingly with non-purified toxin. Non-purified toxin has a good correlation with toxin A and the SLPs extracted with guanidine-HCl but no so much with the rest of the antigens. However, when analysing the carriers individually, there is also a strong correlation with EDTA extraction (whole surface proteins) and the surface lipocarbohydrate (LC) that cannot be seen with cases and controls.

There is a high correlation between the two protein extractions, EDTA (whole surface proteins) and guanidine-HCl (SLPs), but it does not seem to be much correlation between them and *C. difficile* surface lipocarbohydrate.

The core of *E. coli* LPS only seems to have a correlation with EDTA extract in both cases and controls, but not carriers, and the *C. difficile* surface lipocarbohydrate (LC) but only in controls.
3.3.6. Antibody levels by ELISA - IgM

Levels of IgM in serum against six antigens of *C. difficile* (obtained as previously described) were measured by ELISA, as well as against the core of *E. coli* LPS. Plates were coated overnight as described in Chapter 2 and the concentrations of antigens were the same as used in the ELISA to detect IgG with the exception of LPS.

- Non purified toxin: 3.1 mg protein/L
- Purified toxin A: 0.42 mg protein/L
- EDTA extractions of surface proteins of strain 338a: 30 mg protein/L.
- EDTA extractions of surface proteins of their own isolate: 30 mg protein/L
- GHCl extractions of SLPs of strain 338a: 30 mg protein/L.
- *C. difficile* surface lipocarbohydrate (LC): 40 mg/L.
- Core of *E. coli* LPS (core type R1): 31 mg/L

By definition, the group of controls did not have isolates of their own so no data is available for EDTA against their own isolate in this group. The mean and standard error of the mean (SEM) were calculated for each of the three groups (carriers, cases and controls) and are compared in Figure 3.4.
Figure 3.4: ELISA IgM

This Figure shows the mean levels of specific systemic IgM of cases, carriers and controls against seven different antigens as measured by ELISA ($A_{405/620\text{nm}}$): Non-purified toxin ($C.\ diffcile$ dialysis culture filtrate); Toxin A purified by affinity chromatography to bovine thyroglobulin; EDTA extraction of the surface proteins of strain 338a; EDTA extraction of their own isolate; GHCI extraction of SLPs of strain 338a; $C.\ diffcile$ surface lipocarbohydrate (LC) extracted with aqueous phenol; the core of the LPS of $E.\ coli$ core type R1 in polymyxin B.

Error bars represent the standard error of the mean (SEM).

As it can be seen in Figure 3.4 no differences between the groups were seen. The IgM levels against both non-purified toxin and toxin A are not different, though it looked as if in the carriers group levels could be slightly higher, it was also the most variable group.

The IgM levels against the surface proteins, both EDTA (mix of all the surface proteins) and guanidine-HCl (SLPs) extractions of a standard strain, were equal in all
three groups. The same happened with the surface lipocarbohydrate of *C. difficile*, no differences could be seen.

IgM levels against the EDTA extraction of their own isolate were also measured. The controls group could not be analysed since by definition they have no isolates. Again, no differences were found.

The core of *E. coli* LPS, however, gave a picture very similar to that seen in IgG. The IgM levels are highest in the cases, contrary to predicted, but that difference is not statistically significant.

### 3.3.7. Neutralization of the cytotoxicity

These experiments were performed as described in Chapter 2. Cells were challenged with a 118 mg/L concentration of non-purified toxin (*C. difficile* culture filtrate) and undiluted serum from the clinical samples (cases, carriers and controls) was added to each well. Plates were then incubated for 3 days to allow the toxin to act on the cells and after this incubation cell viability was measured by an MTT assay. Viable cells reduce MTT (yellow) to formazan (purple), and the production of formazan can be measured at A<sub>570</sub>. Toxin controls (no serum), serum controls (no toxin) and blanks (no toxin, no serum) were included in every plate.
Figure 3.5: Cytotoxicity Neutralization

This Figure shows the neutralization of the cytotoxicity in both Vero and Caco2 cells. "Unchallenged cells": blanks, no toxin and no serum. "Cells + toxin": cells challenged with toxin and no serum added. "+ serum": cells challenged with toxin and serum of cases, carriers or controls added. *** indicate significant difference with "unchallenged cells": p<0.001. +++ indicate significant difference with "cells + toxin"
Error bars represent the standard error of the mean (SEM).

Vero cells

In Figure 3.5 it can be observed that the sera from each of the three groups (cases, carriers and controls), neutralize the toxin when compared to the toxin control. The cell viability in the three groups is even higher than blanks or the young volunteers’ control (data not shown). However, there were no significant differences between the sera from the three groups.
Caco2 cells

Caco2 cells are a human colon cancer lineage that polarize and differentiate in culture. They are more sensitive to toxin than Vero cells (they die at much lower concentrations of toxin). In Figure 3.5 challenged cells (with toxin only) are less viable than in Vero cells, and although there is neutralization after the addition of serum, the levels of viability are lower than the blanks, and much lower than in Vero cells.

The three groups of sera neutralized the toxin, but not to 100% level, but there were no differences among them. There were also no differences between these and sera from the young volunteers group (data not shown).

3.3.8. Western blots

ImmunobLOTS were performed as described in Chapter 2. EDTA extraction from C. difficile strain 338a was run in a 10% PAGE gel and then transferred to a nitrocellulose membrane. After blocking and washing, the membrane was cut into four strips and each incubated with a mixture of different sera:

- A mixture of 4 young (under 65) healthy (non-hospitalised) volunteers’ sera (data not shown)
- A mixture of 17 sera from “cases”
- A mixture of 19 sera from “carriers”
- A mixture of 23 sera from “controls”
In each of the four cases the final concentration was the same: 1 in 1000. IgG was detected. The blots were repeated several times with similar results. Results are shown in Figure 3.6.

![Western Blot Image](image)

**Figure 3.6: Western blot**

This Figure shows the western blot against an EDTA extract of strain MPRL338a. The first two lanes were incubated with serum from cases, lanes 4 and 5 with serum from carriers and lanes 7 and 8 with serum from controls. Lanes 3, 6 and 9: molecular weight marker (rainbow marker).

As seen in Figure 3.6 the sera from cases give a stronger signal and to more bands than both carriers and controls, particularly strong are two bands around 50kDa and 35kDa respectively that very probably correspond with the S-layer proteins, and a third and much smaller band just below 15kDa. Those symptomatically colonized by *C. difficile* had a stronger reaction to the proteins in the EDTA extracts than those not colonized or colonized but without symptoms.
3.3.9. Antibiotic treatment and development of disease

Information on antibiotic treatment and proton pump inhibitors (PPI) administration was provided retrospectively by both Dr. Maria Corretge and Dr. Rowan McIlhagger. Notes were not available for 33 out of the 69 patients (48%). From the 36 where notes were available, 9 (25%) were cases, 12 (33%) were carriers and 15 (42%) were controls.

From the 9 cases, one of them is recorded as not having been given antibiotics during their stay in hospital before sampling. Four were already on metronidazole treatment for C. difficile, for two of them, this was the only antibiotic recorded. The other two, one was on previous treatment with gentamicin and the other one had had a combination of trimethoprim, metronidazole, amoxicillin (β-lactam) and omeoprazol (PPI). A sixth one was administered both augmentin (β-lactam) and ciprofloxacin (fluoroquinolone), the latter up to the date of sampling. Two were administered two β-lactams (ceftriaxone and coamoxiclav) and the ninth one received only ceftriaxone (β-lactam).

From the 12 carriers, 8 were toxin positive in stool while the other 4 were toxin negative and culture positive. From those 4 isolates, 3 were toxigenic and the fourth was the only non-toxigenic strain. The carrier of the non-toxigenic strain is recorded as not having had any antibiotics or PPI, and that was the case as well for one of the carriers of a toxigenic isolate but toxin negative in stool.

Five of the 12 carriers (4 of the 8 had toxin positive stools) had been administered ciprofloxacin, a fluoroquinolone. For one of them (toxigenic but toxin negative in
stool) it was the only antibiotic recorded. From the other 4 that had been on ciprofloxacin, one was also administered metronidazole, another one was administered lansoprazol (PPI) and the other two had treatments with β-lactams (flucloxacillin and coamoxiclav respectively). Apart from these two, three others were administered β-lactams. One of those three was administered 2 β-lactams (amoxicillin and ceftriaxone) as well as clarithromycin and gentamicin. A second one had had augmentin (β-lactam) as well as metronidazole and clarithromycin and the third one had cefuroxime (β-lactam) as well as trimethoprim and lansoprazol (PPI).

The two remaining ones who are not recorded as having had ciprofloxacin (fluoroquinolone) or β-lactams, one had had clarithromycin (making it the third one) and the other one trimethoprim and omeoprazol (PPI).

Nine out of the 15 controls where data was available had been administered β-lactams, two of them had two β-lactams (coamoxiclav and augmentin). Four of them had been also administered a PPI, and three had had augmentin (β-lactam) and trimethoprim. Three others had had trimethoprim on its own or with a PPI. From the two remaining ones one is recorded to have had just clarithromycin and the other one just metronidazole.
### Table 3.3: Antibiotic intake

This table shows the information available on the antibiotic (and PPI) intake. **NT:** non-toxigenic strain; **toxin:** presence (+) or absence of toxin in fresh stool; +: antibiotic given; ++: two antibiotics of that kind were being administered; **metro:** metronidazole; **B-lac:** β-lactam antibiotics (amoxicillin, coamoxiclav, augmentin, ceftriaxone, cefuroxime, flucloxacillin); **Cipro:** ciprofloxacin (fluoroquinolone); **trim:** trimethoprim; **gent:** gentamicin; **clarith:** clarithromycin. **PPI:** proton-pump inhibitors (omeprazol, lansoprazol); **total:** total number of antibiotics received.
In summary, from the data available carriers seemed to be administered slightly more antibiotics than cases and controls. The average number for cases and controls is 1.7 while in cases is 1.8. They also have the highest variety of antibiotics.

Half of the cases were receiving metronidazole for the treatment of the *C. difficile* episode and the other half were receiving β-lactams. 58% of carriers were receiving either β-lactams or fluoroquinolones (or both), and 60% of the controls were receiving β-lactams but none of them had had fluoroquinolones. Even though the data is partial, this could indicate that ciprofloxacin is making the host susceptible to *C. difficile* colonization.

β-lactams administered in controls were mostly accompanied by clavulanic acid which is a β-lactamase inhibitor. The β-lactamases are the main mechanism of resistance against β-lactam antibiotics. In controls, 8 out of the 11 (73%) β-lactams administered are either coamoxiclav or augmentin (amoxicillin clavulanate), while in carriers, only 2 out of 6 were coadministrated with clavulanic acid and a third one was flucloxacillin, a β-lactam resistant to β-lactamases. In cases, 4 out of the 5 (80%) patients receiving β-lactams were receiving ceftriaxone or amoxicillin without clavulanic acid and the fifth although was administered augmentin it also had had ciprofloxacin. Even though the data available is not complete, it looks as if the administration of β-lactams without an inhibitor of β-lactamases is making the patients susceptible to disease.
3.4. Discussion

The aim of this study was to relate the development of disease with the lack of an antibody response of the host specifically to *C. difficile*.

One of the risk factors traditionally associated with CDAD is advanced age. In this study only elderly patients were considered for the study and no significant differences were found between the groups.

Contrary to what was expected, cases (patients symptomatically colonized with *C. difficile*) do not have lower levels of antibodies than carriers when measured by ELISA, neutralization of the cytotoxicity or Western blots against surface proteins. The hypothesis of this study was that those patients who were colonized by *C. difficile* but did not mount an appropriate antibody response were the ones developing disease, while the patients that were able to produce specific antibodies after the colonization with *C. difficile*, were protected from disease and remained as asymptomatic carriers. This hypothesis, as explained more thoroughly in Chapter 1, was supported by Kyne et al. (2000) but not so by Drudy et al (2004) who did not find significant differences between cases, carriers and controls.

The cases in this group of patients do not lack the ability to produce antibodies both specific to *C. difficile* and specific to other organisms. It would seem likely that antibodies start being produced after colonization and this production of antibodies is triggered by disease itself, and it would seem that certain antigens such as the SLPs induce the highest response. However, it should be noted that everyone seemed to have a certain level of pre-existing antibodies.
Kyne et al. (2000) found that the cases had on average more severe pre-existing diseases than asymptomatic carriers. Since no information is available on the disease that resulted on the hospitalisation of the patients of this study, it is a possibility that cases had more severe diseases than carriers, and that it is this disease that is preventing the immune system from responding against *C. difficile* before CDAD is developed. Maybe carriers are healthier and consequently able to mount a response prior to disease. The key might be when the antibodies are produced rather than on the ability to produce the antibodies or on the quantity of antibodies produced. If individuals are exposed long enough to *C. difficile* before their gut flora is severely affected, they might be able to produce specific antibodies that could be protective even if the bowel flora is afterwards affected. The level of antibodies before infection with *C. difficile* might also be crucially important to stop symptoms developing.

