Studies on the Aetiology, Diagnosis and Epidemiology of *Clostridium difficile*

by

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DECLARATION

I hereby declare that this thesis has been composed entirely by myself and, furthermore, that I have carried out all the work reported within, with the following exceptions.

1. Six of the PCR reactions and gels (from a total of more than 60) were performed by Warren Fawley, Department of Microbiology, University of Leeds. In addition Warren Fawley carried out the environmental screening of wards and typing of these isolates during the clinical trial.
2. During the evaluation of the kit, Oxoid toxin A tests were performed by Hannah Todd and Brian King.
3. The second evaluator of CPE tests in the comparison with the Oxoid toxin A test was Brian King.
4. A small quantity of media and cell culture preparations were prepared by Jane Freeman.

Permission has been obtained from the publisher for inclusion of a photocopy of the article: Comparison of the Oxoid *Clostridium difficile* toxin A detection kit with cytotoxin detection by a cytopathic effect method examined at 4, 6, 24 and 48 h.

I also declare that none of the work included in this thesis has been submitted for any other degree or professional qualification.

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LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

A-B+	toxin A negative, toxin B positive
ADP	adenosine di-phosphate
AP-PCR	arbitrary primer PCR
bp	base pair
BBM	brush border membrane
BHI	brain-heart infusion
BME	basal medium (Eagle) without glutamine
°C	degrees Centigrade
CCEY	cycloserine-cefoxitin egg yolk agar
CCFA	cycloserine-cefoxitin fructose agar
CDI	*Clostridium difficile* infection
CDSC	Communicable Diseases Surveillance Centre
CF	continuous flow
CFA	cellular fatty acid
CIE	counterimmunoelectrophoresis
CHO	Chinese hamster ovary
cm	centimetre
CMA	cycloserine-mannitol agar
CPE	cytopathic effect
CT	computed tomography
CTX	cefotaxime
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylene diamine tetra acetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
°F	degrees Farenheit
g	gram
g	gravitational force
GLC	gas-liquid chromatography
GTP	guanosine triphosphate
h	hour
HEp-2	human epithelial cell line 2
ICU	intensive care unit
IL-	interleukin-
kb	kilobase
kD	kilo Dalton
L	litre
LGI	Leeds General Infirmary
mg	milligram
min	minute
ml	millilitre
mm	millimetre
µm	micrometre
µl	microlitre
NK-1	neurokinin-1
ng	nanogram
nm	nanometre
NT	neurotensin

NNT	number needed to treat
NTP	nucleoside triphosphate
OR	odds ratio
PAGE	polyacrilamide gel electrophoresis
PaLoc	pathogenicity locus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PhD	Doctor of Philosophy
PHLS	Public Health Laboratory Service
PMC	pseudomembranous colitis
PyMS	pyrolysis mass spectrometry
PT	piperacillin-tazobactam
RAPD	randomly amplified polymorphic DNA
REA	restriction endonuclease analysis
RFLP	restriction fragment length poymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RR	rate ratio
RS-PCR	ribo-spacer PCR
SDS	sodium dodecyl sulphate
SJUH	St. James' University Hospital
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TER	transepithelial resistance
TNF-α	tumour necrosis factor-α
UK	United Kingdom
ULTH	United Leeds Teaching Hospitals trust
USA	United States of America
UV	ultraviolet
VFA	volatile fatty acid

ABSTRACT

Clostridium difficile is recognised to be the most common cause of infective diarrhoea in hospital patients. In addition, the incidence of this disease has been increasing throughout the 1990's, which is of particular concern amongst elderly patients. In this climate, it is of great importance to determine what are the most significant factors that predispose to this infection. This knowledge can then be used to prevent cases of the disease arising, by reducing the risk to the individual or to the hospital community as a whole.

Whilst knowledge about the aetiology of *C. difficile* infection (CDI) is very important, it is also valuable to study its epidemiology. This can help with our understanding regarding transmission of the disease, and help to identify epidemic clones. Such strains can then be further examined to try to determine the reasons for them being more frequently associated with disease. It is possible to check whether current diagnostic tests are adequate by monitoring for aberrant strains such as *C. difficile* toxin A negative / toxin B positive isolates. Constant, independent review of newly developed diagnostic tests is essential to ensure that they offer a sufficiently high level of sensitivity and specificity to be clinically useful.

New rapid methods for the diagnosis of CDI are constantly being developed. One of these, the Oxoid toxin A test (Unipath, Basingstoke, Hampshire, UK), was evaluated by comparison with a cytopathic effect (CPE) method, using 100 strains of *C. difficile*. The performance of the Oxoid toxin A test was only as good as the CPE method read at 6 hours, with a sensitivity of 72% and a specificity of 94%. This is not likely to be sufficiently accurate to be relied on as a single test for CDI. Furthermore, with the advent of disease caused by toxin A negative / toxin B positive strains, toxin A tests are no longer to be recommended for CDI diagnosis.

The epidemiology of CDI was studied in two ways. 1) The prevalence of *C. difficile* toxin A negative / toxin B positive strains was measured in 269 isolates from symptomatic hospital patients in Leeds and Bradford, using toxin gene PCR detection. 2) A comparison of *C. difficile* strain types was made between isolates from symptomatic community patients in Leeds and Truro, using ribo-spacer (RS) PCR. There were 15 isolates from Leeds and 39 from Truro. The epidemiological investigations indicated that there were no toxin A negative / toxin B positive strains apparent in Leeds or Bradford, although these strains have been reported from other areas of the country. The comparison of community strains revealed significant differences between Leeds and Truro. Amongst Leeds community patients, 60% of strains isolated were identical to the 'endemic' hospital strain (which accounts for over 80% of sporadic CDI cases in Leeds hospital patients). By contrast, this strain was not present in the Truro population ($p = <0.0001$).

In order to examine the likelihood of CDI following treatment with either cefotaxime (CTX) or piperacillin-tazobactam (PT), a prospective, ward based, crossover study was carried out. This was performed on two well matched care of the elderly wards, with patients who required empirical broad-spectrum antibiotic treatment. Although only 48 patients were enrolled in this study, a highly significant difference was noted in the incidence of CDI and *C. difficile* colonisation between the two groups. The odds ratio (OR) for CDI in CTX treated patients compared with those who received PT was 14.6 (CI= 1.7-124.7). The number needed to treat (NNT) to save one case of CDI was 2.18.

INTRODUCTION

1.1 HISTORICAL BACKGROUND

There have been several stages in the development of our knowledge regarding the aetiology of pseudomembranous colitis (PMC) and its relationship to *Clostridium difficile*. First, came the description by Finney in 1893, of a 'diphtheritic colitis' where membranous plaques were found in the distal small bowel, as well as membranes in the large bowel (Finney, 1893). At the time, the cause of the syndrome was not known. Subsequently, Hall and O'Toole (1935) discovered a bacterium in the stools of healthy newborn children, which they named *Bacillus difficilis* because of their difficulty in isolating it. They also observed it to produce a toxin which was lethal when injected into rabbits and guinea pigs (Hall and O'Toole, 1935); (Snyder, 1937). However, as it seemed to cause no harm to colonised infants, a pathogenic role in humans was not considered until much later.

Over the next few decades, several hypotheses were advanced as to the aetiology of PMC. One of the earliest was a suggestion that intestinal hypoxia, occurring secondary to hypotension could be the cause (Penner and Bernheim, 1939). Later, as antibiotic use became more widespread throughout the 1950's, *Staphylococcus aureus* was implicated because it had been isolated from some patients with diarrhoea (Altermeier *et al.* 1963); (Hummel *et al.* 1964); (Wakefield and Somers, 1953), and vancomycin appeared to have a beneficial effect on the condition (Khan and Hall, 1966). After it was realised that many cases of colitis seemed to have no link with *S. aureus*, (Dearing *et al.* 1960) and it had proved difficult to induce colitic changes in animal models using this organism (Kay *et al.* 1958); (Prohaska *et al.* 1956), the enthusiasm behind the theory waned.

Although some investigation of patients in whom *C. difficile* had been isolated from clinical samples was performed by Smith and King (1962), their conclusion was that the

toxin effects seen in animal studies were not relevant to human disease. Throughout the 1960's the incidence of PMC increased (Hummel *et al.* 1964), and studies in the 1970's indicated that lincomycin and clindamycin use was often associated with subsequent diarrhoea or colitis (LeFrock *et al* 1975). Small, (1968) observed that hamsters given lincomycin developed fatal enterocolitis, but the cause of this was not known. In 1973, Tedesco carried out a prospective study in 200 patients where the rate of diarrhoea following clindamycin therapy was 21% and the rate of PMC 10% (Tedesco *et al.* 1974). Other reports, however, showed lower incidence rates (Gurwith *et al.* 1977); (Ramirez-Ronda, 1974); (Swartzberg *et al.* 1977). At this time the condition was known as 'clindamycin colitis' (Kabins and Spira, 1975). In 1974, Green reported the detection of a cytotoxin in the faeces of guinea pigs following their treatment with penicillin (Green, 1974) (this was the first report of the action of toxin B, although at the time the effect was thought to be due to a virus). Also in this year, a PhD thesis was published by Hafiz (Hafiz, 1974), which was the most comprehensive piece of work on *C. difficile* at the time. It indicated that the organism was widespread in the environment and usually produced a toxin. At the time that these three simultaneous pieces of work were published, it was not appreciated that the organism, the toxin and PMC were related.

A few years later, in 1977, Larson described the first case of a cytopathic toxin in faeces (Larson *et al.* 1977), from a patient who developed colitis following a course of penicillin. Rifkin, in the same year, reported toxic stool filtrates from two patients, and went on to demonstrate that sterile filtrates from these patients produced a fatal enterocolitis when instilled into the peritoneum of hamsters (Rifkin *et al.* 1977). The effect was found to be neutralised by *Clostridium sordellii* antitoxin (Allo *et al.* 1979); (Chang *et al.* 1978a); (Larson and Price, 1977); (Rehg, 1980); (Rifkin *et al.* 1977) and so at the time, *C. sordellii* was felt to be the cause of PMC (Anonymous, 1977). However, in 1978 the true identity of

the aetiological agent in PMC was revealed to *be C. difficile* (Bartlett *et al.* 1978a); (George RH *et al.* 1978); (George *et al.* 1978a); (Larson *et al.* 1978).

1.2 CHARACTERISTICS OF *CLOSTRIDIUM DIFFICILE*

1.2.1 Morphology and cultural characteristics

C. difficile is a Gram-positive to Gram-variable bacillus of 0.3-0.8μm in diameter and 2-9μm in length and is not acid-fast. It has parallel sides, produces large, oval, subterminal spores that swell the cell slightly and is motile in early broth culture by means of peritrichate flagella. *C. difficile* grows optimally at 30-37°C, but can also survive at 25°C or 45°C. Being an obligate anaerobe, it will not grow in the presence of oxygen. Culture is usually performed in a nitrogen/hydrogen atmosphere, using either an anaerobic cabinet or an anaerobic jar. It does not require blood to grow and colonies on most media become apparent, although small, at 24 h of incubation, with typical morphology being achieved by 48 h.

Colonial morphology may be quite variable, especially on blood agar, but the organism does produce a very characteristic smell, variously described as similar to that of horse or elephant manure. After 48 h incubation on selective media, most colonies appear 2-4 mm in diameter, circular, flat and spreading with irregular edges. They are usually a greyish colour with a granular, ground-glass appearance. If scraped from the agar plate onto a swab, the colony appears slightly yellowish in colour and is of a butyrous nature. On blood agar the colonies are non-haemolytic and may appear more smooth and shiny in nature with a whitish colour, whilst retaining irregular edges, although some colonies do still show the

granular, ground-glass appearance. The typical odour is less noticeable when the organism is cultured on blood agar as opposed to a selective medium. Particularly when grown on blood agar, a single strain may exhibit more than one type of colonial morphology simultaneously. Colonies have also been noted to fluoresce a yellow-green colour on exposure to long-wave (360nm) ultraviolet (UV) radiation (George *et al.* 1979). However, this feature is rather medium dependent and some selective media used for *C. difficile* isolation, e. g. cycloserine-cefoxitin fructose agar (CCFA), autofluoresce due to the neutral red indicator (Kauffman and Weaver, 1960). When inhospitable conditions are encountered by *C. difficile*, then spores are produced as a means of survival. Spore production on selective media is not as pronounced as on blood agar, but recovery can be enhanced by the addition of 0.1% sodium taurocholate to solid growth media (Wilson *et al.* 1982).

C. difficile is catalase, oxidase, indole, nitrate, DNAse and urease negative but hydrolyses aesculin and liquefies 2% gelatin. It is lipase and lecithinase negative. Other useful tests include prolyl aminopeptidase (+ve), leucine aminopeptidase (+ve), ß-galactosidase (-ve), and acid phosphatase (-ve) (Aspinall and Dealler, 1992).

1.2.2 Structure

The cellular fatty acids (CFA) of *C. difficile* have been studied (Moss and Lewis, 1967) and more recently CFA profiles have been shown to vary with nutritional conditions (Hopkins and MacFarlane, 2000). Drucker *et al* (1996) reported the phospholipid profiles of 32 outbreak strains and cell-surface carbohydrate antigens have also been investigated (Poxton and Cartmill, 1982). *C. difficile* is known to produce capsular material (Davies and Borriello, 1990) and to possess several peritrichous flagella for movement. The flagellin gene has been sequenced and the flagellin characterised, having a molecular mass of approximately 39kDa (Tasteyre *et al.* 1997); (Tasteyre *et al.* 2000a). Polar fimbriae are also

present on the cell, which are 4-9 nm in diameter and 6 μm in length (Borriello *et al.* 1988a). Several *C. difficile* surface proteins have been proposed as playing a possible role in adhesion. Eveillard *et al.* (1993) and Karjalainen *et al.* (1994) have described 27 kDa and 40 kDa proteins, whilst Waligora *et al.* (1999) described 40 kDa, 50 kDa And 70 kDa proteins which could bind to Vero cells.

1.2.3 Metabolism

C. difficile has a fermentative, proteolytic metabolism, producing acid and gas from glucose, aesculin, fructose, mannitol and mannose. Its major metabolic products are acetate and butyrate, although isobutyrate, isocaproate and isovalerate are produced in lesser amounts. One particular property of *C. difficile* is its deamination of phenylalanine to parahydroxyphenylacetic acid and further decarboxylation of p-hydroxyphenylacetic acid to produce p-cresol (Phillips and Rogers, 1981), which gives rise to a characteristic odour.

1.2.4 Virulence factors

There have been quite a number of established and proposed virulence factors previously described for *C. difficile* (Borriello, 1990); (Borriello, 1998); (Larson *et al.* 1978). The main virulence factors are two toxins, designated toxin A and toxin B. However, other factors are also important, especially in allowing the organism to establish itself in the gut. These include adherence to the intestinal mucosa, chemotaxis and motility, capsule production and hydrolytic and proteolytic enzyme production. Work in the hamster model has demonstrated that not all strains of toxin producing *C. difficile* are equally virulent (Borriello *et al.* 1987); (Delmée and Avesani, 1990). Furthermore, non-toxigenic strains are felt to have little if any pathogenic potential (Lyerly *et al.* 1988). Although

patients with diarrhoea have had such strains detected in their stools, it is not known whether they were involved in the aetiology of the diarrhoea.

1.2.4.1 Toxins A and B

1.2.4.1.1 Physical properties of the toxins

C. difficile manufactures several toxic products. The two most widely known, and generally thought to be the organism's main virulence factors, are toxin A and toxin B (Borriello *et al.* 1990); (Sullivan *et al.* 1982); (Taylor *et al.* 1980); (Taylor *et al.* 1981). Although these have also been described as enterotoxin and cytotoxin respectively, this nomenclature is slightly misleading. Both toxins are able to cause a cytopathic effect, with toxin B being far more potent in this respect (Lyerly *et al.* 1988); (Sullivan *et al.* 1982). However, toxin B does not possess any enterotoxin like properties. Levels of toxin produced can differ between strains by more than 6 logs (a million times) although equal amounts of each toxin are thought to be generated (Lyerly *et al.* 1988). In addition, three other factors have been described.

One appears to be a poorly stable enterotoxin (Banno *et al.* 1984); (Giuliano *et al.* 1988); (Mitchell *et al.* 1987), another has been shown to produce changes in electrical potential in isolated segments of rabbit intestine (Justus *et al.* 1982), whilst the third is described as an actin-specific ADP-ribosyl-transferase (Popoff *et al.* 1988). Torres *et al.* (1990) also described three enterotoxic factors, termed C1, C2, and C3. The precise function of these latter three factors, along with their role in the pathogenesis of *C. difficile* disease, if any, is uncertain.

In contrast, there have been numerous reports characterising both the nature and properties of toxins A and B (Gianfrilli *et al.* 1984a); (Lyerly *et al.* 1982); (Lyerly *et al.* 1986a); (Lyerly *et al.* 1986b); (Meador and Tweten, 1988); (Pothoulakis *et al.* 1986); (Rihn *et al.* 1984); (Rolfe and Finegold, 1979); (Taylor and Bartlett, 1979); (Thelestam and Bronnegard, 1979); (von Eichel-Streiber *et al.* 1990), as well as their effect on gut mucosa in the animal model (Libby *et al.* 1982). Both toxins are found to have a cytotoxic effect, with toxin B being considerably more potent (Knoop *et al.* 1993); (Lyerly *et al.* 1985). They also both result in increased vascular permeability and haemorrhage. In addition, toxin A causes marked fluid accumulation in various animal models (Ketley *et al.* 1987); (Lima *et al.* 1988); (Mitchell *et al.* 1986), resulting in it being called enterotoxin. However, toxin A does not act as a secretagogue, but results indirectly in fluid accumulation, secondary to mucosal damage and release of cytokines from leucocytes (see section 1.2.4.1.6). Kamiya and Borriello, (1992) report detection of a form of toxin A without haemagglutinating capability but which retains cytotoxicity and suggest that it might be a pro-toxin. The toxin genes for *C. difficile* have been sequenced (Barroso *et al.* 1990); (Dove *et al.* 1990) and the toxins characterised, with molecular masses of 308 kDa for toxin A and 269 kDa for toxin B (Johnson JL *et al.* 1990); (Sauerborn and von Eichel-Streiber, 1990). The two toxins are heat labile and are susceptible to proteolytic breakdown, with toxin B being the less stable of the two (Banno *et al.* 1984); (Sullivan *et al.* 1982); (Taylor *et al.* 1981). They have been shown by von Eichel-Streiber *et al.* (1992) to have 63% amino acid homology and both are thought to have receptor binding domains in the carboxy terminal repeat region (von Eichel-Streiber and Sauerborn, 1990); (Pothoulakis, 1996a), with enzymatic domains localised to the amino terminus (Faust *et al.* 1998); (Hofmann *et al.* 1997); (von Eichel-Streiber *et al.* 1995). The carboxy terminal peptide repeat sequence of toxin A (Dove *et al.* 1990) has been shown to be homologous to the carbohydrate binding region of streptococcal glucosyltransferases (von Eichel-Streiber and Sauerborn, 1990).

1.2.4.1.2 Animal model experiments

As discussed in section 1.1, early work indicated that *C. difficile* was associated with PMC and that the organism produced toxic factors. Chang *et al.* (1978b) described the colitis, which develops in hamsters following clindamycin administration, as a model for *C. difficile* colitis in humans. Animal experiments have been conducted using other species but none are as susceptible as hamsters to *C. difficile* disease (Fekety, 1974). Another early observation was that faecal filtrates from patients with PMC, or caecal contents of hamsters injected with clindamycin, caused death in normal hamsters when injected intracaecally (Bartlett *et al.* 1977a); (Bartlett *et al.* 1978b); (Bartlett *et al.* 1980). Following these discoveries, many investigators continued their research by trying to characterise the nature and function of these toxins using both in vitro, as well as in vivo (animal model) methods. Initial attempts at purification and characterisation focussed upon the cytotoxin (Humphrey *et al.* 1979); (Rolfe and Finegold, 1979); (Taylor and Bartlett, 1979). Then came evidence of another distinct toxin with enterotoxin like properties (Banno *et al.* 1981); (Taylor *et al.* 1981). Taylor *et al.* (1981) demonstrated that this enterotoxin was distinct from the previously known cytotoxin, and was capable of inducing haemorrhagic fluid accumulation in ligated rabbit ileal loops. These loops also showed histological changes of haemorrhage and mucosal destruction. Similar effects were seen when the enterotoxin was injected into ligated hamster ileal and large bowel loops. When the enterotoxin was injected into the unligated caeca of hamsters, it was lethal, whereas cytotoxin only caused small areas of focal haemorrhage. This enterotoxin was designated as toxin A, and felt to be the most significant toxin in the pathogenesis of *C. difficile* disease.

Sullivan *et al.* (1982) purified both toxins and demonstrated their cytotoxic effects on Chinese hamster ovary (CHO-K1) cells. They also demonstrated that both toxins were lethal to mice when injected intraperitoneally. The lethality of both toxins has been

demonstrated by a number of investigators, using several animal models, either by intravenous, intraperitoneal or subcutaneous injection (Arnon *et al.* 1984); (Banno *et al.* 1981); (Banno *et al.* 1984); (Lyerly *et al.* 1986b); (Sullivan *et al.* 1982); (Taylor *et al.* 1981). Although both toxins appeared equally potent in animal lethality tests by Sullivan *et al.* and Lyerly *et al.*, other investigators found toxin A to be far more potent than toxin B. The possible explanation for this discrepancy is that the toxin preparations may not have been equally pure, or that because toxin B is less stable (Banno *et al.* 1984); (Sullivan *et al.* 1982); (Taylor *et al.* 1981), storage conditions may have allowed it to deteriorate faster than toxin A.

Lyerly *et al.* (1985) investigated the effects of toxins A and B given intragastrically to rats, mice and hamsters. They demonstrated that hamsters were far more susceptible to *C. difficile* toxins than either mice or rats. Culture filtrates, as well as toxin A given alone, resulted in intestinal pathology and death, whereas neither feature was observed after toxin B administration alone. However, if small quantities of toxin A (too low to cause pathological changes alone) were given with toxin B, hamsters became ill and died. Similarly, if toxin B was administered to animals with bruised caeca, pathological changes and death were observed. This suggests that toxin B is only able to manifest its toxic properties if it has access to deeper tissues via a damaged or disrupted mucosal surface. Furthermore, it is likely that in the presence of significant amounts of toxin A, it can contribute significantly to the development of *C. difficile* colitis. Therefore previous observations that toxin B alone was lethal after intracaecal injection (Libby *et al.* 1982) may have resulted from the fact that associated damage to the mucosa allowed the toxin deeper access than if it had been administered orally. Another observation from this study was that repeated small doses of toxin A appeared to have a cumulative effect, suggesting that the effects of the toxin on the mucosa are not transient. Mitchell *et al.* (1986) and Lima *et al.* (1988) demonstrated that the effects of toxin A were more pronounced on rabbit ileum than on the colon, and their

findings concurred with those of Triadafilopoulos *et al.* (1987) that toxin B alone does not have an enterotoxic effect.

Kim *et al.* (1987) investigated the effect of immunising hamsters against toxin A or toxin B to try and help elucidate the relative contribution of each toxin to disease. They found that immunisation against toxin A alone, but not against toxin B alone was sufficient to protect against clindamycin induced colitis. If lethal doses of either toxin were administered orogastrically however, then only immunisation against the challenging toxin protected against death. The conclusion was that toxin A is the more important toxin in hamsters and that in the course of enterocolitis, levels of toxin B alone do not get high enough to cause death. This allows immunisation against toxin A alone to protect against death because the synergistic interaction between the two toxins is abolished. Further evidence for the hypothesis that both toxins play a part in disease comes from the observation that a greater percentage of infant hamsters suckling from mothers immunised against only toxin A became ill, compared with a group suckling from mothers immunised against both toxins.

1.2.4.1.3 In vitro experiments using human cell lines and tissues

There is evidence that different strains of *C. difficile* produce differing quantities of toxin (Wren *et al.* 1987), and a suggestion that this may affect their pathogenic potential. Other evidence supports the theory that the amount of toxin produced by a strain of *C. difficile* may relate to its virulence. Viscidi *et al.* (1981) measured the toxin concentrations in vitro of *C. difficile* isolates which were obtained from healthy neonates, adults with toxin associated diarrhoea, adults on antibiotics without diarrhoea and adults on antibiotics with toxin negative diarrhoea. They found that the in vitro toxin titres of the strains from neonates and from adults with toxin positive diarrhoea were much higher than those

produced by strains from the asymptomatic adults on antibiotics or individuals with toxin negative diarrhoea. It is possibly because some strains produced lower quantities of toxin that they were less pathogenic. Discussion of why neonates remain healthy despite the presence of *C. difficile* toxins at high titres will take place in section 1.5.2.2.1.1.

Because of the suggestion of a synergistic interaction between the two toxins, and although some evidence in the hamster model indicated that the degree of toxin A production was better correlated with severity of disease than that of toxin B (Borriello *et al.* 1987), further work was carried out using a human in vitro model. This was in order to try to ascertain whether the effects of toxin A and B were different on the human bowel mucosa, compared with the animal models. A study using human colonic mucosal sheets in Ussing chambers demonstrated that toxins A and B cause dose-dependent, electrophysiological changes as well as morphological damage (Riegler *et al.* 1995). Previous work with polarised human intestinal T84 cells indicated that in apical (lumenal) exposure tests, toxin A was the more potent of the two (Hecht *et al.* 1988); (Hecht *et al.* 1992), but this study showed toxin B to be ten times more potent than toxin A. Another finding was that the degree of mucosal damage was correlated with the toxin concentration, suggesting that higher concentrations of toxin may give rise to more serious colitic changes in patients. Work using cultured nonpolarised Don cells indicated that toxin B was 1000 times more potent at intoxicating the cell than toxin A (Chaves-Olarte *et al.* 1997).

Recently, a very elegant study by Stubbe *et al.* (2000) demonstrated the effects of toxins A and B on human colonic carcinoma-derived T84 cell monolayers. Using a system whereby either the lumenal (apical) or serosal (basolateral) surface of the cell layer could be exposed to toxin, they measured transepithelial resistance (TER) (as the most sensitive measure of barrier function) as well as tight junction integrity and monolayer morphology. Toxin A added apically resulted in a substantial reduction in TER, whereas toxin B added

apically showed no effect. When added basolaterally, both toxins exerted a substantial reduction in TER, with toxin B showing the greater fall. The effect of toxin A on tight junctions after 6 h was minimal, but complete disorganisation had occurred by 24 h when added apically. Toxin B added apically had no effect on tight junctions. When added basolaterally, however, both toxins completely disrupted the tight junction network after 6 h. Morphological changes with apical toxins only occurred with toxin A which demonstrated a slight enlargement of the space between cells at 6 h, but complete disorganisation by 24 h. Both toxins added basolaterally caused changes similar to apical toxin A, with a more rapid effect of toxin B. Addition of a very small quantity of toxin A (with scarcely any change in TER) plus toxin B apically resulted in a greater TER drop than with toxin A alone. Finally, using monoclonal antibodies to toxin A Stubbe *et al.* showed that it was possible to protect against the effects of a toxin A plus B mixture added apically but not when added basolaterally. These findings indicate that toxin B cannot penetrate normal tight junctions in the bowel mucosa, but if these are damaged by toxin A, then toxin B will cause severe cytotoxic effects on the lamina propria and lower layers. This work also suggests that there are unlikely to be receptors for toxin B on the apical surface of intestinal mucosal cells, but that they may well exist on the basolateral surface. The apparent lack of a functional site for toxin B on the apical surface of intestinal mucosal cells could be the explanation for the discrepancy between mucosal binding activities of toxins A and B previously noted (Rolfe, 1991).

1.2.4.1.4 Toxin receptors

A proposed receptor for toxin A has been described on rabbit erythrocytes as well as on hamster brush border membranes (BBM) (Krivan *et al.* 1986). This receptor is a glycoprotein which contains the trisaccharide Galα1-3Galβ1-4GlcNAc. The same receptor

has also been identified in the rat ileal BBM (Pothoulakis *et al.* 1991), and a slightly different one in the rabbit ileal BBM (Clark *et al.* 1987); (Pothoulakis *et al.* 1996b). This trisaccharide is not expressed on human cells, so the human toxin A receptor must be different but does appear to be a glycoprotein (Pothoulakis *et al.* 1992). Lewis I, X and Y antigens can bind toxin A (Tucker and Wilkins, 1991) and may act as functional receptors but they are not universally expressed by intestinal epithelial cells. The relative distribution of toxin A receptors in the gut epithelium may be one of the factors which determines the severity of disease in an individual. Toxin A binding activity in the newborn rabbit BBM is absent, with a diminished biological response to toxin. Biological response and binding activity then rise together to reach adult levels by 30-40 days of age (Eglow *et al.* 1992). This finding may result from the absence of receptors at the time of birth, which then develop by the age of 30-40 days. A similar observation has been made with infant hamsters, which can be harmlessly colonised by *C. difficile* between the ages of 4-13 days, despite high toxin titres. Outside this window, they do not become colonised with *C. difficile* unless treated with antibiotics, which results in the development of colitis and death. As proposed by Chang *et al.* (1986), such a deficiency of receptors may also be the reason why neonates and infants are so often asymptomatically colonised by *C. difficile*, even in the presence of high toxin titres. Very little is currently known about toxin B receptors, although from the work by Stubbe *et al.* (2000), it would be reasonable to expect them to be present on the basolateral, rather than the apical surface of the intestinal mucosal cells.

1.2.4.1.5 The cellular mechanism of *C. difficile* toxins

After binding to their respective receptors, toxins A and B enter the cell and result in alterations of actin containing filaments which causes cell rounding. The alterations consist of a decrease in filamentous (F) actin with a reciprocal increase in globular (G) actin (Pothoulakis *et al.* 1986). Calcium is known to be important if these changes are to occur, as

depletion of intracellular calcium abolishes the effect of toxin B on actin microfilaments (Gilbert *et al.* 1995). These changes are now known to be mediated by the effect of toxins A and B on Rho proteins, which are low molecular weight GTP-binding proteins of the *ras* superfamily (Dillon *et al.* 1995); (Just *et al.* 1994); (Just *et al.* 1995a). RhoA, one of the Rho family proteins, is involved in actin assembly regulation (Hall, 1998) and is now known to be glycosylated at threonine[37] (located in its effector domain) by both toxins A and B (Just *et al.* 1995b); (Just *et al.* 1995c). Two other proteins of the Rho family, Rac and Cdc42 are also glucosylated by *C. difficile* toxins. Such a modification of the Rho family proteins renders them inactive and prevents polymerisation of actin filaments, resulting in cell retraction and rounding.

1.2.4.1.6 Mechanism of *C. difficile* toxin mediated inflammation

The precise way in which *C. difficile* toxins cause colitic changes in the colon is not fully understood, but the process appears to be a complicated one. In addition to the direct affects on actin microfilaments described above, which disrupt the intestinal epithelium, subsequent actions on lamina propria neuroimmune cells, T cells, macrophages and eosinophils may be significant. Toxin A is known to be able to stimulate a chemotactic response in human granulocytes (Pothoulakis *et al.* 1988). Branka *et al.* (1997) found that IL-8 production in a polarised homogenous goblet cell line (HT29-Cl.16E) was up-regulated in only 2-3 hours following exposure to low quantities of toxin A. This was associated with down-regulation of mucin exocytosis in the cell line. IL-8 is known to be an important factor in neutrophil chemotaxis and activation (Baggiolini *et al.* 1989). Neutrophil infiltration has also been noted to be a feature following exposure of rabbit distal colon to toxin A (Burakoff *et al.* 1995); (Kelly *et al.* 1994). Several other factors have been shown to be important in *C. difficile* induced enteritis, by experiments where specific inhibitors were used to abolish the effects of *C. difficile* toxins on the bowel mucosa in animal models or in

vitro. Both neurokinin-1 (NK-1) receptors (also known as substance P receptors) and mast cell degranulation have been shown to be involved in *C. difficile* disease in this way (Castagliuolo *et al.* 1998); (Wershil *et al.* 1998). Individuals with *C. difficile* induced PMC have been shown to have higher levels of NK-1 receptors in the small blood vessels and lymphoid aggregates of the bowel than control patients with ulcerative colitis (Mantyh *et al.* 1996). More recently, Castagliuolo *et al.* (1999) have reported that a bioactive peptide, neurotensin (NT) is involved in the early stages of *C. difficile* toxin effects on gut mucosa. Administration of NT to rats has been found to result in mast cell degranulation (Carraway *et al.* 1982) and increases in vascular permeability, histamine and leukotriene C4 levels (Carraway *et al.* 1991). Castagliuolo *et al.* (1999) demonstrated that NT and NT receptors are found at elevated levels in rat colonic mucosa early in the course of *C. difficile* colitis. NT receptor antagonists were found to prevent toxic effects of toxin A on the bowel. Reduced mast cell degranulation was noted and consequently reduced neutrophil infiltration, as mast cell mediators can initiate neutrophil chemotaxis (Wershil *et al.* 1996). NT has also been shown to stimulate the release of NK-1 (Carraway and Mitra, 1994); (Stapelfeldt and Szurszewski, 1989). Additionally, Castagliuolo *et al.* (1999) demonstrated that rat colonic explants did not manifest mast cell degranulation after NT stimulation if NK-1 receptors were blocked. This suggests that NT is causing mast cell degranulation via an NK-1 mediated route. As well as mast cell degranulation, NK-1 is stimulatory to monocytes, resulting in the release of the proinflammatory cytokines interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Lotz *et al.* 1988) and increases neutrophil cytotoxicity (Wozniak *et al.* 1989). Castagliuolo *et al.* (1999) conclude that neuroendocrine N cells in the intestinal mucosa, which are contiguous with the bowel lumen can be stimulated by toxin A to release NT. In turn, this results in the stimulation of cells in the lamina propria to secrete NK, and mast cell degranulation is increased. NK also stimulates other immune cells to release proinflammatory cytokines and neutrophils are

chemotactically attracted to the area. Another effect of these inflammatory mediators may be to up-regulate NT receptors.