However, the fact that the antibody levels are not much higher in the cases might indicate that even if they are not immunocompromised *per se*, their immune system could be exhausted due to age (immunosenescence) and due to other diseases and infections. The significantly higher levels of IgG against non-purified toxin and against SLPs is an indication that at least at one point cases were able to respond to *C. difficile*. However the absence of an increase of IgM (indicators of recent disease) levels at the peak of their disease (the samples from cases were taken during the episode of diarrhoea) might be an indication that the immune system is failing. It could be argued that carriers are producing specific IgM at the same levels as cases due to recent infection even if they are asymptomatic. However, that does not explain why cases do not have higher levels of IgM than the controls, who are not exposed to
the organism. Also, a high IgG and low IgM at the time of infection could be an indication of recurrence. The clinicians in charge of consenting the patients and taking the samples did not indicate that that was the case but this should not be discarded. Even though, this would explain the IgM results, it would not agree with the findings of Kyne et al. (2001). Their data suggested that recurrences happened as a consequence of the inability to produce specific IgG.

The differences seen in Western blots agree with the differences seen in IgG ELISA against SLPs, there is a significant higher response in cases. This reinforces the fact that cases are not unable to produce specific antibodies, but does not give any further information on their immunosenescence status.

The CMV IgG levels were also measured. CMV infections are a characteristic indicator of immunosenescence, the drop of the immune response due to age. Of cases 78% were positive for CMV infection while in carriers and controls it dropped to 65%. These Figures are not surprisingly high since it is expected from elderly patients, but the fact that the percentage is higher in cases, though not significantly (p=0.06), might be an indication that cases are in fact sicker than carriers or controls. More work is needed to support these findings but it is consistent with what Kyne et al (2000) found when they analysed the severity of the primary disease in the patients of their study. C. difficile is after all an opportunistic pathogen, and the drops in the immune response that other authors have found could be related but not necessarily causative of CDAD.
CMV are known to cause PMC in co-infection with *C. difficile* in patients with AIDS and leukaemia (Ives and Smith, 1996; Riva et al., 2005). The immune response in this group of patients is not as low as such patients to explain disease, but it is plausible that the CMV infection is contributing towards the worsening of CDAD. The co-infection of *C. difficile* with CMV and other opportunistic pathogens might be an explanation worth exploring.

The group of controls aimed to represent the hospitalised elderly population from which cases and carriers were taken but without prior exposure to *C. difficile*. If this is the case, then it has to be assumed that the levels of antibodies in the controls are the basal levels in this population. But if this assumption is true, then carriers have not significantly increased their levels of specific antibodies after exposure to *C. difficile*, and cases only their IgG against non-purified toxin and SLPs. It could be argued that the group of controls are not representative of this population, that they are a group of healthier individuals. Maybe the reason they are not colonized with *C. difficile* is that they have not spent as much time in hospital as the ones that are. Maybe their basal levels are higher, maybe after a certain amount in hospital dealing with disease and opportunistic pathogens those basal levels drop and they only increase again after exposure. But then, the basal levels against the core of *E. coli* LPS, a very highly immunogenic and common antigen, would be higher in the controls group. But that is not the case. Even though the difference is not significant (*p*=0.06), cases have higher levels of IgG to the core of *E. coli* LPS than controls. We have to assume that controls are indeed a representation of the population to which cases and carriers belong.
It remains to be seen if a boost of the specific immune response (e.g. by vaccination) will offer protection against first episodes of *C. difficile*, and if affirmative, whether it is cost effective to vaccinate the whole long-term stay hospital population or even just targeted wards to prevent disease from developing.

From the data of this study, it does not seem likely that the immune status at the moment of first colonization is the key factor to be considered on why some develop disease and others remain asymptomatic, at least not the systemic antibodies. In this study mucosal antibodies have not been looked at, but the local immune response in the gut is probably more relevant to CDAD than systemic antibodies as shown by Johal et al (2004b).

It is undeniable the leading role that the commensal gut flora has in the protection of the gut against colonization by pathogens. Those whose bowels have been severely compromised either by antibiotics, surgical procedures, nasogastric tubes, PPI or others, are the ones susceptible to disease, and those with a healthier gut flora at the moment of exposure to the organism can get colonized but will more likely remain asymptomatic.

Unfortunately, there is not enough data available for the patients of this study to make comparisons of their treatments while in hospital. From the data available, it seems that ciprofloxacin, a fluoroquinolone, and β-lactam antibiotics susceptible to β-lactamases might be the ones making the patients of this study susceptible to *C. difficile* colonization.
Harbarth et al (2001) compared the CDAD cases to the non-CDAD patients from a post-operative population and found that those who developed CDAD were significantly older and had had a significantly higher number of antibiotics than those who did not develop CDAD. The length of the antibiotic treatment and the length of hospitalisation were found to be unrelated. Also, when antibiotic restriction policies were applied during different outbreaks of *C. difficile*, the outbreaks were controlled and the number of cases decreased (Quale et al., 1996; Jones et al., 1997; McNulty et al., 1997; Kuijper et al., 2006). There is an historical strong relationship between *C. difficile* and antibiotics, and even when this relationship fails to explain the totality of the cases, it is still undeniable. Alternative therapies such as the toxin-binding agent tolevamer are less aggressive than antibiotics since instead of killing, it captures the toxin and prevents it from damaging the host.

The isolates from cases were not different enough from the isolates from carriers to explain the differences between cases and carriers and 65% of the isolates are typical as described by Mutlu et al (2007).

In conclusion, this study does not relate the systemic immune system to the appearance of disease. However, some authors (Kyne et al., 2001; Drudy et al., 2004) showed that there is a strong relationship between the non-production of specific antibodies after the first episode and recurrences. It is not unreasonable to think that those who are immunocompromised or unable to mount a specific response before the full recovery of the bowel flora (which can take several months), will suffer another episode of disease. The immune response would then be key not to first episodes but to further episodes and worsening of disease.
However, if this hypothesis is true, that recurrences are a consequence of a lack of an antibody response, boosting by vaccination does not seem to be a plausible alternative. These patients have already been exposed multiple times to *C. difficile* and every one if its antigens with no result. Both faecal transplantation and the addition of immunoglobulin seem better treatment options for recurrences.
4. Antibiotics and expression of the PaLoc

4.1. Introduction

This chapter started off as a continuation of the work done by Lisa Drummond during her PhD (Drummond, 2004). As explained in more detail in Chapter 1, she showed that under the presence of sub-inhibitory concentrations of antibiotics, \(C.\)\(\text{difficile}\) grew slower but toxin was being produced faster and at higher levels.

The main aim of this chapter was to relate those changes to changes in the expression of the regulatory genes contained in the PaLoc (see 1.4).

Amoxicillin is a \(\beta\)-lactam antibiotic (penicillin family) that acts by preventing bacterial cell-wall synthesis. It inhibits the cross-linking of peptidoglycan chains. Amoxicillin is one of the main antibiotics associated with the predisposition to \(C.\)\(\text{difficile}\) disease and was chosen as the main antibiotic for this study.

Other antibiotics used were vancomycin, metronidazole and clindamycin. Vancomycin, used in the treatment of CDAD, also acts on the cell wall of Gram positives preventing the formation of the peptidoglycan matrix. Metronidazole is the first choice antibiotic in the treatment of CDAD and other anaerobic bacterial infections. In anaerobic conditions in bacteria its nitro group is reduced and in this form it disrupts the DNA double helix inhibiting nucleic acid synthesis. Clindamycin inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome, and along with lincomycin it was the original antibiotic associated with \(C.\)\(\text{difficile}\) disease.
The sequenced strain 630 was the only one used in this study.

4.1.1. Brief introduction to Real time PCR (and Ct values)

Real time PCR was developed due to the need to quantify differences of mRNA levels. There are other ways of comparing mRNA levels but PCR is considered the most sensitive and discriminative.

After extracting and purifying the RNA from the cells, a reverse transcription is performed. The RNA is transformed into cDNA, and this cDNA is amplified by Real Time PCR. In Real time PCR the amount of DNA is measured at every cycle by fluorescence. Many fluorescent dyes can be used but the most common one (because of its high sensitivity) is SYBR® green. SYBR® green is a cyanide dye that binds specifically to double stranded molecules of DNA. On the downside, it also binds to primers dimers so it is recommended to check the melting temperature of the products (to make sure it is the expected one, and that it is the same for every product) but also to run an agarose gel with the PCR products to check they are the correct size.

The more DNA is amplified the more molecules of dye will be bound to it and therefore will give a stronger signal. The Ct ("cycle threshold") value is the cycle at which the increase of fluorescence detected starts being exponential. The earlier this happens (the lower the Ct value), the higher the level of initial cDNA.
Theoretically, a difference of 3.3 cycles represents a ten fold difference in cDNA concentration (DNA doubles every cycle). To check the efficiency of the PCR, a standard curve with known DNA concentrations is produced each time.

4.2. Materials and Methods

In her thesis, Drummond (2004) related the optical density at 600nm to the viable count at different time points. In the absence of antibiotics and other growth inhibitors, she showed reference strain 630 to grow exponentially during the first 18-20h, at which it reached its peak and entered the stationary phase. She also measured the production of toxin A in strain 630 which was not occurring until after 24 h of uninhibited growth.

Strain 630 was grown in PPY medium in the presence and absence of sub-inhibitory concentrations of four different antibiotics: amoxicillin, metronidazole, vancomycin and clindamycin. The MICs of 630 to these antibiotics were calculated by Drummond (2004) and confirmed by broth macrodilution as described in Chapter 2. These are stated at the beginning of each section in the Results of this chapter.
In this study, amoxicillin was the main antibiotic used. Growth and toxin under different concentrations of amoxicillin were measured every 6 h over 24 h. The yields of RNA after 24 h were too low to compare. Both toxins A and B were measured as described at each of the time points and growth was measured by optical density at 600nm. At each of the same time points, samples were taken and total RNA was extracted and purified as described in Chapter 2. Equal amounts of RNA were retro-transcribed and Real time PCR was performed on the resulting cDNA to test for the expression of the five PaLoc genes: *tcdA* (toxin A), *tcdB* (toxin B), *tcdC*, *tcdR* and *tcdE*, as well as of a sixth gene involved in the metabolism of glucose, triose phosphate isomerase (*tpi*), a housekeeping gene that served as a control gene. The exact methodology is described in Chapter 2.

For the other three antibiotics (metronidazole, vancomycin and clindamycin), growth and toxin were measured as described for amoxicillin but the RNA was extracted only at the 18 h time-point. Hammond et al (1997) extracted total RNA from *C. difficile* cultures and found that both before 7 h and after 21 h, the amount of toxin mRNA was very low. The preliminary results for this study showed very low yields of total RNA after 24 h. It was also shown that there was more variation at 18 h than at 12 h and therefore 18 h was considered to be the optimal time point to study.

The positive control was the standard curve performed with known concentrations of genomic DNA to guarantee the presence of the gene. The standard curve also serves as a PCR efficiency control. The negative controls were a blank and a non-Rt control which consisted of RNA extracted and purified at the same time but that had not
been reverse-transcribed. Primers should not bind to RNA and the presence of a signal on this control implies the presence of genomic DNA.

4.3. **Results**

4.3.1. No Antibiotic

The results of all the non-antibiotic controls were grouped to get a general picture of what is happening in the untreated population of *C. difficile*. As can be seen in Figure 4.1, in the absence of antibiotics, the stationary stage is reached by 12 h. Toxin A+B can be detected after 12 h but by 24 h the levels are still low.

**Figure 4.1: Growth and Toxin production in Controls.**

This graph shows the growth (O.D. 600nm) and the toxin (A+B) detection (O.D. 450/620 nm) of strain 630 in the absence of antibiotics over 24 hours. Error bars represent the standard error of the mean (SEM). At each timepoint, n=6.
As can be seen in Figures 4.2 and 4.3, the peak of expression of the PaLoc genes occurred at 12 h. There was a drop of all five genes at 18 h, and at 24 h four of the genes (excluding *tcdA*) they had a second peak.

The same was true for the expression of the *tpi* gene, giving a pattern of expression almost identical to the PaLoc genes.

**Figure 4.2: Toxins and tpi**

This graph shows the Ct values of the toxins (A and B) genes as well as the *tpi* gene of strain 630 in the absence of antibiotics. Error bars represent the standard error of the mean. At each timepoint, n=4.
Figure 4.3: tcdC, tcdR and tcdE

This graph shows the Ct values of the toxins (A and B) genes as well as the tpi gene of strain 630 in the absence of antibiotics. Error bars represent the standard error of the mean. At each timepoint, n=5.
4.3.2. Amoxicillin

The MIC for Amoxicillin ranges from 2 to 4 mg/L. As it can be seen in Figure 4.4, at 2 mg/L growth is retarded but not completely inhibited. At 1 mg/L growth is slightly lower than the non-antibiotic control and at concentrations lower than 1 mg/L, growth is almost identical to the control.

![Figure 4.4 - Growth amoxicillin](image)

**Figure 4.4: Growth in the presence of amoxicillin**

This graph shows the growth of strain 630 in the presence of amoxicillin compared to the growth in the absence of antibiotics over 24 hours. Error bars represent the standard error of the mean. At each timepoint, n=3.

Looking at toxin A+B production (fig. 4.5) it can be seen that in the absence of amoxicillin, the toxin levels detected are low, even at 24 h. However, in the presence of 1 mg/L toxin A+B can be detected as early as 6 h and by 18 h the levels detected are much higher. In the presence of 0.5 mg/L amoxicillin the levels of toxin are almost identical to the control.
Figure 4.5: Detection of toxin (A+B) in the presence of amoxicillin

This graph shows the toxin detected in the medium of strain 630 grown in the presence of amoxicillin compared to the toxin detected in the absence of antibiotics over 24 hours. Error bars represent the standard error of the mean. At each timepoint, n=4.

When the expression of the PaLoc genes was analysed, there were differences between the control (absence of amoxicillin) and the presence of 1 mg/L amoxicillin. The qPCR for tcdA was not fully optimised due to the fact that the product is too large, so data was considered only when it produced a linear standard curve. There is a lack of data so conclusions cannot be drawn but it seems like (fig. 4.6a) there is a difference of 5 to 9.5 cycles in the Ct value at 18 h which will translate in approximately 100 fold difference. At 18 h and in the presence of amoxicillin, tcdA seems to be expressed 100 times more than in the control. The levels of cDNA in the presence of amoxicillin dropped at 24 h while the control remained at the same Ct.
value, suggesting the translation of the cDNA into protein, which would be consistent with the elevated values seen in toxin (A+B) detection.

**Figure 4.6a – Ct values for tcdA in the presence of amoxicillin**

This graph shows the Ct values of the toxin A gene in the absence and in the presence of amoxicillin. n/a: data not available; n/d: gene not detected. Error bars represent the standard error of the mean. At 6 and 12 hours, n=2; At 18 and 24 hours, n=1.

The levels of tcdB do not seem to be altered by the presence of amoxicillin (fig. 4.6b) which could be implying that the high levels of toxin detected in the medium are due to an increase of toxin A exclusively.
Figure 4.6b – Ct values for tcdB in the presence of amoxicillin

This graph shows the Ct values of the toxin B gene in the absence and in the presence of amoxicillin. Error bars represent the standard error of the mean. At every timepoint, n=3 except 1/4 MIC at 18 hours, n=1.

Figure 4.7 – Ct values for tcdC in the presence of amoxicillin

This graph shows the Ct values of the tcdC gene in the absence and in the presence of amoxicillin. Error bars represent the standard error of the mean. At every timepoint, n=5.
When the PaLoc accessory gene, \( tcdC \) (fig. 4.7) was looked at, it did not seem to be affected by the presence of 1 mg/L of amoxicillin in the growth medium. However, in the presence of 0.5 mg/L and at 24 h there was a decrease of about 5.5 cycles in the Ct value. This difference represented a 100-fold difference in cDNA levels. However, the error bars can narrow this difference to 0.7 cycles and thus not much can be concluded, particularly when the one growing in the presence of 0.5 mg/L had, up to 24 h, the growth curve and toxin detection levels almost identical. In this study, the presence of toxin in the medium was measured only up to 24 h.

**Figure 4.8 - Ct values for tcdR in the presence of amoxicillin**

This graph shows the Ct values of the \( tcdR \) gene in the absence and in the presence of amoxicillin. Error bars represent the standard error of the mean. At every timepoint, \( n=5 \).
Figure 4.9 – Ct values for tcdE in the presence of amoxicillin

This graph shows the Ct values of the tcdE gene in the absence and in the presence of amoxicillin. Error bars represent the standard error of the mean. At every timepoint, n=5.

Both tcdR (fig 4.8) and tcdE (fig 4.9) are clearly affected by the presence of amoxicillin, particularly in the presence of 0.5 mg/L of amoxicillin where growth is not altered. The Ct value of tcdR is decreased by 7 cycles in the 1mg/L amoxicillin and more than 10 in the 0.5 mg/L which translated into a 100 and 1000 fold increase on the cDNA respectively. The Ct value of tcdE is also decreased by 5 cycles in the 1 mg/mL and 10 cycles in the 0.5 mg/L, which translated into 30 and 1000-fold increase of the cDNA respectively.
When the levels of the \textit{tpi} (triose phosphate isomerase) gene expression (a gene involved in the metabolism of glucose and not related to virulence) were analysed (fig. 4.10) there were no differences seen between the presence and the absence of amoxicillin.

\begin{center}
\textbf{Figure 4.10 - Amoxicillin: Ct values for \textit{tpi}}
\end{center}

This graph shows the Ct values of the tpi gene in the absence and in the presence of amoxicillin. n/d: gene not detected. Error bars represent the standard error of the mean. At 6, 12 and 24 hours, n=4; at 18 hours n=1 except no amox n=5.
4.3.3. Metronidazole

The MIC for metronidazole was estimated to be 0.125 mg/L. As seen in Figure 4.11, growth was not seen at 0.125 mg/L, however late growth was noticeable in the presence of 0.25 mg/L suggesting that the MIC was in fact 0.5 mg/L. However, RNA was extracted only for 0.0625 mg/L because it was the only one besides the non-antibiotic control with considerable growth at 18 h.

![Figure 4.11 - Metronidazole: Growth](image.png)

**Figure 4.11: Growth in the presence of metronidazole**

This graph shows the growth of strain 630 in the presence of metronidazole compared to the growth in the absence of antibiotics over 24 hours. At every timepoint, n=1.
Figure 4.12: Detection of toxin (A+B) in the presence of metronidazole

This graph shows the toxin detected in the medium of strain 630 grown in the presence of metronidazole compared to the toxin detected in the absence of antibiotics over 24 hours. At every timepoint, n=1.

Figure 4.12 shows that the toxin A+B levels detected were low both in absence and in the presence of metronidazole, however the toxin seemed higher in the presence of 0.063 mg/L but only at 18 h.

When the expression of the PaLoc genes were analysed, it was noted that the toxin genes tcdA and tcdB were very low in the controls (absence of antibiotics) and in the presence of metronidazole (Fig. 4.13) they were not detected.
**Fig 4.13 - Metronidazole:**
Ct values tcdA and tcdB

![Graph showing Ct values for tcdA and tcdB in the presence of metronidazole (18 h)](image)

This graph shows the Ct values of the toxins (A and B) genes in the absence and in the presence of metronidazole. n/d: gene not detected. Error bars represent the standard error of the mean. No metronidazole, n=5; 1/2 MIC, n=1.

**Figure 4.13 – Ct values for tcdA and tcdB in the presence of metronidazole (18 h)**

**Fig 4.14 - Metronidazole:**
Ct values tcdC, R and E

![Graph showing Ct values for tcdC, tcdR and tcdE in the presence of metronidazole (18 h)](image)

This graph shows the Ct values of the tcdC, tcdR and tcdE genes in the absence and in the presence of metronidazole. n/d: gene not detected. Error bars represent the standard error of the mean. No metronidazole, n=5; 1/2 MIC, n=2.

**Figure 4.14 – Ct values for tcdC, tcdR and tcdE in the presence of metronidazole (18 h)**
When the expression of the accessory genes was analysed (Fig. 4.14), it was seen that the Ct value for \textit{tcdC} increased in 5.5 cycles in the presence of metronidazole which is around 45 times less cDNA. The Ct value for \textit{tcdR} did not show differences with the control and \textit{tcdE} was undetectable in the presence of metronidazole.

4.3.4. Vancomycin

The MIC for vancomycin was estimated to be 2 mg/L. Although there was some late growth at 24 h in the presence of 2 mg/L vancomycin and the real MIC should have been 4 mg/L, the RNA was extracted at 18 h for the 1 mg/L and 0.5 mg/L.

![Fig. 4.15 - Vancomycin: Growth](image)

**Figure 4.15: Growth in the presence of vancomycin**

This graph shows the growth of strain 630 in the presence of vancomycin compared to the growth in the absence of antibiotics over 24 hours. At every timepoint, \( n=1 \)
Figure 4.16: Detection of toxin (A+B) in the presence of vancomycin

This graph shows the toxin detected in the medium of strain 630 grown in the presence of vancomycin compared to the toxin detected in the absence of antibiotics over 24 hours. At every timepoint, n=1.

As seen in Figure 4.15, growth was inhibited in the presence of 1 mg/L. However, toxin levels were higher in the presence of vancomycin than the control but only at 24 h (fig 4.16). The presence of 0.5 mg/L did not inhibit growth considerably but the levels of toxin A+B detected were clearly higher than the control at 24 h.

However, these differences did not translate at a genetic level, at least not at the time point chosen (18 h). The levels of tcdA were very low and the levels of tcdB undetectable (fig 4.17). The levels of the accessory genes (tcdC, tcdR and tcdE) did not differ from those of control either (fig. 4.18).
Fig. 4.17 - Vancomycin: 
Ct values *tcdA* and *tcdB*

![Graph showing Ct values for tcdA and tcdB](image)

**Figure 4.17 – Ct values for tcdA and tcdB in the presence of vancomycin (18 h)**

This graph shows the Ct values of the toxins (A and B) genes in the absence and in the presence of vancomycin. n/d: gene not detected. Error bars represent the standard error of the mean. No vancomycin, n=5; 1/2 and 1/4 MIC, n=1

Fig 4.18 - Vancomycin: 
Ct values *tcdC*, R and E

![Graph showing Ct values for tcdC, tcdR, and tcdE](image)

**Figure 4.18 – Ct values for tcdC, tcdR and tcdE in the presence of vancomycin (18 h)**

This graph shows the Ct values of the *tcdC*, *tcdR* and *tcdE* genes in the absence and in the presence of vancomycin. Error bars represent the standard error of the mean. No vancomycin, n=5; 1/2 MIC, n=2.
4.3.5. Clindamycin

Strain 630 is resistant to clindamycin. The MIC was determined to be 520 mg/L, the highest concentration tested. As seen in Figure 4.19, growth is considerably lower in the presence of clindamycin, the higher the concentration, the lower the growth.

![Figure 4.19 - Clindamycin: Growth](image)

**Figure 4.19: Growth in the presence of clindamycin**

This graph shows the growth of strain 630 in the presence of clindamycin compared to the growth in the absence of antibiotics over 24 hours. At every timepoint, n=1.
**Figure 4.20: Detection of toxin (A+B) in the presence of clindamycin**

This graph shows the toxin detected in the medium of strain 630 grown in the presence of clindamycin compared to the toxin detected in the absence of antibiotics over 24 hours. At every timepoint, n=1.

As can be seen in Figure 4.20, the levels of toxin (A+B) are all similar to the control (no clindamycin) except for in the presence of 32.5 mg/L of clindamycin at 24 h where the levels of toxin are extremely high. Even though this experiment was only repeated once, data is supported by the findings of Drummond (2004).
When the expression of the PaLoc genes was analysed, differences could be seen. As can be seen in Figure 4.21, the levels of both tcdA and tcdB are much higher in the presence of clindamycin than in the absence of antibiotics, although the less dramatic change was in the presence of 260 mg/L which is the one that grew the least. In the presence of 130 mg/L, the Ct value for tcdA increased 9 cycles and for tcdB 7.5 cycles, which is the equivalent to approximately 500 and 180 times more cDNA than the control.

![Fig 4.21 - Clindamycin: Ct values tcdA and tcdB](image)

**Figure 4.21 – Ct values for tcdA, tcdB and tpi in the presence of clindamycin (18 h)**

This graph shows the Ct values of the toxins (A and B) genes in the absence and in the presence of clindamycin, as well as of the tpi gene. n/d: gene not detected. Error bars represent the standard error of the mean. The lack of error bars indicate there were not enough repeats. No clindamycin, n=5; the rest, n=1.
When the expression of the three accessory PaLoc genes was analysed (fig 4.22), it could be observed that there were higher levels of expression in the presence of clindamycin. In the presence of 130 mg/L, the Ct value for tcdC was increased 5 cycles, for tcdR almost 11 cycles and for tcdE, 7 cycles, which corresponds to 40, 1500 and 150 times more cDNA respectively.

![Graph](image.png)

**Figure 4.18** – Ct values for tcdC, tcdR and tcdE in the presence of clindamycin (18 h)

This graph shows the Ct values of the tcdC, tcdR and tcdE genes in the absence and in the presence of clindamycin. Error bars represent the standard error of the mean. The lack of error bars indicate there were not enough repeats. No clindamycin, n=5; 1/2 and 1/4 MIC, n=2; 1/8 and 1/16, n=1

When the expression of the tpi (triose phosphate isomerase) gene was analysed (fig 4.21), a gene involved in the metabolism of glucose and therefore growth, it was seen that while in the controls the expression of this gene was undetectable, in the presence of 260 mg/L and 130 mg/L of clindamycin, the levels were moderately high.
4.4. **Discussion**

The aim of the work described in this Chapter was to relate the presence of antibiotics in the growth medium to overproduction of toxins, and to relate these changes in toxin production to changes in the expression of the PaLoc genes.