Toxin B has also been shown to have some direct effects on lamina propria white cells including macrophages (Sousa *et al.* 1997) and mast cells (Wex *et al.* 1997), resulting in the further release of inflammatory cytokines. Access of toxin B to the lamina propria is achieved after the destruction of tight junctions by toxin A, as earlier discussed in section 1.2.4.1.3.

Apart from the local inflammation which results from macrophage and neutrophil release of proinflammatory mediators, both toxins A and B are able to induce apoptosis in intestinal epithelial cells (Fiorentini *et al.* 1998); (Mahida *et al.* 1998). When due to toxin A, the release of interleukin-8 (IL-8) is another feature. Apoptosis secondary to toxin B may also result in the increased release of cytokines that can increase local tissue damage (Castagliuolo *et al.* 1999).

1.2.4.1.7 Importance of both toxins

For a long time toxin A was felt to be the most important factor in the development of symptomatic *C. difficile* disease, primarily because of evidence from the hamster model mentioned above. More recent evidence has shown toxin B to play a significant part in damaging the intestinal mucosa (Riegler *et al.* 1995). In addition, strains of *C. difficile* which do not produce normal toxin A have been associated with disease in children (Depitre *et al.* 1993); (Kato *et al.* 1998) and adults (al-Barrak *et al.* 1999); (Brazier *et al.* 1999a); (Kato *et al.* 1997); (Limaye *et al.* 2000); (Sambol *et al.* 2000). This further reinforces the importance of toxin B. Both toxins are felt to act in a synergistic fashion to cause the pathological changes that result from *C. difficile* infection.

It was generally thought that *C. difficile* strains were either toxigenic, with both toxins produced, or non-toxigenic with neither (Lyerly *et al.* 1988). However, Gianfrilli *et al.* (1984a) did report that 30% of strains of *C. difficile* from patients with diarrhoeal symptoms or PMC appeared to have detectable levels of toxin B only. Haslam *et al.* (1986) described a cytotoxigenic strain that did not produce toxin A when grown in media lacking in some amino acids. Fluit *et al.* (1991), investigated 59 strains of *C. difficile* and demonstrated that toxigenic strains had both toxin genes whilst non-toxigenic strains had neither. In 1991, McMillin *et al.* described a strain which, whilst possessing the genetic material for toxin A and B production, was not found to be toxigenic under the conditions tested (McMillin *et al.* 1991). This suggests that the organism can possess both toxin genes without necessarily expressing them. Subsequent work has demonstrated that some strains have a deletion in the toxin A gene and produce a modified toxin A, which is lacking in its carboxy terminal repeat sequences (Torres, 1991); (Borriello *et al.* 1992a); (Depitre *et al.* 1993); (Lyerly *et al.* 1992); (Sambol *et al.* 2000). Such a deletion renders it undetectable by traditional ELISA methods, which rely on this region for toxin detection, but still capable of causing *C. difficile* disease (Kato *et al.* 1997); (Limaye *et al.* 2000); (Sambol *et al.* 2000). In addition, one such strain (8864) has been noted to produce slightly different toxin B to normal strains (Borriello *et al.* 1992a); (Lyerly *et al.* 1992); (Torres, 1991) and this toxin B has been found to be more cytotoxic than normal by 5-10 fold (Lyerly *et al.* 1992). Conversely, a similar toxin A negative strain, known as 1470 has been shown to have a toxin B which is less potent than that from type strains of *C. difficile* (von Eichel-Streiber *et al.* 1995). One report of a strain producing toxin A but lacking toxin B production has been made (Smith *et al.* 1992), however this has not subsequently been confirmed by other groups. Cohen *et al.* (1998) report a case of recurrent CDI where the strain causing disease for the third time was lacking at least part of the toxin B gene.

1.2.4.2 Adherence to intestinal mucosa

Adherence to the intestinal mucosa is thought to be an important step in the progression towards disease caused by *C. difficile*. It was first noted to occur when *C. difficile* was isolated from a washed biopsy sample, taken from a patient with PMC (Borriello, 1979). Later work by Borriello *et al.* (1988b) in a hamster model, indicated that a virulent strain was more adherent to the bowel mucosa than a poorly virulent strain or an avirulent strain. *C. difficile* is known to possess fimbriae although their possible role in adhesion remains unclear at present (Borriello, 1998a). Several workers have described cell surface proteins, which could be involved with attachment to the gut mucosa (Eveillard *et al.* 1993); (Karjalainen *et al.* 1994); (Waligora *et al.* 1999). Environmental factors such as sodium, calcium or iron concentration, as well as pH appear to affect the adherence of *C. difficile* to vero cells in anaerobic conditions (Waligora *et al.* 1999). Other physical properties have also been proposed as being contributory towards mucosal attachment. *C. difficile* cells are found to be moderately hydrophobic (Wood-Helie *et al.* 1986) and have an evenly distributed, net positive charge (Krishna *et al.* 1996). This may facilitate interaction with negatively charged cells in the gut mucosa as well as with gut mucus, and contribute towards the known phagocytosis resistance of *C. difficile* (Dailey *et al.* 1987). Adherence of an avirulent strain to large and small bowel mucosa in a hamster model was increased to the level seen in a virulent strain when co-administration of toxic culture filtrate was performed (Borriello *et al.* 1988b). Heat inactivated toxin had no effect on adherence. This suggests that adherence may be facilitated by toxins or some other secreted factor, and not that avirulent strains lack some important cell surface structure or attachment protein.

1.2.4.3 Chemotaxis and motility

Gut mucus has been demonstrated to be a chemoattractant for *C. difficile* in humans as well as in animal models (Borriello and Bhatt, 1995). In a hamster model, the degree of attraction correlated positively with the virulence of the strain used (Borriello and Bhatt, 1995). The flagella of *C. difficile* may play a part in adherence, as without these, directed movement towards the mucosa would not be possible. However, Tasteyre *et al.* (2000b) found that an antiserum raised against purified flagellin did not inhibit adherence to cultured cells. This cannot be taken as proof that flagella do not aid adherence as even without flagella, bacteria may sediment down to the cell monolayer spontaneously and then become attached.

1.2.4.4 Capsule production

C. difficile is known to be resistant to phagocytosis (Dailey *et al.* 1987), and one of the mechanisms by which this is achieved may be its production of a polysaccharide capsule (Davies and Borriello, 1990). Davies and Borriello noted the capsular material to be either loose-knit and branching, extending 350 nm from the cell wall, or dense and compact extending only 100 nm. Growth on agar appeared to demonstrate microcolonies covered by glycocalyx. Polymorphonuclear leucocytes, attracted to the mucosal surface but unable to ingest *C. difficile*, may contribute to local tissue damage by release of digestive enzymes.

1.2.4.5 Proteolytic and hydrolytic enzyme production

Enzyme production has been known to be a feature of *C. difficile* since as long ago as 1975, when Hafiz and Oakley (1976) described the production of hyaluronidase and gelatinase in 100% of 30 strains tested. Subsequent investigators have described the

production of other hydrolytic enzymes, including chondroitin-4-sulphatase and collagenase (Steffen and Hentges, 1981). Further detailed study of the breakdown of connective tissue (Seddon *et al.* 1990) confirmed that most strains produce hyaluronidase, chondroitin-4-sulphatase and heparinase, although the activity of the latter is generally weak. *C. difficile* has also been demonstrated to produce proteases (Seddon and Borriello, 1992), and along with the previously described hydrolytic enzymes, these may play a significant part in causing mucosal damage. It is also suggested that such proteases might be involved in the activation of pro-toxin A (Seddon and Borriello, 1992). Enzymes such as hyaluronidase may play an important part in the production of nutritional factors, such as N-acetylglucosamine from the breakdown of hyaluronic acid.

1.2.5 Genetic structure

Most of the work on the genetic structure of *C. difficile* has centred around the genes for its two main toxins. The toxin A (*tcdA*) and toxin B (*tcdB*) genes have both been identified and sequenced (Barroso *et al.* 1990); (Dove *et al.* 1990); (von Eichel-Streiber *et al.* 1992), the former being 8.1-kb, whilst the latter is 7.1-kb in length. It has now been determined (Braun *et al.* 1996); (Hammond and Johnson, 1995) that they are both in the same vicinity on the bacterial chromosome, as part of a 19.6-kb pathogenicity locus (PaLoc). Some of the other genes in the PaLoc (*tcdC-E*) are thought to be involved in the regulation of the toxin genes *tcdA-B* (Moncrief *et al.* 1997). Moncrief *et al.* (1997) suggest that *tcdC* may have a negative regulatory effect on toxin production and that *tcdD* seems to have a positive regulatory role (see also Hundsberger *et al.* 1997). Initial studies suggested that the PaLoc was present in toxigenic strains and lacking in non-toxigenic strains. It has since been discovered that some strains, which produce abnormal toxins, have alterations in the PaLoc.

Analysis of a recently identified strain (8864) which produces toxin B but not toxin A (Borriello *et al.* 1992a); (Lyerly *et al.* 1992); (Torres, 1991) has demonstrated a 5.9-kb deletion and a 1.1-kb insertion in the PaLoc. This corresponds to the production of a truncated form of toxin A and possible production of another protein of up to 22 kDa (Song *et al.* 1999), coded for by a new gene named *tcdF*. Furthermore, *tcdB* in this strain is not identical to that of the type strain simultaneously tested. Analysis of strain 1470 (serotype F), another toxin A-negative, toxin B-positive strain described by Depitre *et al.* (1993), has indicated that it has a 1.8-kb deletion in the PaLoc affecting *tcdA*. This appears to result in a complete lack of toxin A production (von Eichel-Streiber *et al.* 1999). Interestingly, this strain does not cause disease in hamsters whereas strain 8864 remains active in that model. Investigation of 48 toxin A negative, toxin B positive strains from around the world, demonstrated them all to have a deletion in the toxin A gene of ~1.7 kb, similar to strain 1470. Differences in the toxin B gene between different strains have also been noted by von Eichel-Streiber *et al.* (1995). Cohen *et al.* (2000) describe a strain of *C. difficile* (P-829) isolated from a symptomatic patient, which had no detectable production of either toxin A or B. However, on analysing the PaLoc, they discovered deletions in *tcdB*, *tcdC*, *tcdD* and *tcdE* whilst *tcdA* was detectable. Therefore, strain P-829 may produce a truncated toxin A, which lacks part of its terminal repeat region and be is therefore undetectable by current ELISA testing. Their work does confirm the hypothesis that most toxigenic strains have a highly conserved PaLoc, whilst non-toxigenic strains are lacking this region entirely. The only strains that appear to have an altered PaLoc, are those which produce modified toxins.

The flagellin gene (fliC) has been described, producing a protein of 290 amino acids with an estimated molecular mass of 31 kDa. Flagellin extracted using SDS-PAGE has an apparent molecular mass of 39 kDa (Tasteyre *et al.* 2000a). Both non-flagellated and flagellated strains have been shown to possess the fliC gene. Analysis of fliC genes from different *C. difficile* strains demonstrates conservation of N- and C-terminal domains with a

variable central region (Tasteyre *et al.* 2000b). Plasmids have also been described in *C. difficile*, although their presence or absence does not appear to be associated with toxin production or virulence (Hayter and Dale, 1984); (Lyerly *et al.* 1988).

1.3 AETIOLOGY AND CLINICAL MANIFESTATIONS OF *CLOSTRIDIUM DIFFICILE* DISEASE

1.3.1 Acquisition of the organism

The first stage in the process leading towards disease due to *C. difficile* is acquisition of the pathogen. Despite the organism having been isolated from many different places (see table 1), it appears much more likely to be encountered in hospital than anywhere else (see section 1.5.2.2.2). Acquisition is thought to be mainly via the hand to mouth route, with the spores often being found to contaminate the environment. This is especially noticeable in the vicinity of patients with CDI (Chang and Nelson, 2000); (Fekety *et al.* 1980); (Kim *et al.* 1981); (Mulligan *et al.* 1979); (Mulligan *et al.* 1980). Spores are particularly likely to build up in poorly or infrequently cleaned sluice areas. Due to the persistent nature of spores once in the environment, careful attention to frequent cleaning is important in order to prevent their accumulation and minimise the risk of dissemination throughout the ward environment. Once the environment is contaminated, it is relatively easy for health care professionals to spread *C. difficile* to patients, or other ward areas, if they do not pay careful attention to handwashing between patient interactions (Fekety *et al.* 1980); (McFarland *et al.* 1989). The isolation of symptomatic patients also seems to represent a reasonable approach, although some authorities challenge its utility (Sanderson and Richardson, 1997). Cases of *C. difficile* infection due to nosocomial transmission are well documented (Heard *et al.* 1986); (Johnson

et al. 1990a); (McFarland *et al.* 1989), (Testore *et al.* 1988) and this is thought to be the main method of acquisition for patients in hospital.

Table 1: Environmental sources from which *C. difficile* has been isolated

Camel dung	River water
Donkey dung	Sand
Horse dung	Sea water
Lake water	Soil
Mud	Swimming pools
Raw vegetables	Tap water

This table is adapted from Al Saif and Brazier, (1996) and Levett, (1986).

1.3.2 Proliferation of the organism

Our main defence against *C. difficile* is the normal bowel flora of the gut. This has been demonstrated in experiments which have indicated the phenomenon of 'colonisation resistance', and its disturbance by antibiotics (see section 1.3.2.1). The role of the immune system and antibody responses to toxin in the bowel is yet to be fully understood, but it seems likely that it does contribute to protection from disease (see section 1.3.2.2). Antibiotics are, without doubt, the one factor that is most likely to influence the ability of normal gut flora to resist colonisation by *C. difficile* (see section 1.3.2.3). This is because they disturb the natural balance of protective organisms. Other modalities such as chemotherapeutic agents have also been shown to predispose towards development of *C. difficile* disease, presumably by compromising the normal bowel flora (see section 1.3.2.4).

1.3.2.1 Colonisation resistance

Work using a hamster model showed that animals given antibiotics, or exposed to toxigenic *C. difficile* alone, did not develop disease. However, those given antibiotics and then exposed to toxigenic *C. difficile* almost always died (Larson and Borriello, 1990) (Larson *et al.* 1980). Wilson *et al.* (1981) demonstrated that *C. difficile* counts were reduced in hamsters that were given normal hamster caecal homogenates, and that animals given such homogenates after exposure to antibiotics did not develop caecitis. Other authors have also observed the inhibitory effect of some components of faecal flora on the establishment of *C. difficile* (Borriello and Barclay, 1985); (Malamou-Ladas and Tabaqchali, 1982); (Rolfe *et al.* 1981). Suppression of *C. difficile* by isolates of indigenous hamster flora was demonstrated by Wilson *et al.* (1986). Borriello and Barclay, (1986) devised an in-vitro model of colonisation resistance to *C. difficile* infection, where the organism was seeded into faecal emulsions. Using this model, they determined that bacteria played an important role

in 'colonisation resistance'. They found that Gram-negative facultative organisms appeared to have no protective role, whereas anaerobes seemed very important. If all organisms were removed and only the spores of endogenous Clostridia left, there was also no protective effect. By using this model it was also possible to confirm that faecal emulsions from young adults were inhibitory to *C. difficile* colonisation, those from elderly patients were less so, and those from neonates were usually not inhibitory at all. These observations would seem to fit with the hypothesis that neonates have a very rudimentary alimentary tract flora which may allow easy colonisation with *C. difficile* (Larson *et al.* 1982). Wilson *et al.* (1986) also demonstrated the effect of colonisation resistance, using gnotobiotic mice. Once these mice were colonised by *C. difficile*, inoculation with caecal flora from 'normal' mice resulted in loss of *C. difficile* within 2 weeks. The interaction of both *Escherichia coli* and non-toxigenic *C. difficile* with toxigenic *C. difficile* has been investigated using a continuous flow (CF) culture or an animal model in both hamsters and gnotobiotic mice (Borriello and Barclay, 1985); (Borriello and Barclay, 1986); (Wilson and Freter, 1986); (Wilson and Perini, 1988); (Wilson and Sheagren, 1983). Many other workers have also carried out animal experiments which have demonstrated the ability of *C. difficile* to induce a colitic illness in the absence of adequate protective flora, or have reported *C. difficile* disease in other species (Abrams *et al.* 1980); (Chang *et al.* 1978b); (Corthier *et al.* 1985); (Czuprynski *et al.* 1983); (Fekety *et al.* 1979); (Humphrey *et al.* 1979); (Jones *et al.* 1987); (Knoop, 1979); (Lusk *et al.* 1978); (Onderdonk *et al.* 1980); (Orchard *et al.* 1983); (Price *et al.* 1979); (Rehg, 1980); (Rehg and Pakes, 1982); (Sugiyama *et al.* 1985); (Wilson *et al.* 1986).

In vivo evidence in humans for the beneficial effect of protective bowel flora is available in the form of the reported beneficial effect of reintroduction of faecal flora or use of probiotic agents in the treatment of *C. difficile* disease. Several authors have reported the beneficial effect of using rectal infusions of faeces from normal hosts in the treatment of recurrent *C. difficile* infection (Bowden *et al.* 1982); (Schwan *et al.* 1983); (Schwan *et al.*

1984); (Tvede and Rask-Madsen, 1989), although Tjellström *et al.* (1993) document the unsuccessful use of such therapy in one case of relapsing *C. difficile* diarrhoea. The use of *Lactobacillus casei* GG has also been attempted for the treatment of relapsing CDI with some success in two small, open trials (Biller *et al.* 1995); (Gorbach *et al.* 1987). The yeast *Saccharomyces boulardii* has also been used, in conjunction with vancomycin, for the treatment of relapsing *C. difficile* infections (McFarland *et al.* 1994); (Surawicz *et al.* 1989). In the first study, a small open trial, 11 out of 13 patients had no further disease following treatment with vancomycin for 10 days and *S. boulardii* for 28 days. In the second study, a placebo controlled trial with 124 patients, a benefit was only seen in patients who were suffering from relapsing disease. The recurrence rate in those who were being treated for their first episode of CDI was the same in both test and control groups. Seal *et al.* (1987) report the use of a non-toxigenic strain of *C. difficile* to treat relapsing diarrhoeal disease.

Several explanations have been proposed as to the reason why *C. difficile* colonisation and proliferation is reduced in the presence of normal colonic flora. A reduction in pH has been cited by Borriello and Barclay, (1986) as the possible mechanism, but this was not confirmed by a more recent study using continuous flow (CF) culture by Yamamoto-Osaki *et al.* (1994). In this study, the pH of the CF culture system was not seen to alter despite *C. difficile* growth being inhibited. Interestingly, when a *C. difficile* positive stool sample was used to start the CF culture system, viable numbers of *C. difficile* fell to zero in around 4 days as the other organisms proliferated. Upon re-introduction, once a steady state had been reached (after 10 days), counts of *C. difficile* were seen to increase. In contrast, when a *C. difficile* negative stool sample was used as the starter, viable numbers of *C. difficile* were seen to fall when introduced after a steady state was reached (10 days). The flora of the *C. difficile* positive stool was noted to be less complex than the *C. difficile* negative one but nevertheless, whilst actively proliferating, it was still able to inhibit *C. difficile* growth. This ability was lost once the steady state was reached. When *C. difficile*

negative faeces was used as a starter culture, the more complex flora was able to inhibit *C. difficile* growth even once a steady state was reached. This phenomenon may be a demonstration that competition for nutrients can suppress growth and prevent the establishment of *C. difficile*. However, to achieve this requires either complex flora at a steady state, or more rudimentary flora that is rapidly proliferating.

Wilson and Perini, (1988) investigated the way in which competition for nutrients in mouse colonic flora may suppress the growth of *C. difficile*, and found that an unidentified component of faecal flora was able to out compete *C. difficile* for nutrients. Haslam *et al.* (1986) demonstrated that for growth in vitro, *C. difficile* required proline, valine, leucine, *iso*-leucine and tryptophan. Using a CF culture system with infant faeces, they demonstrated that a significant, almost complete reduction of some amino acids occurred, and this could be related to the inhibition of *C. difficile* seen. In addition, *C. difficile* was inoculated into a dialysed culture filtrate (harvested after incubation for 7 days with intestinal bacteria). Five different amino acids were added singly to this filtrate, as well as none or all five at once. Standard anaerobic broth was used for a growth control. The pH of the dialysed filtrates with or without amino acids was lower than in the control broth. No proliferation of *C. difficile* was observed in any of the filtrates where a single amino acid had been added. When all five were added then *C. difficile* was able to proliferate and the pH was found to rise. This suggests that lack of amino acids can play a significant role in the inhibition of *C. difficile* growth although reduced pH may also be a factor.

Another factor, which has been mooted to give rise to inhibition of *C. difficile* proliferation, is volatile fatty (VFA) acid concentrations in the gut. Rolfe, (1984) suggested that VFAs played a significant part in colonisation resistance, however, other investigators have demonstrated no such link when using an in-vitro model, gnotobiotic mice or a CF culture system (Borriello and Barclay, 1985); (Su *et al.* 1987); (Yamamoto-Osaki *et al.*

1994). It has also been suggested that lack of a carbon source may result in limited growth of organisms in the colonic ecosystem (Freter *et al.* 1983), or that an individual's diet may influence *C. difficile* toxin production (Mahe *et al.* 1987). Presence of non-toxigenic or intermediate level toxin producing strains in the bowel of gnotobiotic mice has been shown to protect against *C. difficile* disease (Corthier and Muller, 1988).

1.3.2.2 Immune response

The immune response, and in particular antibody production, appears likely to play a significant part in protection from CDI. Lishman *et al.* (1981) was one of the first groups to describe an antitoxin response to *C. difficile* disease. Later, in a study of 340 patients, serum antibodies to toxin A and toxin B were shown to be present in more than 60% of those aged over 2 years (Viscidi *et al.* 1983). Aronsson *et al.* (1983) also described an antibody response to these two toxins, which appeared to be reduced in those with the most severe colitis. In another study, antibody responses were more marked in patients with mild disease than in those who were severely affected, and those with relapsing disease had the lowest antibody levels (Aronsson *et al.* 1985a). Kelly *et al.* (1992) showed that IgA with activity against toxin A was detectable in colonic aspirates of patients, although levels did not appear to correlate with serum concentrations. However, it is thought that intestinal IgA responses may parallel serum IgG responses (Johnson *et al.* 1992a). Serum antibodies of the IgG class with activity against a *C. difficile* surface protein of 36 kD have also been reported in patients with PMC (Pantosti *et al.* 1989). This surface protein has been purified and partially characterised but its function remains uncertain (Cerquetti *et al.* 1992); (Cerquetti *et al.* 2000).

Increased serum levels of antitoxin A antibodies have been described by several investigators after episodes of CDI (Aronsson *et al.* 1983); (Aronsson *et al.* 1985a); (Johnson

et al. 1992a); (Viscidi *et al.* 1983). Johnson *et al.* (1992a) described increased antibody levels even in patients with relapsing disease and suggested that high antibody levels correlate with severity of illness rather than with protection from it. Mulligan *et al.* (1993) demonstrated detectable levels of both IgA and IgM in serum, which were reactive against somatic cell antigens of *C. difficile*. Furthermore, they noted that these levels were significantly higher in asymptomatically colonised patients than in symptomatic patients or controls, which suggests a possible protective role for an adequate immune response. Similarly, Kyne *et al.* (2001) found that serum levels of IgM and IgG antibodies to toxin A were higher in individuals who experienced a single episode of CDI compared with those suffering multiple episodes. When Nakamura *et al.* (1981) investigated the toxin neutralising activity of serum, they found that in young adults, neutralising ability was often present, whereas it was almost always absent in samples from elderly patients. The utility of an adequate immune response is indicated by the observation that hypogammaglobulinaemia may predispose children to *C. difficile* disease (Gryboski *et al.* 1991) and that relapsing *C. difficile* colitis may respond to intravenously administered gamma globulin (Leung *et al.* 1991); (Salcedo *et al.* 1997); (Warny *et al.* 1995). A study by Warny *et al.* (1994) suggested that an adequate immune response does play a significant protective role against CDI. They found that serum IgG and faecal IgA levels in patients with prolonged, relapsing CDI were lower than those in patients with a short duration of symptoms. Serum IgA, rather than IgG has been reported to be responsible for toxin neutralising ability (Johnson, 1997). Oral IgA has been reported to be of some use, in conjunction with standard antibiotic therapy, for the treatment of persistently relapsing CDI (Tjellström *et al.* 1993).

The importance of the immune response in minimising symptoms from CDI is further reinforced by animal work which has demonstrated the efficacy of both active and passive immunisation against *C. difficile* toxins. Early work using hamsters showed that active immunisation against toxins A and B could protect against *C. difficile* colitis (Libby *et*

al. 1982). The effectiveness of active (and passive) immunisation in hamsters, for the prevention of antibiotic associated caecitis, was also demonstrated by Fernie *et al.* (1983). Kim *et al.* (1987) immunised hamsters against toxins A and B, and discovered that toxoid A alone was protective whilst toxoid B alone was not. In addition, they demonstrated effective passive immunisation, in that mothers with protective antibody levels were able to pass this resistance to disease on to their offspring via breast milk. Passive immunisation in gnotobiotic mice given monoclonal antibodies against toxin A has also been found to be effective (Corthier *et al.* 1991). In the animal model it is thought that toxin A is the main determinant of progression to disease. More recently, animal experiments using both rats and hamsters have suggested that bovine immunoglobulin containing antibodies against toxins A and B can be used to protect against *C. difficile* disease (Kelly *et al.* 1996); (Lyerly *et al.* 1991). In-vitro experiments on both human fibroblasts and rat ileal loops have demonstrated that antibodies to toxins A and B in bovine colostrum can effectively neutralise their toxic effects (Kelly *et al.* 1996). Bovine anti *C. difficile* toxin antibodies from colostrum have also been given to human volunteers to see whether they can survive passage through the stomach and small bowel. The results indicate that significant amounts of immunoglobulin survive transit through the stomach and small bowel, with residual concentrations still capable of neutralising the cytotoxicity of toxins A and B (Kelly *et al.* 1997); (Warny *et al.* 1999). Stubbe *et al.* (2000) discovered that human colonic carcinoma derived T84 monolayers were protected against cytopathic effects of toxins A and B by monoclonal antibodies of the IgA and IgG classes. They also found that polymeric IgA was more effective, and its effect more prolonged than that of monomeric IgA or IgG.

1.3.2.3 The effect of antibiotics on bowel flora and *C. difficile*

Administration of antibiotics is thought to be the most significant predisposing factor for CDI and this has been demonstrated when risk factors for it have been studied (Brown *et al.* 1990); (Gerding *et al.* 1986); (Hutin *et al.* 1997); (Lai *et al.* 1997); (Schwaber *et al.* 2000); (Thibault *et al.* 1991); (Zimmerman, 1991). Katz *et al.* (1997) suggest that a lack of antibiotic exposure in patients with no other clinical predictor could be used as a criterion for not testing stool samples for *C. difficile* toxin. Most antibiotics have been associated with *C. difficile* disease to some extent, with some agents being more often implicated than others (see table 2). The frequency of association of disease with one agent rather than another may be misleading as to its likelihood of inducing symptoms, because the relative frequencies of use must be taken into account. In some instances patients have received antibiotics as long as four weeks prior to symptoms, whilst some authorities recognise that antibiotics up to ten weeks prior to illness may be a significant factor (Tedesco, 1982). Apart from the direct effects of antibiotics on 'colonisation resistance', there may be other ways in which antibiotics could influence the likelihood of developing CDI. It is possible that antibiotics could affect the virulence of *C. difficile* directly. Addition of sub MIC concentrations of antibiotics to cultures of *C. difficile* in brain heart infusion broth, have been shown to increase toxin production. Vancomycin and penicillin are described as inducing this phenomenon by Onderdonk *et al.* (1979). Clindamycin and cephaloridine were found to augment toxin A and B production, whilst tetracycline had no effect (Honda *et al.* 1983). Other investigators have also reported an increase in the cytotoxic activity of *C. difficile* cultures when clindamycin is added (George *et al.* 1980); (Nakamura *et al.* 1982). Contrary to this finding, Barc *et al.* (1992) investigated toxin A and B production in the presence of clindamycin, both in vitro and in vivo but were unable to find any increase in production. Onderdonk *et al.* (1979) did not find any increase in toxin production with subinhibitory

concentrations of clindamycin. This suggests that not all C. *difficile* strains respond to the same stimuli with an increase in toxin production.

Table 2: Risk of *C. difficile* infection associated with various antibiotics

Risk of association	Antibiotics
High	clindamycin, 'second' and 'third generation' cephalosporins
Moderate	'first generation' cephalosporins, amoxycillin, ampicillin, macrolides, tetracyclines, co-trimoxazole
Low	quinolones, rifampicin, ureidopenicillins, trimethoprim, aminoglycosides, metronidazole, vancomycin

This table is adapted from Wilcox, (2000)

When the effects of PT, CTX and ciprofloxacin on toxin production were studied in-vitro (Freeman and Wilcox, 2000), no increase in toxin B production was found. As discussed in section 1.2.4.2, adherence plays an important part in the ability of *C. difficile* to colonise the gut. This ability may also be affected by antibiotics, as some are known to be able to affect bacterial surface components (Schifferli and Beachey, 1988). Little work in this area has been performed, but Krishna *et al.* (1996) carried out an experiment on hamster caecal flora from animals either administered or not administered clindamycin. They found

no subsequent difference in cell surface hydrophobicity of *C. difficile* cultured in either type of caecal flora.

1.3.2.3.1 Clindamycin

As previously mentioned in section 1.1, Tedesco (1974) first brought the link between clindamycin and pseudomebranous colitis to public attention. In 1978 several investigators made the link between 'clindamicin colitis', as PMC was previously known, and its causative agent *C. difficile* (Bartlett *et al.* 1978a); (George *et al.* 1978a); (George RH *et al.* 1978); (Larson *et al.* 1978). At this time it was also observed that if hamsters were given clindamycin, they suffered from a fatal enterocolitis also found to be due to *C. difficile* (Chang *et al.* 1978b); (Lusk *et al.* 1978). Not only were caecal filtrates from such animals cytotoxic to cells in tissue culture, but if filtered and injected into the caeca of healthy hamsters, these animals would go on to develop pseudomembranous lesions (Bartlett *et al.* 1977a). The duration of perturbation of faecal flora in hamsters following clindamycin administration is extremely prolonged and was found to be between 1-2 months by Fekety *et al.* (1980). Further work with the hamster model (Larson and Borriello, 1990) has confirmed that when compared to ampicillin, flucloxacillin or cefuroxime, treatment with clindamycin results in a much more prolonged period of subsequent susceptibility to CDI. The use of clindamycin in hospitals today has declined markedly since the early reports of its association with PMC (Aronsson *et al.* 1985b); (Bartlett, 1981); (Golledge *et al.* 1989); (Le Frock *et al.* 1975), but there have been recent publications which have suggested that some outbreaks of CDI may still be related to its use (Brown *et al.* 1990); (Johnson *et al.* 1999). Pear *et al.* (1994) describe the control of an outbreak of CDI by restriction of clindamycin use, after this was noted to be correlated with patients developing *C. difficile* diarrhoea. Similarly, another outbreak of CDI was noted to be associated with increased clindamycin use, and the causative strain to be clindamycin resistant. Hospital wide reduction in the use

of clindamycin reduced the incidence of CDI from 11.5 cases/month to 3.33 cases/month. Clindamycin susceptibility of *C. difficile* isolates was then seen to increase from 9% to 61% (Climo *et al.* 1998). Local use of clindamycin has also been linked to CDI, with reports following treatment with a vaginal cream preparation (Meadowcroft *et al.* 1998); (Trexler *et al.* 1997); (Vikenes *et al.* 1999). Although it appears to be one of the most potent predisposing factors to CDI (Gerding *et al.* 1995), currently, clindamycin is not frequently the cause due to its reduced frequency of use.

1.3.2.3.2 Cephalosporins

There is widespread use of cephalosporins in hospitals today and they often provide the mainstay of antimicrobial therapy, both for prophylaxis as well as for treatment of infections. As with most classes of antibiotics, their use has been associated with the development of CDI. This was noted as long ago as 1981, when Bartlett reviewed 329 patient records to determine what predisposing antibiotics they had taken. He noticed that over time clindamycin became less frequently implicated, but that the frequency of association with cephalosporins increased. This probably related to the changing patterns of antibiotic usage (Bartlett, 1981). Since then, a great number of reports on the associations of cephalosporins with subsequent *C. difficile* disease have been published. Ebright *et al.* (1981) showed that eight commonly used cephalosporins were capable of inducing colitis in hamsters, which was indistinguishable from that caused by clindamycin. Bodey *et al.* (1983), found that the bowel flora in recipients of ceftazidime and particularly ceftriaxone was significantly disturbed, with reductions in both anaerobic and aerobic flora. This was particularly marked with ceftriaxone, where all Gram-negative bacilli, 90% of anaerobes and 76% of aerobic gram-positive organisms were eradicated. The increased deleterious effects of ceftriaxone on bowel flora may be due to its greater degree of biliary excretion. Another investigator who evaluated the effect of antibiotics on human faecal flora was Mulligan, who

found that five out of six patients who were monitored before, during and after therapy with cefoxitin developed ingrowth of *C. difficile*. Marked reductions in components of normal flora were also noted (Mulligan *et al.* 1984). Nord, (1987), whilst investigating the effects of prophylactic antibiotics on 'colonisation resistance', discovered that cephalosporins were more likely than penicillins to have a significant deleterious effect. Silva *et al.* (1984) examined the records of 130 patients with presumed or proven *C. difficile* colitis and determined that in 55% of cases, cephalosporins were associated with the onset of symptoms. They also carried out experiments using the hamster model and demonstrated that disease could be induced by administration of a cephalosporin. Similarly, Aronsson *et al.* (1985b) reported data from Sweden between 1980-1982 indicating that cephalosporins were implicated in cases of *C. difficile* colitis 40 times more often than narrow-spectrum penicillins. A small study on the association of extended spectrum cephalosporins with *C. difficile* diarrhoea, by Golledge *et al.* (1989), found that the relative risk of disease was highest after taking clindamycin. The relative risk for cefotaxime, cefamandole and ceftriaxone were not significantly different from each other and were only just lower than that of clindamycin. When de Lalla *et al.* (1989) looked at 40 patients who had developed CDI or PMC, they found that consumption of 'third generation' cephalosporins amongst this group was significantly greater than consumption of ureidopenicillins. Similar findings were also made in a case control study by Nelson *et al.*, who found that the greatest risk factor for the development of CDI was 'second' or 'third generation'cephalosporin exposure (Nelson *et al.* 1994). Cartmill *et al.* (1994) showed that exposure to cephalosporins played a major role in the development of a *C. difficile* outbreak, with 76.3% of 169 patients having received antibiotics including a cephalosporin prior to developing symptoms of CDI. Cephalosporin use was again significantly associated with CDI in a study on elderly patients with community-acquired lower respiratory tract infection by MacGowan *et al.* (1997).

The development of susceptibility to CDI does not necessarily require a long course of antibiotics. One investigator, who looked at the effect of just a single dose of antibiotic on the faecal flora of volunteers, showed that whilst cephalosporins were associated with the emergence of *C. difficile* in the stools, penicillins were not (Ambrose *et al.* 1985). A control group who did not receive antibiotics also showed no emergence of *C. difficile*. Cephalosporins are often used as prophylactic agents and reports of subsequent CDI or colitis have been made by several investigators (Arsura *et al.* 1985); (Block *et al.* 1985); (Crabtree *et al.* 1999); (Keighley *et al.* 1983); (Roberts and Hughes, 1985); (Tan *et al.* 1979). When single dose prophylactic antibiotics were investigated by Privitera *et al.* (1991), they discovered that *C. difficile* was detectable in the stools of 23% of patients where a cephalosporin was used, only 3.3% of samples from patients who had been given mezlocillin, and in none of those given no antibiotic. Although all cephalosporins have been implicated as incitory agents for CDI, most recent concerns have centred on the effects of the 'third generation' cephalosporins. This is partly because of the recent increase in use, and partly because it is now felt that these agents in particular are especially likely to render an individual susceptible to CDI. Anand *et al.* (1994), performed a large study evaluating CDI over a two year period and found that ceftriaxone or ceftazidime use was highly correlated with its development, whilst ticarcillin-clavulanate was not. In addition, they found many more actual versus expected cases of CDI (based on usage) following 'third generation' cephalosporins, slightly more actual versus expected cases for 'second generation' cephalosporins and fewer actual versus expected cases for 'first generation' cephalosporins. However, in their study, there was no data on *C. difficile* exposure for each patient group, no information regarding duration of treatment with particular agents and no adjustment made for age of recipient or cases where multiple antibiotics were received.

Cefotaxime has also been seriously linked with the subsequent development of CDI, particularly in elderly patients (Impallomeni *et al.* 1995); (Lesna and Parham, 1996); (Starr

and Impallomeni, 1997). A letter to the BMJ from the Corporate Drug Safety and Development Department of Roussel (who manufacture cefotaxime) cast doubt over the validity of the study by Impallomeni *et al.*, although their evidence in favour of cefotaxime appears scant (Rothschild *et al.* 1996). The figures that they quote for diarrhoea and PMC developing in healthy volunteers given cefotaxime would be hard to apply to an ill, elderly, more vulnerable population. When an outbreak of *C. difficile* diarrhoea in Sheffield was investigated, cephalosporins were again implicated (Zadik and Moore, 1998). Rates of CDI were calculated for each antibiotic divided by the quantity used, and corrections were made to account for combinations. The rate of CDI for ampicillin/amoxycillin was used as the baseline and rates for other antibiotics were divided by this to obtain a 'rate ratio' (RR). The greatest association with CDI was for cefotaxime where the RR was 27.5, ceftriaxone RR, 15.1, cefuroxime RR, 8.6 and ceftazidime RR, 6.4. When adjustments for combinations were made the ratios were 16.9, 8.6, 5.3 and 4.8 respectively. The increased risk of CDI following cefotaxime when compared with the risk after ampicillin/amoxycillin was significant at the $p=<0.000001$ level. Further evidence for the association between cephalosporins and CDI comes from a recent study by Schwaber *et al.* (2000), in which receipt of a cephalosporin (particularly of the 'third generation') was significantly associated with CDI but not with nosocomial diarrhoea of other aetiologies.

In addition to the numerous publications citing cephalosporins, particularly those of the 'third generation', as potent inducers of CDI, there is also work describing the reduction of the incidence of such disease by reducing prescriptions of these drugs (Brown *et al.* 1990); (Jones *et al.* 1997); (Ludlam *et al.* 1999); (McNulty *et al.* 1997); (Quale *et al.* 1996). Other reports indicate that increased rates of *C. difficile* disease were noted following relaxation of antibiotic policy, or change of policy resulting in greater cephalosporin use (Ho *et al.* 1996); (Impallomeni *et al.* 1995). Increases in the use of cephalosporins such as cefuroxime and cefotaxime were quite noticeable following the revised British Thoracic

Society guidelines (1993) for the treatment of severe community-acquired pneumonia. Cephalosporins are now probably the most significant risk factor for patients in hospital today, with 'third generation' drugs being the highest risk group. This is because of their extremely frequent use for many infections, and the fact that 'third generation' cephalosporins appear to be second only to clindamycin in their ability to predispose patients to the development of CDI. The future impact of 'fourth generation' drugs such as cefixime, which is administered orally, is still yet to be fully investigated. However, oral cefixime is known to have significant effects on bowel flora, reducing the amount of many organisms including anaerobes (Finegold *et al.* 1987); (Nord *et al.* 1988). An association with increased colonisation by *C. difficile* has also been described by several groups (Chachaty *et al.* 1993); (Finegold *et al.* 1987); (Nord *et al.* 1988). In a group of 51 healthy volunteers, Chachaty *et al.* (1993) found that colonisation with *C. difficile* rose from 6% before cefixime to 57% after. It can therefore be seen that such drugs have the potential to cause serious problems when administered to a more susceptible population, such as ill elderly patients. Levy *et al.* (2000), investigated the use of antibiotics in an ambulatory care setting. Despite CDI having a low incidence in this group, a significantly increased association with such illness was only found with two drugs, both cephalosporins, cephalexin and cefixime. Due to the lower frequency of testing for the possibility of CDI in patients outside hospital, the number of cases could be underestimated and more care may be needed to diagnose such individuals with diarrhoea in future. Currently cephalosporins, and especially 'third generation' drugs, are probably the most significant contributor towards CDI in hospitals today.

1.3.2.3.3 Penicillins

Commonly used antibiotics such as amoxycillin and ampicillin (aminopenicillins) have often been associated with subsequent CDI, but this must be qualified by the number of

doses of such treatments given to patients in comparison to other drugs. Whilst Bartlett (1981) found that 80% of cases of CDI occurred in patients following therapy with either ampicillin, clindamycin or cephalosporins, to accurately estimate the risk for each agent we must divide the number of cases by the total amount or number of doses of each antibiotic given. This would result in a rate per dose, or per gram of antibiotic. If the rate, so calculated, for ampicillin is then standardised to 1, the other antibiotics can have a relative risk (compared with ampicillin) calculated. The true accuracy of these figures does depend upon all patients so considered having been at similar risk of developing CDI (susceptibility) with regard to factors other than antibiotics, as well as having been equally exposed to the organism. Other investigators have described aminopenicillins as often being implicated as incitory agents for CDI, but without taking account of their frequency of prescription. Silva *et al.* (1984) carried out a study on 130 patients with CDI, as mentioned in section 1.3.2.3.2, and discovered that 29% of cases were associated with prior ampicillin. When account is taken of frequency of use of individual antibiotics, such as in the work by Zadik and Moore (1998), a different picture emerges, with cephalosporins being far more likely than amoxycillin or ampicillin to induce CDI (see section 1.3.2.3.2).

Ureidopenicillins, such as piperacillin or mezlocillin appear to have an even lower risk than ampicillin of inciting *C. difficile* related diarrhoea. Several studies have shown marked differences between the likelihood of developing CDI after ureidopenicillins versus after cephalosporins (Ambrose *et al.* 1985); (Anand *et al.* 1994); (de Lalla *et al.* 1989); (Golledge *et al.* 1989); (Keighley *et al.* 1983); (Privitera *et al.* 1991). The most interesting of these studies is by Anand and colleagues. They carried out a retrospective study of patients with toxin A positive stools over a 2 year period. Antibiotic usage, expressed as doses administered was also recorded. They found that 'third generation' cephalosporins were significantly associated with subsequent CDI, leading to many more cases than would be expected based on quantity used alone (if all antibiotics are equally likely to render

patients susceptible to CDI). Fewer cases than expected were associated with amoxycillin and, significantly, no cases of CDI were noted following treatment with ticarcillin/clavulanate, which was the most frequently used antibiotic by a factor of 1.5 ($p=0.00001$). Unfortunately, there is no information as to whether patients who were given different antibiotics had similar illnesses, or were equally debilitated. Nor is there any indication of whether courses of the different agents were for similar periods of time or if patients had similar degrees of exposure to *C. difficile*. Another penicillin/beta-lactamase inhibitor combination therapy, amoxycillin/clavulanic acid (co-amoxyclav), has also seldom been associated with CDI. Mitchell *et al.* (1996) studied a group of children being treated with co-amoxyclav for otitis media. They found that no-one had toxin A or B in their stools at enrolment, or when diarrhoea commenced. Whilst on therapy, 30% of patients studied developed diarrhoea and 13% were found to have toxins present on exit testing (between 8-34 days, mean 13 days). It is not clear whether the cause of diarrhoea in any of the 13% was *C. difficile*. Hirschorn *et al.* (1994) describe CDI in association with co-amoxyclav, but only when it was given with either cefaclor or cefuroxime. One of the possible explanations for ureidopenicillins having a lesser likelihood of leading to *C. difficile* disease is that they are more active than cephalosporins against *C. difficile* (Chow *et al.* 1985).

1.3.2.3.4 4-Fluoroquinolones

Quinolones, such as ciprofloxacin and norfloxacin have only been introduced relatively recently as antibiotic therapies. Nevertheless, they have undergone some scrutiny to try to ascertain the likelihood of their use resulting in CDI. This investigation has generally led to the conclusion that they do not often lead to CDI (Golledge *et al.* 1992); (Schacht *et al.* 1988). Several reports even suggest that ciprofloxacin may be used in the successful treatment of CDI (Daniels and Pristas, 1992); (Lettau, 1988). Some investigators have reported an association between ciprofloxacin and CDI (Bates *et al.* 1990);

(Bauwens *et al.* 1997); (Cain and O'Connor, 1990); (Crabtree *et al.* 1999); (Hillman *et al.* 1990); (Jones *et al.* 1997); (Loge, 1989); (Low and Harries, 1990); (McFarland *et al.* 1995), but in several of these reports there may be other explanations as to the predisposing factor. Salmonella enteritis predated the cases of CDI in three reports (Bates *et al.* 1990; Hillman *et al.* 1990; Low and Harries, 1990) and enteric infection has been cited as a possible factor leading to *C. difficile* disease (Falsen *et al.* 1980); (Riley *et al.* 1986). In the other report (Cain and O'Connor, 1990), the patient had also been given a course of co-trimoxazole five weeks before developing CDI which may have been more likely to have provoked the illness. In work by Golledge *et al.* (1992), 213 patients receiving monotherapy with ciprofloxacin were investigated and although 44 did have diarrhoea, none was found to have *C. difficile*. A further 73 of the patients without diarrhoea were also screened for *C. difficile* and found to be negative. Golledge and colleagues continued their investigations by carrying out tests on human faecal samples in vitro, to see whether faecal emulsions 'treated' with antibiotics would support the growth of *C. difficile*. They found that whilst clindamycin used in this way rendered the faecal emulsions permissive to *C. difficile* growth, non-treated samples or those treated with ciprofloxacin remained inhibitory to the organism. These findings led them to the conclusion that ciprofloxacin is unlikely to render individuals susceptible to CDI. On a theoretical basis, this finding may not be too surprising as ciprofloxacin may not be bactericidal in anaerobic environments (Smith and Lewin, 1988), however nor is it particularly active against *C. difficile* (Delmée and Avesani, 1986); (Edlund and Nord, 1986).

1.3.2.3.5 Other antimicrobial agents

Although the antibiotics most commonly associated with CDI have been discussed above, almost all antibiotics have been implicated at one time or another. Antibiotics that are in common use, such as erythromycin, tetracycline and trimethoprim-sulphamethoxazole

(co-trimoxazole) have been associated more frequently than agents such as chloramphenicol and rifampicin. Gantz *et al.* (1979) report the association of erythromycin therapy with CDI. Riley *et al.* (1991a) describe the implication of tetracyclines and co-trimoxazole in the development of CDI in patients from a community setting. Silva *et al.* (1984) report the association of chloramphenicol, erythromycin and co-trimoxazole with CDI. Another report of co-trimoxazole associated with CDI is in HIV positive patients on prophylactic therapy against *Pneumocystis carinii* (Gordin *et al.* 1994) and malaria prophylaxis with doxycycline has also lead to CDI (Golledge and Riley, 1995). Colardyn *et al.* (1984) report a case CDI associated with imipenem therapy. Infrequently, there have been occasions where antimicrobials usually used for the treatment of CDI have been said to induce disease. Both metronidazole (Saginur *et al.* 1980); (Thomson *et al.* 1981) and vancomycin (Hecht and Olinger, 1989); (Miller and Ringler, 1987); (Schenfeld and Pote, 1995) have been implicated. Antituberculous drugs have also been noted to be associated with CDI on occasion (Bartlett *et al.* 1981), particularly rifampicin (Byrd *et al.* 1997); (Fekety *et al.* 1983); (Fournier *et al.* 1980); (Prigogine *et al.* 1981). Newer antibiotics have been linked to cases of CDI, including clarithromycin (Braegger and Nadal, 1994); (Guyot *et al.* 2000) and pristinamycin (Talon *et al.* 1995). One of the few classes of antibiotic to be very seldom associated with *C. difficile* diarrhoea is the aminoglycosides (George, 1988). Silva *et al.* (1984) report that aminoglycosides were implicated in quite a large number of cases, but they were usually administered with other antibiotics, often cephalosporins. Several other antimicrobial agents have been mentioned in association with CDI, including oxacillin and dicloxacillin (Brook, 1980), spiramycin (Decaux and Devroede, 1978), miconazole (Bartlett, 1981), and albendazole (Shah *et al.* 1996).

1.3.2.3.6 Prophylactic antibiotic use in surgery

As discussed in section 1.3.2.3.2, antibiotics that are only given for short durations, such as for surgical prophylaxis have still been associated with CDI. In two studies (Ambrose *et al.* 1985); (Privitera *et al.* 1991), cephalosporins were demonstrated to result in more cases of CDI than ureidopenicillins. In one of these, development of CDI was noted in situations where only one dose of antibiotic had been given (Privitera *et al.* 1991). However, Kreisel *et al.* (1995) investigated the development of CDI after prophylactic antibiotics and found that patients who developed disease had received significantly longer courses of treatment than those who did not. They also noted that the inapropriate use of prolonged prophylaxis significantly predisposed towards CDI, and that affected patients stayed in hospital for significantly longer periods. It should be appreciated that prophylactic antibiotics can predispose to CDI, sometimes after only one dose, but that prolonged courses are even more likely to result in such iatrogenic illness.

1.3.2.4 The effect of other agents on bowel flora and *C. difficile*

Apart from the well-known association between administration of antibiotics, reduction of colonisation resistance and subsequent CDI, other agents have also been reported to occasionally contribute towards such illness. Most common amongst such reports are those linking antineoplastic agents to CDI (Anand and Glatt, 1993). Amongst such drugs, methotrexate (Miller and Koornhof, 1984) and 5-fluorouracil (Sriuranpong and Voravud, 1995) appear to be most often implicated. Silva *et al.* (1984) identified 4 cases out of a total of 130 where antineoplastic drugs alone were implicated in *C. difficile* colitis. These were cyclophosphamide, doxorubicin (adriamycin), 5-fluorouracil and methotrexate. Roda, (1987) describes a case of PMC following treatment with cytarabine. More recently, cisplatin has been associated with CDI (Emoto *et al.* 1996); (Park *et al.* 1999).

Jarvis *et al.* (1997) describe the association between mitoxantrone and etoposide with CDI, but the patient concerned had also received antibiotics six weeks earlier. Paclitaxel, used for both breast and ovarian cancer treatment has been linked to some cases of CDI (Ang *et al.* 2000); (Chi *et al.* 1999); (Husain *et al.* 1998). Other authors report CDI following chemotherapy for various tumours (Cudmore *et al.* 1982); (Fainstein *et al.* 1981); (Kamthan *et al.* 1992); (Paterson, 1997); (Satin *et al.* 1989). Ramos *et al.* (1997), report a case of CDI in a patient having chlorambucil therapy for rheumatoid arthritis. Other medications which have been cited as giving rise to CDI include: diclofenac (Gentric and Pennec, 1992), sulphasalazine (Bartlett *et al.* 1981), silver sulphadiazine (Jennings and Hanumadass, 1998), tacrolimus (Sharma and Holder, 1998) and tyrothricin (Demols *et al.* 1996). There have been cases of CDI described where no prior antibiotic has been given (Moscovitz and Bartlett, 1981). Contributory factors in such cases may include diabetes mellitus, hepatic and renal failure, malnutrition and cystic fibrosis (Silva, 1989).

1.3.3 Clinical features of *C. difficile* infection

In common with disease due to other intestinal pathogens, *C. difficile* is associated with a continuous spectrum of symptoms, ranging from asymptomatic colonisation to fulminant PMC with toxic megacolon, possibly resulting in bowel perforation and even death. As previously discussed, CDI most commonly ensues after antimicrobial or antineoplastic therapy, although a very small number of cases are not associated with such predisposing factors (Bartlett, 1992). Symptoms classically develop 5-10 days after antibiotic therapy, but may not occur until 10 weeks have elapsed (Tedesco, 1982).

1.3.3.1 Intestinal infection

The most prominent symptom associated with CDI is diarrhoea, which may be present in varying degrees, from a couple of loose motions a day, to 20 or more profuse watery stools per day. Mucus is often present but blood is found in only 5 to 10% of cases. In cases that progress beyond mild diarrhoeal symptoms, other systemic features become apparent. These include fever, leucocytosis and cramping abdominal pain. Patients with colitis will often have a fever of more than 100°F and peripheral white cell count of 12,000 to 20,000/mm^3. Abdominal distension, nausea, vomiting and dehydration are not uncommon when disease persists. In very severe cases, diarrhoea may cease and abdominal distention with tenderness become more prominent as the continuing inflammation of the colon results in a paralytic ileus and development of a toxic megacolon. This is a very serious complication and may presage bowel perforation and death. When colitis develops, endoscopic investigation at the outset of disease usually reveals whitish/yellow mucosal plaques 1-2 mm in size. As the disease progresses, these enlarge in size, coalescing to form larger lesions whilst mucosal appearance between lesions may be normal or slightly erythematous. Pseudomembranes, which are composed of fibrin, white cells, mucus and cellular debris may be seen adhering to the mucosa. The pathological changes in the bowel mucosa are usually limited to the colon in *C. difficile* disease, although rarely the terminal ileum may be affected (Bartlett *et al.* 1979).

Presence of cytotoxin in the stools has been documented to occur in 10-25% of antibiotic associated diarrhoea, 50-70% of antibiotic associated colitis and 90-100% of antibiotic associated PMC (Bartlett, 1990) (see table 3). In cases of severe PMC, cytotoxin may gain access to the circulation and was detected in the blood of two children who suffered fatal *C. difficile* associated PMC (Qualman *et al.* 1990). Recurrence of symptoms

following treatment is also a common feature of CDI, occurring on up to 24% of occasions (Wilcox and Spencer, 1992).

Table 3: Rates of *C. difficile* isolation and toxin detection from stool samples of various populations

SOURCE OF SPECIMEN	ISOLATION RATE (%)	POSITIVE TOXIN ASSAY (%)
Patients with antibiotc-associated PMC	95-100	95-100
Patients with antibiotic-associated colitis	50-70	50-70
Patients with antibiotc-associated diarrhoea	15-25	10-25
Patients without diarrhoea (antibiotic exposure)	10-25	5-10
Hospitalised patients	10-25	2-8
Patients with gastrointestinal disease unrelated to antibiotic exposure	2-3	0.5
Healthy adults	2-3	0
Healthy neonates	5-70	5-63

Table adapted from (Bartlett, 1994), Table 2

1.3.3.2 Extra-intestinal disease

Other manifestations have been reported in association with *C. difficile* apart from diarrhoeal illness or colitis. These include septicaemia, osteomyelitis, peritonitis, urogenital infections, wound infections and splenic abscess (Levett, 1986) (see table 4). Protein-losing enteropathy has also been reported (Rybolt *et al.* 1989) as well as production of ascites (Jafri and Marshall, 1996). One case has been described where *C. difficile* was the cause of septic arthritis in the prosthetic hip joint of a woman who 12 months earlier had suffered an episode of CDI whilst in hospital having her hip replacement (McCarthy and Stingemore, 1999). Another case was reported in a prosthetic knee joint (Pron *et* al. 1995). C. *difficile* has also been associated with reactive arthritis (Bolton *et al.* 1981); (Lofgren *et al.* 1984).

Table 4: Sites of extra-intestinal *C. difficile* disease

EXTRA-INTESTINAL MANIFESTATION	REFERENCE
Abscess (soft tissue)	(Beerens and Tahon-Castel, 1965) (Danielsson *et al.* 1972) (Eastwood, 1980)
Bacteraemia	(Alpern and Dowell, 1971) (Gorbach and Thadepalli, 1975) (Rampling *et al.* 1982) (Saginur *et al.* 1983) (Smith and King, 1962)
Empyema	(Simpson *et al.* 1996) (Smith and King, 1962)
Gangrene	(Chmelar and Tulachova, 1984)
Intra-abdominal infection after penetrating trauma	(Thadepalli *et al.* 1972)
Osteomyelitis	(Riley and Karthigasu, 1982) (Towns *et al.* 1984)
Peritonitis / ascitic infection	(Genta *et al.* 1984) (Klinger *et al.* 1984) (Saginur *et al.* 1983) (Smith and King, 1962)
Reactive arthritis	(Bolton *et al.* 1981) (Hannonen *et al.* 1989) (Lofgren *et al.* 1984)
Septic arthritis	(McCarthy and Stingemore, 1999) (Pron *et* al. 1995)
Splenic abscess	(Saginur *et al.* 1983)
Urogenital infection	(Smith and King, 1962) (Thadepalli *et al.* 1973)
Wound infection	(Smith and King, 1962)

1.4 DIAGNOSIS OF *CLOSTRIDIUM DIFFICILE* DISEASE

The diagnosis of *C. difficile* disease generally requires the combination of an appropriate clinical picture coupled with detection of *C. difficile*, its metabolites or toxins. Alternatively, a typical appearance upon endoscopic investigation of the bowel, coupled with biopsy and histological examination may be used. The detection of *C. difficile*, its metabolites or toxins in the faeces of a patient with no clinical evidence of disease is usually felt to represent *C. difficile* colonisation.

1.4.1 Specimen transport and characteristics

C. difficile is readily recoverable from most faecal samples due to its ability to produce spores. However, when attempts are being made to detect *C. difficile* toxins, it is preferable to use as fresh a sample as possible, and to avoid storage at room temperature. This is because of the documented deterioration of *C. difficile* toxins which occurs at ambient temperatures in stool samples (Bowman and Riley, 1986); (Brazier, 1993); (Chang *et al.* 1979) and even at 4°C (Borriello *et al.* 1992b). It is not advisable to process solid samples as toxin is seldom found, and its presence would be of uncertain significance in the absence of symptoms (Department of Health and Public Health Laboratory Service Joint Working Group, 1994).

1.4.2 Methods for detection of *C. difficile* or its metabolic products

1.4.2.1 Microscopy

Although microscopy for leucocytes has been recommended by some as being of help in diagnosing CDI (Bowman and Riley, 1988), its sensitivity and specificity are

extremely poor (Shanholtzer *et al.* 1983). This is due to the many other possible causes of faecal leucocytes. In addition, Gram staining is unable to distinguish one type of *Clostridium* sp. from another. Other techniques, such as using fluorescence microscopy to detect labelled *C. difficile* cell wall antibodies adhering to *C. difficile* in faecal smears (Wilson *et al.* 1982b) are too non-specific to be used as sole diagnostic tests.

1.4.2.2 Gas-liquid chromatography

High-pressure gas liquid chromatography (GLC) has been studied as a means of detecting *C. difficile* in stool samples (Levett, 1984a); (Pepersack *et al.* 1983), whilst others used frequency pulsed electron capture GLC (Brooks *et al.* 1984). Metabolic products which most reliably indicated *C. difficile,* when detected, were isocaproic acid and *p*-cresol (Levett and Phillips, 1985); (Phillips and Rogers, 1981). However, results from GLC testing have proved too variable to be relied on diagnostically, some investigators reporting encouraging findings, (Pepersack *et al.* 1983); (Potvliege *et al.* 1981), whilst others found poor results (Levett, 1984a). A low positive predictive value makes the test unsuitable as a diagnostic tool.

1.4.2.3 Culture

Early attempts at producing a medium on which to select *C. difficile* from other faecal flora included the use of *p*-cresol (Hafiz and Oakley, 1976), neomycin and sodium azide (Bartlett *et al.* 1977b), kanamycin (Bartlett *et al.* 1978a), and clindamycin (Larson *et al.* 1978). These all proved suboptimal, but a satisfactory medium containing cycloserine, cefoxitin and fructose (CCFA) was developed by George *et al.* (1979). Further modifications to this medium have since occurred, with the cycloserine and cefoxitin concentrations being reduced by half to 250 mg/L and 8 mg/L respectively to reduce

inhibition of *C. difficile* (Clabots *et al.* 1991); (Levett, 1985); (Willey and Bartlett, 1979). Contrary to this, Peterson *et al.* (1996) found that if pre-reduced for 4 hours prior to inoculation, CCFA with the original 500 mg/L and 16 mg/L performed better than CCFA with the modified concentrations. Other selective media have been tried such as cycloserine-mannitol agar (CMA) and cycloserine-mannitol blood agar, but whether these are better than CCFA or not is uncertain. Some research suggests better isolation rates (Bartley *et al.* 1991); (Marler *et al.* 1992), whilst others found worse rates (Iwen *et al.* 1989); (Mundy *et al.* 1995). Egg yolk containing media has an advantage over that using blood, in that it can help distinguish lipase/lecithinase producing species such as *Clostridium sordellii* from *C. difficile*. In order to enhance recovery of *C. difficile* from spores, taurocholate addition has been found to increase spore germination (Buggy *et al.* 1985); (Wilson *et al.* 1982a); (Wilson, 1983). Another more recent finding is that addition of lysozyme 5 mg/L to bile salt containing selective media further enhances recovery of *C. difficile*, presumably by enhancing spore germination (Wilcox *et al.* 2000).

Enrichment broths for *C. difficile* isolation have also been described, with cycloserine, cefoxitin and fructose (Buchanan *et al.* 1984); (Riley *et al.* 1987), cycloserine, cefoxitin and gentamicin (Carroll *et al.* 1983), or cycloserine, cefoxitin and sodium taurocholate (O'Farrell *et al.* 1984). They appear to offer a slight advantage over standard Robertson's cooked meat broth.

1.4.2.4 Enrichment methods

Apart from enrichment broths, two other methods for improving *C. difficile* recovery from stool samples are alcohol and heat shock techniques. These are designed to kill all vegetative cells, leaving only spores, and thus reduce competing flora, making *C. difficile* recognition easier. Alcohol shock involves mixing the stool specimen with an equal volume

of 80% alcohol and leaving to stand for 1 h before subculture onto non-selective media. Heat shock involves heating the sample to 70°C, in a water bath, for 20 min prior to culture. Although not entirely clearcut, heat shock, and probably alcohol shock also, appear better than culture onto solid media alone. Some studies indicate that alcohol shock (Clabots *et al.* 1989); (Riley *et al.* 1987) and heat shock (Hanff *et al.* 1993) improve sensitivity, whilst one showed alcohol shock to be worse than direct plating onto selective agar (Borriello and Honour, 1981), and another that heat shock was worse than such direct culture (Marler *et al.* 1992).

Culture methods have a very high sensitivity, approaching 100% in samples containing cytotoxin (DeMaio and Bartlett, 1995); (Wilson *et al.* 1982a), but suffer from a much lower specificity. Culture does not distinguish toxigenic from non-toxigenic strains, so further work is then needed to determine toxin status. Consequently, culture of *C. difficile* alone does not equate to CDI. Culture of the organism is of potential use when epidemiological studies are being carried out, or information about epidemic strains is required. It also allows sensitivity testing, although this is not really of importance in *C. difficile* disease as drug resistance is not thought to account for treatment failure or relapses (DeMaio and Bartlett, 1995).

1.4.2.5 Identification

Once an organism with the cultural characteristics of *C. difficile* has been isolated, confirmatory evidence is required to ensure correct identification. Most authorities would not consider the distinctive smell alone to be sufficient, although with experience this can be very accurate. Similarly, fluorescence under UV light is not a reliable enough feature to be depended on. There are several possibilities short of full biochemical testing which may be

considered adequate. A latex agglutination kit (Microgen Ltd, Guildford, UK) which detects somatic antigens may be considered useful, but cross-reacts with three other *Clostridial* species (Bowman *et al.* 1986); (Brazier, 1990). Nevertheless, in conjunction with specific cultural characteristics (see table 5), this may form a useful confirmatory test (Brazier, 1998). Production of L-proline-aminopeptidase is another characteristic feature of *C. difficile*, and has been used by some as a useful confirmatory test on isolates with typical growth features (Fedorko and Williams, 1997). Another rapid kit test for *C. difficile* incorporating five tests, including L-proline-aminopeptidase, has been designed but is not commercially available at present (Aspinall and Dealler, 1992).

Table 5: Differential tests for *Clostridial* species commonly mistaken for *C. difficile*

SPECIES	UV FLUORESCENCE	LATEX*	LECITHINASE	ODOUR
C. difficile	+	+	-	+
C. innocuum	+	-	-	-
C. glycolicum	-	+	-	-
C. bifermentans/ sordellii	-	+	+	-

*Slide agglutination for somatic antigen (Microscreen, Microgen Ltd, Guildford, UK)

This table is adapted from (Brazier, 1995), Table 1

One alternative identification method, which may be considered by institutions with cell culture facilities, is to test for cytopathic effect, neutralisable by *C. difficile* or *C. sordellii* antitoxin (see section 1.4.3.1). This can be performed on broth supernatants, with high level toxin producing strains giving positive results in 4-6 h. Alternatively, the technique can be attempted directly from colonies, by first suspending 1-2 colonies in 0.5 ml of phosphate buffered saline (PBS) and then using this for the cytopathic effect test after filtering. Centrifugation of the suspension can also be used to minimise any non-specific toxic effects that may occur when a bacterial suspension is added to a cell culture line. Positive results with this method can often be achieved in less than 4 hours (Settle, unpublished observations). Screening of isolates in this way, from either broths or colonies, can significantly reduce the number of expensive confirmatory kit tests required when large numbers of isolates are being cultured for studies where confirmation of identity is mandatory. Further tests need only then be performed on non-toxigenic strains.