Strain 630 was used due to the fact that it has been sequenced and this ensured that the primers would recognise the targeted sequence. Hundsberger et al (1997) found that tcdC was the only gene of the PaLoc that was transcribed at early stages and that this reversed in stationary phase suggesting that the presence of tcdC was an inhibitor of the transcription of the other four genes. That was not observed in this study where all five genes had the same pattern of expression.

It is undeniable that the presence of sub-lethal concentrations of antibiotics (in particular amoxicillin, vancomycin and clindamycin) change the pattern of toxin production. Toxin (A+B) is produced at an earlier stage of growth in the presence of antibiotics although the reasons why this is happening are not entirely clear and it is possible that every type of antibiotic acts in a slightly different way.

Onderdonk et al. (1979) described the effect that stress had on toxin production. Among the stress factors they tested were the presence of sub-inhibitory concentrations of vancomycin and penicillin, which in both cases resulted on a 1000-fold increase of toxin within 4h. Hennequin et al. (2001b) showed that the expression of groEL (Hsp60), a heat shock protein of *C. difficile* with adhesin properties, was increased during stress and one of the stress factors they tested was the presence of ampicillin. The presence of antibiotics at non-lethal levels is a stress to *C. difficile* to
which it responds by increasing its virulence. However, this increase of the virulence (increase of toxins and adhesion) could be a side effect of early sporulation due to stress. Kamiya et al (1992) correlated sporulation and the release of toxin, and the showed that if sporulation was retarded, so was the the expression of the cytotoxin. It is not unreasonable then to presume that if sporulation is initiated earlier due to environmental factors such as the presence of antimicrobials, toxin would be secreted earlier.

The yields of RNA obtained after 24 h of growth were very low and therefore expression was not analysed past 24 h.

The data of this study show that the release of toxin in the presence of amoxicillin and vancomycin is occurring while in the exponential phase of growth so it might not necessarily be related to sporulation, which is traditionally a phenomenon occurring in the stationary phase. No spore count was carried out to support this and should not be discarded. However, there could be another explanation.

Amoxicillin seems to be affecting the expression of tcdR and tcdE which will result in an overexpression of the toxins and an earlier release. However, only tcdA seems to be overexpressing while tcdB remains the same. This has not been confirmed by detection in the medium since only toxin A+B was measured. Hammond et al (1997) proposed a model for the transcription of the PaLoc in which both monocistronic and polycistronic transcripts co-existed. The presence of a polycistronic transcript (a full length transcript that was then processed into individual transcripts) would allow for the co-regulation of both toxins. The presence of monocistronic transcripts would
mean that each toxin is also subject to its own regulation and would explain why
toxin A and toxin B can be found in different concentrations, which would not be
possible if only a polycistronic transcription was occurring and both toxins were
equally regulated. The presence of monocistronic transcripts was also described by
Hundsberger et al (1997). Amoxicillin could very well be affecting only the
individual regulation of tcdA and explain why the levels of tcdB are not affected.

Differences in cDNA in amoxicillin were only seen at 18 h while differences in toxin
detected in the medium were observed as early as 6 h. It could be that the time
intervals were too long and that differences would be appreciated if these intervals
were shortened. For vancomycin only the 18 h time point was studied and no
differences in the expression of the PaLoc were observed even though the toxin
levels in the media at 24 h were increased in the presence of vancomycin. It could
very possibly be that the time point chosen was not the optimal one and that data at
more time-points is needed. Or maybe, the phenomenon is not entirely due to
modifications in the genetic regulation.

Amoxicillin and vancomycin are antibiotics that affect the cell wall. At
concentrations close to the MIC, C. difficile grows at a slower pace. However, at
these concentrations the cell wall would be a functional one at least partially or C.
difficile would not be growing at all, but that does not necessarily mean that the cell
wall is fully intact. The cell wall could still be non-lethally damaged by the
antibiotic and as a result, have a less densely cross-linked peptidoglycans creating
"holes" or "pores" that do not exist on the intact cell wall. These holes could be the
reason for the leakage of toxin. This would explain why no differences were found in
the expression of the PaLoc genes at early stages despite the differences seen in the
detection of the toxins in the medium.

It is unknown whether a more frequent monitoring of the expression of the PaLoc
genes (which was done on a single time point in the case of vancomycin) would offer
more information and show more differences. It is likely however, that the effect of
amoxicillin is not at a single but at different levels.

Experiments with clindamycin gave the most interesting results. The reason why this
antibiotic was not chosen as the main antibiotic of this study was the high
concentrations needed in order to achieve inhibition of growth of strain 630.

Clindamycin acts by inhibiting protein synthesis in sensitive bacteria. *C. difficile*
strain 630, a resistant strain, seems to be reacting to the presence of very high
concentrations of clindamycin by increasing gene expression which might result in
an increase in protein synthesis. If this is indeed the case, clindamycin would not
only be failing to prevent protein synthesis but it would be causing the opposite
effect.

Whether the increase of expression in the presence of clindamycin is specific to the
PaLoc genes or is a generalised phenomenon is not known. When the levels of *tpi*, a
gene involved in the metabolism of glucose, were measured there was an increase as
well, but only on the two highest concentrations. Considering, these two were where
growth was most affected, it could be that growth is retarded and not that
clindamycin is promoting the increase of *tpi*. Also, the one with the highest
concentration (and lowest growth) did not show differences in the PaLoc expression
when compared to control, and the two lowest concentrations that did show
differences in the PaLoc expression, did not show differences in the expression of
*tpi*. This would suggest that the overexpression of the PaLoc is specific but more data
is needed to confirm this.

After the addition of clindamycin, the five genes of the PaLoc were overexpressed
compared to the control, particularly in the three concentrations where growth was
less affected. In the experiments with clindamycin the RNA was only analysed on a
single point basis, so it is possible that the differences seen are not increases of the
expression as such but shifts of the expression pattern.

When the toxin released in the medium was analysed, of the three concentrations
where changes in expression were seen, only the concentration that affected growth
the least (the lowest concentration: 32.5 mg/L) showed presence of toxin before 24 h.
However the levels of toxin detected suggest that toxin release was not only
occurring earlier but also at much higher levels. If these experiments had been
extended beyond 24 h, it is likely that the toxin levels detected for the other two
concentrations would be as high. However, it might happen that despite the notable
increase in gene expression, this does not translate into a higher presence of toxin in
the medium since it is possible that clindamycin while not lethal might still affect
protein synthesis. This would be in accordance with the fact that it was only the
lowest concentration of clindamycin tested that affected toxin production before 24 h.
It has to be taken into consideration that although 32.5 mg/L was the lowest concentration tested, it is still a high concentration and possibly more achievable in vivo than the higher ones and therefore the results for 32.5 mg/L are more concerning than the results for higher concentrations.

The implications of the data from clindamycin are more worrying than that from amoxicillin and vancomycin. With the latter two, there was an earlier production of toxin which implies less time for the host to react to *C. difficile* before the toxin is released and can therefore explain why β-lactams have been related to host susceptibility to *C. difficile*. The presence of clindamycin, however, seems to increase the expression and release of toxins to much higher levels, so even when the release is occurring in the stationary phase *C. difficile* is much more toxigenic. Though it was not studied, clindamycin might also alter the expression of other genes associated with virulence. This could be a reason that explains reports of highly virulent strains that are not higher toxin producers *in vitro*. The stress levels that clindamycin induces on *C. difficile* are particularly strong forcing it to react the way it does. It is then a possibility that higher virulence is related to higher levels of environmental stress to *C. difficile*.

In this study, metronidazole was the one that affected growth the most. The MIC was the lowest and the differences of growth against the control in sub-inhibitory concentrations of metronidazole were considerable larger than in the case of the other three antibiotics tested. Only minor differences were seen in the presence of toxin in the medium. However differences were seen in the expression of *tcdC* which could potentially lead to an overexpression of toxins that could be detected after 24h.
It has to be remembered that the RNA experiments with metronidazole were done only on a single time point basis, and more importantly at a concentration that was later found to be much lower than 1/2 MIC. However, according to the data of this study, metronidazole is more effective (less antibiotic needed) against strain 630 than vancomycin and has fewer side effects in terms of how *C. difficile* reacted to the presence of it.

The relationship between antibiotics and CDAD is clear and well established. Antibiotics disrupt the protective flora allowing the selection of resistant strains and the colonization of *C. difficile*. However the role of antibiotics proposed in this study is currently being overlooked despite all the evidence available.

Alternatives to antibiotics are being suggested (see Chapter 1) but they still remain the first choice of treatment for many illnesses including CDAD. Maybe efforts to create stronger and better antibiotics should be redirected to the development of less aggressive therapeutic options.
5. Enhancement of cytotoxicity by cellular antigens

5.1. Introduction

Toxins A and B are the major virulence factors of \textit{C. difficile}. The absence of both of them leads to the non-virulence of the strain. However, there is not a strict correlation between the toxigenicity of the strain (the amount of toxin produced \textit{in vitro}) and the degree of disease (Borriello et al, 1987). Other virulence factors (see Chapter 1) have been considered and although the role of these factors is not fully known, it is understood that they contribute to pathogenesis.

Despite the fact that the two main toxins (A and B) are exotoxins and therefore secreted into the medium, it is not usual to find the toxins without the presence of the bacterium. In other clostridia which produce exotoxins, such as \textit{C. botulinum}, disease is exclusively caused by the toxin and the presence or absence of the bacterium is irrelevant for diagnosis, but this is not typical of \textit{C. difficile}. Even though toxin detection is the standard mechanism of diagnosis, culture is been said to be more discriminative (Delmée et al., 2005) and samples that were considered negative due to the lack of toxin detection were positive for toxin-producing \textit{C. difficile} when cultured.

The focus in this study was to investigate the combination of toxin and cell surface antigens since these are the most exposed to the host. The aim of this chapter was to test if the cytotoxicity of \textit{C. difficile} is enhanced by the presence of cellular antigens
in two cell lines: Vero and Caco2. Purified toxin A was used mainly for this study along with four other antigens:

1) A mixture of *C. difficile* strain MPRL338a surface proteins extracted with 10mM EDTA

2) *C. difficile* strain MPRL338a SLPs extracted with 5M guanidine-HCl (GHCl)

3) *C. difficile* surface lipocarbohydrate (LC), which is analogous to LTA of other Gram-positive bacteria, extracted with aqueous phenol.

4) *E. coli* O18K' LPS already extracted, lyophilised and stored at minus 20°C.

*E. coli* LPS (endotoxin) is used as a representative of Gram-negative bacterial LPS which is highly immunogenic and present in every human gut (see Introduction of Chapter 3). It is not known if there is any interaction between endotoxin and *C. difficile* exotoxins but since both share the same habitat, the possibility of a synergy between both should be taken into account. The fact that this laboratory also works with LPS makes it very accessible to this project.
5.2. Materials and Methods

Toxin A was purified from a *C. difficile* culture filtrate by affinity chromatography as described earlier in Chapter 2 and the concentration of total protein was measured by the Bradford method. This purification was thought necessary to minimise the content of cellular antigens in the toxin preparation. Every toxin concentration stated in this Chapter should be understood as μg of total protein per mL of toxin solution.

Some of the experiments were performed with *C. difficile* dialysis culture filtrate that was not put through chromatography. In those cases it will be referred to as “non-purified toxin”. When not specified, it should be assumed that purified toxin was used.

After considering different types of stains to quantify the cytopathic effect or CPE, it was decided to focus on cell viability. Cell viability was measured by an MTT assay as described in Chapter 2. Viable cells are able to reduce MTT (yellow) to formazan (purple). The presence of formazan can be detected by spectrophotometry ($A_{570}$). The higher the O.D. is, the higher the proportion of viable cells present in the well.

Incubation times of challenged cells before the MTT assay must be of at least 3 days as it was observed that toxin, even though it produced a characteristic cytopathic effect (CPE) within 24 hours, did not significantly reduce cell viability unless it was incubated for a minimum period of 3 days. Periods longer than 5 days affected cell viability considerably and made it more difficult to determine the effect of the toxin.
Each set of results (a column in the Figures) was the mean of between 10 and 28 replicates for the Vero cells and 6-37 for the Caco2 cells, normally 10-12 repeats. Differences were considered statistically significant for p values less than 0.0500.

5.3. Results

5.3.1. Vero cells

The first experiment was to test that the antigens on their own were not toxic to the cells. Both EDTA and GHCl extracts were dialysed to remove the chemical agent. Antigens used were:

- 10 µl per well of 380 µg/mL dialysed EDTA extraction
- 10 µl per well of 770 µg/mL dialysed GHCl extraction (SLPs)
- 10 µl per well of 1000 µg/mL LC solution
- 10 µl per well of 1000 µg/mL E. coli O18K− LPS solution.

Cells were challenged with a non-purified EDTA extraction of the surface proteins, with the SLPs, the LC and E. coli O18K− LPS. Vero cells were incubated for three days. Results are shown in Figure 5.1a.