1.4.2.6 Other methods

An assay of faecal lactoferrin levels has been considered as a possible aid to the diagnosis of *C. difficile* disease (Yong *et al.* 1994). However, owing to its poor levels of sensitivity and specificity is not appropriate as a diagnostic test in this milieu. Most polymerase chain reaction methods have been aimed at toxin gene detection, but one early method was designed to detect part of the 16s RNA gene of *C. difficile* (Gumerlock *et al.* 1991). This method was claimed to have a detection threshold of ten *C. difficile* organisms amongst 10^6 *E. coli*. It could also distinguish between *C. difficile* and other species such as *C. sordellii* or *C. bifermentans*. Asymptomatic patients were found to be negative by this assay, whilst 23 patients with antibiotic-associated colitis were positive, as well as 4 patients with diarrhoea who were *C. difficile* culture negative. The method is unable to distinguish toxigenic from non-toxigenic strains.

1.4.3 Toxin detection methods

1.4.3.1 Cytotoxin assay by cytopathic effect (CPE)

Cytotoxin detection, using a mammalian cell line in tissue culture, is generally accepted to be the gold standard single diagnostic test for *C. difficile* disease (Bond *et al.* 1995); (Fekety and Shah, 1993); (Brazier, 1998a). Its combination with culture for the organism probably represents the optimal combination of methods for investigation of this illness (Fang and Madinger, 1994); (Peterson and Kelly, 1993). The principle of the test is that toxin B, and to a much lesser extent toxin A, produce an actinomorphic-like cytopathic effect (CPE) in tissue culture monolayers exposed to them. This usually manifests as a 'rounding up' of the cells (see section 1.2.4.1.5). A number of different cell lines have been investigated to try to determine which is most sensitive, with varying results (Chang *et al.* 1979); (Donta *et al.* 1982); (Murray and Weber, 1983); (Thelestam and Bronnegard, 1980). Currently, fibroblast cell lines (America) or Vero cell lines (Europe) are preferred for CPE testing (Brazier, 1998a). It is estimated that the tissue culture assay can detect 1pg of toxin B (Lyerly *et al.* 1988).

The test itself involves initial suspension of 1-2 g of faeces, in an equal volume of PBS. This is centrifuged to remove large debris and then filtered through a 0.2 or 0.45μm membrane filter. The sterile faecal supernatant thus produced is then added to the cell monolayer culture (20μl of supernatant with 180μl of growth medium) and incubated at 37°C. To ensure specificity, each specimen is tested in parallel with and without *C. difficile* or *C. sordellii* antitoxin. A known weak positive extract is used as a positive control and PBS only added to one well as a negative control. Examination for CPE is traditionally performed at 24 and 48 h, although if high titres of toxin are present an effect can be

observed at 6 h. Systems for CPE testing when laboratories do not have cell culture facilities also exist, such as the Tox-Titer microtitre plate system (Bartels Immunodiagnostic Supplies Inc., Bellevue, WA, USA) which uses human foreskin cells (Nachamkin *et al.* 1986); (Wu and Gersch, 1986). Cytotoxin detection has become even more important now that strains without detectable toxin A have been found to be associated with disease (Depitre *et al.* 1993); (Kato *et al.* 1997); (Kato *et al.* 1998); (Sambol *et al.* 2000).

1.4.3.2 Counterimmunoelectrophoresis (CIE)

As CPE testing in most institutions was found to require 24-48 h to obtain a result, efforts were made to try to develop faster toxin detection methods. CIE was one such approach, and was designed to detect toxin in the stool (Ryan *et al.* 1980). Initial reports were encouraging (Welch *et al.* 1980), but later work reported poor sensitivity and specificity for the method (Kurzynski *et al.* 1983); (Wu and Fung, 1983). This may have been due to a possible cross-reaction with *C. sordellii* (Poxton and Byrne, 1981a), or detection of non-toxigenic *C. difficile* strains (West and Wilkins, 1982). Attempts were made to reduce false positivity rates by absorbtion of unwanted antibodies to whole *C. difficile* cells (Ryan *et al.* 1983), but the method has still not become widely used. Several reports indicate that the method has poor sensitivity and specificity as a diagnostic test (Jarvis *et al.* 1983); (Levine *et al.* 1982); (Tilton *et al.* 1982).

1.4.3.3 Enzyme-linked immunosorbent assay (ELISA)

Another method that was developed to provide faster detection of *C. difficile* toxin(s), was the ELISA. In this test, antibodies bind to toxin A or B in the first stage, and then a second antibody with some means of spectrophotometric detection (for example conjugated with horseradish peroxidase) binds to the first antibody. Then a substrate is

added which will change colour in the presence of the second antibody, the intensity of the colour indicating the quantity of toxin present. This allows automated reading of the result, which can improve turnaround time and reduce test to test variability. Numerous different manufacturers developed ELISA kits for toxin(s) A or A+B, and a comparison of these is summarised below (table 6). It should be remembered that the figures for sensitivity and specificity were usually generated in comparison to a CPE test. Consequently, if the CPE method used in the comparison did not use the most sensitive cell line, the sensitivity and specificity figures of the kit compared to it would be improved. This may in part explain the enormous range of results for ELISAs, even when the same kit was being tested. It is unlikely that ELISA methods can achieve the same sensitivity as CPE testing techniques because their limit of toxin detection is in the nanogram range compared to the picogram range for CPE methods (Lyerly, 1988). Nevertheless, ELISAs do provide a same day result and can be particularly useful in situations where cell culture facilities are not available. Because it is known that *C. difficile* strains without detectable toxin A can cause disease (section 1.2.4.1.7), it is important to use an ELISA which can detect both toxins.

Table 6: Published specificities and sensitivities for commercial *C. difficile* toxin detection ELISA kits (compared with cytotoxin detection)

KIT	TOXIN DETECTED	SAMPLE SIZE	SPECIFICITY (%)	SENSITIVITY (%)
Premier	A	101	100	71
		170	98	90
		504	99	87
		285	99.6	65
		320	99.2	67.6
		313	98.9	84.1
		301	95	91
		160	99.1	84.1
		329	97	84
		228	95	88
		700	98	87
		410	100	93
VIDAS CDA	A	194	75	63
		285	100	65
		329	95	71
		945	98.5	73
Bartels	A	329	92	94
		463	95.5	95.1
		700	96	87
Tox-A test	A	329	92	93
		463	93.7	86.6
		700	95	87
		355	100	84.6
		410	93	99
CD-TOX	A	160	88	92.3
Cytoclone	A+B	285	97.8	75.5
		945	99.1	83.6
		160	93.5	96.2
		700	99	89

This table is adapted from (Brazier, 1998a)

1.4.3.4 Dot-immunobinding assay

This method involves initial binding of toxin A to a membrane. Mouse monoclonal antibody to toxin A is then added. Then, anti-mouse antibodies conjugated to horseradish peroxidase are added. Finally, a chromogenic substrate is added which develops a blue-green colour if horseradish peroxidase is present. One such assay, C. diff-CUBE (Difco Laboratories, Detroit, MI, USA) was shown to have poor sensitivity and specificity by one study (Kurzynski *et al.* 1992), but better results were demonstrated by Woods and Iwen, (1990). Another method based on the same principle is the Immunocard (Meridian Diagnostics Inc., Cincinnati, OH, USA). However, this test detects *C. difficile* glutamate dehydrogenase and so is not specific for toxigenic strains. Reports of its performance vary, with some being reasonable (Staneck *et al.* 1996), and some poor (Jacobs *et al.* 1996). More recently, another similar toxin A test has become available. The Oxoid *C. difficile* toxin A test kit (Unipath, Basingstoke, UK) demonstrates toxin A bound to a membrane by complexing blue latex-coated antibody with the immobilised toxin. Sensitivity and specificity figures claimed by the manufacturer are based on a multicenter evaluation of the test (Bentley *et al.* 1998). The results showed a sensitivity and specificity of 83.1% and 96.9%, rising to 91% and 98% when discrepant results were resolved by culture. Independent assessment of this kit demonstrated a sensitivity of 72% and specificity of 94%, with false negative results occurring particularly in specimens with low toxin titres (see sections 3.2.1 and 3.2.3). The kit is easier to use than ELISAs and more amenable to single tests. Positive results appear accurate, although negative results are not as reliable and so confirmatory tests may be prudent for negative results if *C. difficile* disease is clinically felt to be likely.

1.4.3.5 Polymerase chain reaction (PCR)

The most recent diagnostic technique to be developed is PCR. Most of the methods designed so far are aimed at detecting toxin genes directly from clinical samples, but early methods were attempted on cultures of *C. difficile*. These were shown to compare very well with cytotoxin detection methods (Alonso *et al.* 1997); (Kato *et al.* 1991), although some were also positive with toxigenic *C. sordellii* strains (Wren *et al.* 1990). One alternative method described replica plating of a culture followed by toxin B gene probing (Wolfhagen *et al.* 1993).

Direct detection by PCR of *C. difficile* in stool samples was developed by a number of investigators (Arzese *et al.* 1995); (Boondekhun *et al.* 1993); (Green *et al.* 1994); (Gumerlock *et al.* 1993); (Kato *et al.* 1993a). These tests have been demonstrated to correlate well with cytotoxicity testing, and can be positive in cases where cytotoxicity tests are negative. This is claimed to be due to increased sensitivity, but there have been no large enough studies performed to truly determine the false positivity rate of such sensitive techniques, which can detect as little as 1 pg of DNA (Gumerlock *et al.* 1993). Inhibitory substances in stool specimens have been found to cause problems, requiring complex neutralisation and extraction procedures (Gumerlock *et al.* 1993); (Kato *et al.* 1993a). One investigator used monclonal antibodies bound to magnetic material to remove any *C. difficile* present in the sample prior to DNA extraction and PCR (Wolfhagen *et al.* 1994). This method was called magnetic immuno-PCR assay (MIPA) and compared well with isolation of a toxigenic strain of *C. difficile*. The most beneficial use for PCR technology at present is probably for the epidemiological typing and investigation of the variant toxin genes (see sections 1.6.2.4, 1.6.2.5 and 1.6.2.6).

1.4.4 Clinical detection methods

In addition to standard history taking and examination, several further investigations have shown some benefit in the diagnosis of CDI. Sometimes, experienced nursing staff will indicate that a patient is suspected to have CDI due to a characteristic smell of the diarrhoea (Brazier, 1998a). More technological help can be obtained by using radiology and endoscopy. Plain abdominal X-rays may reveal colonic wall thickening, 'thumbprinting' and in severe cases 'toxic megacolon'. These features, along with nodular mucosal thickening, pericolonic oedema and ascites, may also be detected by CT scanning (Boland *et al.* 1995). Several investigators have found CT scanning useful as a diagnostic aid (Kawamoto *et al.* 1999); (Wilcox *et al.* 1995); (Zamora *et al.* 1996) including in CF patients without symptoms of diarrhoea (Binkovitz *et al.* 1999). However, specific features such as the 'accordion sign' are not specific to CDI but merely indicate severe colonic inflammation (Macari *et al.* 1999). In addition, presence of abnormal CT findings has not been found to correlate with increased severity of CDI (Boland *et al.* 1995). Sigmoidoscopy and colonoscopy can provide useful information about the severity of colitis and may suggest the diagnosis if classical, raised, yellow-white pseudomembranes are visualised (Fekety and Shah, 1993); (Wei *et al.* 1997).

1.5 EPIDEMIOLOGY OF *CLOSTRIDIUM DIFFICILE* COLONISATION AND INFECTION

1.5.1 Distribution of *C. difficile*

C. difficile is a fairly ubiquitous organism and can be found in many environments. It is noted to have been isolated from soil, sand and mud, as well as camel, horse and donkey dung (Hafiz, 1974). More recently, detailed environmental analysis was carried out in South Wales, resulting in *C. difficile* isolation from river water, sea and lake water, swimming pools and even tap water (Al Saif and Brazier, 1996). Notably 14 of 16 (87.5%) samples of river water were positive compared to 7 of 15 (46.7%) samples of sea water and the same proportion of lake water specimens. It was found in soil and on raw vegetables. Nevertheless, some investigators have been unsuccessful in attempts to isolate *C. difficile* from soil (Kim *et al.* 1981); (Riley, 1994), and Oishi *et al.* (1983) failed to detect *C. difficile* in foods. Discussion regarding the presence of *C. difficile* in the hospital environment can be found in section 1.5.2.2.2.

1.5.2 Colonisation with *C. difficile*

1.5.2.1 Animals

Carriage has been demonstrated in the faeces of dogs and cats, horses, sheep and poultry, but not in cattle, pigs or fish (Al Saif and Brazier, 1996). A range of other animals have also been noted to be potential reservoirs of *C. difficile* (Riley, 1994) (see table 7). When isolates from both animals and humans were compared in one study by restriction

endonuclease analysis (REA) and restriction fragment length polymorphism (RFLP) typing, no common types were demonstrated (O'Neill *et al.* 1993). It remains unclear whether there is an epidemiological link between carriage in animals and subsequent acquisition in humans, but cats and dogs appear to be the animals most frequently colonised with *C. difficile* (Borriello *et al.* 1983); (Riley *et al.* 1991b). Such carriage does however, seem to relate to antibiotic use (Madewell *et al.* 1999); (Riley *et al.* 1991b) and this observation forms a parallel with the situation in humans. Although Borriello *et al.* (1983) found most strains recovered in pets to be non-toxigenic, Riley *et al.* (1991b) found both cytotoxigenic and non-cytotoxigenic strains.

Table 7: Animal reservoirs of *C. difficile*

Camels	Seals
Cattle	Snakes
Donkeys	Deer
Horses	Hares
Antelopes	Native cats
Kodiak Bear	Domestic cats
Dogs	Quokka*
Hamsters	Numbat*

*Small Australian marsupials

Table adapted from (Riley, 1994), Table 2

In addition to carriage of *C. difficile*, animals may also suffer with *C. difficile* disease and a great deal of research has been performed using hamster, guinea pig, rabbit, mouse and rat models to try and understand more about this illness in humans. CDI has been reported in horses (Madewell *et al.* 1995); (Teale and Naylor, 1998), ostriches (Frazier *et al.* 1993), and in one report dogs (Berry and Levett, 1986) , although this was not corroborated by the work of two other investigators (Borriello *et al.* 1983); (Struble *et al.* 1994).

1.5.2.2 Humans

Colonisation rates with *C. difficile* amongst human subjects differ significantly with age, relative exposure to the organism, antibiotic administration, and competence of immune function.

1.5.2.2.1Age

1.5.2.2.1.1 Neonates

Neonatal carriage of *C. difficile* has been recognized since 1935, when Hall and O'Toole isolated a bacterium, known then as *Bacillus difficilis*, from the stools of 4 out of 10 asymptomatic newborn infants (Hall and O'Toole, 1935). Numerous studies have since been conducted, and the colonisation rates reported amongst neonates range from 2-71% (Al-Jumaili *et al.* 1984); (Blakey *et al.* 1982); (Bolton *et al.* 1984); (Donta and Myers, 1982); (el Mohandes *et al.* 1993); (Larson *et al.* 1978); (Larson *et al.* 1982); (Lishman *et al.* 1984); (Phua *et al.* 1984); (Richardson *et al.* 1983); (Rotimi and Duerden, 1981); (Snyder, 1940); (Tabaqchali *et al.* 1984a); (Viscidi *et al.* 1981). Rates of colonisation at birth are low, rising during hospital stay (Bolton *et al.* 1984), presumably due to nosocomial transmission

(el Mohandes *et al.* 1993); (Phua *et al.* 1984); (Sherertz and Sarubbi, 1982); (Zedd *et al.* 1984). Further evidence to support this theory was provided by the isolation of *C. difficile* from environmental sources (Larson *et al.* 1982); (Malamou-Ladas *et al.* 1983), whilst the mothers of colonised infants were shown not to be the source of the organism (Larson *et al.* 1982). There is some work to suggest that maternal transmission plays a part in infant colonisation with *C. difficile* (Tabaqchali *et al.* 1984a), when 8 out of 9 babies from *C. difficile* colonised mothers were found to be colonised compared with 23 out of 41 babies from non-colonised mothers. However, this also suggests that cross-infection may be occurring in a large number of those babies born to non-colonised mothers. Others have not found evidence for maternal colonisation to be significant (Bolton *et al.* 1984), although this may be due to differences in culture methodology.

Colonised neonates are often found to have high toxin titres in their stools, but are usually asymptomatic (Al-Jumaili *et al.* 1984); (Donta and Myers, 1982); (el Mohandes *et al.* 1993); (George, 1986); (Viscidi *et al.* 1981), despite these toxin levels being equal to those in adults with PMC (Stark *et al.* 1982). The reasons for this finding still remain somewhat unclear but several hypotheses have been proposed.

Lower rates of colonisation have been seen by some investigators in breast fed versus bottle fed infants (Cooperstock *et al.* 1982); (Cooperstock *et al.* 1983); (Tullus *et al.* 1989), suggesting a possible protective role for immunoglobulin in the mother's milk. Other studies, however, did not find any difference between colonisation rates in breast or bottle fed infants (Bolton *et al.* 1984); (Stark *et al.* 1982); (Viscidi *et al.* 1981), although Stark and Lee, (1982) report a delay in colonisation in breast fed babies. Breast milk was shown to have neutralising activity against *C. difficile* toxins in 30-60% of the samples tested in two studies (Kim *et al.* 1984); (Wada *et al.* 1980). Kim *et al.* (1984) demonstrated that anti toxin A activity was due to IgA, whilst anti toxin B activity was not due to IgA, IgG or IgM.

Subsequent work has also demonstrated a non-immunoglobulin component to the anti toxin A activity of human colostrum (Rolfe and Song, 1995). Although protective factors in human milk may play a role in the protection of infants from CDI, they cannot account for protection in infants who are not breast-fed. Rolfe and Song (1995) have observed that infant formula based on cows milk can also inhibit the binding of toxin A to hamster BBMs, which could provide the explanation.

A second possible mechanism for protection is that the intestinal mucosa of the neonate may be devoid of the receptors for toxin A (Kotloff *et al.* 1988). This phenomenon has been found to explain the non-susceptibility of newborn rabbits to the effects of toxin A (Eglow *et al.* 1992) and proposed as the mechanism for a similar observation in hamsters (Borriello, 1990).

Other suggested protective mechanisms in neonates include reduced sensitivity of neonatal intestinal cells to toxins compared with adult cells (Chang *et al.* 1986), or production of a thicker layer of protective mucus at the mucosal surface interfering with toxin binding (Lyerly *et al.* 1988).

1.5.2.2.1.2 Infants

Colonisation rates amongst children under the age of one remain high at 30-65% (Cooperstock *et al.* 1982); (Hall and O'Toole, 1935); (Holst *et al.* 1981); (Stark *et al.* 1982), but these rates fall sharply after the first year, reaching adult levels by the age of three (Stark *et al.* 1982).

1.5.2.2.1.3 Adults

In young, asymptomatic adult subjects, gastrointestinal tract colonisation rates with *C. difficile* have been generally found to range from 0-3% (Aronsson *et al.* 1985b); (Bartlett *et al.* 1980); (George *et al.* 1978b); (Falsen *et al.* 1980); (Larson *et al.* 1978); (Marrie *et al.* 1982), although in one Japanese study, colonisation rates were 15.4% in healthy adults (Nakamura *et al.* 1981). It is not clear whether this group had been exposed to antibiotics, or had more exposure than average to *C. difficile*. The use of different culture methodologies may also lead to discrepancies between the results of colonisation studies performed in different centres. Colonisation of adults at other sites, such as the genitourinary tract remains a contentious issue. Some investigators have shown antenatal carriage rates of 18-24% using enrichment broth culture techniques (O'Farrell *et al.* 1984); (Tabaqchali *et al.* 1984a); (Thirkell *et al.* 1984), whilst others demonstrated carriage rates of zero (Al-Jumaili *et al.* 1984); (Bolton *et al.* 1984); (Holst *et al.* 1981). Similarly, discrepancies exist between the results of screening genitourinary clinic patients by Hafiz *et al.* (1975), O'Farrell *et al.* (1984), and Thirkell *et al.* (1984) who found carriage rates of 12-100%, and those of Levett, (1984b) and Moss, (1983) who found no carriers. These diverse results may reflect varying geographical colonisation rates, the variable sensitivity of enrichment broths for isolating small numbers of organisms or possibly indicate the presence of spores.

Elderly adults demonstrate higher asymptomatic colonisation rates with *C. difficile* than their younger counterparts (Aronsson *et al.* 1985b); (Brazier *et al.* 1999b); (Rudensky *et al.* 1993); (Settle *et al.* 1999), with rates of 10-12% not being uncommon. Interestingly in the study by Nakamura *et al.* (1981) in which the colonisation rate in healthy adults was so high, healthy elderly patients had a colonisation rate of 7.0%, whilst elderly patients with cerebrovascular disease had a rate of 15.9%. Nevertheless, these rates are still much higher than colonisation rates for adults in many countries. Two reports did indicate low rates of

C. difficile colonisation amongst elderly patients (Campbell *et al.* 1988); (Corrado *et al.*1990), although they were both on small groups. Such findings are very different from the norm and may just relate to the specific circumstances in those units at that time. High rates of colonisation in elderly patients may result from their possibly reduced immune responses to infection, or because their bowel flora is less able to resist colonisation by *C. difficile*, which has been demonstrated by Borriello *et al.* (1986).

Hospitalised patients are the group who demonstrate the highest rates of *C. difficile* colonisation, with reports of 20% or more being common (Bender *et al.* 1986); (Bennett *et al.* 1984); (Burdon, 1982); (Gerding *et al.* 1986); (Johnson *et al.* 1990a); (McFarland *et al.* 1989); (Pierce *et al.* 1982); (Rudensky *et al.* 1993); (Varki and Aquino, 1982). This may be as a result of the fact that they are often treated with antibiotics, and are more likely to be exposed to *C. difficile* in hospital than at home. However, some of the higher rates were observed in outbreak situations.

1.5.2.2.2 Exposure to *C. difficile*

Exposure to the organism, or more usually its spore is a prerequisite for developing *C. difficile* colonisation or disease. Acquisition of the organism from the environment was suggested by the early reports of clusters of cases of PMC (Kabins and Spira, 1975); (Keighley *et al.* 1979); (Ramirez-Ronda, 1974); (Tedesco *et al.* 1974). Animal studies also indicated the significance of environmental contamination (Fekety *et al.* 1979); (Larson *et al.* 1978). More recently, the presence of *C. difficile* in the hospital environment has been well documented. Mulligan *et al.* (1980) documented that 32.5% of environmental culture sites were positive in areas where symptomatic patients with *C. difficile* diarrhoea were being cared for compared with 1.3% of sites in control areas. The most common positive sites for *C. difficile* were bathroom floors, toilet seats, toilet bowl rims and bed handrails.

Kim *et al.* (1980) also found that environmental sites in the vicinity of symptomatic *C. difficile* infected patients were more likely to become contaminated than similar sites in control rooms. Fekety *et al.* (1980), screened environmental sites in rooms of patients with symptomatic CDI, asymptomatic *C. difficile* colonisation and *C. difficile* negative diarrhoea. They found *C. difficile* on the patients' blankets, the floor of the patients' rooms, the bathroom floor, the toilet seat and the sink cabinet, but not in the air or on food. *C. difficile* was isolated from the hands of staff. In the room of a patient with symptomatic CDI, 19.6% of environmental sites were positive. Only 6.8% of sites were positive in the room of an asymptomatic *C. difficile* colonised patient and just 2.6% of sites were positive in the room of a patient with *C. difficile* negative diarrhoea, suggesting that greater environmental contamination is likely when patients are symptomatic.

Another significant study was performed by McFarland *et al.* (1989). This group prospectively monitored *C. difficile* acquisition and transmission on a medical ward, using rectal swabs rather than relying on faecal specimens. Over an 11-month period, 428 patients were studied and 6.7% were *C. difficile* positive on admission. Of those who were negative on admission, 21% had nosocomial acquisition of *C. difficile* with 63% of these exhibiting asymptomatic carriage only. Patients were found to be at higher risk of aquiring *C. difficile* if they were co-occupying a room with a colonised patient. In total, on 87% of occasions where acquisition by one patient was felt to be due to exposure to another positive patient, the strains were identical using an immunoblotting method described by Mulligan *et al.* (1988). In these cases, the initially positive patient who was thought to have led to another patient aquiring *C. difficile* was a carrier on 61% of occasions and had diarrhoea 39% of the time. This discrepancy may just reflect the fact that carrier status was 1.7 times more common than infection. Whenever an initially negative environment became positive, it was with an identical strain to that of the patient already in that area. This work confirmed the findings of other investigators, who recognised that carriage of *C. difficile* on the hands of

health care workers occurs (Fekety *et al.* 1981); (Gerding *et al.* 1986); (Heard *et al.* 1988); (Kim *et al.* 1981); (Malamou-Ladas *et al.* 1983); (Mulligan *et al.* 1980). Such colonisation was demonstrated on the hands of 59% of personnel following patient contact. Environmental contamination of the ward environment was also found to be significant in this study (29% of sites overall), again in agreement with previous findings (Al-Jumaili *et al.* 1984); (Fekety *et al.* 1980); (Fekety *et al.* 1981); (Gerding *et al.* 1986); (Heard *et al.* 1986); (Kim *et al.* 1980); (Larson *et al.* 1980); (Larson *et al.* 1982); (Mulligan *et al.* 1979); (Savage and Alford, 1983); (Walters *et al.* 1982). Furthermore, a greater degree of environmental contamination was noted in association with patients who had diarrhoea (49% of sites tested), as previously described (Kim *et al.* 1981); (Mulligan *et al.* 1979) (Mulligan *et al.* 1980). One early study of an outbreak of PMC suggested that environmental contamination played little or no part in such incidents, but perhaps poor infection control measures allowed direct person to person transmission (Keighley *et al.*1979). Johnson *et al.* (1990) found no spatial clustering within three wards studied, however, they detected *C. difficile* in 60 of 282 (21%) patients, over a 9-week period. There is no overall indication of whether patients were *C. difficile* positive on admission or acquired it nosocomially, apart from the observation that 7 out of 9 patients who developed *C. difficile* diarrhoea had previously negative swabs. No environmental screening for *C. difficile* was performed. Consequently, it seems difficult to be certain that hand to hand transmission alone and not environmental contamination led to nosocomial acquisition of *C. difficile*. It is now recognised that environmental contamination with *C. difficile* spores plays an important role in the development of this predominantly nosocomial disease. Such spores are also able to persist for months in the environment (Kim *et al.* 1981); (Mulligan *et al.* 1979), due to their resistant nature. At present, the infective dose in humans has not been determined, but may be very low, as work in the hamster model indicates that administration of as few as 2 colony forming units can lead to disease (Larson *et al.* 1978).

In addition to hospitals, care facilities such as residential or nursing homes for the elderly are at risk of becoming heavily colonised with *C. difficile*, which may in turn lead to outbreaks occurring. McFarland *et al.* (1989) showed that 82% of patients who became positive for *C. difficile* in hospital remained positive at the time of discharge. Of the patients investigated in the study, 17% who were negative for *C. difficile* on admission were positive when discharged. Patients with *C. difficile* positive stools were noted to be discharged significantly more often to extended care facilities than *C. difficile* negative patients (27% vs. 12%, $p<0.01$), thus highlighting the risk to these facilities. Outbreaks of *C. difficile* infection in nursing homes have been described (Bender *et al.* 1986).

1.5.2.2.3 Antibiotic exposure

Exposure to antimicrobial agents is usually the inciting agent for CDI as discussed in section 1.3.2.3. It is also associated with increased risk of *C. difficile* colonisation, as indicated by Johnson *et al.* (1990) in their work on nosocomial *C. difficile* colonisation and disease. Viscidi *et al.* (1981) report a rate of asymptomatic colonisation with *C. difficile* of 21% in patients receiving antibiotics. Kyne *et al.* (2000), in a study on acute medical patients, found that 31% of those who received antibiotics were colonised by *C. difficile* and 56% of this group developed CDI. Several other investigators have reported increased asymptomatic colonisation rates in patients who have received antibiotics (Bartlett *et al.* 1978c); (George *et al.* 1982); (Greenfield *et al.* 1983); (Viscidi *et al.* 1981); (Wu *et al.* 1983). Studies of the effects of antibiotics on the gastrointestinal flora of healthy volunteers have also indicated an increase in *C. difficile* colonisation rates, see section 1.3.2.3.2. One study showed an asymptomatic *C. difficile* colonisation rate of 48% in subjects following administration of antibiotics (George, 1988).

1.5.2.2.4 Immune function

As discussed in section 1.3.2.2, an effective immune response is thought to be an important factor in protection from CDI and higher serum antibody levels have correlated with milder illness or colonisation (Aronsson *et al.* 1983); (Aronsson *et al.* 1985a); (Mulligan *et al.* 1993). Secretory IgA is also capable of neutralising toxin A (Kelly *et al.* 1992) and Stubbe *et al.* (2000) demonstrated that monoclonal IgA antibodies could protect human colonic carcinoma derived T84 monolayers from the effects of toxins A and B. In the very elderly, the immune system is not thought to be as effective, which may reduce their protection against *C. difficile* disease or colonisation (Mulligan *et al.* 1993). This is supported by the observation of Viscidi *et al.* (1983) that although antitoxin antibodies were detected in those aged over 70, they had no toxin neutralising capability.

1.5.2.2.5 Other risk factors

Apart from elderly patients, other groups who appear more likely to develop *C. difficile* colonisation than normal include patients with cystic fibrosis (Peach *et al.* 1986); (Wu *et al.* 1983), and those with inflammatory bowel disease (Dorman *et al.* 1982); (Greenfield *et al.* 1983); (Keighley *et al.* 1982); (Meyers *et al.* 1981). It is possible that these findings relate to an increased antibiotic usage in such groups. Presence of a nasogastric or gastrostomy tube has also been linked with an increased risk of asymptomatic *C. difficile* acquisition (Simor *et al.*1993). This may relate to an increased risk of transmission via the hands of caregivers, who become colonised due to environmental contamination. One study found a significant link between H_2 antagonists and *C. difficile* colonisation in elderly patients (Walker *et al.* 1993), but this finding needs to be confirmed.

1.5.3 *C. difficile* infection

1.5.3.1 Hospitals and extended care facilities

C. difficile is currently the most important cause of nosocomially acquired, diarrhoea-associated infection (Djuretic *et al.* 1996); (Silva, 1994); (Wilcox, 1996). It has been reported to account for 75% or more of such cases (Gerding *et al.* 1995) and is of great importance in both hospitals as well as nursing or residential homes. Detailed discussion regarding the epidemiology of *C. difficile* colonisation in hospitals and extended care facilities takes place in the sub-sections of section 1.5.2.2 and also relates to CDI. One additional finding of interest is that patients who have asymptomatic colonisation with *C. difficile* on admission to hospital, albeit with toxigenic strains, appear less likely to develop *C. difficile* disease (Shim *et al.* 1998). This observation may be explained on the basis that some patients with robust immune responses could inactivate toxin produced in the intestine and thereby arrest the development of symptoms. Mulligan *et al.* (1993) described serum IgA and IgM levels in asymptomatic carriers which were significantly higher than levels in either patients with disease or in controls. Secretory IgA levels in this group of patients were not determined.

In addition to significant morbidity, CDI results in significant extra costs to care institutions and may also lead to mortality (Eriksson and Aronsson, 1989); (Lesna and Parham, 1996); (Monti *et al.* 1992); (Spencer, 1998); (Wilcox *et al.* 1996). As mentioned in section 1.5.2.2.1.1, children under the age of one appear relatively immune to the effects of *C. difficile* toxins, but disease can occur (Adler *et al.* 1981). Older children often have diarrhoea after taking antibiotics, but PMC is rare (Gryboski *et al.* 1991). However, cases of PMC have been described (Donta *et al.* 1981); (Mandal *et al.* 1982); (Qualman *et al.* 1990). CDI in adults is predominantly an illness of those aged over 60 (Aronsson *et al.* 1984a);

(Nash *et al.* 1982). The Communicable Diseases Surveillance Centre (CDSC), UK (2000) reported that 80% of cases occur in patients over the age of 65. Kyne *et al.* (1999) reported that the patients at highest risk of CDI are those over the age of 75 with severe co-existing illness.

Rates of *C. difficile* disease have increased markedly in the last two decades (Brown *et al.* 1990); (CDSC, 1998); (Olson *et al.* 1994), and although increased reporting may account for part of this, a true increase in the incidence of CDI in various hospitals appears to have occurred (Wilcox and Smyth, 1998). As discussed in section 1.5.2.2.2, environmental contamination appears to be the main factor leading to subsequent patient acquisition. The increase in patient susceptibility is probably a combination of factors and may include increasing antibiotic usage, particularly of 'third generation' cephalosporins and increasing age of the patient population. An increased exposure to *C. difficile* may also arise due to understaffing of hospital facilities, with consequent suboptimal levels of infection control. Acquisition rates in hospitals have been reported to be from 6-20% (Brazier *et al.* 1999b); (McFarland *et al.* 1989); (Rudensky *et al.* 1993); (Samore *et al.* 1994a).