No significant differences were found between only cells (no toxin, no antigens) and cells with GHCl extract (SLPs) or E. coli LPS added. Cell viability was higher, however, after the addition of EDTA extract (p=0.0051) or LC (p=0.0003).
Figure 5.1: Viability of Vero cells after the addition of antigens (a) or toxin (b).
Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Error bars show the standard error of the mean. A) No antigen: cells that have not been challenged with antigens; EDTA: addition of EDTA extract; GHCI: addition of GHCl extract; LC: addition of extracted LC; LPS: addition of extracted LPS. ** and *** shows an enhancement of cell number compared to no-antigen control where ** p<0.01 and *** p<0.001. B) Titration of toxin A. * and *** are significantly different from no-toxin control. * p<0.05. *** p<0.001.

Once it was determined that the extracts were not reducing cell viability on their own, they were tested to see if they had any enhancing influence on the toxin. Toxin was added in different concentrations ranging from 21 µg/mL or 10.5 µg/mL the concentrations at which cell viability was drastically reduced (toxin=10.5µg/mL vs only cells: p=0.0006) to lower concentrations which did not significantly reduce cell viability. Results of the toxin titration are shown in Figure 5.1b.

Surface protein extracts (EDTA and GHCl extracts) were then added to each toxin concentration. Results are shown in Figure 5.2.
Figure 5.2: Titration of Toxin A with or without addition of surface-associated protein antigens. Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Blanks: no toxin, no antigen; only toxin: cells challenged with toxin but no antigen; +edta: cells challenged with toxin and EDTA extract; +ghcl: cells challenged with toxin and GHCl extract. * indicates significant difference with only toxin: p<0.05. Error bars show the standard error of the mean.

At the higher concentrations of toxin (21 or 10.5 µg/mL), the reduction of cell viability with and without the presence of surface proteins is not significantly different. However, when the concentration of toxin is not enough to reduce cell viability when compared to the controls (2.63 µg/mL and 0.66 µg/mL), a reduction of viability is observed after the addition of EDTA extract but this reduction is only significant at 2.63 µg/mL (vs only toxin p=0.0341). Lower concentrations are too low to affect cell viability before and after the addition of the EDTA extract. As seen in Figure 5.1, dilution 2.63 µg/mL was not enough to reduce viability, but after the addition of EDTA extract, the reduction of viability compared to the blanks is clearly significantly lower (p=0.0040). It should be remembered that EDTA extract on its
own did not reduce cell viability, in fact, as seen in Figure 5.1a, cell viability was increased. The addition of the GHCH extracts which only contain the SLPs, do not alter cell viability suggesting that the two SLPs are not the ones responsible for this enhancement of the toxin activity.

The same experiments were performed with the surface carbohydrate of *C. difficile* (LC) which is analogous to LTA, and *E. coli* LPS. Cells were left unchallenged, or were challenged with just the antigens, just the toxin preparation or both. Results are shown on Figure 5.3.

**Figure 5.3: Titration of Toxin A with or without addition of surface-associated carbohydrate antigens.** Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Blanks: no toxin, no antigen; only toxin: cells challenged with toxin but no antigen; +lc: cells challenged with toxin and LC; +lps: cells challenged with toxin and LPS. * ** and *** indicate significant difference with only toxin: *p*=<0.05, **p*=<0.01 and ***p*=<0.001. Error bars show the standard error of the mean.
As shown in Figure 5.1, the addition of these concentrations of LC and LPS were not detrimental to the cells. However, when added in combination with the higher concentrations of toxin (dilutions 21 μg/mL and 10.5 μg/mL) which already affected cell viability, the addition of LC significantly reduced even further the cell viability (p=0.0016 and p=0.0362). This was not observed after the addition of LPS. At lower concentrations of toxin (2.63 μg/mL and 0.66 μg/mL) where cell viability was not affected in the presence of toxin alone, the addition of either LC or E. coli LPS significantly enhanced the toxic activity. This reduction was highly significant for both LC (p<0.0001 and p=0.0003) and LPS (p=0.0071 and p=0.0004).

**Experiments with non-purified toxin**

These experiments were repeated with non-purified toxin solution which contains both toxins A and B as well as some other products secreted by C. difficile.

As shown in Figure 5.4, none of the antigens has a detrimental effect on its own. All four of the non-purified toxin concentrations tested reduced cell viability (p=0.0014 to p=0.0374). The addition of each of the four antigens to each concentration of non-purified toxin had very little effect. Further reduction of cell viability was only seen at 77.5 μg/ml of non-purified and after the addition of the LC (p=0.0216) or after the addition of the SLPs extracted with guanidine-HCl (p=0.0040).
Figure 5.4 - Effect of surface proteins (extracted with EDTA and GHCl), C. difficile surface carbohydrate and LPS on Vero cells challenged with non-purified toxin. Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Only cells: no toxin, no antigen; only toxin: cells challenged with toxin but no antigen; +edta: cells challenged with toxin and EDTA extract; +ghcl: cells challenged with toxin and GHCl extract; +lc: cells challenged with toxin and LC; +lps: cells challenged with toxin and LPS. * and ** indicate significant difference with only toxin: * p=<0.05; ** p=<0.01. ++ and + indicate significant difference with only cells: + p=<0.05; ++ p=<0.01. Error bars show the standard error of the mean.

5.3.2. Caco2 cells

The Caco2 cell line is a human colon cancer cell line that can spontaneously differentiate in culture. In these experiments, it was aimed for confluent non differentiated monolayers before challenge with toxin and/or antigens to facilitate a more even acquisition of both the toxin and the antigens by the cells. The main problem found with performing these experiments on differentiated cells was ensuring that every layer of cells was exposed to the same concentration of toxin and/or antigens. For this reason, only monolayers were studied.
Caco2 cells were found to be more sensitive to toxin and therefore the concentrations needed to get the same level of reduction of cell viability were lower.

Antigens used were:

- 10 µl per well of 190 µg/mL dialysed EDTA extraction
- 10 µl per well of 385 µg/mL dialysed GHLI extraction
- 10 µl per well of 500 µg/mL LC solution
- 10 µl per well of 500 µg/mL E. coli 018K' LPS solution.

Cells were challenged with a non-purified EDTA extraction of the surface proteins, with the SLPs, the LC and E. coli O18K' LPS and then incubated for three days. No significant differences were found between the blanks (no toxin, no antigens) and the addition of any of the antigens, suggesting there is no detrimental effect produced by any of these four antigens when added on their own. Results are shown in Figure 5.5a.

**Figure 5.5:** Viability of Caco2 cells after the addition of antigens (a) or toxin (b). Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Error bars show the standard error of the mean. A) No antigen: cells that have not been challenged with antigens; EDTA: addition of EDTA extract; GHLI: addition of GHLI extract; LC: addition of extracted LC; LPS: addition of extracted LPS. B) Titration of toxin A. *** are significantly different from no-toxin control: p=<0.001.
Once it was determined that the extracts were not reducing cell viability on their own, they were tested to see if they had any enhancing influence on the toxin. Toxin was added in different concentrations ranging from those (0.42 - 21 μg/mL) which drastically reduced cell viability (difference toxin vs blank: p<0.0001), to lower concentrations (4.2*10^-4 - 0.04 μg/mL) which did not significantly reduce cell viability. Results of the toxin titration are also shown in Figure 5.5b.

Surface protein extracts (EDTA and GHCl extracts both dialysed to eliminate the chemical agent) were then added to each toxin concentration. Results are shown in Figure 5.6.

**Figure 5.6 Titration of Toxin A with or without addition of surface-associated protein antigens.** Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Blanks: no toxin, no antigen; only toxin: cells challenged with toxin but no antigen; +edta: cells challenged with toxin and EDTA extract; +ghcl: cells challenged with toxin and GHCl extract. ** indicates significant difference with only toxin: p=<0.01. Error bars show the standard error of the mean.
As observed with Vero cells, at the higher concentrations of toxin (21 µg/mL and 4.2 µg/mL), there was no significant differences in the reduction of the cell viability with and without the presence of surface proteins. In fact the addition to 0.42 µg/mL toxin to any of the protein extracts seemed to be counteracting the effect of the toxin (p=0.0020 for EDTA and p<0.0001). However, when the concentration of toxin was not enough to reduce cell viability when compared to the controls (0.04 µg/mL and 4.2*10^-3 µg/mL), a reduction of viability was observed after the addition of EDTA extract (vs toxin alone p=0.0061 and p=0.0007). As seen in Figure 5.5b, these concentrations were not enough to reduce viability, but after the addition of EDTA extract, the reduction of viability compared to the blanks was clearly significantly lower (p=0.0027 and p=0.0230). The addition of the GHCl extracts which only contained the SLPs did not seem to reduce cell viability further. The cell viability after the addition of GHCl extract to the lowest of concentrations tested (4.2*10^-3 µg/mL), was significantly lower than toxin alone (p=0.0148) but not significantly lower than the blanks (p=0.0919), probably due to the lack of number of repeats at this toxin concentration.

The same experiments were performed with the surface carbohydrate of C. difficile (LC) which is analogous to LTA, and E. coli LPS. Cells were left unchallenged, or were challenged with just the antigens, just the toxin preparation or both. Results are shown on Figure 5.7.
As shown in Figure 5.7, the addition of these concentrations of LC and LPS were not detrimental to the cells. When they were added in combination with the higher concentrations of toxin (21 μg/mL and 0.42 μg/mL) which already affected cell viability, the addition of either LC or LPS significantly increased cell viability (p=0.0141 and p=0.0011) suggesting a protective effect. At lower concentrations of toxin (4.2*10^{-4} μg/mL) where cell viability was not affected in the presence of toxin alone, the addition of LC significantly enhanced the toxic activity (p=0.0029). This reduction was not seen after the addition of *E. coli* LPS.
Experiments with non-purified toxin

Figure 5.8 - Effect of surface proteins (extracted with EDTA and GHCl), *C. difficile* surface carbohydrate and LPS on Caco2 cells challenged with non-purified toxin. Cell viability is measured by the transformation of MTT to formazan (O.D.570nm). Only cells: no toxin, no antigen; only toxin: cells challenged with toxin but no antigen; +edta: cells challenged with toxin and EDTA extract; +ghcl: cells challenged with toxin and GHCl extract; +lc: cells challenged with toxin and LC; +lps: cells challenged with toxin and LPS. * and ** indicate significant difference with only toxin: * p=<0.05; ** p=<0.01. +++ and + indicate significant difference with only cells: + p=<0.05; +++ p=<0.001. Error bars show the standard error of the mean. n/a: data not available.

When these experiments were performed with non-purified toxin (contains both toxin A and B as well as any other product secreted by *C. difficile*), no detrimental effect was observed with any of the antigens tested when added to the cells on their own.

The non-purified toxin concentrations used ranged from those which reduced the cell viability (0.31 - 31 µg/ml) when compared to the blanks (p<0.0001 to p=0.0161) to those which did not affect cell viability (0.031 and 3.1*10^{-3}).
The addition of the cell antigens to the different concentrations of antigens had very little effect on cell viability. No effect was seen for any of the antigens tested when the toxin concentration was enough to reduce cell viability. At 0.031 μg/ml, which did not reduce cell viability, the addition of EDTA extract (mix of surface proteins), guanidine-HCl extract (SLPs) or LC reduced the cell viability when compared to toxin alone (p=0.0032, p=0.0183 and p=0.0168 respectively) but not when compared to the blanks.

At 3.1*10^{-3} μg/ml of non-purified toxin, which was not enough to reduce cell viability on its own, the addition of EDTA extract (surface protein mixture) reduced cell viability when compared to toxin alone (p=0.0368) and when compared to the blanks (p=0.0053). The addition of guanidine-HCl was not tested on this concentration of non-purified toxin and the addition of LC and LPS was done too few times to be analysed statistically.

### 5.4. Discussion

The aim of this study was to determine if the surface antigens of *C. difficile* have a role in pathogenesis. The correlation between severity of disease and quantity of toxin produced is weak (Borriello et al., 1987), with the exception of hyper-virulent strain 027 which was reported to be producing around 20 times more toxin than the classical strains (Warny et al., 2005).

The hypothesis of this study was that other antigens might be enhancing the toxin effect. The cell wall of *C. difficile* is a complex structure containing several proteins and for most of them, their function remains uncertain. Some of these proteins show
certain variability in different strains, such as the SLPs, FliC, FliD, Cwp66 or Cwp84 (McCoubrey and Poxton, 2001; Pechine et al, 2005). These proteins might have a role in adhesion, evasion of the immune response or entry of the toxin into the cell, and the variability they present could help explain different grades of virulence of strains that are equals in terms of toxin production.

*C. difficile* surface proteins and surface lipocarbohydrate in solution were added to both Vero and Caco2 cells with and without toxin. Neither of the antigens had a detrimental effect on the cells on their own, discarding any effect that traces of the chemicals used in extraction might have and indicating that the effect shown on the viability of the cell when both toxin and antigen are added is due to a synergy between the toxin and a product of the extraction.