1.5.3.2 Community

Far less information is available on *C. difficile* disease and carriage in the community, but it does still appear to be a significant pathogen. Samore *et al.* (1994b) investigated patients admitted to a medical ward, a surgical ward or an ICU. Cultures were performed within 72 h of admission and an asymptomatic colonisation rate of 7% was found. The mean age of patients was 61 years. A review of 73 cases of *C. difficile* infection in Ireland revealed that 10.9% were thought to be community acquired. Two larger studies have been performed in Australia, one demonstrated that 4.7% of diarrhoeal stool samples from this source were *C. difficile* positive accounting for 30.3% of all enteropathogenic

organisms isolated (Riley *et al.* 1986). The other showed a *C. difficile* positivity rate of 5.5% (16 of 288 samples), which represented the most common enteric pathogen found (Riley *et al.* 1991a). More recently, the Swedish *C. difficile* study group carried out a prospective study of all cases of CDI during 1995. They found that 28% of cases were apparently of community origin (Karlstrom *et al.* 1998). When Wheeler *et al.* (1999) prospectively studied the causes of infectious intestinal disease in the community, they found relatively low levels of CDI at less than 1% of all diarrhoeal cases. However, there are far more individuals in the community than in hospitals. Consequently, 1% of the total number of patients with diarrhoeal symptoms in the community might represent a large percentage of people with CDI overall, when hospital cases are added. These reports suggest that there is a significant burden of *C. difficile* disease in general practice.

1.5.3.3 Risk factors

Exposure to antibiotics is the greatest risk factor for development of CDI, with 'third generation' cephalosporins or multiple courses of antibiotics being most dangerous. Duration of treatment may also be a factor (Brown *et al.* 1990). Antibiotics as a risk factor are comprehensively discussed in section 1.3.2.3 and its subsections, whilst environmental contamination is discussed in section 1.5.2.2.2. Age is an important risk factor (Aronsson *et al.* 1984); (Borriello and Larson, 1981); (Brown *et al.* 1990); (McFarland *et al.* 1990), with 80 % of cases of CDI occurring in those aged over 75 (see section 1.5.3.1). Analysis of other risk factors suggests that gastrointestinal procedures and intensive care residence (Brown *et al.* 1990), as well as tube feeding (Bliss *et al.* 1998); (Talon *et al.* 1995), are significantly associated with *C. difficile* disease. Similarly, increased length of hospital stay (Gerding *et al.* 1986) and more severe underlying illness (McFarland *et al.* 1990), have also been associated with an increased risk of CDI development.

Starr *et al.* (1997) proposed what they termed the 'herd immunity' model. This states that the collective susceptibility of a ward group is the important factor in determining whether an outbreak will occur. Four groups of patients exist in this model: resistant uncolonised, resistant colonised, susceptible uncolonised and susceptible colonised. Disease can only progress from the susceptible colonised state, but all the states are in continual flux. The theory is that provided there are not very many susceptible, uncolonised patients then exposure to *C. difficile* will not result in many cases of disease. Reduction of exposure to *C. difficile* will also reduce the chance of susceptible uncolonised patients becoming colonised. If, on the other hand, levels of susceptible uncolonised patients rise, then once they are exposed to *C. difficile* there is the risk of an outbreak occurring. Risk of susceptibility is highest whilst on antibiotics, and according to Starr's estimate, falls relatively rapidly after they are stopped. Hence, he argues that it is of greatest benefit to stop antibiotics as early as possible, thereby minimising the number of patients who are very susceptible to colonisation and so to disease. In reality this seems to be a complicated way of explaining a phenomenon with which we are already acquainted, namely that it is wise to minimise the use of antibiotics. However, there is slightly more to the theory than that, as Starr argues that keeping patients in hospital for longer, after their antibiotics are finished, would be helpful as it would potentially elevate the 'herd immunity' to CDI.

1.5.4 Prevalence of different *C. difficile* types in the UK and abroad

Most of the typing data for *C. difficile* in England and Wales is co-ordinated by the PHLS Anaerobe Reference Unit , Cardiff PHL, where 117 different ribotypes (see section 1.6.2.6) have been described. Their data indicate that 90% of hospital patient isolates belong to one of 16 types. Ribotype 1 isolates represented 57% of the total number of over 1000 strains. This ribotype was also demonstrated to be endemic in 33 of 58 hospitals surveyed (Brazier, 1998b). Upon testing 40 ribotype 1 isolates from 20 different hospitals, Brazier's

group, using 11 different restriction enzymes, were unable to distinguish between isolates (Brazier *et al* 1997a). The international typing study (Brazier *et al.* 1997b), indicates that this ribotype is also causing problems in the USA. Ribotype 1 has been found to correspond to Delmée's serogroup G, but this serogroup has not been found to be the predominant strain in Belgium (Van Dijck *et al.* 1996) or France (Barbut *et al.* 1996).

Increasing numbers of reports are being made about toxin A-B+ strains in association with clinical disease. These strains have been found to correspond mostly to serogroup F (Depitre *et al.* 1993), but a few strains appear to be serogroup X (Kato *et al.* 1998); (von Eichel-Streiber *et al.* 1999). They also correspond to ribotype 17 (O'Neill *et al.* 1996). Kato *et al.* (1997), report a high prevalence of 33% toxin A-B+ strains amongst 143 toxigenic isolates tested. The small amount of data available from England and Wales suggests a much lower incidence rate of 3% amongst hospital isolates submitted to the PHLS Anaerobe Reference Unit, Cardiff PHL (Brazier, 1998b).

In contrast to the findings with hospital strains, the PHLS Anaerobe Reference Unit, Cardiff PHL reports that the most prevalent community strain was non-toxigenic (ribotype 10) and accounted for 15.9% of 390 isolates typed. The most frequent hospital isolate, ribotype 1 accounted for only 7.4% of these community strains (Brazier, 1998b).

1.5.5 Cross Infection

As discussed earlier in section 1.5.2.2.2, environmental contamination has been well documented in the ward environment, particularly near patients with diarrhoea. Carriage of *C. difficile* on the hands of staff is also well known to occur and cross-infection has been well described by research using epidemiological typing methods (Heard *et al.* 1986); (Magee *et al.* 1993); (McFarland *et al.* 1989); (Savage and Alford, 1983);

(Testore *et al.* 1988). Strategies aimed at reducing the risk of cross infection are based on two principles. Firstly, reducing environmental contamination by a combination of patient isolation and frequent cleaning of at risk clinical areas, and secondly by reducing person to person transmission. Barrier nursing and isolation of CDI cases has been shown to be effective in some, but not all reports (Bender *et al.* 1986); (Brown *et al.* 1990); (Nolan *et al.* 1987). Nevertheless, it does originate from sound basic principles, as we know that local environmental contamination with spores is worst around diarrhoeal patients and that environmental contamination can play a part in the perseveration of *C. difficile* outbreaks (McFarland *et al.* 1989); (Samore *et al.* 1996). Environmental cleaning using hypochlorite seems most effective (Kaatz *et al.* 1988), and when other disinfectants are used it is the dilutional effect which seems important (Struelens *et al.* 1991), along with physical removal of spores. Reducing person to person transmission usually involves the the use of gloves and aprons for staff involved in physical patient care and rigorous attention by everyone to handwashing between all patient contacts. The use of gloves for those directly involved in patient care has been recommended in several studies as beneficial (Johnson *et al.* 1990b); (McFarland *et al.* 1989). Handwashing after patient contact has also been demonstrated to be an important control measure (Kaatz *et al.* 1988); (McFarland *et al.* 1989), although liquid soap appears as useful as chlorhexidine (Bettin *et al.* 1994). Another practice which has been found to reduce cross infection is the use of disposable thermometers (Brooks *et al.* 1992); (Jernigan *et al.* 1998).

1.5.6 Relapse

Rates of recurrence following successful treatment of CDI with either metronidazole or vancomycin reported in the literature range from 5-24% (Wilcox and Spencer, 1992). Whilst these were originally felt to be due to persistence of spores and subsequent true 'relapse' of the inadequately treated infection, this no longer appears to be the case. Several

studies have now demonstrated that in a large proportion of cases, such 'relapses' are due to genetically different strains and are therefore really re-infections (Barbut *et al.* 2000); (Johnson *et al.* 1989); (O'Neill *et al.* 1991). Furthermore, whilst endemic strains of *C. difficile* exist at some institutions, some of the 'relapses' with identical genetic strains may also be reinfections, merely with the same strain as before (Wilcox *et al.* 1998). Re-infection rather than relapse in now felt to be the most likely cause of recurrent CDI.

1.6 TYPING OF *CLOSTRIDIUM DIFFICILE*

Since the identification of *C. difficile* as the cause of most cases of antibiotic associated colitis (Bartlett, 1990), there have been numerous attempts to try and develop a satisfactory method for typing the organism. Many different typing and fingerprinting methods have been tried and have been shown to be extremely useful for epidemiological typing. However, no standardised method was accepted which could be employed anywhere in the world allowing comparison of results. Now, a method may have been achieved which will be universally accepted as the best way of typing *C. difficile*, allowing comparison of results worldwide.

1.6.1 Phenotypic methods

1.6.1.1 Antibiotic sensitivity pattern

Antibiotic sensitivities were one of the first methods used to try to relate strains of *C. difficile* in potential outbreak situations. Investigation of the sensitivities of *C. difficile* revealed that most strains were either highly sensitive or highly resistant, with MICs falling

into a narrow range (Burdon, 1982); (Dzink and Bartlett, 1980); (Gianfrilli *et al.* 1984b). Use has been made of resistance patterns during the investigation of outbreaks (Burdon, 1982); (Climo *et al.* 1998); (Wust *et al.* 1982). Although being a relatively simple test to perform, its use is limited by the fact that there is little variability in sensitivity between *C. difficile* strains. Wust *et al.* (1982) used antibiograms along with plasmid analysis, counterimmunoelectrophoresis of toxins and polyacrilamide gel electrophoresis (PAGE) of soluble proteins to show that 12 of 16 isolates in an outbreak were identical.

1.6.1.2 Bacteriophage and bacteriocin susceptibility

The use of phages and bacteriocins to type *C. difficile* was described by Sell *et al.* (1983). At least 40 different patterns are known to exist (Tabaqchali, 1990). This system has been used to study the epidemiology of an outbreak in a hospital in Zurich (Hachler and Wust, 1984) as well as a neonatal unit outbreak (Zedd *et al.* 1984), where 30 out of 31 isolates were found to be identical. This included environmental as well as staff and patient isolates. Bacteriocin, bacteriophage and plasmid profile typing methods were combined by Mahony *et al.* (1991), but were only able to type 84% of 114 isolates. There is no widespread expertise in the use of these techniques.

1.6.1.3 Electrophoretic protein profiles

This technique relies on variations in cellular and surface proteins to distinguish different strains of *C. difficile*. Proteins are first extracted and then separated by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE). Various detection methods of the protein profiles are described depending on whether [^{35}S] methionine is incorporated into the cellular proteins before separation. This method, known as radio-PAGE, requires autoradiographic detection methods. Other methods rely on staining

techniques such as Coomassie blue to visualise the separated protein bands. Staining methods were reported by Poxton *et al.* (1984), Wexler *et al.* (1984) and radio-PAGE methods by Tabaqchali *et al.* (1984b). Further weight was added to the suggestion that radio-PAGE was a useful typing scheme when newer detection methods also confirmed the specific radio-PAGE types as distinct. This was described using DNA restriction endonuclease analysis (Wren and Tabaqchali, 1987), and arbitrary primer PCR (AP-PCR) patterns (Wilks and Tabaqchali, 1994). Some radio-PAGE types have also been reliably demonstrated to be toxigenic (Wren *et al.* 1987). Despite radio-PAGE being heralded as a very useful technique, it does suffer from the disadvantage of involving radioactivity, and whole cell EDTA-extracted protein SDS-PAGE patterns are claimed to be even more discriminatory (Costas *et al.* 1994). Ogunsola *et al.* (1995a) found that SDS-PAGE of EDTA-extracted cell surface proteins compared favourably with serogrouping for 61 isolates tested. All these methodologies produce profiles with numerous bands and can prove difficult to interpret without computer assistance (Ehret *et al.* 1988); (Tabaqchali *et al.* 1987).

1.6.1.4 Immunoblotting

Western blotting (immunoblotting) techniques involve protein antigen extraction and PAGE electrophoresis followed by blotting onto a nitrocellulose membrane, with detection by a second antibody conjugated to an enzyme which allows chromogenic detection. Immunoblotting was compared by Mulligan *et al.* (1988) with plasmid fingerprinting, serotyping and PAGE. They determined immunotyping to be the most discriminatory method of this group, however it has not been widely adopted for the typing of *C. difficile*. The technique is likely to be of greater benefit when more than one or two antisera are used (Kato *et al.* 1993b). However, they noted that not all strains of 114 tested were typeable

even using all 10 currently available antisera. New antisera require to be made before this method can type all strains.

1.6.1.5 Serogrouping

This method uses antibodies, which are raised in laboratory animals, to cell surface proteins. The sera are then mixed with formol-treated cells with agglutination indicating a positive result. Ten different serogroups have been described and all groups except serogroup A appear homogenous by PAGE typing, which splits serogroup A into 12 different strains (Delmée *et al.* 1986). Serogroup D was found to be non-toxigenic, whilst serogroup F and X produce toxin B but no detectable toxin A (Delmée and Avesani, 1990); (von Eichel-Streiber *et al.* 1999). The lack of standardised reagents between laboratories means that it is difficult to compare serogrouping results from different centres.

1.6.1.6 Pyrolysis mass spectrometry (PyMS)

PyMS is a highly discriminatory method that is capable of testing a high throughput of samples, making it useful for outbreak investigation (Cartmill *et al.* 1992); (Magee *et al.* 1993). It involves the production of volatile bacterial products by thermal degradation, known as the pyrolysate, and then analysis using a mass spectrometer. It does not type but rather 'fingerprints' strains because whilst it can compare all the isolates in one batch, distinguishing well between them, the results cannot be compared to earlier or later batches. Inclusion of previously isolated strains of known types can overcome this drawback to a certain extent. This technique has also shown good correlation with both SDS-PAGE profiles of outer membrane proteins (Ogunsola *et al.* 1995b), and PCR ribotyping (O'Neill *et al.* 1996). One drawback is that the machinery needed to perform the technique is extremely expensive, effectively limiting its use to reference laboratories.

1.6.2 Genotypic methods

Genotypic methods are generally more discriminatory than phenotypic ones because whilst organisms may change their phenotypic characteristics fairly readily, it is much harder to alter their genetic make-up. Therefore, it is normally possible to delineate a greater number of types in a species with genotypic tools. Various such methods have been applied to the typing of *C. difficile*.

1.6.2.1 Plasmid analysis

Plasmid analysis was one of the first genotypic methods to be attempted for typing *C. difficile* (Wust *et al.* 1982) and has been used for *C. difficile* typing on several occasions (McKay *et al.* 1989); (Steinberg *et al.* 1987). For strains that carried plasmids it was found to be very discriminatory (Mulligan *et al.* 1988), but they are only found in 18-60% of strains (Clabots *et al.* 1988a); (Clabots *et al.* 1988b); (Muldrow *et al.* 1982). Reproducibility may suffer due to the unstable nature of plasmids and consequently it is of limited use in typing *C. difficile*.

1.6.2.2 Restriction endonuclease analysis (REA)

REA involves DNA extraction followed by restriction endonuclease digestion and then electrophoresis of the product, staining or using autoradiography to visualise the banding patterns according to the method being used. REA using *Hin*dIII was able to demonstrate cross-infection between two patients in a study by Kuijper *et al.* (1987). Other investigators have found REA to be a discriminatory method (Devlin *et al.* 1987); (Peerbooms *et al.* 1987); (Samore *et al.* 1994b) and when used by O'Neill *et al.* (1991) to investigate recurrent CDI, more than half the cases were shown to be caused by new strains.

One of the reasons that this method is so discriminatory, is that it produces digests with up to 50 or more bands. Consequently, these are very difficult to analyse with the naked eye and computer assistance is required (Tabaqchali *et al.* 1987). The method is also time-consuming and laborious, so is best performed by laboratories with plenty of experience.

1.6.2.3 Restriction fragment length polymorphism (RFLP) analysis

This method involves the same procedures as for REA but instead of staining the digests immediately after electrophoresis, a blotting procedure is performed and 16S or 23S ribosomal RNA probes are used to hybridise with the digest. This results in far fewer bands to be interpreted, making analysis easier. The method was described by Bowman *et al.* (1991) for the typing of *C. difficile*. Subsequently, a comparison with REA was made on 116 strains and RFLP analysis was found to be less discriminatory, with only 6 types demonstrated vs. 34 types for REA (O'Neill *et al.* 1993). RFLP analysis is therefore not regarded as particularly useful for *C. difficile* typing.

1.6.2.4 PCR with arbitrary primers (AP-PCR)

Arbitrarily primed PCR refers to the use of a single, short primer that is not targeted at any specifically known sequence in the genome, and which hybridizes at random to complementary sites. This process occurs in both directions along the DNA, and produces a number of amplified DNA fragments. These differ in number and size, depending on how genetically similar the different test strains are, and can be visualised by electrophoretic separation. This type of PCR is usually performed using conditions of low stringency, with low annealing temperatures and high $MgCl_2$ concentrations. Due to the arbitrary nature of the primer, no knowledge of the target nucleotide sequence is required for AP-PCR. However, because both ends of the amplimer are complementary (palindromic), hairpin loop

formation is possible instead of primer binding and this may reduce the efficiency of the PCR reaction. It can also result in a reduction in the reproducibility of the method. Consequently, primer and template concentrations are very important and can influence the success of the technique.

This method was first described as a technique for typing *C. difficile* by McMillin and Muldrow, (1992), who demonstrated that differentiation between six *C. difficile* strains was possible. Later, the technique was modified and used as a rapid typing method on colonies of *C. difficile* directly, without DNA extraction (Wilks and Tabaqchali, 1994). The genotypes demonstrated using this method by Wilks and Tabaqchali coincided with nine previously described radio-PAGE types. Other investigators have also described the use of AP-PCR for typing *C. difficile* (Silva *et al.* 1994); (Tang *et al.* 1995).

Another method, closely related to AP-PCR is randomly amplified polymorphic DNA (RAPD) PCR. This method uses two short arbitrary primers rather than the single longer primer used in AP-PCR. In some publications, these terms have been used interchangeably regardless of primer length or number (Power, 1996). RAPD PCR was used by Killgore and Kato, (1994) to type 41 *C. difficile* isolates from a hospital outbreak, and was found to be more discriminatory than immunoblot typing methods.

1.6.2.5 Standard PCR methods

Early work on PCR methodology was performed by Kato *et al.* (1991), who used paired primers derived from both the repeating and non-repeating regions of the toxin A gene. Using three different primer pairs, fragments of 546- 242- and 1266-bp were generated when toxin positive strains were tested. It was possible to differentiate 26 non-toxigenic from 35 toxigenic strains of *C. difficile*. These methods did not produce any

product with 20 other clostridial species, including *C. sordellii*. Wren *et al.* (1990) also tested a method that relied upon amplification of an area in the repeating region of the toxin A gene. They successfully distinguished between 17 non-toxigenic and 58 toxigenic strains of *C. difficile* but did obtain a positive result with a strain of *C. sordellii*.

1.6.2.6 Ribo-spacer polymerase chain reaction (RS-PCR)

The use of a PCR method, which demonstrates differences between the16S-23S ribosomal RNA intergenic spacer region of *C. difficile* strains was first described by Gürtler (1993). Using a paired oligonucleotide PCR system, he demonstrated 14 reproducible DNA fingerprints amongst 24 test strains. Cartwright, *et al.* (1995) modified this technique and went on to demonstrate 41 distinct ribotypes amongst the 102 strains of *C. difficile* tested. Strains were classified as genetically distinct if their PCR profiles differed by more than one band. Another group to successfully type various bacterial species using RS-PCR was Jensen *et al.* (1993). Subsequent work comparing pyrolysis mass spectrometry and serotyping with PCR ribotyping has shown favourable results (O'Neill *et al.* 1996), and this method is now in use by the PHLS Anaerobe Reference Unit, Cardiff PHL to type *C. difficile* (Brazier, 1998b). It is this method which could be universally accepted for *C. difficile* typing in future.

1.6.2.7 PCR on faecal specimens

In contrast to the methods described in sections 1.6.2.4, 1.6.2.5 and 1.6.2.6, which are usually performed on culture isolates, some investigators have explored the possibility of direct testing of stool specimens. Some of the earliest work on these methods was carried out by Gumerlock *et al.* (1991), who used a pair of primers complementary to regions in the 16S rRNA gene, to amplify a 270-bp fragment of genomic DNA. Production of the correct

fragment was confirmed by gene probing a Southern blot. The method was reportedly very sensitive. Green *et al.* (1994) performed an oligonucleotide probe method to detect toxigenic *C. difficile* in stool specimens. It was found to compare favourably with stool cytotoxicity, as well as with a commercial EIA kit. The 33-bp probe used detected a sequence in the toxin B gene. Arzese *et al.* (1995) and Boondeekhun *et al.* (1993) both designed methods to detect the toxin A gene and Arzese claimed improved detection of toxigenic strains, whilst Boondeekhun found one discrepant result. Similarly, Gumerlock *et al.* (1993) developed a toxin B gene detection method and found two out of 18 cytotoxin negative samples to be PCR positive. The patients concerned were symptomatic and it was claimed that the PCR method was 10-100 times more sensitive than cytotoxin (CPE) testing. However, it is also possible that a very sensitive detection method could detect the presence of an organism that is colonising a patient rather than causing disease. Another potential drawback of PCR methods on stool samples rather than on cultures of *C. difficile*, is that the presence of inhibitory factors may require a complicated preparation stage before the test can be performed. Kato *et al.* (1993), describe a PCR method in which a segment of the toxin A gene was amplified, where prior treatment of the stool sample using an ion-exchange process was required.

1.7 THE POLYMERASE CHAIN REACTION (PCR)

The following sections are derived from information obtained from the following references (Anderson, 1990; Persing, 1993).

Having been developed by Kary Mullis in 1983, the PCR allows the exponential amplification of specific target sequences of genomic DNA. After further improvements,

including automated oligonucleotide synthesis, automated thermal cycling and a heat stable DNA polymerase from *Thermus aquaticus*, publication of a practical application for the technique occurred in 1985 (Saiki *et al.* 1985). Since this time, the importance of PCR as a research tool has increased significantly, along with its more frequent application in clinical diagnostic microbiology.

1.7.1 Principles of the polymerase chain reaction

In this technique, DNA synthesis is achieved by repeated cycles of oligonucleotide directed DNA replication. When the genome of the organism to be tested is known, a pair of oligonucleotide primers is designed which can bind to specific sites on the 3' and 5' DNA strands. The primers are usually around 20-bp in length and the distance between annealing sites determines the amplimer fragment size. Fragments of between 50 and 1500-bp are generally preferred for diagnostic assays.

The PCR cycle is usually composed of three stages. Firstly, denaturation of the target DNA, which usually requires a temperature of around 90°C. This is required to form single strands of DNA and expose the sites where the primer can anneal. The second stage is annealing of primers to the DNA strand, which usually occurs at 40-60°C. Lower annealing temperatures reduce the stringency of the reaction by increasing the possibility of non-specific primer binding. Finally, comes the extension phase, where the primer is extended along the target by a thermostable DNA polymerase. This generally takes place at 72°C. In a typical PCR protocol, there will be 30-50 of these cycles performed, producing a double stranded DNA amplimer identical to the DNA sequence between the two primer binding sites on the original target.

1.7.2 Oligonucleotide primers

The choice of primer sequence will be depend on the region of the gene that is to be amplified. For PCR where a specific genetic element is to be amplified, the primer needs to be long enough to be specific to that section of DNA, and not to bind to multiple regions along the genome. This usually requires a primer of 18-25 bp length. Longer primers will increase cost significantly without any significant increase in specificity. In addition, it is wise to avoid primers which include areas of self complementarity, as this can lead to binding of the primers to each other and elongation along their length. This results in double stranded fragments of a length similar to the sum of the primers, known as primer dimers. Formation of primer dimers will tend to reduce the efficiency of the PCR in producing the desired amplimer.

1.7.3 Application of the polymerase chain reaction to *C. difficile* typing

This is discussed in the following sections: 1.6.2.4, 1.6.2.5, 1.6.2.6, and 1.6.2.7.

1.8 AIMS OF THE RESEARCH

1.8.1 Antibiotics and their contribution towards CDI

As discussed in section 1.3.2.3 and its subsections, antibiotics are known to have a significant impact on the bowel flora of a recipient. Exposure to antibiotics is one of the most important factors in the development of CDI and some antibiotics are thought to be more likely than others to lead on to CDI (see section 1.3.2.3 and subsections). Levels of

CDI were very high on the care of the elderly wards at our institution and at the same time, concern was being raised in the scientific community over 'third generation' cephalosporins and their contribution towards the development of CDI (see section 1.3.2.3.2). It was decided that it would be useful to ascertain whether an alternative antibiotic would pose less of a threat to elderly patients. Some data indicate that anti-pseudomonal penicillins, with or without beta-lactamase inhibitors such as clavulanic acid or tazobactam, may have a reduced propensity to induce CDI compared with 'third generation' cephalosporins. (see section 1.3.2.3.3). However, there have been no prospective studies to date, controlled for exposure to *C.difficile,* which directly compare two broad spectrum antibiotic agents, in order to determine their influence on the risk of developing CDI. Consequently, piperacillin-tazobactam (PT) was selected as the alternative agent to cefotaxime (CTX), for the empirical treatment of serious infection in the elderly. A study in which environmental exposure to *C. difficile* was controlled for was designed, in order to compare the propensity of elderly patients to develop *C. difficile* colonisation or CDI after therapy with CTX or PT.

1.8.2 Methods of diagnosing CDI

New methods continue to be developed for the detection of *C. difficile* toxins, in order to diagnose CDI. These are usually designed to be simple to use and allow a rapid result to be obtained. One such test is the Oxoid toxin A test (Unipath, Basingstoke, UK), which is a dot-immunobinding assay method, capable of producing a result in 30 min. There was no published evaluation of this test, although the multicentre trial originally used to evaluate the kit was published in 1998 (Bentley *et al.* 1998). A study was designed to evaluate the accuracy of the Oxoid toxin A test, using a cytopathic effect method to detect toxin B as the comparator. The CPE method used HEp-2 cells and toxin B titres of the stool specimens were measured. This was to see whether there was a correlation between the toxin B level (assumed to be similar to the toxin A level) and the ability of the Oxoid test to

detect toxin A. In addition, all samples were cultured and any non-toxigenic isolates identified. Combining the information from all tests carried out was intended to allow the reasons for any discrepancies between tests to be understood.

1.8.3 Epidemiological study of CDI

As discussed in sections 1.2.4.1.7 and 1.5.4, there is increased recognition of *C. difficile* strains that produce toxin B but no detectable toxin A (A-B+). These strains have, nevertheless been associated with clinical disease. This gives rise to concern regarding the diagnosis of CDI, because tests for toxin A alone will be negative on stools from such patients, perhaps leading to misdiagnosis. Data from the PHLS Anaerobe Reference Unit, Cardiff PHL indicates that such strains account for 3% of those referred to them, however, from one hospital these strains did represent 10% of the number submitted (Brazier *et al.* 1998b). Determination of the percentage of A-B+ strains in Leeds and Bradford has not been performed to date, and this was the aim of the author's investigation.

Another area of *C. difficile* epidemiology that is not well researched is that of infection and colonisation rates in the community (see section 1.5.3.2). Neither is it currently clear whether the strains responsible for CDI in hospital patients also cause disease in the community. Cases of CDI from two elderly care wards at our institution were previously shown to be due to a single strain (known as p24) on 87% of occasions, (Fawley and Wilcox, 2001). In addition, this strain has been shown to predominate throughout the UK, and is designated ribotype 1 (Stubbs *et al.* 1999). However, limited data from PHLS Anaerobe Reference Unit, Cardiff PHL indicates that strains from community patients are diverse and do not mimic strains causing hospital infection (see section 1.5.4). Furthermore, it is not known whether disease in the community results from the same strains in different parts of the country. In particular, it was of interest to compare strains from an urban centre

like Leeds which were causing disease in the community with similar strains from a rural area. A study was in progress to compare rates of community CDI in Leeds and Truro, and so permission was obtained from Dr Mark Wilcox and Dr Richard Bendall to allow genotypic comparison of the strains from each centre. The author sought to assess whether strains isolated from community patients with disease in Truro were the same as those producing CDI in community patients from Leeds.

MATERIALS AND METHODS

2.1 REAGENTS AND CHEMICALS

All chemicals used were purchased from BDH (Poole, Dorset, UK) unless otherwise stated and were of the highest grade available. Oligonucleotide primers from previously published methods were used (Gumerlock *et al.* 1993); (Jensen *et al.* 1993); (Kato *et al.* 1998); (Killgore and Kato, 1994); (Tang *et al.* 1994). Primers were purchased from MWG-Biotech AG, (Ebersberg, Germany), and were stored at -70°C prior to use.

2.2 STRAINS AND MEDIA

All materials for media preparation were purchased from Oxoid (Basingstoke, Hampshire, UK), unless otherwise stated and media was prepared according to the manufacturers directions. A toxin A-B+ strain of *C. difficile* (Bz 17) was kindly supplied by Dr J.S. Brazier, PHLS Anaerobe Reference Unit, Cardiff PHL, UK. Human epithelial (HEp-2) cells were supplied by the routine diagnostic microbiology laboratory at Leeds General Infirmary, where they were used routinely for cytotoxin detection. Green monkey kidney (Vero) cells were supplied by the Public Health Laboratory Service (PHLS), Leeds branch.

Stool samples for the study of the Oxoid toxin A kit (Oxoid, Basingstoke, Hampshire, UK) were obtained from the diagnostic laboratory of the United Leeds Teaching Hospitals Trust (ULTH). These totalled 100 in number and were collected during 1996. After having been clinically tested, they were stored at -20°C in original containers, until required. Stool samples for the clinical trial were collected from patients at the ULTH and were processed immediately on receipt. Following this they were frozen, in their original containers, at -20°C. These were collected from 1996 to 1997. Isolates of *C. difficile* for toxin A gene determination were obtained from the diagnostic laboratory of St James's

University Teaching Hospital (SJUH), Leeds as well as stool samples from ULTH and Bradford Royal Infirmary during 1999 and 2000. Following diagnostic testing, stool samples were stored at -20°C until required. The total number of *C. difficile* isolates obtained for testing was 269. Stool samples from general practices were used in order to characterise community strains of *C. difficile*. These 15 samples were received at the diagnostic laboratory of the ULTH during 1999 and 2000 and stored at -20°C, after clinical testing, until required. Further strains of *C. difficile* were obtained from Dr Richard Bendall, PHLS, Truro, UK. These 39 isolates had been obtained during 1999 and 2000 from specimens submitted by GP's.

Once strains were isolated from stool samples, they were stored in glycerol broth (20% glycerol (w/v) in nutrient broth) at -70°C.

2.2.1 Cycloserine-cefoxitin egg yolk (CCEY) agar preparation

Preparation of CCEY media was carried out according to manufacturer's recommendations. Standard CCEY was supplemented with 1% lysed horse blood (E&O Laboratories, Bonnybridge, Stirlingshire, UK), 250mg/L cycloserine and 8mg/L cefoxitin. Egg yolk supplement was omitted. Lysozyme CCEY was supplemented with 2% lysed horse blood (E&O Laboratories, Bonnybridge, Stirlingshire, UK), antibiotics as above and lysozyme (5mg/L) (Sigma-Aldrich Company Ltd, Poole, Dorset, UK). Egg yolk supplement was omitted.

2.3 ISOLATION AND CHARACTERISATION OF CLINICAL STRAINS

Faecal samples submitted by GP's and those from hospital in-patients were cultured according to the standard operating procedure of the respective laboratory. Standard procedures for *C. difficile* isolation were essentially identical at both diagnostic laboratories. A pea-sized piece of faeces was spread on lysozyme CCEY or Brazier's CCEY agar (Lab M, Bury, Lancashire, UK) which had the egg yolk omitted (modified CCEY). A similar amount was placed into a 5ml bijou bottle containing 2ml of 80% alcohol and left for 1 h before being plated out in a similar fashion. Incubation was performed at 37°C, in an anaerobic cabinet (Don Whitley Scientific, Shipley, West Yorkshire, UK), with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Plates were inspected at 48 h, and single colonies resembling *C. difficile* were picked off for purity on lysozyme CCEY or modified CCEY agar (Lab M, Bury, Lancashire, UK), as well as full blood agar plates. Colonies resembling *C. difficile* on purity plates and producing the characteristic 'elephant dung' odour were subcultured into pre-reduced brain heart infusion (BHI) broth (section 2.4.1). After 48 h anaerobic incubation, the broth was tested by cytopathic effect (CPE), for presence of cytotoxin (see sections 2.4.3, 2.4.5). Isolates failing to produce cytotoxin were then further identified using the RapID ANAII kit (Innovative Diagnostics Systems, Norcross, GA, USA), which were used in accordance with the manufacturer's instructions.