When the antigens were added to the cells in conjunction with purified toxin A, it was seen that they had some synergistic effect towards the reduction of cell viability. At high concentrations of toxin, those which reduced cell viability, only LC further reduced cell viability of Vero cells. This was not seen in Caco2 cells with any of the antigens or in Vero cells with any of the other antigens. When the toxin concentration was not high enough to reduce cell viability, the addition of either LC or *E. coli* LPS had a dramatic effect on the viability of Vero cells, which also occurred after the addition of EDTA extract but not after the addition of GHCl extract. In Caco2 cells however, this effect was noticeable after the addition of LC but not *E. coli* LPS, and it was much better seen after the addition of EDTA extract and to a less extent (not significant) after the addition of the SLPs (GHCl extract).
When these experiments were done with non-purified toxin, this effect basically disappeared in Caco2 cells and was reduced in Vero cells. No toxin concentrations that did not reduce viability in Vero cells were used, which were the ones that gave the most interesting results with purified toxin A. However, the effect that LC had at higher concentrations of purified toxin was much reduced in non-purified toxin experiments. The addition of GHCl extract to Vero cells challenged with high concentrations of non-purified toxin, showed an effect that was nonexistent in the purified toxin A experiments at any of the concentrations tested.

Non-purified toxin was obtained from a *C. difficile* culture grown in a dialysis bag for six days after which cells were removed by centrifugation. The contents of the bag were dialysed to get rid of all the medium components that had gone into the bag and then filter-sterilised. The solution contained both toxins as well as anything secreted or released by *C. difficile* in those six days, but big enough to remain after dialysis and small enough to remain after filter-sterilisation. The effect of non-purified toxin was obviously bigger since it contained toxin B which has higher cytotoxic properties than toxin A. The reduction of the synergistic effect of the cell antigens in the non-purified toxin experiments could be explained by the presence of the causative antigen in the solution.

The addition of any of the antigens of *C. difficile*, particularly the LC, made lower concentrations of toxin equivalent to higher concentrations, in other words, more effective. EDTA extracts were mainly protein but no purification was done so it is probable that they contained LC, but it remains a possibility that the effect seen with EDTA was due to a cell wall protein. It is unlikely that this effect is caused by the
SLPs, since these were tested at high concentrations and the effect was minimal. There are a variety of cell wall proteins whose functions are not well established that could be the cause of this effect.

Caco2 cells are more sensitive cells than Vero cells, and as a consequence, antigens used were less concentrated. Preliminary results showed some effect of EDTA and GHCl extracts on cell viability on their own and it was feared that this was caused by traces of the chemicals used to extract the protein. Antigen concentrations were then reduced, but this reduction might have reduced the synergistic effect.

There was no effect of *E. coli* LPS in Caco2 cells but it was high in Vero cells. Since both LC and LPS are surface carbohydrates, they probably have common properties and could be substrate to the same receptor or enzyme used in the enhancement of toxin. The mechanism by which this enhancement occurs remains unknown, but if this effect seen *in vitro* is translated *in vivo*, it would mean that the virulence of a particular strain would vary depending on the number of *C. difficile* cells colonizing the gut, and not entirely because of a higher concentration of toxin. It could also mean that a non-toxigenic strain could potentially enhance the toxicity of a toxigenic strain or even just the toxicity of traces of toxin left in the gut.
6. Conclusion

This Chapter summarizes the main conclusions that can be extracted from this study and makes suggestions to investigate these matters further.

As stated at the end of Chapter 1, there were three main hypotheses that were being tested:

1) The inability to produce specific antibodies to \textit{C. difficile} makes the colonized patient susceptible to disease.

2) The presence of sub-inhibitory concentrations of antibiotics induces a stress on \textit{C. difficile} that results on the overproduction of toxins due to changes in the expression of the PaLoc genes.

3) The presence of cell antigens of \textit{C. difficile} increases the cytotoxicity of the toxins (\textit{in vitro}).

As explained more thoroughly in the Results chapters, some answers were obtained.

\textbf{Patients that present CDAD are not unable to produce specific antibodies to \textit{C. difficile} as demonstrated by the fact that they did not have lower levels of antibodies in serum when measured by ELISA, Western blot and neutralization of the cytotoxic effect. The levels of antibodies in Cases tended to be higher than both Carriers and Controls, but the fact that this tendency was not clear might have been an indicator that they are in fact partially impaired to mount an appropriate immune response. There is a possibility that the amount of systemic antibodies produced was insufficient but would not explain why Carriers did not develop disease. It is a}
possibility that systemic antibodies in Carriers were produced earlier or quicker than in Cases, and therefore even though the quantity of antibodies produced in Cases was enough, disease had already developed. The level of antibodies before infection with *C. difficile* might be crucially important to stop symptoms developing. The reason why Cases had a slower response could be due to the host itself or to other factors such as other or more severe diseases. Another explanation could be that Cases are not slower in producing antibodies but that external factors had hastened the colonization of the gut by *C. difficile*. It should be taken into account that the levels of mucosal antibodies (which were not measured in this study) might be more relevant to disease than systemic antibodies.

**Susceptibility to *C. difficile* is determined by multiple factors.** It is very difficult to determine what makes a patient susceptible to *C. difficile* since many factors seem to be interacting. Traditional risk factors such as age and antibiotics usage should always be taken into consideration. Age was not a factor in this study because all the patients were elderly but it is undeniable that the frailty associated with advanced age is a contributor to disease. Elderly people are more likely to be hospitalised and therefore exposed to nosocomial pathogens.

The antibiotics administered were not fully studied due to unavailability of records, but from the data gathered, disease was not associated to the number of antibiotics administered but to the type of antibiotic given. Ciprofloxacin, a fluoroquinolone, and β-lactams without β-lactamases inhibitors seemed to be to blame for the appearance of CDAD in the population of this study.
Cytomegalovirus infection is not traditionally considered as a risk factor for CDAD. CMV infections are easily overlooked in the hospitalised population because the symptoms that present are easily explained by the reason of their hospitalisation. In this study a tendency was shown for the Cases to have had a CMV infection in a higher proportion than Carriers or Controls. The exact role played by CMV in CDAD, if any, needs further study but it could be by cooperation with \textit{C. difficile} as well as by swaying the immune response, making it inefficient or “slower” as suggested previously.

\textbf{The presence of sub-inhibitory concentrations of antibiotics induces a stress on} \textit{C. difficile} \textbf{that results on the overproduction of toxins} or at least an earlier production of toxin. In this study, toxin production was only analysed in a 24 h period since Drummond et al (2004) had already studied this matter. The aim of this study was to relate those changes at the genetic level by measuring the expression of the five PaLoc genes over those 24 h.

It seemed that antibiotics not only disrupted the commensal flora of the gut facilitating colonization by \textit{C. difficile}, but it seemed to be altering the pattern of expression of \textit{C. difficile}. Each of the four antibiotics tested were affecting \textit{C. difficile} in different way, with the most dramatic effect produced by clindamycin and the least by metronidazole. Only the PaLoc genes were analysed. This study should be extended to a larger number of genes to get a clearer picture of how gene expression is being affected.
The length of days when toxin was being produced was not measured but it would be interesting to see if antibiotics have any effect on that matter.

The presence of cell antigens of *C. difficile* increases the cytotoxicity of the toxins particularly the surface carbohydrate of *C. difficile* (LC). When cells were challenged with non-lethal concentrations of toxin in conjunction with the surface carbohydrate, the effect was as if the toxin concentration had been much higher. This was shown in both Vero and Caco2 cell lines, although the effect was better seen on Vero cells. The mechanism by which this antigen is helping the toxin become more effective is not known, and it has not been shown to happen in vivo. However, this revelation might be a turning point in the understanding of the pathogenesis of *C. difficile*.

This enhancing effect was also produced by the LPS of *E. coli*, and to a certain extent also by a mixture of *C. difficile* surface proteins. This mixture, although formed mainly of protein, was not purified and will contain the LC. However, it should not be discarded the fact that another protein of the *C. difficile* wall (different from the SLPs) might be causing the effect described and that its concentration in the mixture is simply too low to be fully appreciated.

In all, CDAD is a multi-factorial disease caused by a multi-resistant organism that responds to environmental stress by increasing its virulence. The aim of this study was to investigate the host response to *C. difficile* as well as the response of *C. difficile* to antibiotics, and the contribution of other virulence factors to disease.
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Molecular characterization and antimicrobial susceptibility patterns of Clostridium difficile strains isolated from hospitals in south-east Scotland

Esvet Mutlu, Allison J. Wroe, Karla Sanchez-Hurtado, Jon S. Brazier and Ian R. Poxton

INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus. It is commonly associated with a spectrum of disease referred to as C. difficile-associated disease (CDAD), which can range from uncomplicated mild diarrhoea to lethal toxic megacolon and possible colon perforation (Johnson & Gerding, 1998). It is considered to be the leading cause of nosocomially acquired diarrhoea in adults and can be responsible for large outbreaks (Kelly & LaMont, 1998). There is a view that the severity of the disease is increasing. The new hypervirulent type (ribotype 027, toxinotype III, pulse-field NAP1) in North America and several European countries has been associated with more severe and fatal cases (McDonald et al., 2005; Kuijper et al., 2006a; Hubert et al., 2007).

As elsewhere, C. difficile is rarely cultured in Scotland and laboratory diagnosis depends on the detection of toxins A and/or B in faeces. Several phenotypic and molecular methods have been applied to determine the relatedness of strains of C. difficile. All have their advantages and disadvantages. Methods based on whole-genome analysis are more discriminatory, but they are technically demanding and labour-intensive (Brazier, 2001). PCR ribotyping is commonly used in Europe as it has been reported to be
highly discriminative, reproducible, relatively rapid and easy to perform (O’Neill et al., 1996; Stubbs et al., 1999). Toxinotyping is a PCR-RFLP method that depends on changes in the toxin genes and other regions of the pathogenicity locus of C. difficile. It has been reported to correlate well with restriction endonuclease analysis, serotyping and PCR ribotyping and it also gives the advantage of determining toxin variant strains (Rupnik et al., 1998; Johnson et al., 2003). Flagellin gene RFLP analysis has been described as an additional typing method that can be used in conjunction with other typing methods (Tasteyre et al., 2000).

An actin-specific ADP-ribosylating binary toxin CDT is produced by some strains of C. difficile. Its role in pathogenesis is currently unclear, but its presence has been correlated with severity of disease in some studies (Barbut et al., 2005) and it is present in the 027 hypervirulent strain (McDonald et al., 2005). The prevalence of binary toxin in clinical isolates of C. difficile is generally low, with frequencies ranging between approximately 2 and 20% (Barbut et al., 2005).

The aims of this study were to (i) characterize 149 C. difficile isolates from toxin-positive faecal samples collected between August and October 2005 by molecular typing methods, PCR ribotyping, toxinotyping and flagellin gene RFLP analysis and by the S-layer typing method, together with the detection of the binary toxin genes cdtA and cdtB; and (ii) determine the susceptibility of isolates to seven different antibiotics. The objectives were to show which strains were currently present locally, to determine whether there were any clonal relationships between isolates and to examine the antimicrobial susceptibility profiles of the different types.

METHODS

Bacterial isolates. C. difficile isolates (n=149) from toxin-positive faecal samples (determined using a Toxin A+B ELISA kit; TechLab) were collected between August and October 2005 and stored at −20 °C. All specimens were from different unsellected cases of CDAD in different hospitals in the Edinburgh area (Lothian University Hospitals National Health Service Trust) consisting of acute and long-stay hospitals. They were collected on a purely random basis with no selection for hospital or patient type. During this period, we were not aware of any outbreaks. Stool samples were cultured on Brazier’s cefoxitin/cycloserine/egg yolk agar (LabM) and incubated for 48 h at 37 °C in an anaerobic chamber. The isolates were identified by characteristic colony morphology, smell, fluorescence under long-wave UV light and appearance on a Gram film. Subcultures were stored in anaerobic investigation medium containing cooked meat particles for maintenance (Brown et al., 1996). Control strains were NCTC 11223, VPI 10463, 338A (a locally isolated strain of ribotype 01; Mc Courage, 2002), the sequenced strain 630 and a 027 strain from Amsterdam provided by E. Kuijper (Leiden, The Netherlands).

DNA extraction. Colonies from overnight anaerobic cultures on fastidious anaerobe agar (LabM) supplemented with 6% horse blood were resuspended in 100 μl of a 5% solution of Chelex-100 resin (Bio-Rad). After incubating in a boiling bath for 10 min, the cell debris was removed by centrifugation for 2 min at 18 000 g. The supernatant was used as the crude DNA template for PCRs except for toxinotyping. For toxinotyping, pure DNA isolation was required. DNA was extracted using a Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions.