2.4 TOXIN TESTING

2.4.1 Preparation from colonies

Single colonies, morphologically resembling *C. difficile* on CCEY agar (Lab M, Bury, Lancashire, UK) were subcultured in Wasserman tubes containing 2ml of pre-reduced

BHI broth. These were then incubated in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 inside an anaerobic cabinet (Don Whitley Scientific, Shipley, West Yorkshire, UK) at 37°C. After 48 h incubation, 0.5 ml of broth was removed and was centrifuged at 1200 x **g** for 10 minutes in a 1.5 ml eppendorf tube, before the supernatant was used for toxin testing.

2.4.2 Preparation from stool samples

Approximately 0.5g of faecal specimen was suspended in 2ml of phosphate buffered saline (PBS) producing an effective dilution of 1 in 5. Liquid specimens were diluted to the same degree. The well-mixed suspensions were then centrifuged for 10 minutes at 1200 x **g**. If the supernatants were subsequently cloudy then they were filtered through a 0.45 μm millipore filter (Nalge, Rochester, NY, USA). The supernatants were then used for toxin testing.

2.4.3 HEp-2 cell monolayer preparation and cytopathic effect test

All materials and media for cell culture were obtained from Life Technologies, (Paisley, Strathclyde, UK) unless otherwise stated. Toxin testing was performed using HEp-2 cell monolayers in 96 well microtitre trays. HEp-2 cells were first cultured in 25 cm^2 flat bottomed flasks, using basal medium (Eagle) without glutamine (BME). A 100ml bottle of BME was supplemented with 5ml newborn calf serum, 1ml L-glutamine (200mM), 1ml antibiotic/antimycotic agent (see below) and 1ml gentamicin (4 mg/ml) (Hoechst Marion Roussel, supplied by pharmacy Leeds General Infirmary (LGI). The proprietary 100x antibiotic/antimycotic agent contained 10,000 units of penicillin, 10,000μg of streptomycin and 25μg of amphotericin B per ml and 1ml was added to 100ml of BME.

Once confluent growth was achieved, growth medium was decanted and the cell layer was exposed to 5ml of a 2.5g/L solution of trypsin in Earle's balanced salt solution. This solution was left to stand for one minute before being decanted and the flask then incubated for approximately 15 minutes at 37°C. Once the cells had become detached they were re-suspended in BME (with enrichments as above) and microtitre trays were inoculated with the suspension. Test wells had 180µl dispensed into them, whilst control wells had 160µl, to allow for 20µl of antitoxin to be added. After 2-3 days incubation aerobically, in a wet box, at 37°C, confluent monolayers were obtained which could be used for cytopathic effect (CPE) testing.

Tests were performed by adding 20µl of supernatant (see sections 2.4.1, 2.4.2) to 180µl of culture medium in a microtitre well. Supernatants were then stored at 4°C in case further testing was subsequently required. All microtitre trays were controlled using a known weakly positive cytotoxin containing filtrate in one well, no additions to one well, and just antitoxin to another. Incubation of the microtitre trays was carried out in a moistened box in a 37°C incubator. Inspection for CPE was carried out at 24 h and 48 h unless otherwise stated. All supernatants were simultaneously tested with *C. sordellii* antitoxin (Pro-Lab Diagnostics, Neston, Cheshire, UK) protected controls. Positive results were apparent as actinomorphic changes in cell structure comprising rounding and detachment from each other. Greater than 50% of cells were required to have been affected at or before 48 h for a result to be classified as positive. The changes also had to be neutralised in the control well for the result to be accepted. In cases where non-specific toxic effects were seen, a 1 in 10 dilution of the supernatant using PBS was usually sufficient to suppress this.

2.4.4 Toxin titres

Toxin titres were measured by carrying out serial dilutions from the original toxin test well. Following addition of supernatant to a well and mixing, 20µl was removed and added to the well vertically below (also containing 180µl of culture media). After mixing, the process was repeated for a total of 7 dilutions. This resulted in a final dilution of $2x10^{-8}$ (including the initial five-fold dilution of the faecal specimen). The toxin titre was expressed as the reciprocal of this dilution. It should be remembered that even in the initial test well there was a dilutional effect of 10x on the supernatant added.

2.4.5 Vero cell line culture

Vero cell culture was performed in a very similar fashion to HEp-2 cell culture, except that some of the reagents used were different. The growth medium was Dulbecco's modified Eagle medium, cell sheets were trypsinised with 0.5g/L trypsin/EDTA in Hank's balanced salt solution and gentamicin was not added to the culture medium. In all other respects, culture and passage of the cell line was carried out in the same manner as with HEp-2 cells. Manipulation of Vero cell lines, prior to use for toxin tests was carried out in a class II microbiological safety cabinet (Envair (UK) Ltd, Haslingden, Lancashire, UK). Vero cell monolayers were used for *C. difficile* cytotoxin testing during the year 2000 and thereafter, using the same procedures as for HEp-2 cells (see section 2.4.3).

2.5 DEVELOPMENT OF *C. difficile* COLONISATION AND INFECTION IN ELDERLY PATIENTS, FOLLOWING CEFOTAXIME (CTX) OR PIPERACILLIN-TAZOBACTAM (PT) THERAPY

2.5.1 Study design

Initially, a randomised, double-blind pilot study was planned, and ethical approval was obtained. Recruitment was extremely poor (one patient in six months), because most eligible patients were found to be unable to give informed consent due to confusion and this led to a change in study design. With ethical approval, the protocol was changed to a ward crossover design without randomisation, so that individual patient consent would not be required.

Patients on one elderly care ward (A), received intravenous (IV) cefotaxime (CTX), 1g tds when broad spectrum antibiotic therapy was deemed to be clinically required, as was normal hospital practice. On a second, similar ward (B), IV piperacillin-tazobactam (PT) 4.5g tds was prescribed instead. Broad spectrum IV antibiotics were generally only prescribed where clinical suspicion of moderate to severe sepsis of unknown origin was high. No other changes were made to antibiotic prescribing protocols. Patients with a history of penicillin allergy were given CTX instead of PT.

The two wards were of similar size (ward A, 32 beds, ward B, 28 beds) and had comparable admission policy and patient mix. Screening in the 8 months prior to study commencement indicated that ward B had twice the level of environmental *C. difficile* contamination and the incidence of CDI was 47% greater than on ward A. Ward B was

therefore selected for initial PT use in order to minimise bias in favour of PT. Study end points were discharge or death. CDI was defined as documented loose stools (once or more per day for at least two days), which was not attributable to another cause, in patients with concurrent *C. difficile* cytotoxin-positive faeces. The initial aim was to study around 40 patients and then carry out analysis to determine whether or not it was worth continuing to obtain larger numbers. Ward cross-over took place after 10 months instead of one year because plans to move the wards became apparent. Following cross-over the wards were moved to another location after only 4 months, due to a major hospital building development. An analysis of results was performed at this time, and the study was terminated due to ethical considerations. The plan for a change in ward location did not become apparent until late in the study.

2.5.2 Study implementation

Any patient who was prescribed CTX or PT was eligible for study entry unless they had previously been treated with one of those agents. A faecal specimen was obtained from patients as soon as possible after the prescription of the study antibiotic, and then weekly during their hospital stay where feasible. Records were kept of patient's daily maximum temperature, nature and frequency of stools, biochemistry, haematology and microbiology results, and all drugs received. Culture of stool samples for *C. difficile* was performed as described in section 2.3. Cytotoxin detection and any further identification procedures were performed as described in section 2.4.3 or 2.4.5.

Environmental contamination with bacterial spores was also monitored in order to assess the relative risk of *C. difficile* exposure of patients on each ward. This was achieved by swabbing certain environmental sites on each ward on a monthly basis (Fawley and Wilcox, 2001). Sites selected were: floor of patient bays, floor of toilets, floor of sluices,

commodes, radiators and air vents. The same sites on each ward were sampled each month, by swabbing a 10cm x 10cm area, using a cotton swab moistened with 0.25% Ringer's solution (Oxoid, Basingstoke, UK). Swabs were then cultured immediately onto modified CCEY as well as into Robertson's cooked meat broth, and incubated anaerobically at 37°C for 48 h. Sub-culture of broths was performed onto modified CCEY agar, as described in section 2.3. All environmental and patient strains were fingerprinted using PCR amplification of 16S-23S ribosomal RNA gene interspacer region (see section 2.7.2.3).

Two-tailed Fisher's exact probability and Mann-Whitney U tests were used for statistical analyses of data.

2.6 COMPARISON OF THE OXOID TOXIN A TEST WITH CYTOTOXIN DETECTION USING A CYTOPATHIC EFFECT METHOD

2.6.1 Sample preparation

As mentioned in section 2.2, previously tested, frozen stool samples were used in an evaluation of the Oxoid Toxin A test (Unipath, Basingstoke, Hampshire, UK). Samples were selected so that 50% were previously positive for *C. difficile* cytotoxin and 50% were previously negative. Samples had been stored at -20°C since original CPE testing and were tested immediately after thawing. All samples were from patients with diarrhoeal illness, where no other infective cause was identified. The 100 samples were randomly arranged to ensure that positives were indistinguishable from negatives. They were then thawed in batches of 10 and roughly 0.5g was suspended in the kit diluent or in 2ml of PBS (see section 2.4.2). The well-mixed suspensions were then centrifuged for 10 minutes at

1200 x **g**, before being used for either the kit test (according to the instructions), or kept in a refrigerator at 4°C (for < 30 min) until the CPE test was set up.

2.6.2 *C. difficile* culture and identification

In addition to testing for toxins A and B, each specimen was cultured for *C. difficile* (see section 2.3) and isolates from CPE negative stool samples were tested for cytotoxin production as described in section 2.4.3. Further identification was carried out if non-toxigenic strains were encountered (section 2.3).

2.6.3 Oxoid toxin A test

The principle of the test is that monoclonal anti toxin A antibody, labelled with blue latex particles, binds to any toxin A in the specimen when it is added to the sample well. The complex diffuses along the test strip and is bound to an immobilised line of anti toxin A monoclonal antibody, forming a blue line in a positive result. Immobilisation of unbound latex particles occurs in a second window to indicate that diffusion past the test window has occurred. The kit was used to test faecal specimens in accordance with the manufacturer's recommendations. A 125µl volume of faecal supernatant was added to the sample window of the test strip. This was left for 30 min and was then examined for evidence of a blue line in the result window. Tests where the control window had a blue line and there was any sign of a blue line in the result window were classified as positive.

2.6.4 Cytopathic effect test (CPE)

This test makes use of the cytotoxic nature of toxin B (see section 1.2.4.1.1). CPE testing was performed using a HEp-2 or Vero cell line, as described in sections 2.4.3 and 2.4.5. Cell monolayers were examined for CPE at 4, 6, 24 and 48h, independently by two individuals. CPE consisted of a discernible, neutralizable rounding-up of cells, often quite subtle at 4h and sometimes 6h, but always affecting >10% of cells initially and >50% of cells by 48h.

2.6.5 Toxin titre estimation

The supernatants used for CPE testing in this study were frozen at -20°C immediately after the cytotoxin test was set up. Cytotoxin titres in these samples were determined after 4 weeks, due to time constraints at the time of initial cytotoxin testing. They were tested for CPE neat (1 in 50 dilution) and at seven further 10-fold dilutions. The toxin titre was designated as the reciprocal of the highest dilution which caused a readily discernible, classical CPE at 48h (although in a few cases, at the highest dilution, these changes did not quite affect 50% of the cell monolayer).

2.7 PCR TYPING AND TOXIN GENE DETECTION OF *C. difficile*

PCR toxin A gene detection was performed on 269 *C. difficile* strains using primers NK9/NK11, described by Kato *et al.* (1998). Some strains were additionally tested using primers described by Gumerlock *et al.* (1993), and Tang *et al.* (1994). Ribo-spacer (RS) PCR typing of 54 *C. difficile* isolates from community patients was carried out using primers described by Jensen *et al.* (1993) and randomly amplified polymorphic DNA (RAPD) primers described by Killgore and Kato, (1994).

2.7.1 Extraction of DNA

Isolates of *C. difficile* were obtained from clinical specimens as described in section 2.3. Subculture was performed by picking a single colony from the selective medium and culturing it on a non-selective medium, incubated under optimal conditions. A single colony from the non-selective plate was transferred to the extraction solution using a sterile loop. Culture of *C. difficile* strains that had been saved in broth was performed on selective and non-selective media. Once a pure culture was obtained on non-selective medium, a single colony was selected for extraction as above.

2.7.1.1 Boil extraction

A single colony was emulsified in 25µl of sterile water in a 0.5ml microcentrifuge tube (Eppendorf-Netheler-Hinz-GmbH, supplied by Merck, Poole, Dorset, UK), giving a slightly milky suspension. This was heated at 99°C for 10 minutes. New extractions were performed for each PCR. If PCR was not being performed immediately, then the extract was stored at -20°C.

2.7.1.2 Lysozyme and proteinase K extraction

25µl of lysozyme (0.1 mg/ml) in 0.1 M TRIS buffer (Tris base 0.53g/L, Tris HCl 0.88g/L, EDTA 0.37g/L) adjusted to pH 8.0, was placed in a 0.5 ml microcentrifuge tube (Eppendorf-Netheler-Hinz-GmbH, supplied by Merck, Poole, Dorset, UK). A single colony of *C. difficile* was emulsified in this, to give a slightly milky suspension. The tube was then incubated for 10 minutes at 37°C, before 25µl of proteinase K (0.1 mg/ml) in 0.1 M TRIS buffer (as above) at pH 8.0 was added and the tube agitated to allow mixing. Incubation for

a further 10 minutes at 37°C was followed by incubation for 10 minutes at 99°C to denature the proteinase K and lysozyme.

2.7.2 Preparation of PCR pool (mastermix)

Preparation of mastermix was performed in a clean area away from sites of bacterial culture and DNA extraction to minimise potential for contamination. Aerosol resistant pipette tips (Molecular BioProducts Inc., supplied by Merck, Poole, Dorset, UK) were used for all PCR preparation steps. The mechanics of preparation were the same for all mastermixes, although quantities of primer, *taq* polymerase and magnesium ($MgCl_2$) differed for each particular method. During mastermix preparation, sufficient was prepared for all the PCR reactions required on that day. If any tubes of mastermix were not used, then they were stored at -20°C, having been adequately labelled.

2.7.2.1 Toxin gene detection PCR (Kato)

For this PCR method, described by Kato *et al.* (1998), each 23µl aliquot of mastermix contained 0.2µl primer mix (10 ρmoles of each primer), 1.0µl $MgCl_2$ (2.0mM, HT Biotechnology Ltd, Cambridge, UK), 2.5µl 10x Super*taq* reaction buffer (HT Biotechnology Ltd, Cambridge, UK), 4µl dNTP mix (made up in sterile water for injection from the Ultrapure dNTP set, Pharmacia Biotech UK Ltd, Little Chalfont, UK) yielding 1.25 mM of each dNTP, 0.2µl (1 unit) Super*taq* (HT Biotechnology Ltd, Cambridge, UK) and 15.1µl sterile water for injection. Initial attempts at the method were made using Amplitaq gold *taq* polymerase and buffer (Perkin Elmer Applied Biosystems, Bucks, UK), but this did not produce reliable results and so Super*taq* and buffer (HT Biotechnology Ltd, Cambridge, UK) were substituted. Late in 2000, new batches of Super*taq* (HT Biotechnology Ltd, Cambridge, UK), were also found to be unreliable and so a ready-prepared 1.1x mastermix

(Abgene Ltd, Epsom, Surrey, UK) was used. For each PCR tube, 22.5µl of 1.1x Master Mix with 1.5mM $MgCl_2$ (Abgene Ltd, Epsom, Surrey, UK), 0.2µl of primer mix (NK11-NK9) and 0.3µl of water for injection were added, to bring the final volume to 23µl. Again, sufficient PCR pool was made up for all the reactions to be performed on that day and 23µl aliquots were placed into 0.5ml microcentrifuge tubes ready for use. Once aliquots were measured out, tubes were stored at -20°C until required.

2.7.2.2 Toxin gene detection (Cohen)

A multiplex PCR reaction to detect the presence of *tcd*A and *tcd*B, as described by Cohen *et al.* (2000) and using primers described by Gumerlock *et al.* (1993) and Tang *et al.* (1994), was performed on two strains with unusual results from the Kato toxin gene detection PCR. The PCR pool for this method contained 1.2µl primer mix (30 ρmoles of each primer), 1.25µl $MgCl_2$ (2.5mM, HT Biotechnology Ltd, Cambridge, UK), 5.0µl 10x Super*taq* reaction buffer (HT Biotechnology Ltd, Cambridge, UK), 6µl dNTP mix (made up in sterile water for injection from the Ultrapure dNTP set, Pharmacia Biotech UK Ltd, Little Chalfont, UK) yielding 0.94 mM of each dNTP, 0.4µl (2 units) Super*taq* (HT Biotechnology Ltd, Cambridge, UK) and 34.15µl sterile water for injection, giving a 50µl reaction volume (with the addition of 2µl template DNA).

2.7.2.3 16-23S ribosomal interspacer region (RS-PCR)

This method was described by Jensen *et al.* (1993) and 50µl reaction volumes were used. There were 54 strains of *C. difficile* which were recovered from specimens sent for testing by General Practitioners. Of these, 15 were from Leeds and 39 from Truro. Mastermix was prepared and 48µl aliquots dispensed into 0.5ml microcentrifuge tubes (Eppendorf-Netheler-Hinz-GmbH, supplied by Merck, Poole, Dorset, UK). Each 48µl

aliquot contained 0.75µl primer mix (37.5 ρmoles of each primer), 1.75µl $MgCl_2$ (3.5mM, HT Biotechnology Ltd, Cambridge, UK), 5.0µl 10x Super*taq* reaction buffer (HT Biotechnology Ltd, Cambridge, UK), 8µl dNTP mix (made up in sterile water for injection from the Ultrapure dNTP set, Pharmacia Biotech UK Ltd, Little Chalfont, UK) yielding 1.25 mM of each dNTP, 0.5µl (2.5 units) Super*taq* (HT Biotechnology Ltd, Cambridge, UK) and 32µl sterile water for injection.

2.7.2.4 RAPD PCR

RAPD PCR was used to determine whether community isolates demonstrating the same profile as our endemic *C. difficile* strain (p24) were indeed identical or not. The method was performed using primers described by Killgore and Kato, (1994). Reaction volumes were once again 50µl, with each 48µl aliquot containing 1.5µl primer mix (25 ρmoles of each primer), 1.5µl $MgCl_2$ (3.0mM, HT Biotechnology Ltd, Cambridge, UK), 5.0µl 10x Super*taq* reaction buffer (HT Biotechnology Ltd, Cambridge, UK), 8µl dNTP mix (made up in sterile water for injection from the Ultrapure dNTP set, Pharmacia Biotech UK Ltd, Little Chalfont, UK) yielding 1.25 mM of each dNTP, 0.5µl (2.5 units) Super*taq* (HT Biotechnology Ltd, Cambridge, UK) and 31.5µl sterile water for injection.

2.7.3 Preparation of the PCR reaction

To each 0.5ml microcentrifuge tube (Eppendorf-Netheler-Hinz-GmbH, supplied by Merck, Poole, Dorset, UK) containing a mastermix aliquot was added 2µl of extracted template DNA. This brought the reaction volume to either 25µl or 50µl, depending on which method was being used. For toxin gene detection, boil extractions were adequate but for RAPD and RS-PCR, lysozyme/proteinase K extractions were preferred. Each PCR series was controlled with a reagent control (no target DNA added to mastermix), an extraction

control (extraction buffer only added to mastermix) and an organism control (extract of known of *C. difficile* strain added to mastermix). The control strain used was dependent upon the particular PCR being performed. For toxin gene detection PCR, a toxin A+B+ strain, a toxin A-B+ strain (Bz 17) and a non-toxigenic strain were used. For RS and RAPD PCR methods, our local endemic strain (p24) was used for reference. The reaction tubes were then placed in the thermocycler (Techne Genius, Techne (Cambridge) Ltd, Duxford, Cambridge, UK) and cycling conditions programmed according to which method was being used. For toxin gene detection (Kato), cycling conditions were: one cycle at 94°C for 3 min followed by 35 cycles of 95°C for 20 sec and 62°C for 2 min (two step only). Tubes were then held at 4°C until the product was electrophoresed or frozen at -20°C. For toxin gene detection (Cohen), cycling conditions were: one cycle at 94°C for 3 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. For RS-PCR (Jensen), cycling conditions were: one cycle at 94°C for 3 min followed by 34 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min and finally one cycle of 72°C for 7 min. For RAPD PCR (Killgore and Kato), cycling conditions were as follows: one cycle at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min.

2.7.4 Optimisation of PCR reactions

The PCR reactions were optimised by performing magnesium titrations from 1.0mM to 3.0mM.

2.7.5 Detection of the amplimer

The PCR products were visualised following electrophoresis of a 15µl aliquot, by staining with ethidium bromide. For the toxin gene detection methods, where only one or two bands were being detected, 2% (w/v) molecular biology grade agarose (Life Technologies, Paisley, UK) was made with TAE buffer (Sigma-Aldrich Company Ltd,

Poole, Dorset, UK) containing 0.2mg/L of ethidium bromide. For the PCR typing methods, where numerous bands were produced, 2% (w/v) Metaphor™ agarose (Flowgen, Lichfield, Staffs, UK) was made up with TBE buffer (Tris base 10.8g/L, orthoboric acid 5.5g/L, plus 4ml of 0.5M EDTA solution). The buffer was made up using distilled water and adjusted to pH 8. The gels were stained for 10 minutes with ethidium bromide solution (0.5mg/L) after electrophoresis. The stained gels were viewed with either a Chromato-Vue TM-20 UV transilluminator (UVP, San Gabriel, CA, USA), when photographs were taken using a Polaroid DS34 camera fitted with an orange (Wratten 16) filter and using Polaroid 667 film or by using an ImageMaster VDS camera system (Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). For interpretation purposes, AP-PCR profiles differing by 3 or more bands were considered to be gentically distinct, whilst 2 or more bands difference was adequate for RS-PCR (Cartwright *et al.* 1995).

2.7.5.1 Agarose gels

For the standard agarose gels, 60ml of TAE buffer (Sigma-Aldrich Company Ltd, Poole, Dorset, UK) was used with 1.2g of agarose. A microwave oven was used to melt the agarose, which was cooled to around 55°C before being poured into the 11 x 12.8cm gel tray (Northumbria Biologicals Ltd, Cramlington, UK) which was sealed at both ends with autoclave tape. One or two 16 well combs were used, allowing 14 or 28 reactions per gel (A 100 bp ladder was included in the first and last wells of each gel to allow sizing of the product). The gel was allowed to set at room temperature and was then cooled to 4°C in a refrigerator. On removal from the refrigerator, the combs were carefully removed followed by the autoclave tape. The gel tray (Northumbria Biologicals Ltd, Cramlington, UK) was then placed in the electrophoresis tank (Northumbria Biologicals Ltd, Cramlington, UK) and TAE buffer (Sigma-Aldrich Company Ltd, Poole, Dorset, UK) added until the gel was covered. Care was taken to avoid air bubbles remaining under the gel tray. The gel was

placed with the wells nearest the anode end of the tank. Once in position, 15µl aliquots of PCR product were loaded under the buffer, after having been mixed with xylene cyanol-bromophenol blue loading dye (Helena Biosciences, Sunderland, UK). A 100 bp ladder (Helena Biosciences, Sunderland, UK) was included at each end of the gel as mentioned above. Electrophoresis was carried out at 250 Volts and 200 Amps for 45 min using an E455 power pack (Flowgen, Lichfield, Staffs, UK) or an EPS 200 power pack (Pharmacia Biotech UK Ltd, Little Chalfont, UK).

Some standard agarose gels were also made in a larger 14 x 23cm gel tray (Owl Separation Systems, Portsmouth, NH, USA) allowing 25 large or 50 small wells per gel. This enabled either 20 or 44 strains to be tested on one gel, as it had to be split into two for staining, and required a ladder at both ends of each half, as well as the loss of one large or two small lanes for cutting of the gel. The larger gels were made in exactly the same way as the smaller ones but required 150ml of TAE buffer (Sigma-Aldrich Company Ltd, Poole, Dorset, UK) and 3g of agarose (Life Technologies, Paisley, UK). Electrophoresis was performed in a large electrophoresis tank (Owl Separation Systems, Portsmouth, NH, USA) with the same current and voltage as above. Large gels were cut in half between the two central lanes containing 100 bp ladder, prior to staining, UV transillumination and photography.

2.7.5.2 Metaphor™ gels

Metaphor™ (Flowgen, Lichfield, Staffs, UK) gels for the RS and RAPD PCR methods were manufactured in a very similar way to the standard agarose gels mentioned above. Significant differences were that cooled TBE buffer was used in their manufacture, and the gel was kept at 4°C for a minimum of 30 min before use. Ethidium bromide was not added to the gel during manufacture. Electrophoresis with these gels was performed using

TBE buffer. Gels of both sizes were used. Gel loading was performed under buffer and the electrophoresis was performed at 200 Amps, 180 Volts for 2-4 hours depending on the time taken for the leading edge of the loading dye band to reach the end of the gel.

RESULTS

3.1 DEVELOPMENT OF *C. difficile* COLONISATION AND INFECTION IN ELDERLY PATIENTS, FOLLOWING CEFOTAXIME (CTX) OR PIPERACILLIN-TAZOBACTAM (PT) THERAPY

3.1.1 Clinical trial

Using the initially designed randomised controlled trial method, only one patient was enrolled over a six-month period and it was decided to review the trial design. Under the new non-randomised, ward crossover design, forty-eight patients were enrolled over a 14-month period. Of these, 34 received cefotaxime (CTX) and 14 piperacillin-tazobactam (PT) (Table 8). The two groups were well matched for age (median 82 and 84.5 years, for CTX and PT groups respectively), and primary diagnosis. The most frequent two diagnoses in both groups were chest infection and stroke. The proportion of females in each group was higher than that of males, but did not differ significantly between groups (23/34 (CTX) vs. 13/14 (PT), p= 0.13). Patients who received CTX had a significantly shorter total hospital stay compared with the PT patients (median 33 (CTX) vs. 69 days (PT), p=0.04). However, this was mostly accounted for by the length of stay before study entry (median 1 (CTX) vs. 11 days (PT), p=0.07). Duration of hospital stay after study entry was similar in each group (median 27.5 (CTX) vs. 34.5 days (PT), p=0.51). The total number of days before study admission on which patients received an antibiotic did not differ significantly between groups (117 (CTX, n=34) vs. 35 (PT, n=14), p=1.00), and antibiotic days in the 72h immediately prior to study entry were also similar (20 (CTX, n=34) vs. 8 (PT, n=14), p=0.93). (When patients were treated concurrently with more than one antibiotic, one day was recorded for each antibiotic taken for a full day). The antibiotics most frequently administered in the CTX group before study entry were ciprofloxacin, ampicillin and erythromycin, compared with ampicillin, trimethoprim and cephradine in PT patients.

Mortality rates in the two groups did not differ significantly (11/34 CTX vs. 3/14 PT, p=0.44). Response to therapy was also similar (2/34 CTX vs. 1/14 PT had bacteriological failure of therapy, p= 1.00).

Table 8: *C. difficile* colonisation and CDI before and after the crossover on each ward

	Before crossover			After crossover			Totals	
Ward[1,2]	A (CTX)	B (PT)		A (PT)		B (CTX)	A+B (CTX)	A+B (PT)
Number of patients	22 (CTX)	10 (PT)	1 (CTX)	4 (PT)	4 (CTX)	7 (CTX)	34 (CTX)	14 (PT)
C.difficile colonisation[3]	17 (77%)	2 (20%)	1 (100%)	1 (25%)	3 (75%)	5 (71%)	26* (76%)	3* (21%)
CDI	11 (50%)	1 (10%)	1 (100%)	0 (0%)	3 (75%)	3 (43%)	18† (53%)	1† (7%)

[1] CTX = cefotaxime, PT = piperacillin-tazobactam.

[2] Some patients on the ward using PT received cefotaxime due to penicillin allergy

[3] Figures for *C.difficile* colonisation include patients with CDI.

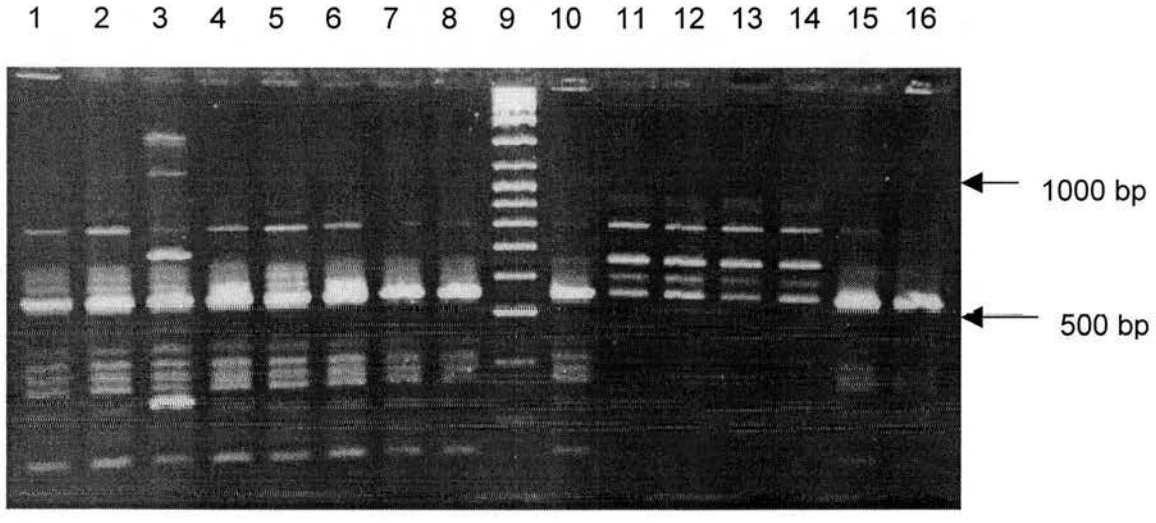
Using Fisher's exact probability test, *p=0.001; †p=0.006

Of 34 CTX patients, 26 were colonised with *C. difficile* during the study, of whom 18 developed CDI (Table 8). In the PT group, three of 14 patients were noted to be colonised with *C. difficile*, of whom one developed CDI. There was a significant difference between the groups for development of CDI (18/34 vs. 1/14 p=0.006) and for *C. difficile* colonisation (26/34 vs. 3/14 p=0.001). Of the 18 CTX patients who developed CDI, 14 were treated, 2 died of other causes (with diarrhoea), and 2 recovered spontaneously. The patient who developed CDI after receiving PT did not receive specific treatment and the symptoms resolved after 2 days.

In the 10 months before the crossover, 77% (17/22) of study patients on ward A (CTX) were found to be colonised by *C. difficile*, whilst 11 developedCDI. In contrast to this, on ward B (PT), 20% (2/10) were discovered to be colonised by *C. difficile* and one developed CDI. This represents a CDI incidence of 50% following CTX compared with 10% following PT. After the crossover, the incidence of CDI remained high (75%) on ward A (now PT), in the patients who still received CTX (due to penicillin allergy), but was low (0%) in those receiving PT. On ward B (now CTX), the *C. difficile* colonisation rate increased from a previously low level of 20% to 71%. The incidence of CDI was 43% for patients who received CTX (Table 8). During the study period, there was greater consumption of oral cephalosporins (cephradine, 78 days and cefaclor, 19 days) in the CTX group, usually as follow-on therapy, compared with in the PT patients (0 days). However, of the 18 patients who developed CDI in the CTX group, seven received one of these cephalosporins while 11 did not (p=0.76). Use of cephalosporins in the pre study period was higher in the PT group. Overall, comparing patients who received cephalosporins other than CTX either before or during the study, with those who did not, there was no significant difference between the two groups in incidence of CDI (p=0.91).

In 34% (10/29) of the patients colonised with *C. difficile* during the study, a culture- and toxin-negative faecal sample had been obtained before any positive sample, implying that the strains were hospital-acquired. Eight of these strains (80%) were the endemic strain (p24). For the 19 patients whose initial sample was *C. difficile* positive, molecular typing methods (see section 2.7 and its subsections) showed that the strain first isolated was identical to strain p24 in 74% (14/19), and a non-endemic strain in 26% (5/19) of cases. Figure 1 demonstrates discrimination between endemic and non-endemic strains. For patients whose first sample was positive by culture or toxin detection, the average length of time from admission until collection of the specimen was 6.6 days (median 6 days).