PCR ribotyping. All 149 isolates were typed by PCR ribotyping according to the method described by O’Neill et al. (1996). Specific oligonucleotide primers 5’-CTGGGGTGAAGTCTAAGAACG-3’ (nt 1445–1466 of the 16S RNA gene) and 5’-GCCGCCCTTGTAGCTGACC-3’ (nt 20–21 of the 23S RNA gene) complementary to the 3’ end of the 16S RNA gene and the 5’ end of the 23S RNA gene were used to amplify the variable-length intergenic spacer region. The 338a and 027 strains were used as controls for ribotypes 001 and 027. Patterns that were different from these two ribotypes were compared with the library of PCR ribotypes already established at the Anaero Research Unit, Cardiff, UK.

Toxinotyping. All 149 isolates were subjected to toxinotyping by the methods developed by Rupnik et al. (1997, 1998). The first 3 kb of tcdB (PCR fragment B1) and the 3 kb repetitive region of tcdA (PCR fragment A3) were detected and characterized by RFLP. The primers 5’-AGAATAATTGATTTGATTAAAGAA-3’ and 5’-CAGATAATGAAGTAAGTCTATAGGC-3’ for the B1 fragment and 5’-TATGTAACAGCATGGATTATATACAG-3’ and 5’-TATTGAAACATATTGACCATAATC-3’ for the A3 fragment were used as described by Rupnik et al. (1997). PCRs were performed in a final volume of 50 μl with a reaction mixture containing 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 1% W-1, 3 mM MgCl2, 1 U Taq polymerase (Invitrogen), 200 μM each dNTP, 15 pmol each primer and 5 μl template DNA. For amplification of A3 fragments, tetramethylammonium chloride (Sigma) was added to a final concentration of 10−4 M. After initial denaturation at 93 °C for 3 min, B1 products were amplified for 30 cycles and A3 products for 35 cycles of annealing and extension at 47 °C for 8 min and denaturation at 93 °C for 4 s. Final extension was at 47 °C for 10 min. Amplified fragments were visualized on a 1% agarose gel and subjected to restriction enzyme digestion using the restriction enzymes AciI, HincII (B1) and EcoRI (A3). After electrophoresis of the digestion products, the toxinotypes of all tested isolates were determined using the toxinotyping schema described by Rupnik et al. (1997, 1998).
35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. A final step of extension for 10 min at 72 °C was performed. Products of 870 bp were digested with the restriction enzymes HpaI, HindIII and Rsal. Digested products were electrophoresed on a 1.2 % agarose gel to determine their RFLP groups. The restriction enzyme HindIII was used for further differentiation between group I and group III flagella types.

S-layer typing. The S-layer typing of C. difficile isolates was performed as described previously (McCoubrey et al., 2003). Briefly, the isolates were subcultured and S-layer proteins were extracted with 5 M guanidine hydrochloride. The resulting two major and several minor bands were visualized by SDS-PAGE (Invitrogen) with Coomassie staining. Mark 12 molecular mass standards were used as calibrations for the calculation of molecular masses. Banding patterns were compared with the previous types.

Antibiotic susceptibility testing. The MICs of 116 of the isolates for six antibiotics were determined using the agar dilution protocol in the NCCLS guidelines (NCCLS, 2001). The antibiotics and concentrations used were as follows: 16-0.125 μg ml⁻¹ for vancomycin, 32-0.5 μg ml⁻¹ for metronidazole, 256-4 μg ml⁻¹ for ceftriaxone, 32-0.5 μg ml⁻¹ for clindamycin, 32-0.5 μg ml⁻¹ for erythromycin and 64-1 μg ml⁻¹ for tetracycline (all from Sigma). The isolates were subcultured from cooked meat broth into pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K₁ and 1 mg NaHCO₃ ml⁻¹ and incubated overnight in an anaerobic chamber at 37 °C. After adjusting the turbidity to a 0.5 McFarland standard, aliquots (1-2 μl) of the cultures were spotted onto Brucella agar (Oxoid) supplemented with haemin, vitamin K₁ and 5 % lysed horse blood plus antibiotic of a given concentration using a multipoint inoculator and incubated anaerobically at 37 °C for 48 h. Control plates were also inoculated and incubated aerobically to check for aerobic growth. Strains NCTC 11223, 336a and 630 were used as control strains as their MICs were known from our previous study (Drummond et al., 2003).

The MICs of the isolates for moxifloxacin were determined by the Etest (AB Biodisk) as we were unable to obtain the pure substance from Bayer. The isolates were grown in pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K₁ and 1 mg NaHCO₃ ml⁻¹ and incubated overnight in an anaerobic chamber at 37 °C. The Etest was carried out by inoculating the surface of pre-reduced fastidious anaerobe agar (LabM) plates containing vitamin K₁, haemin and 5 % lysed horse blood with a 1 McFarland standard-matched inoculum. The inoculation was performed with cotton-tipped swabs and Etest strips were applied to the agar surface according to the manufacturer's instructions. Sufficient growth was obtained after 24 h and the ellipse was clearly visible. The end points were read at complete inhibition of all growth, including hazes and isolated colonies. Strains 630 and 027 were used as sensitive and resistant controls, respectively.

Breakpoints of susceptibility for each drug were chosen at the levels listed by the NCCLS. MIC₉₀ and MIC₅₀ values for each isolate were calculated using Microsoft Excel.

RESULTS AND DISCUSSION

Molecular typing

The 149 isolates included in this study were collected over a period of 2 months between August and October 2005. The results of the different typing methods are summarized in Table 1. All of the isolates were typable by the PCR ribotyping method and 15 different ribotype patterns could be discriminated. Ribotype 001 was the most common (n = 113, 75.8 %), followed by ribotype 106 with 12 isolates (8.1 %). The other ribotypes identified were 005 and 014 with four isolates each, ribotype 002 with three isolates and ribotypes 013 and 126 with two isolates, whilst the other ribotypes (020, 023, 042, 049, 070, 171) were represented by single isolates. Three isolates belonging to two different ribotypes were not able to be allocated a specific ribotype and may represent new types. No 027 strain was found. However, during the preparation of this manuscript the first case of 027 in Scotland was reported from the Glasgow area. These findings are consistent with other reports from

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*One isolate from each of these ribotypes could not be typed by fliC restriction analysis.

Table 1. Numbers and types of isolates determined by PCR ribotyping, toxinotyping and fliC restriction analysis.

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the UK prior to the recognition of the 027 strains in several areas of England. PCR ribotype 001 has been reported to be the most common type (53%) among hospitalized patients in the UK (Stubb et al., 1999). In an earlier study by our group, ribotype 001 was responsible for 78% of CDAD infections locally (McCoubrey et al., 2003). Recently, ribotype 106 has become prominent in England. In the period 1995–2003 it was at similar levels to those found in our study (8%); however, in 2005 it was the predominant strain at 26%, just above 027 strains and surpassing the 001 strains, which were both at 25% (Health Protection Agency, 2006). Prior to the recognition of 027 strains in Europe, particularly in The Netherlands and Belgium, there are only a few reports from other countries in Europe. In a Polish hospital, all environmental isolates and 11 of 31 neonatal isolates were found to belong to ribotype 001 (Martirosian et al., 1995), whereas ribotype 067 accounted for 39% of all isolates in Hungary (Urban et al., 2001). In one report from the Middle East, ribotypes 097 and 078 were reported to be responsible for over one-third of the cases of CDAD in Kuwaiti hospitals (Rotimi et al., 2003).

All of the isolates were subjected to toxinotyping. The B1 and A3 fragments of tcdA and tcdB were amplified as they have been reported to be the most variable fragments and good markers when searching for variant strains (Rupnik, 2001). C. difficile VPI 10463 (which has been defined as toxinotype 0; Rupnik et al., 1998) was used as a reference strain. This toxinotype 0 was observed in the majority of the isolates (96.6%, n=144). The five remaining isolates were of four different known toxinotypes: toxinotypes I, IV and XIII with one isolate each and toxinotype V with two isolates. No toxinotype III strains were found. There was no previous local information on the prevalence and distribution of different toxinotypes. When compared with the studies from Europe, the rates of variant (not 0) toxinotypes in our study were lower (Rupnik et al., 1998, 2001; Spigaglia & Mastrantonio, 2002). The profiles of the strain collections in these studies were different but non-toxigenic strains were not included in our study. The prevalence of variant toxinotypes has been reported to be 21.5% among the selected C. difficile isolates from 22 serogroups tested by Rupnik et al. (1998) and was estimated for the Cardiff collection as 8.8% among the toxigenic strains (Rupnik et al., 2001). The percentage of variant strains from Asia has been reported to be 23.5%, whilst 25% of the toxigenic strains from Italy were found to show variation (Spigaglia & Mastrantonio, 2002; Rupnik et al., 2003a). Of the strains from an American hospital, 11.1% belonged to variant toxinotypes. The most frequent variant toxinotypes in two European collections were toxinotypes III, IV and VII (Rupnik et al., 1998, 2001). Our variant strains were of toxinotypes I, IV, V and XIII. The toxinotype IV strain was of ribotype 023, whilst two toxinotype V strains were of ribotype 126. Toxinotypes I and XIII were of ribotype 001. In our study, among the 15 ribotypes that were determined, all isolates within a ribotype except ribotype 001 belonged to a single toxinotype. This was similar to the findings of Rupnik et al. (2001) where PCR ribotyping and toxinotyping were shown to correlate well. In the case of different toxinotypes within a PCR ribotype, these toxinotype profiles were found to be similar. In our study, only five isolates of toxinotypes other than 0 were found and isolates of ribotype 001 belonged to three different toxinotypes. Toxinotype I and XIII strains have been reported to differ from toxinotype 0 only at the 3' end of the tcdA gene (Rupnik et al., 1998).

PCR amplification of the flic gene from 147 isolates produced an 870 bp fragment. Two different restriction profiles were obtained when the amplification products were digested using the enzymes HpaI, HindIII and Rsal. All isolates but two were of restriction pattern VII, the exceptions being restriction pattern I, and were of ribotypes other than 001 (ribotypes 106 and 042) but were of toxinotype 0. Taste et al. (2000) compared PCR-RFLP analysis of the flagellin gene and serogroups in a collection of strains representing all of the 12 known serotypes from widely different geographic areas. They reported that this method could constitute an additional typing method to be used in conjunction with other methods. They found RFLP type VII as the most frequent RFLP type, followed by types I and VIII. RFLP type VII strains were mostly toxin-positive strains, whereas type I strains were either toxin positive or negative. They found that RFLP types II, III, IV, V and VI were uncommon and only associated with single serogroups. Our study is the first to compare ribotyping and toxinotyping with flagellin gene typing. However, only two flagella types were detected (I and VII): type VII strains contained different ribotypes and toxinotypes and type I strains were of different ribotypes but all of toxinotype 0. Thus a larger number of strains from different ribotypes and toxinotypes are needed to be able to demine the relationships between these typing methods.

All strains (n=149) were tested for the presence of binary toxin genes (cdtA and cdtB), but only three (2%) harboured these genes. Similar to the number of variant strains encountered above, the presence of binary toxin genes was low compared with other studies. It has been reported that 6.4% of toxigenic isolates of C. difficile referred to the Anaerobe Reference Unit from UK hospitals had both binary toxin genes (Stubbs et al., 2000) and 4.5, 5.8 and 8.6% prevalence of binary toxin-positive strains was detected in Spain, America and Poland, respectively (Geric et al., 2004; Alonso et al., 2005; Pituch et al., 2005). Pituch et al. (2005) found that all binary toxin-positive strains from Poland were of the same toxinotype, type IV, and were all of the same ribotype. In our study, two of the binary toxin-positive isolates were of ribotype 126, toxinotype V, and one isolate belonged to ribotype 023, toxinotype IV. In most studies, it has been shown that only strains belonging to variant toxinotypes that have significant changes in tcdA and tcdB possess binary toxin genes (Stubbs et al., 2000; Rupnik et al., 2003b). Geric et al.
(2003) reported A- B- strains with binary toxin genes. We had only toxigenic strains in our study. Our strains of toxinotypes IV and V with changes in both the tcdA and tcdB genes had binary toxin genes, whereas strains of toxinotypes I and XIII with minor differences only in the 3' end of the tcdA gene did not have cdtA or cdtB.

**S-layer typing**

In the past, we have used S-typing as our primary method for epidemiological studies of *Clostridium difficile* (McCoubrey et al., 2003). We were interested in correlating the different molecular types with S-type. However, as it is relatively labour-intensive, we selected a sample of only 62 isolates to represent the range of molecular types described above with the result that six different S-types were recognized. Most isolates (64.5%; n=40) belonged to the common S-type, known as type 4939, named for the molecular masses of the two S-layer peptides. In an earlier paper (McCoubrey et al., 2003), this common S-type, which is that of ribotype 001, was referred to as 5336. We have recently changed our SDS-PAGE system to a commercial system (Invitrogen) employing 10% gels. With this new method, the molecular masses of the S-layer proteins are found to be different. Most of the others were of S-types 4741 (21%; n=13) and 4640 and 4938 with three isolates each (4.8%). Of the remainder, two isolates (3.2%) were of S-type 4639 and one isolate (1.6%) was of S-type 4837. All ribotype 001 isolates (n=37) were of the same S-type (4939), whilst three of the isolates from different ribotypes were also of this S-type. All but one of the ribotype 106 isolates (n=11) were of S-type 5242, the other being of the common 4939 type. Ribotypes 002 and 014 also contained different S-types. The toxinotype 0 isolates (n=59) belonged to all six S-types, with the most common being S-type 4939 (n=38). Toxinotype I and XIII isolates, which were also of ribotype 001, were of S-type 4939.