Figure 1: Genotypic typing using RS-PCR, demonstrating patient isolates with endemic (p24) strain pattern and another genotypically distinct pattern (by kind permission of Warren Fawley)



lanes 1-2, 4-8, 10, 15-16: endemic strain pattern
lane 3: endemic pattern with other bands also (suspected mixed extract used)
lane 9: 100 bp ladder
lanes 11-14: genotypically distinct strain pattern

The average time until collection of the first specimen for those patients who were initially culture and toxin negative, but subsequently positive, was 6.5 days (median 6 days). The length of time until *C. difficile* acquisition in patients with initially negative samples was 15.5 days on average (median 13.5 days). Amongst the 6 who developed CDI, time to acquisition was on average 12 days (median 10.5 days). For *C. difficile* colonisation average time to acquisition was 20.75 days (median 17 days). On the two wards overall, CDI rates were almost identical over a 22 month period, 9.2 and 8.9 cases per 100 admissions for wards A and B respectively. This period included the 8 months before the study as well as the study period.

Environmental screening over the 22 month period resulted in 1122 swabs being processed, with 572 from ward A and 550 from ward B (Fawley and Wilcox, 2001). Over the whole period, 34% of sites on ward A and 36% of sites on ward B were positive. In the 8 months prior to study commencement, ward B was more heavily contaminated with *C. difficile* (average of 26% vs. 13% of sites positive). In the first 10 months of the study, contamination rates increased on both wards, averaging 56% of sites (from 13%) on ward A (CTX) and 40% of sites (from 26%) on ward B (PT). In the last 4 month period (after crossover), average environmental *C. difficile* contamination rates were 31% on ward A (PT) and 38% on ward B (CTX). Despite the general increase in environmental contamination on both wards during the first study period, the degree of change was highly significant on the ward using CTX (13% to 56%, $p=<0.0001$, ward A) but not significant on the ward using PT (26% to 40% $p=0.17$, ward B). Additionally, there was a significant decrease in environmental contamination on ward A after changing from CTX to PT, from 56% (over 10 months) to 31% (over 4 months) of sites positive ($p=0.03$). There was no significant difference in environmental contamination levels between the two wards either before ($p=0.16$), during the first 10 months ($p=0.08$) or the last 4 months of the study ($p=0.84$) (Table 9).

Table 9: Environmental contamination with *C. difficile* on the two wards before and during the study (averaged over time stated)

LOCATION	% COLONISED SITES		
	BEFORE STUDY (8 MONTHS)	FIRST STUDY PERIOD (10 MONTHS)	SECOND STUDY PERIOD (4 MONTHS)
Ward A (CTX then PT)	13%[a]	56%[a, b]	31%[b]
Ward B (PT then CTX)	26%[c]	40%[c, d]	38%[d]
Difference in colonisation rates between wards	p=0.16	p=0.08	p=0.84

All probabilities in table calculated using two-tailed Fisher's exact probability test

[a] Change in colonisation rate from before study to first study period p=<0.0001

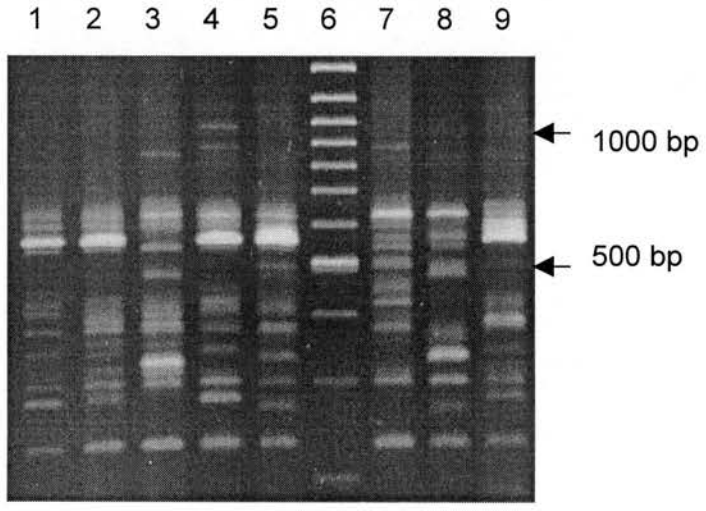
[b] Change in colonisation rate from first to second study period p=0.03

[c] Change in colonisation rate from before study to first study period p=0.17

[d] Change in colonisation rate from first to second study period p=1.00

The strains isolated from either ward environment demonstrated 6 different DNA fingerprints, with the endemic strain (p24) accounting for 93% of isolates. Figure 2 shows a comparison between some of the distinct patient and environmental strains with strain p24. This strain accounts for most sporadic cases of CDI at this hospital. *C. difficile* with the endemic DNA fingerprint was isolated from 22/29 patients. In ten patients, non-endemic strains were recovered during the study, although in three cases this was following previous isolation of the endemic strain.

Figure 2: RS-PCR of environmental and patient isolates, demonstrating some genotypically distinct strains, with p24 control (by kind permission of Warren Fawley)



lane 1: strain p24 control
lanes 2,3,4: genotypically distinct patient isolates
lane 6: 100 bp ladder
lanes 5,7,8,9: genotypically distinct environmental strains

3.2 COMPARISON OF THE OXOID TOXIN A TEST WITH CYTOTOXIN DETECTION USING A CYTOPATHIC EFFECT METHOD

3.2.1 Toxin testing

Of the 100 samples tested, fifty-four were CPE negative and 46 were CPE positive. Two of the CPE positive samples, which were also found to be toxin A test positive, had been CPE negative when originally tested. There were also six of the originally positive samples that were found to be CPE negative in this study, although one was toxin A test positive. This specimen, was culture positive with a toxigenic strain of *C. difficile*, and was classified as a true positive. Therefore, for the purposes of specificity and sensitivity determination, 47 samples were true positives, and 53 were true negatives.

At 4h incubation the CPE test showed 26 positive results, with 21 false negatives (sensitivity 54%) and 5 false positives (specificity 90%). At 6h incubation the CPE test showed 32 positive results, with 15 false negatives (sensitivity 67%) and 3 false positives (specificity 94%). At 24h incubation the CPE test showed 44 positive results, with 3 false negatives (sensitivity 92%) and no false positives (specificity 100%). At 48h incubation the CPE test showed 46 positive results, with one false negative (sensitivity 98%) and no false positives (specificity 100%).

The Oxoid Toxin A kit demonstrated 37 positive results, of which 3 were false positives (specificity 94%). Negative results were recorded for 63 specimens, of which 13 were false negatives (sensitivity 72%). One of the samples which was toxin A test positive had no detectable toxin B by CPE (it was originally CPE positive and was included as one of the 50 positive specimens). Sensitivities and specificities were calculated using the CPE

result at 48h as the gold standard, with the addition of the above-mentioned toxin A test positive/CPE negative specimen as a true positive (see Tables 10 and 11).

There was a high degree of correlation (100% agreement at 48h) between the two individuals who read the CPE test. At 6h one investigator (the more experienced of the two) identified 3 more positive specimens than the other investigator. One of these proved to be a false positive.

3.2.2 *C. difficile* culture

All 46 CPE positive samples were *C. difficile* positive by culture, as well as the six samples that were negative on CPE testing in this study, but were previously CPE positive. One of these six samples was toxin A test positive. In addition, eight specimens that had originally been CPE negative and were again negative in this study, were *C. difficile* culture positive. Of these eight strains, all but one was toxigenic by CPE testing of culture filtrate. In summary, *C. difficile* was isolated from 50 (100%) of the originally CPE positive stool samples and from 10 (20%) of the originally CPE negative stool samples.

Table 10: Sensitivity and specificity of CPE test for toxin B at various time intervals and for Oxoid toxin A test

CPE at incubation times (h)	false test negatives	false test positives	sensitivity (%)	Specificity (%)
4	21	5	54	90
6	15	3	67	94
24	3	0	92	100
48	1	0	98	100
Oxoid toxin A test	13	3	72	94

Table 11: Results for Oxoid toxin A test and toxin B detection by cytopathic effect at 48 hours

Oxoid toxin A test	CPE +ve	CPE -ve
Positive	33	4*
Negative	13	50

*One of these specimens was classified as a true positive

3.2.3 *C. difficile* toxin B titres in faecal supernatants

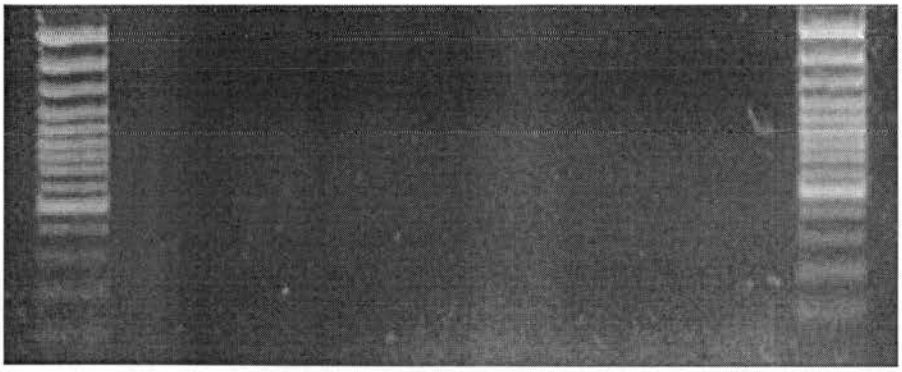
After storage of faecal supernatants at -20°C for 4 weeks, *C. difficile* toxin B titres (calculated as the reciprocal of the stool filtrate dilution) were found to range between $2x10^1$ and at least $2x10^8$. In seven supernatants that were previously CPE positive, no toxin B was detected at this time. None of the samples that had been CPE negative 4 weeks earlier had positive toxin titres. There were 11 samples with toxin B titres $>2x10^4$ and all were toxin A test positive, although not all toxin A positive samples had titres above this level. One toxin A test positive sample had a toxin B titre of only $2x10^1$. Toxin A negative samples had toxin B titres of between $2x10^1$ and $2x10^4$.

3.3 PCR TYPING AND TOXIN GENE DETECTION OF *CLOSTRIDIUM DIFFICILE*

3.3.1 Problems encountered with reagents

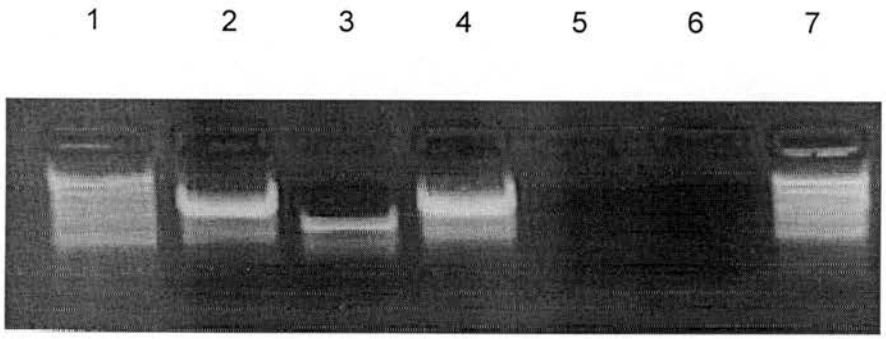
When toxin gene determination of *C. difficile* strains from Leeds and Bradford was first attempted, the results were rather mixed. The first attempts followed the method previously described by Kato *et al.* (1998) and were made using Amplitaq gold *taq* polymerase and buffer (Perkin Elmer Applied Biosystems, Bucks, UK). This yielded no detectable product, and it was not apparent whether inadequate extraction or amplification was the problem (see figure 3). Using the lysozyme/proteinase K extraction method, but PCR reagents from a colleague who had previously been successful with the method, both the 1200 bp product and 700 bp were demonstrated (see figure 4).

Figure 3: Initial result of Kato PCR, with no detectable products



100 bp ladder at each end of the gel

Figure 4: Gel using Kato PCR reagents that had previously been used successfully, showing two distinct products (only run for 30 min)



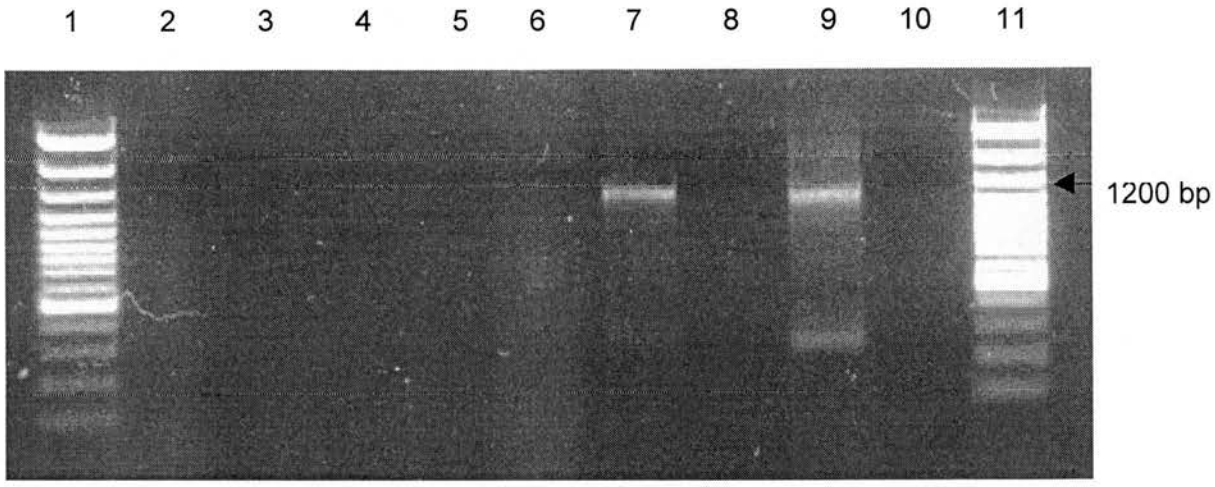
lanes 1,7: 100 bp ladder
lanes 2,4: A+B+ control strains
lane 3: A-B+ strain
lane 5: non-toxigenic strain
lane 6: negative control

Evidently, the previous problem was not due to failure of extraction and so the reason for the failure in amplification had to be found.

Perkin Elmer had provided two different PCR buffer solutions and so the alternative buffer was tried. This resulted in the production of 1200 bp fragments from A+B+ strains, but no 700 bp product could be detected from A-B+ strains (see figure 5). The strain used as non-toxigenic in fact proved to be toxigenic on CPE testing, after the PCR result showed a 1200 bp product. This was due to an incorrectly labelled stock sample. A magnesium titration was performed in case amplification of the 700 bp product was being prevented by a sub-optimal $MgCl_2$ concentration (see figure 6). Alternative extraction methods did not improve results, nor did using the primer that had worked adequately in the earlier experiment (figure 4) to demonstrate whether extraction was working.

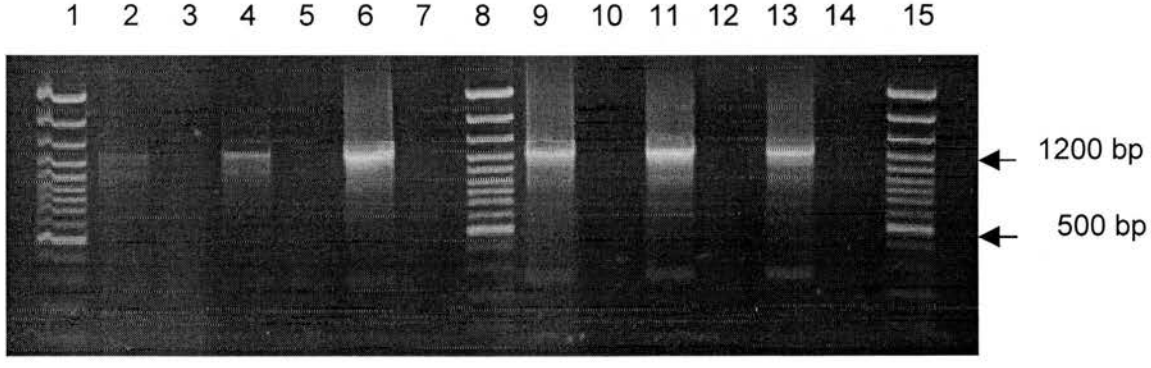
Finally a comparison of two different *taq* polymerases with appropriate PCR buffer (Amplitaq gold *taq* polymerase (Perkin Elmer Applied Biosystems, Bucks, UK) and Super*taq* (HT biotechnology Ltd, Cambridge, UK)) was performed with other reagents being the same. Despite some non-specific bands, the 1200 bp and 700 bp products were detected with the new *taq* polymerase but only the 1200 bp product was seen with the old *taq* polymerase (see figure 7). Work then continued satisfactorily with the new brand of *taq* polymerase (see figure 8).

Figure 5: Results of Kato PCR using Amplitaq gold *taq* polymerase (no products with standard buffer, lanes 2-6; 1200 bp product but no 700 bp product using Amplitaq gold buffer, lanes7-10)



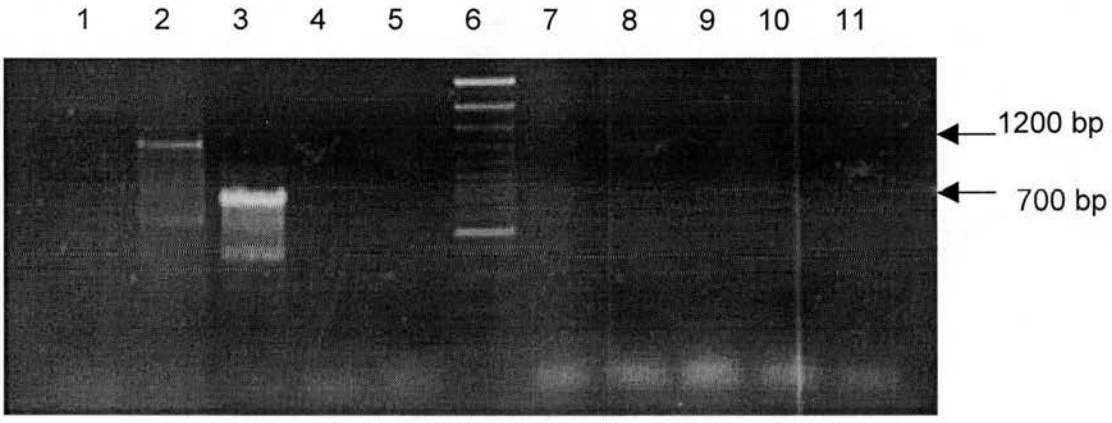
lanes 1,11: 100 bp ladder
lanes 2,7: A+B+ strains
lanes 3,8: A-B+ strains
lanes 4,9: presumed A-B- strains
lanes 6,10: negative controls

Figure 6: Kato PCR gel demonstrating magnesium titration with no 700 bp products seen



lanes 1,8,15: 100 bp ladder
lanes 2-4: 1mM $MgCl_2$
lanes 5-7: 1.5mM $MgCl_2$
lanes 9-11 2.0mM $MgCl_2$
lanes12-14: 2.5mM $MgCl_2$

Figure 7: Kato PCR gel showing results with Super*taq* (lanes 1-5) or Amplitaq gold *taq* (lanes 7-11)



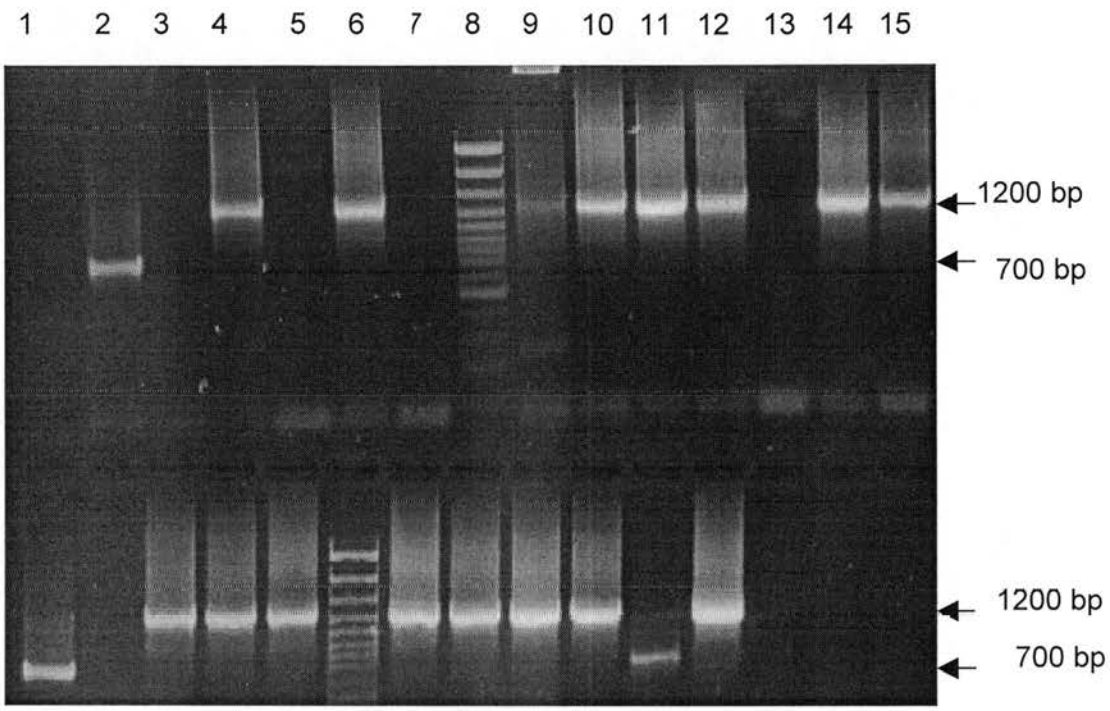
Lanes 1,5,7,9: negative controls,

lanes 2,8: A+B+ strain,

lanes 3,9: A-B+ strain,

lanes 4,10: A-B- strain.

Figure 8: Kato PCR gel showing detection of 700 bp and 1200 bp products



Top lanes:

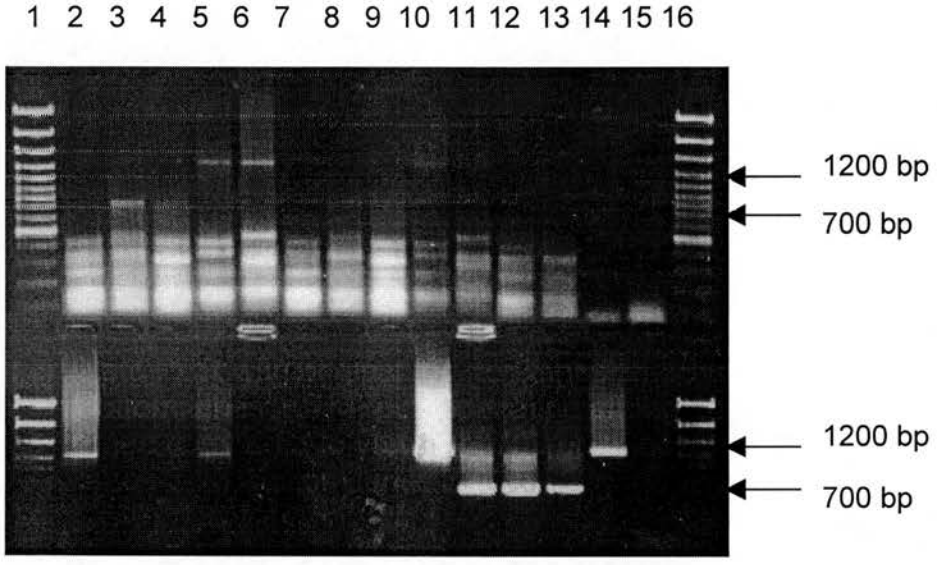
lane 1: negative control
lane 2: A-B+ control strain
lane 3 A-B- control strain
lane 4: A+B+ control strain
lanes 5,7,13: A-B- test strains
lanes 6,9,10,11,12,14,15: A+B+ test strains
lane 8: 100bp ladder

Bottom lanes:

lane 1,11: A-B+ control strains
lane 2: A-B- control strain
lanes 3,4,5,7,8,9,10,12: A+B+ test strains
lane 6: 100 bp ladder

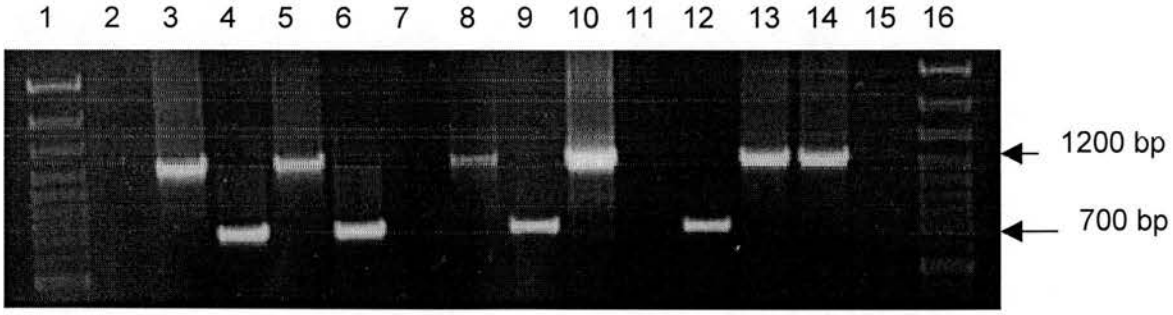
After a break of several months in performing the PCR testing, new *taq* polymerase (from the same manufacturer whose product was working previously) was obtained in order to continue. Unfortunately, the 700 bp fragment could not be detected when using the A-B+ control strains. Extraction methods and reaction volumes were adjusted, but without success. After performing another magnesium titration, a faint 700 bp band was just discernible, but the band was much stronger with the original batch of *taq* polymerase (see figure 9). Note that a faint band is also seen at 1200 bp in the A-B+ control sample with the old *taq*, suggesting contamination with a fully toxigenic strain. Two alternative *taq* polymerases (one as a ready-made mastermix, Abgene Ltd, Epsom, Surrey, UK) were tried, with successful results using both boil and lysozyme/proteinase K extractions (see figure 10). The ready-made master mix (Abgene Ltd, Epsom, Surrey, UK) was then used for the toxin gene detection PCR (Kato) and found to perform faultlessly (see figure 11).

Figure 9: Kato PCR with original *taq* (top lanes 14,15 (neg controls) and bottom lanes 10-14) demonstrating strong 700 bp band visible when compared to very faint 700 bp bands using new *taq* (top lanes 2-13, bottom lanes 2-9, 15)



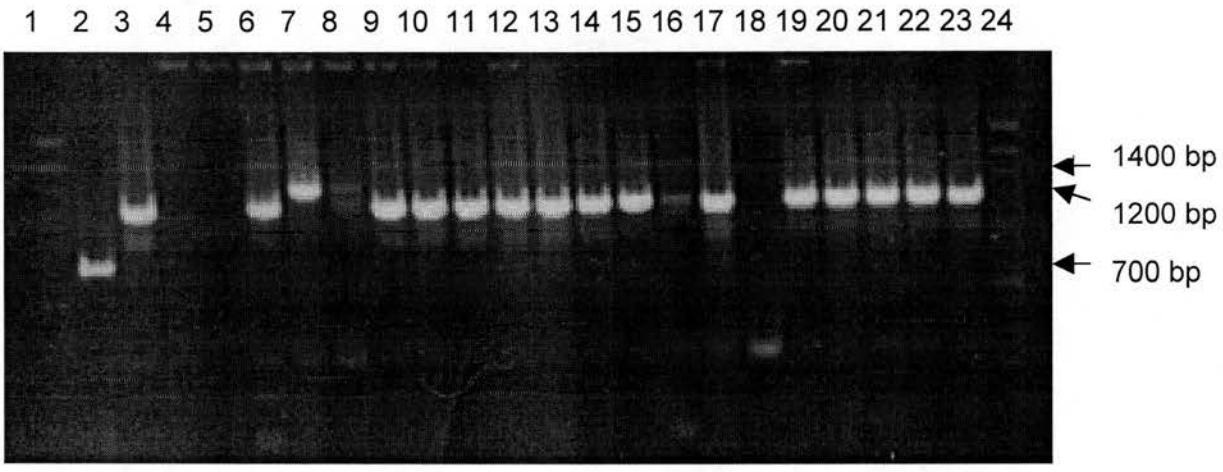
Top Lanes	Strain	Mg^{2+} conc^n	Bottom lanes	Strain	Mg^{2+} conc^n
1	ladder		1	ladder	
2	A-B+	1.5mM	2	A+B+	1.5 mM
3	A-B+	1.5mM	3	A-B+	2.5mM
4	A-B+	1.5mM	4	A+B+	2.5mM
5	A+B+	1.5mM	5	A+B+	3.0 mM
6	A+B+	2.0mM	6	A-B+	3.0 mM
7	A-B+	2.0mM	7	A-B+	3.0 mM
8	A-B+	2.0mM	8	A-B+	3.0 mM
9	A-B+	2.0mM	9	A+B+	3.0 mM
10	A+B+	2.0mM	10	A+B+	1.5mM
11	A+B+	2.5mM	11	A-B+	1.5mM
12	A-B+	2.5mM	12	A-B+	1.5mM
13	A-B+	2.5mM	13	A-B+	1.5mM
14	neg control		14	A+B+	1.5mM
15	neg control		15	empty	
16	ladder		16	ladder	

Figure 10: Kato PCR with two new *taq* polymerases to demonstrate whether 700bp product reliably produced



Lanes:	Strain	*Taq*
1	100 bp ladder	
2	neg control	Promega
3	A+B+ control	Promega
4	A-B+ control	Promega
5	A+B+ test strain	Promega
6	A-B+ control	Promega
7	neg control	Promega
8	A+B+ test strain	Promega
9	A-B+ control	Abgene mastermix
10	A+B+ control	Abgene mastermix
11	neg control	Abgene mastermix
12	A-B+ control	Abgene mastermix
13	A+B+ test strain	Abgene mastermix
14	A+B+ test strain	Abgene mastermix
15	empty	
16	100 bp ladder	

Figure 11: Demonstration of 1400 bp product using Kato PCR



lanes1,24:	100 bp ladder
lane 2:	A-B+ control
lane 3:	A+B+ control
lane 4:	A-B-control
lane 5:	neg control
lane 7:	1400 bp product from strain 72
lane 8:	strain 72 (boil extract)
lane 18:	extraction failure
other lanes:	A+B+ test strains

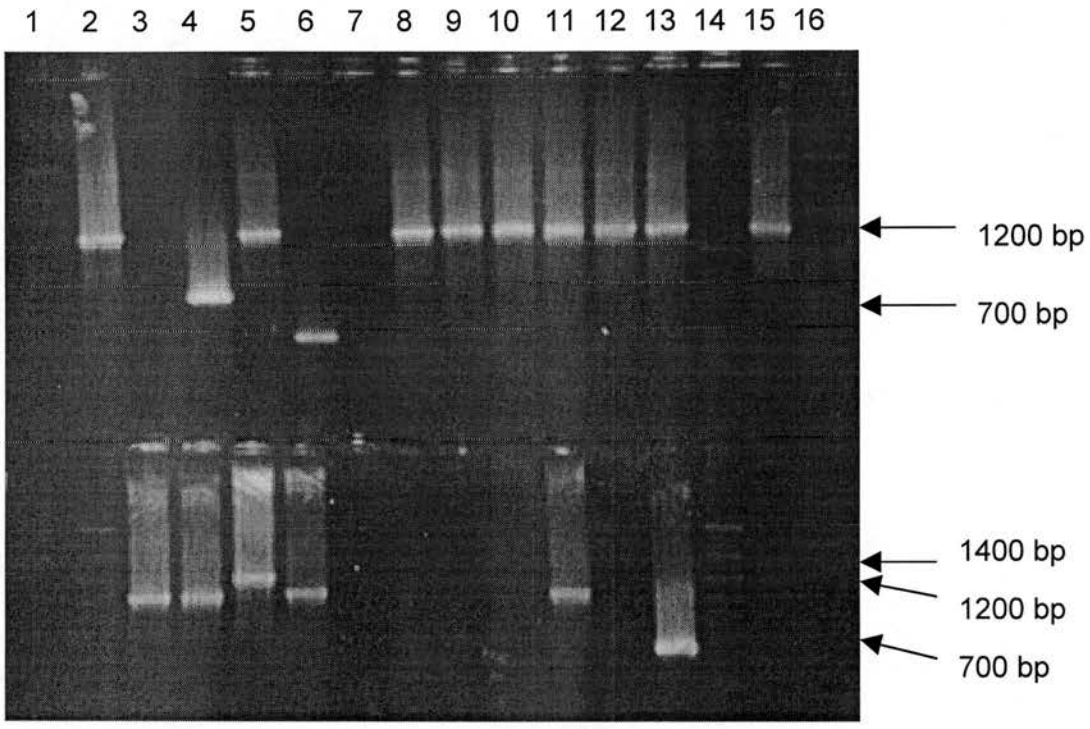
3.3.2 Toxin gene detection

Out of the 269 strains of *C. difficile* that were tested for presence of the toxin A gene, all possessed either the toxin A gene or neither gene, with no toxin A negative, toxin B positive strains being detected. The PCR findings were confirmed by culture and toxin testing by CPE. All 9 toxin negative strains were found to produce no product on PCR (Kato) for toxin A gene. The identity of each of these strains was confirmed to be *C. difficile* by RapID ANAII kit (Innovative Diagnostics Systems, Norcross, GA, USA).