The discriminatory power of different typing methods is an important consideration when selecting which method to use. The usual gold standard of PFGE is generally considered to have a higher degree of discrimination than PCR ribotyping. However, in the past, the typing ability of PCR ribotyping was higher than that of PFGE because DNA degradation occurred as a result of endogenous restriction enzymes in strains from serogroup G, which corresponds to PCR ribotype 001 (Collier et al., 1996; Bidet et al., 2000).

Currently, PCR ribotyping is preferred because of the ease and speed of the technique and because it is reported to be highly discriminatory and reproducible. Toxinotypes are reported to correlate well with the types obtained by two other typing schemes, serogrouping and PFGE typing (Rupnik et al., 1998), whilst toxinotyping and ribotyping methods correlate well. Most strains within a PCR ribotype belonged to a single toxinotype. Strains in toxinotypes I, III, IV, VI and VIII could be differentiated into several PCR ribotypes (Rupnik et al., 2001).

**Antibiotic susceptibility testing**

A major aim of this study was to determine the current antibiotic susceptibility patterns of the *Clostridium difficile* isolates in our region and to find out whether there was any relationship between the types and antibiotic susceptibilities. The susceptibility to antibiotics was investigated in a sample of 116 isolates of our collection by determining the MICs for seven antibiotics: metronidazole, vancomycin, erythromycin, clindamycin, ceftizoxime, moxifloxacin and tetracycline. Table 2 shows the ranges of MICs and resistance rates among the isolates for the seven antibiotics used, together with MIC50 and MIC90 values and breakpoints for the antibiotics. All isolates were sensitive to the two agents commonly used to treat CDAD, metronidazole and vancomycin, with a narrow range of MICs.

In our previous study (Drummond et al., 2003), no resistance to metronidazole or vancomycin was reported. MIC ranges and MIC50 and MIC90 values for these antibiotics were similar to those in the present study. However, the number of isolates with an MIC of 4 µg ml-1 for vancomycin increased from 5 out of 186 isolates (2.7%) in our earlier study to 25 out of 116 isolates (21.6%) in the present study. Vancomycin and metronidazole are the most common antibiotics used in the treatment of CDAD and, in most studies, isolates of *Clostridium difficile* have generally been found to be susceptible to these (Drummond et al., 2003; Aspervall et al., 2006). However, a few studies have reported strains resistant to metronidazole or with reduced susceptibility to vancomycin (Brazier et al.,

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (µg ml-1)</th>
<th>MIC50 (µg ml-1)</th>
<th>MIC90 (µg ml-1)</th>
<th>Breakpoint (µg ml-1)</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>1-4</td>
<td>2</td>
<td>4</td>
<td>≥8</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>≦0.5-4</td>
<td>1</td>
<td>2</td>
<td>≥8</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5-32</td>
<td>≥32</td>
<td>≥32</td>
<td>≥8</td>
<td>94.8</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5-32</td>
<td>8</td>
<td>16</td>
<td>≥8</td>
<td>62.9</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>32-256</td>
<td>64</td>
<td>64</td>
<td>≥64</td>
<td>87.1</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.25-32</td>
<td>≥32</td>
<td>≥32</td>
<td>≥4</td>
<td>87.1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≦1-64</td>
<td>≦1</td>
<td>2</td>
<td>≥16</td>
<td>4.3</td>
</tr>
</tbody>
</table>
2001; Peláez et al., 2005). The first UK isolate of C. difficile with reduced susceptibility to metronidazole was reported in 2001 (Brazier et al., 2001).

Resistance to clindamycin was seen in 73 isolates (62.9%). Tetracycline resistance was low, with only five isolates with MICs $\geq 16$ pg ml$^{-1}$. MIC$_{50}$ and MIC$_{90}$ values for erythromycin were $\geq 32$ pg ml$^{-1}$, showing that the majority of the isolates were highly resistant to this antibiotic ($n=110$). All of these isolates were resistant to erythromycin. Similar high resistance rates to the antibiotics ceftriaxone and moxifloxacin (87.1%) were also found. Their MIC$_{50}$ values were high and the same as their MIC$_{90}$ values.

Previously, moxifloxacin was reported to have good activity against Gram-positive bacilli including C. difficile (Hoogkamp-Korstanje & Roelofs-Willems, 2000), but reduced susceptibility to this antibiotic has been shown in several studies (Wilcox et al., 2000; Leroi et al., 2002). Clindamycin and ceftriaxone resistance rates did not show much difference from our previous study (Drummond et al., 2003).

**Antibiotic susceptibility in relation to molecular type**

Of the 116 isolates for which MICs were measured, 87 were of ribotype 001, 10 were of ribotype 106 and 19 were of other ribotypes. In terms of toxinotypes, 112 were of toxinotype 0, with one isolate each of toxinotypes I, IV, V and XIII. The antibiotic resistance patterns of these 116 isolates are detailed in Table 3.

In a study from the UK, PCR ribotypes 001 and 106 were found to be more resistant to erythromycin (98 and 100%, respectively) than other PCR ribotypes (John & Brazier, 2005). All of our ribotype 001 and 106 ribotypes were resistant to this antibiotic. We also found higher resistance levels to the antibiotics ceftriaxone and moxifloxacin, which were not tested in that study, among ribotype 001 and 106 isolates than among the other ribotypes. John & Brazier (2005) reported that clindamycin resistance was lower than erythromycin resistance in ribotypes 001 and 106, whilst ribotypes 015, 014, 005 and 002 had a higher frequency of resistance to clindamycin than to erythromycin. Of our isolates, only ribotype 002 had a higher clindamycin resistance level than erythromycin, but the frequency of clindamycin resistance was lower for ribotype 014, whilst both resistance rates were the same for ribotype 005. In a study in which clindamycin and fusidic acid resistances were determined (Aspevall et al., 2006), no particular relationship between PCR ribotypes and antibiotic resistance was found. The clindamycin resistance frequency in our study was lower than that found by Aspevall et al. (2006) (83%). In a study from Australia, the MIC range for moxifloxacin (0.75 to $\geq 32$ pg ml$^{-1}$) was found to be close to the resistance breakpoint with MIC$_{50}$ and MIC$_{90}$ values of 2 and 4 pg ml$^{-1}$, respectively (Leroi et al., 2002). Susceptibilities of clonal and distinct C. difficile strains from the UK to newer fluoroquinolones including moxifloxacin have been tested (Wilcox et al., 2000). Trovafloxacin and moxifloxacin were the most active fluoroquinolones with three- to fourfold more activity than older agents such as ciprofloxacin among genotypically distinct strains. Clonal strains that were epidemic ribotype 001 strains were sevenfold less susceptible to moxifloxacin compared with the distinct strains. The MIC range for this antibiotic was 0.12-16 pg ml$^{-1}$ and MIC$_{50}$ and MIC$_{90}$ values were 1 and 16 pg ml$^{-1}$, respectively (Wilcox et al., 2000). We found higher MIC$_{50}$ and MIC$_{90}$ values for moxifloxacin in our study. Most of the ribotype 001 (98.9%, $n=86$) and ribotype 106 (90%, $n=9$) isolates were resistant to moxifloxacin, whereas only six isolates (31.5%) from other ribotypes were resistant in our study.

Only two isolates of flagellin gene restriction pattern I were observed. All of these restriction type I isolates were sensitive to tetracycline and moxifloxacin and resistant to erythromycin. One was resistant to both clindamycin and

**Table 3. Percentage antibiotic resistance rates by ribotype and toxinotype**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Percentage of isolates resistant to antibiotics based on:</th>
<th>Ribotype</th>
<th>Toxinotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>001 ($n=87$)</td>
<td>106 ($n=10$)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td></td>
<td>95.4</td>
<td>100</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td>98.9</td>
<td>90</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*As toxinotypes I, IV, V and XIII are represented by single isolates, the designations R (resistant) and S (sensitive) have been used rather than percentages.
cephtriaxone, whilst the other was sensitive to both antibiotics.

One of the two isolates carrying the binary toxin genes was resistant to erythromycin, clindamycin and tetracycline, whilst the other one was only resistant to ceftriaxone. Both isolates were sensitive to moxifloxacin.

Fifty-six of the 62 isolates that were typed by S-layer were tested for antibiotic susceptibility. Most of the S-type 4939 isolates (38/39) and all of the S-type 4741, 4938 and 4639 isolates (n=10, n=3 and n=2, respectively) were resistant to erythromycin. Most of the S-type 4939 and 4741 isolates were resistant to clindamycin, ceftriaxone and moxifloxacin. One isolate that belonged to type S-4837 was sensitive to all antibiotics. This isolate was of ribotype 070, toxinotype 0 and fliC restriction type VII.

**Multi-resistant strains**

Seventy-two isolates were resistant to both erythromycin and clindamycin in our study, with resistance to both antibiotics being 62, 70 and 52.6% in ribotypes 001, 106 and the others, respectively. Of toxinotype 0 and other toxinotype isolates, 61.6 and 75% were resistant to both antibiotics, respectively. Only one fliC restriction pattern I isolate and one binary toxin gene-positive isolate resistant to these antibiotics were encountered. Macrolide-lincosamide-streptogramin B resistance in *Clostridium difficile* is mostly encoded by the ermB resistance determinant. This gene encodes a 23S rRNA methyltransferase that modifies the target site for the antibiotic and is a mobilizable, conjugative transposon, Tn5398 (Farrow et al., 2001). In recent years, some ermB-negative isolates with erythromycin and clindamycin resistance have been reported (Ackermann et al., 2003; Spigaglia & Mastrantonio, 2004; Pituch et al., 2006). Spigaglia & Mastrantonio (2004) could not find any erm genes of other classes such as ermA, ermC, ermF, ermQ and mefA among these isolates. It has been suggested that resistance in ermB-negative resistant strains could be due to mutations within the target sequences in the 23S rRNA or efflux mechanisms or a new mechanism of resistance (Ackermann et al., 2003; Spigaglia & Mastrantonio, 2004; Pituch et al., 2006). We did not test our isolates for resistance genotypically.

A total of 66 isolates were resistant to the three antibiotics erythromycin, clindamycin and ceftriaxone and 96 isolates were resistant to erythromycin, ceftriaxone and moxifloxacin. Ribotypes 001 and 106 had higher resistances (95.4 and 90%, respectively) to the antibiotics erythromycin, ceftriaxone and moxifloxacin when compared with other PCR ribotype groups (21%). Ackermann *et al.* (2001) suggested that resistance to moxifloxacin might be due to amino acid substitution in the DNA gyrase. They also found that moxifloxacin-resistant strains that were selected *in vitro* had wild-type gyrA sequences.

In our study, only one isolate was resistant to five antibiotics: erythromycin, clindamycin, moxifloxacin, ceftriaxone and tetracycline; it was of ribotype 001 and toxinotype 0. Ackermann *et al.* (2003) reported resistances to the antibiotics erythromycin, clindamycin and moxifloxacin as 27, 36 and 12%, respectively, among 192 isolates tested. They found that moxifloxacin resistance was almost always detected together with resistance to erythromycin and clindamycin (12.5%). In our study, 110 erythromycin-resistant isolates were found of which 100 (90.9%) were resistant to moxifloxacin and only one out of six erythromycin-sensitive isolates was resistant to moxifloxacin. Among the 73 clindamycin-resistant isolates, 64 (87.7%) were also resistant to moxifloxacin. Of the 72 isolates resistant to both erythromycin and clindamycin, 64 (88.9%) were resistant to moxifloxacin. Additionally, we found that all but one of the 64 isolates that were resistant to these three antibiotics were also resistant to ceftriaxone and these multi-resistant isolates were mostly of ribotype 001 (n=53, 84.1%).

As the 027 type has a characteristic antibiotic resistance pattern – resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin (Kuijper *et al.*, 2006b) – this could be part of an algorithm to identify 027 strains. However, in our study we identified 36 strains that were resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin. Thirty-two of these strains were of ribotype 001, three were of ribotype 106 and one was of 014, and all of them were of toxinotype 0. This questions the usefulness of this approach to detect 027 strains.

We are aware that a Europe-wide surveillance study has been performed (F. Barbut and others, unpublished) and some strains for this study were collected in Scotland. However, there was no overlap in strains between these studies as those for the European surveillance study were collected earlier in 2005.

The results obtained from this study demonstrate clearly the complexity of the strains of *Clostridium difficile* in our area. If characterized purely on ribotyping, it would appear that most of the strains are closely related. However, the use of other typing methods, especially antibiotic resistance patterns, demonstrates wide variation among strains.

**ACKNOWLEDGEMENTS**

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