Because only St James's University Hospital (SJUH) carried out culture for *C. difficile* as a routine diagnostic measure, rates of non-toxigenic carriage for patients with diarrhoea could only be estimated from 103 samples. There were 8 non-toxigenic *C. difficile* strains detected, representing 7.8% of the total number of strains from SJUH. One non-toxigenic strain was also identified amongst the other 166 strains tested. Testing colonies of this isolate on several occasions by PCR (Kato, Gumerlock or Tang) produced no toxin gene products. It is of interest that the CPE test of the stool sample from which this non-toxigenic strain was isolated was positive. On further investigation, a strain of *C. difficile* which generated toxin B in broth culture and produced a 1200 bp product on toxin gene PCR (Kato) was also detected in this sample.

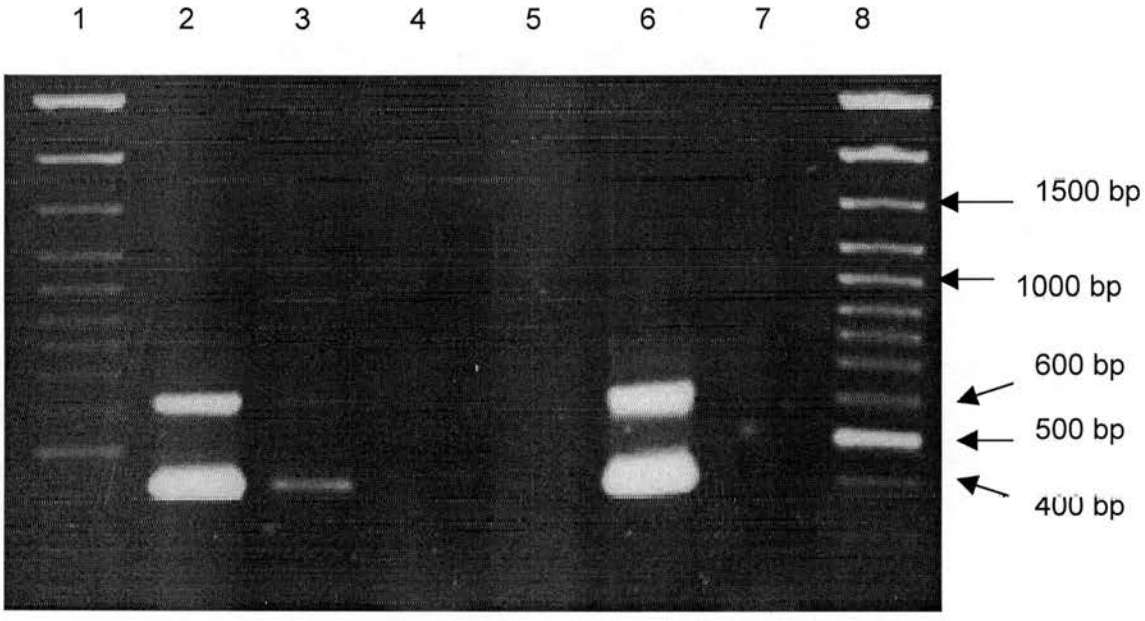
One of the strains positive for toxin B by CPE testing produced a PCR toxin gene product (Kato) of approximately 1400 bp, rather than the expected 1200 bp. This finding was reproducible and not felt to be a non-specific phenomenon (See figures 11 and 12). When alternative toxin gene detection primers (Gumerlock, Tang) were used, the correct size products were generated, demonstrating that both toxin genes were present (See figure 13). This strain was also found to produce toxin A, detectable by the Oxoid toxin A test.

Figure 12: Confirmation of 1400 bp product from strain 72 by Kato PCR



Top:	lanes 1,16:	100 bp ladder
	lanes 2-5:	control strains
	lane 6:	non *C. difficile*
	lanes 7,14:	A-B- strains
	lanes 8-13,15:	A+B+ strains
Bottom:	lanes 2,14:	100 bp ladder
	lanes 3-4,6,11:	A+B+ test strains
	lane 5:	strain 72
	lane 13:	A-B+ control strain
	lanes 7-10:	A-B- strains
	lane 12:	1/100 dilution of A+B+ extract (no product)

Figure 13: Cohen PCR for *tcdA* and *tcdB*, showing correct size products for strain 72



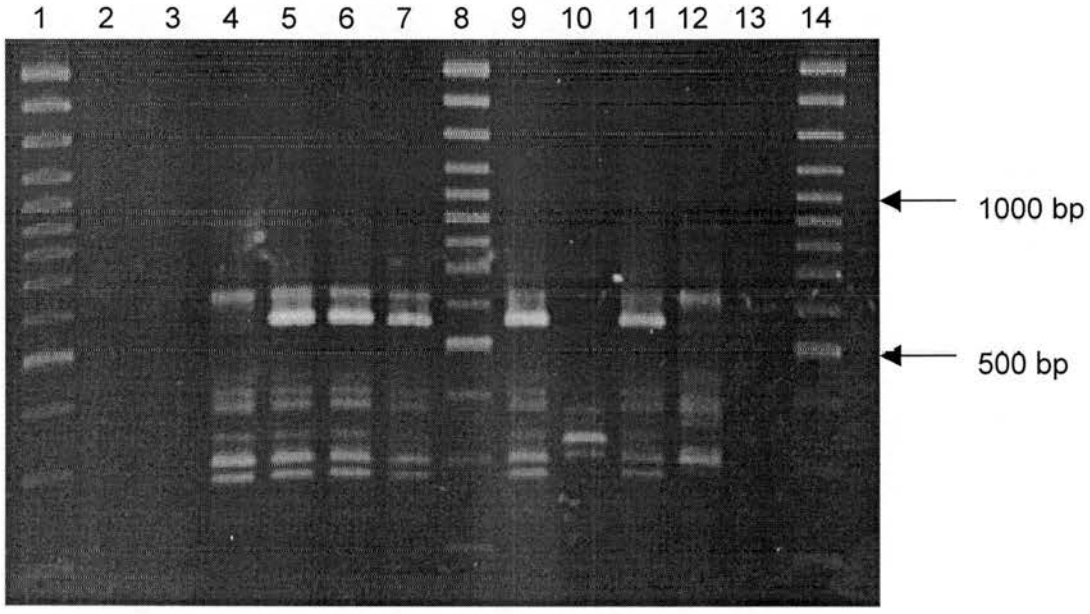
lanes 1,8:	100 bp ladder
lane 2:	A+B+ control strain
lane 3:	A-B+ control strain
lane 4:	negative control
lane 5	A-B- control strain
lane 6:	strain 72
lane 7:	A-B- test strain

3.3.3 Typing PCR

PCR profiles of all the community strains were obtained using RS-PCR, previously described by Jensen *et al.* (1993). Notable differences were demonstrated when the Leeds isolates were compared with those from Truro. Amongst 15 isolates from community patients in Leeds, nine (60%) produced an identical RS-PCR profile (<2 bands difference) to that of the hospital endemic clone in Leeds (strain p24). In contrast, out of 33 toxigenic isolates from community patients in Truro, only five (15%) showed this PCR profile (see figure 14). To further clarify whether or not these strains were indeed the same as strain p24, RAPD PCR was carried out on all those with identical RS-PCR profiles to that strain (see figure 15). This demonstrated that all nine strains from Leeds were identical (< 3 bands difference) to strain p24 (see figure 16), whilst none of the strains from Truro were identical (3 or more bands different). This difference in the incidence of p24 between populations is highly significant (p= <0.0001).

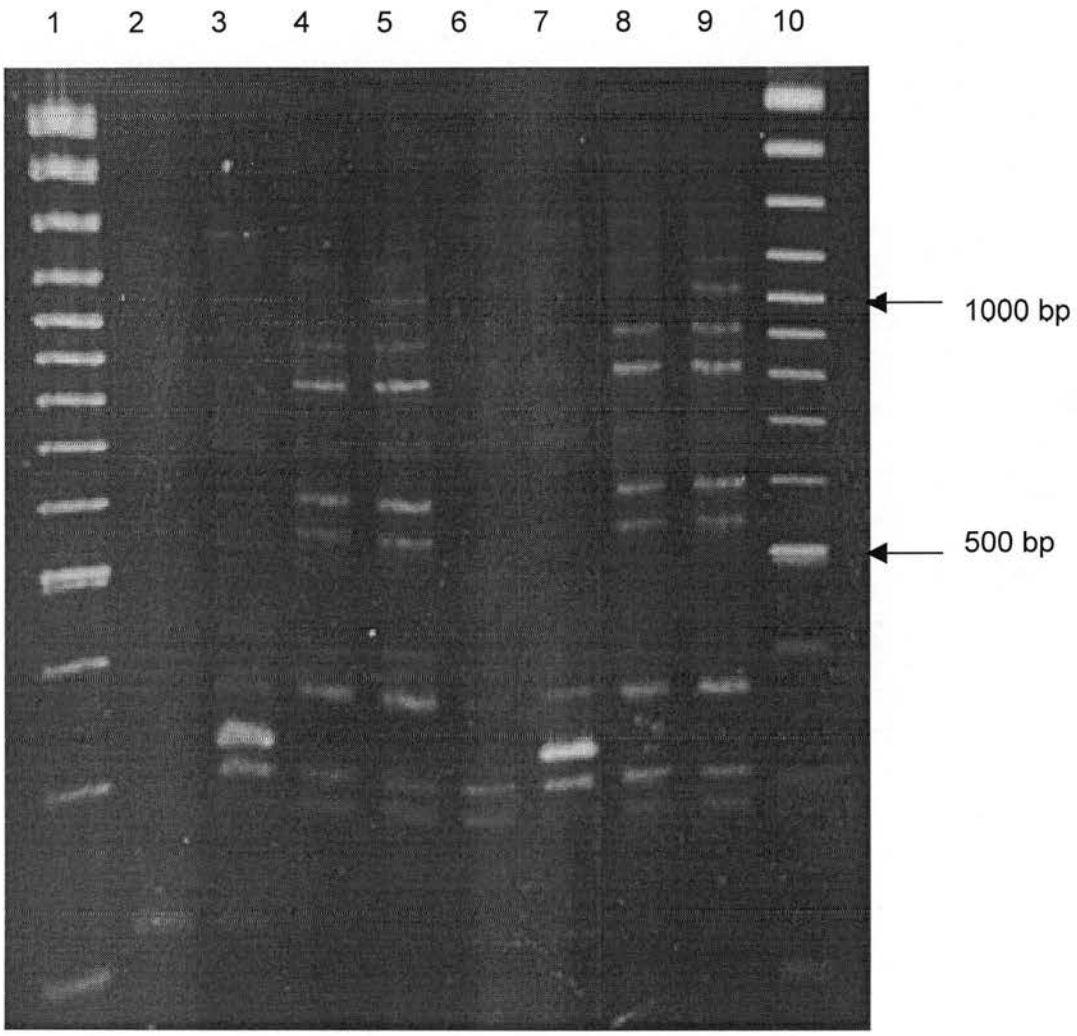
Culture and toxin B testing of the community strains by CPE confirmed that all those from Leeds were toxigenic, however these samples had been selected on the criterion of toxin B positivity. Of 39 strains from Truro, 33 (85%) were found to be toxigenic. The rate of non-toxigenic *C. difficile* isolation (15%) from Truro community patients was higher than the rate of isolation from either Leeds hospital patients (7.8%, p>0.05, <0.10), or elderly admissions to Leeds General Infirmary (12%, Settle *et al.* 1999). However, these differences do not reach statistical significance.

Figure 14: RS-PCR gel, showing five Truro strains with identical profiles to strain p24



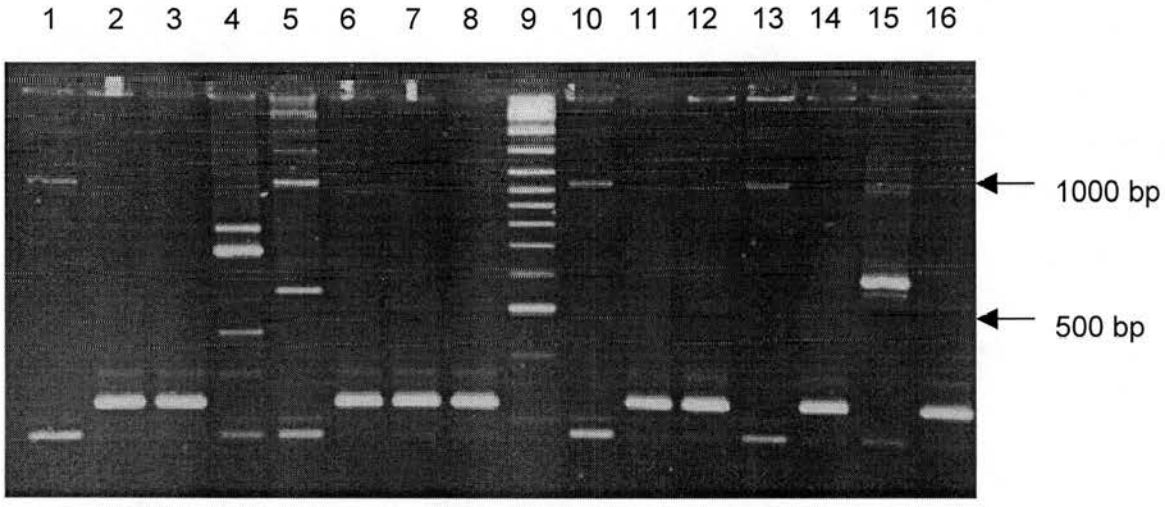
lanes 1,8,14:	100 bp ladder
lanes 2,3,13:	negative controls
lanes 4,5,7,9,11:	Truro strains
lane 6:	strain p24
lane 10:	strain p24 (RAPD profile)
lane 12:	Leeds strain (non p24)

Figure 15: RAPD PCR gel showing that the five Truro strains which had identical RS profiles to p24 are not p24



lanes1,10:	100 bp ladder
lane 2:	negative control
lanes 3,7:	strain p24
lanes 4,5,6,8,9:	Truro strains which had identical RS profiles to p24

Figure 16: RAPD PCR gel of Leeds community isolates, which had identical p24 like RS profiles, to confirm their identity. (their RAPD profile was confirmed as identical to that of strain p24 on another gel) (by kind permission of Warren Fawley)



lane 9:	100 bp ladder
lanes 2,3,6,7,8,11,12,14,16:	Leeds strains with p24 RAPD profile
lanes 1,4,5,10,13,15:	Leeds strains with non-p24 RAPD profiles

DISCUSSION

4.1 THE SIGNIFICANCE OF *CLOSTRIDIUM DIFFICILE* INFECTION

Over the last two decades, the number of cases of CDI being reported have increased sharply (see 1.5.3). Although greater awareness and reporting is likely to account for part of this increase, CDI itself appears to be more common within hospitals and a genuine increase in incidence seems to have occurred. Development of CDI has been shown to prolong patients' hospital stay (Wilcox *et al.* 1996) and cause additional morbidity, so an increase in the number of cases must be taken seriously. Amongst hospital patients, the elderly account for the majority of CDI cases. Consequently, it is reasonable to concentrate on this group when trying to find ways of reducing the risk of developing CDI. It is clearly important to optimise the diagnosis of CDI as well as to find ways of reducing the risk of the infection. Furthermore, an improved understanding of the epidemiology of the pathogen may enable better strategies for managing CDI to be developed. In particular, a more comprehensive understanding of how the infection is spread may allow appropriate preventive strategies to be formulated.

4.2 AETIOLOGY OF *CLOSTRIDIUM DIFFICILE* INFECTION

Much is already known about the aetiology of CDI, and as previously outlined in sections 1.5.3.1 and 1.5.3.3, elderly, hospitalised patients are at the highest risk. Antibiotics are thought to be the most important factor that renders patients susceptible to CDI, with clindamycin and 'third generation' cephalosporins likely to be the most incitory agents (see 1.3.2.3 and subsections). It was therefore decided to compare the incidence of CDI and *C. difficile* colonisation in elderly patients who had received either cefotaxime (CTX) or piperacillin-tazobactam (PT) for the treatment of serious infection.

4.2.1 Findings of the clinical trial

The initial attempt at a randomised, double blind, prospective study was unsuccessful, due to the difficulty in obtaining consent for trial entry. This was because most of the patients suitable for study entry were confused and did not have relatives with them who could give consent. This study was redesigned so that consent was no longer required. However, this meant that no rectal swab could be taken at study entry and so the carriage rate of *C. difficile* for patients at the time of admission could not be precisely calculated. Instead, a stool sample was collected as soon as possible after hospital admission. Inevitably this was sometimes several days after admission and when positive results were then obtained it was no longer possible to determine when the organism had been acquired. Asymptomatic carriage of *C. difficile* amongst elderly patients admitted to our hospital has previously been shown to be approximately 12% (Settle *et al.* 1999). This equates to an expected number of 6 patients in our study group being colonised with *C. difficile* at the time of admission. As 29 patients (60%) were found to be colonised during their hospital stay, it seems likely that most of them acquired the organism in hospital rather than being colonised prior to admission. This theory is supported by the observation that the proportion of environmental isolates found to be the endemic strain (93%) is higher than the proportion of patient isolates shown to be the same strain in this study (76%). Nevertheless, it should be noted that the median time until a first specimen was collected amongst patients who were initially negative was the same as for those who were initially positive at 6 days. Futhermore, in patients who were initially negative, the median period of time to *C. difficile* acquisition was 13.5 days. This tends to suggest that patients whose first sample was positive at 6 days might still have acquired the organism before admission to the study ward. If this were the case, it would correspond to a rate of colonisation on admission of 39.6% (19/48). This is far in excess of the 12% figure, previously generated from a larger number of elderly admissions to this hospital. These patients were screened on admission, during the

same time period. The possibility that such a large proportion of the patients in the study may have been colonised on admission, might be accounted for if they had recently been hospitalised before admission to the study ward. Alternatively, perhaps the relatively small number of patients involved biases the figures.

In this hospital the great proportion of cases of CDI in elderly patients (>80%) are caused by one 'endemic' strain, designated p24. The overall incidence of CDI in this hospital has been high during the late 1990's, due to the organism being endemic on many wards. The policy of using 'third generation' cephalosporins for the treatment of elderly patients has done nothing to reduce this burden.

There was no significant difference between patient groups with respect to age, sex distribution, diagnosis on study admission or quantity of antibiotics taken before study entry. Although there was a difference between the types of antibiotics taken before study entry by the patients of each group, PT patients may have been at greater risk of CDI because of this. Those receiving PT had received more cephradine prior to study entry, which may be expected to be more likely to lead to CDI than the ciprofloxacin which CTX patients had received more frequently (see 1.3.2.3.2 and 1.3.2.3.4). Ideally, patients would only have been considered for study entry if they had received no antibiotic within the previous 10 weeks, however enforcing this condition would have ruled out most potential patients, so a more realistic criterion had to be accepted. Consequently, the total amount of antibiotic received by patients before study entry was noted along with a record of which antibiotics were taken. According to current understanding of susceptibility to CDI following receipt of antibiotics (see 1.3.2.3 and subsections), if one group was at greater risk than the other of developing CDI on study entry, it was the PT group. Use of cephalosporins other than CTX was more prevalent overall in the CTX patients, primarily because of follow on therapy (usually cephradine). However, analysing the receipt of non-study cephalosporins revealed

no significant increase in the subsequent likelihood of developing CDI. This serves to emphasize that the risk of CDI following cefotaxime is probably much greater than the risk after cephradine, because although a relatively small number of patients was studied, there was a highly significant effect following cefotaxime, whilst cephradine use did not correlate with subsequent CDI.

The most interesting finding of the study is that patients who received CTX had a significantly greater risk of subsequent *C. difficile* colonisation and CDI than did patients who had received PT. The relative risk of CDI in CTX treated patients was 7.4 (95% CI= 1.7-33) compared with those who received PT. The odds ratio (OR) for CDI in CTX treated patients compared with those who received PT was 14.6 (CI= 1.7-124.7) and the number needed to treat (NNT) to avoid one case of CDI was 2.18.

It is perhaps unexpected that PT is less likely to induce *C. difficile* colonisation or CDI than CTX, given its broad-spectrum activity, particularly against anaerobes. This observation may cast doubt on the theory that the anaerobic gut flora is a critical determinant of 'colonisation resistance' (see section 1.3.2.1). Alternatively, the relative *C. difficile* sparing effect of PT may reflect the limited penetration of this antibiotic into the gut lumen in some patients. Of 20 patients given PT, when sampled on one occasion during treatment, six had detectable faecal concentrations of piperacillin, of whom four had measurable levels of tazobactam, using a relatively insensitive antibiotic detection method. The effect on anaerobic flora was noted to be minor, with anaerobic Gram-positive cocci and *Bacteroides* sp. being unaffected (Nord *et al.* 1992). Alternatively, it may be because piperacillin and tazobactam are secreted in bile at the incorrect relative proportions that PT is not as active as would theoretically be expected. A report by Westphal *et al.* (1997), indicates that rather than the 8:1 ratio present in serum, bile concentrations of piperacillin and tazobactam are on average approximately 50:1. It should be noted that biliary levels of piperacillin and

tazobactam in the 15 subjects studied did vary considerably (Westphal *et al.* 1997). Conversely, as *C. difficile* is more susceptible to PT than CTX, it may be that the organism is more likely to be killed in PT treated patients. Data on the gut levels of PT in patients receiving the drug remain fairly limited. Preliminary in-vitro data using *C. difficile* inoculated faeces spiked with either PT or CTX, or taken from antibiotic treated patients, do not demonstrate a difference in the growth of *C. difficile*. (Freeman and Wilcox, 2000). One possible explanation for the difference in rates of CDI following CTX or PT therapy is that CTX is metabolised to an active compound, desacetylcefotaxime (Wise *et al.* 1980); (Jones *et al.* 1982). This metabolite has also been shown to interact synergistically with cefotaxime against many organisms, including enterobacteriacae and *Bacteroides fragilis*. These findings are extensively reviewed by Jones, (1995). Not only is desacetylcefotaxime an active metabolite with an antimicrobial activity and spectrum greater than the 'second generation' cephalosporins, it has been shown to exceed cefotaxime concentrations in bile (Novick, 1982). Consequently, it will be secreted into the bowel along with cefotaxime and possibly interact in a synergistic fashion against bowel flora.

It is also of interest that patients who developed CDI did not have significantly longer hospital stays, which is in contrast to some earlier findings (Wilcox *et al.* 1996). However, the explanation for this could be that as patients in the PT group had longer in hospital prior to study admission than their counterparts in the CTX group, they might have been more severely ill. No formal assessment of severity of illness was made in this study and such a factor should be controlled for if length of stay of patients in each group is to be properly compared. That patients in the PT group were in hospital longer before study entry than those in the CTX group would also be expected to leave them more susceptible to *C. difficile* acquisition.

One important factor, which has not been controlled for in earlier work (Anand *et al.* 1994); (de Lalla *et al.* 1989), is exposure to *C. difficile* spores in the environment. By monitoring the environmental contamination of each ward on a monthly basis, it was possible to determine whether increased ward contamination was leading to an increase in cases of CDI or vice-versa. At the outset, the ward with the greater environmental load of *C. difficile* was chosen for the PT group, so that these patients were not favoured in any way. Despite this, and the other factors mentioned above, which might have resulted in the PT group being at higher risk of developing CDI, it was the CTX group who demonstrated the highest incidence of *C. difficile* colonisation and CDI in the first 10 months of the study. Environmental contamination on the CTX ward increased significantly ($p=<0.0001$) but not on the PT ward ($p=0.17$), subsequently falling after the cross-over from CTX to PT ($p=0.03$) but not after the cross over from PT to CTX ($p=1.00$). If the incidence of CDI were purely to reflect environmental contamination, then the rate of CDI on ward A after changing from CTX to PT should have remained high instead of falling significantly. Higher rates of CDI in patients receiving CTX compared with PT, when patients are assumed to have had equivalent exposure to *C. difficile* also indicates an effect of antibiotic rather than of environment.

A further factor pointing to the role antibiotics in driving CDI, rather than environmental contamination, is that during the study, the ward which used CTX always started off as the cleaner of the two and became the most contaminated during the period of CTX use. Conversely, the ward which used PT always commenced as the more contaminated of the two and became the cleanest whilst using PT.

One alternative explanation for the greater degree of CDI on a ward when using cefotaxime would be if the ward population (herd) were generally more susceptible to CDI due to an overall greater use of antibiotics. It is noticeable that not as many patients received

PT as did CTX over the study period. If this led to an increased 'herd' susceptibility, then the number of cases of CDI on the ward using CTX (in patients not enrolled in the study) should be greater than in the same group on the PT ward. Therefore the overall incidence rate of CDI should be higher on ward with the poorest 'herd immunity'. In fact, the overall incidence rate of CDI was very similar on both wards, being slightly higher on ward A, which used CTX for the 10 month period (9.2 versus 8.9 cases per 100 admissions). This is in contrast to a slightly higher overall environmental contamination on ward B (34% versus 36% of sites over 22 months). Despite the overall incidence rate of CDI being so similar on both wards, CDI in those who had received PT was very uncommon. This suggests that it is specifically the patients who received PT and not all patients on the ward who have a reduced risk of developing CDI (which may explain the lower rate of CDI on ward B, in the face of greater environmental contamination).

The decision to end the study was taken when the wards were due to be relocated, because data analysis at this time showed a marked difference in *C. difficile* colonisation and more importantly infection rates. It was therefore considered unethical to continue using cefotaxime for treating elderly patients. Clinical response to treatment with CTX or PT was similar, as might be expected in such patients, considering the spectra of the two antibiotics. Although the study was neither blinded nor randomised, we are confident in the accuracy of our findings given the absence of significant, identifiable confounding factors and the objective measurements of *C. difficile* colonisation and infection used. Furthermore, randomisation was found to be impractical because of the confused elderly patient cohort under investigation.

The adoption of a block cross-over design rather than individual randomisation is a weakness, as environmental factors such as 'herd immunity' and environmental contamination with spores can differ between wards. Similar CDI incidence rates between

wards coupled with environmental screening for spores serve to minimise the deficiencies of the model used. Nevertheless, a more detailed statistical analysis of the data, using logistic regression methods would perhaps have added further weight to the findings. Using such a technique would have allowed the interrelationships between several risk factors to be investigated and determine more accurately the contribution of factors such as environmental contamination to the development of CDI.

Starr and colleagues recently speculated that the selective pressure resulting from cephalosporin prescribing might increase the proportion of *C. difficile* susceptible patients in a ward or unit (Starr *et al.* 1997). In this setting administration of narrow spectrum antibiotics, with otherwise relatively low propensities to select for *C.* difficil,e may subsequently induce symptomatic infection. Alternatives to cephalosporins, for example the combinations of penicillin and either trimethoprim (McNulty *et al.* 1997) or ciprofloxacin (Jones *et al* 1997) for the empirical antibiotic treatment of infection have been shown in uncontrolled studies to be associated with a reduced incidence of CDI. Guidelines for the treatment of community-acquired pneumonia, a common cause of hospital admission in the elderly, cite cephalosporins, including cefotaxime, as antibiotics of choice for severe as opposed to mild-moderate infections (British Thoracic Society, 1993). These guidelines were implicated in the increased incidence of CDI in a department of medicine for the elderly (Impallomeni *et al.* 1995). However, it should be noted that there was some evidence that incidence of CDI was on the increase before the introduction of the BTS guidelines (Wilcox, 2000).

In the light of current beliefs regarding the use 'third generation' cephalosporins and risk of subsequent CDI, it may not have been justified to carry out the study based on the null hypothesis that risk was equal for both antibiotics. If independent monitoring of the study outcome had been performed, then any early indication of highly significant

differences in risks between groups could have been obtained. This may have allowed an earlier end to the study and reduction in any excess risk to patients.

PT may serve as a useful alternative antibacterial agent to 'third generation' cephalosporins for the treatment of elderly patients. If the enormous apparent difference between the incidence of CDI following treatment with either CTX or PT is confirmed by use on larger numbers of patients, analysis suggests that only 2-3 patients would require treatment with PT rather than CTX to avoid a case of CDI.

4.3 DIAGNOSIS OF *CLOSTRIDIUM DIFFICILE* INFECTION

Although the generally accepted gold standard test for *C. difficile* toxin B detection is using CPE methodology, facilities do not always exist to perform this method and many manufacturers have produced kits to allow more widespread availability of *C. difficile* toxin B testing. Many of these tests are ELISA based (see section 1.4.3.3), and can give results in 2-4h, however attempts have been made to further simplify the testing procedure and reduce the time taken to obtain a result. One recent test of this type is the Oxoid Toxin A test (Unipath, Basingstoke, Hampshire, UK), the principle of which is described in section 2.6.3. This test is designed to be very straightforward to perform, with a minimum of additional equipment required (centrifuge capable of 9800x **g**), and produces a result in around 30 min. For small laboratories, with low numbers of specimens to be tested for *C. difficile* toxin, this type of test represents a more practical solution than CPE or ELISA methods. Analysis of the performance of such new tests is important to determine their role in the diagnosis of CDI.

4.3.1 Performance of the Oxoid toxin A detection kit

In this comparison of the the Oxoid Toxin A test (Unipath, Basingstoke, Hampshire, UK) and CPE testing for toxin B using HEp-2 cells, the rapid test (sensitivity 72%, specificity 94%) was similar in accuracy to the CPE method at 6h (sensitivity 67%, specificity 94%). These figures are at odds with those published by Bentley *et al.* (1998), which were used by Oxoid for their kit insert (83.1% sensitivity and 96.9% specificity, resolved to 91% and 98% respectively). In resolving the figures, cytotoxin positive but toxin A negative faecal samples were reclassified as true negatives if culture for *C. difficile* was negative. However, as the CPE testing was controlled with *C. sordellii* antitoxin, positive results would indicate the presence of *C. difficile* or *C. sordellii* regardless of whether culture was positive. It is extremely unlikely that 10% of positive results could occur due to *C. sordellii* as this is not commonly isolated from faeces (presence of *C. sordellii* toxin in stool specimens has not been descibed). Failure to grow *C. difficile* from the specimen should not be grounds to reclassify the toxin A result as a true negative rather than a false negative and doing so would serve to improve the sensitivity of the test in question. Detection of toxin A is not expected to be as sensitive as toxin B detection, due to the difference in sensitivity between the two methods. Toxin B is extremely potent and can cause a cytopathic effect at concentrations as low as a few pg/ml, whereas the threshold for toxin A detection kits is in the ng/ml range (Lyerly *et al.* 1988).

Two other issues arising from the CPE testing methods in this study and in that of Bentley *et al.* should be noted. Firstly, in the method used by the author, HEp-2 cell lines were used for toxin B detection. This cell line has been reported to be inferior to others by some researchers (Murray and Weber, 1983), although direct comparisons with Vero cells are lacking. Vero cells have been shown to be the most sensitive for toxin B detection when

compared with other cell lines (Maniar *et al.* 1987); (Torres *et al.* 1992), although there is some evidence that human foreskin fibroblasts are also very sensitive (Tichota-Lee *et al.* 1987). Consequently, the results of the CPE testing in the author's study may have favoured the Oxoid Toxin A test (Unipath, Basingstoke, Hampshire, UK) when compared to the method used by Bentley *et al.* Secondly, the final dilution of stool filtrate in the author's CPE method is 1 in 60, compared with 1 in 160 for Bentley *et al.* This could result in some reduction in sensitivity for the CPE method, as used by Bentley *et al.*, allowing a greater chance of false negative results. Perhaps this may explain in part (along with their method of resolving discrepant results) the poor sensitivity of the CPE method in their hands.

The overall performance of the Oxoid Toxin A test (Unipath, Basingstoke, Hampshire, UK) in this study is not adequate for it to be recommended for the primary diagnosis of CDI. If used for diagnostic purposes, then testing of repeat specimens would be advisable in cases with negative results where the patient remains symptomatic. It should also be emphasised that there are increasing numerous reports of toxin A negative, toxin B positive (A-B+) clinical isolates of *C. difficile*, and these will not be detected by toxin A kits (see 1.2.4.1.7). Brazier reported that *C. difficile* A-B+ isolates account for 3% of the total number of strains received from laboratories (but 10% of the strains submitted from one laboratory) in England and Wales by the Anaerobe Reference Laboratory (see section 1.5.4). In Japan, 33% of isolates collected in one hospital were of this phenotype (Kato *et al.* 1997). As A-B+ isolates of *C. difficile* have been recovered from symptomatic patients (Brazier *et al.* 1999a); (Kato *et al.* 1997); (Sambol *et al.* 1998); (Sambol *et al.* 2000), previously held beliefs that toxin A is the most important toxin in the pathogenesis of human antibiotic associated diarrhoea must be called into question.

4.3.2 Toxins A and B in stool specimens

The great majority of *C. difficile* strains are thought to produce equimolar quantities of toxins A and B. The observation that one sample was toxin A test positive, but CPE test negative is not likely to be due to differential toxin production, but possibly to unequal rates of breakdown of the two toxins, as toxin B is known to be more labile than toxin A (see section 1.2.4.1.1). The culture results indicate that it is possible for a toxigenic strain of *C. difficile* to be present in a sample and yet toxins A and B remain undetectable. This may be explained in several ways. Degradation of toxins in the faecal sample may occur prior to testing due to proteolytic enzymes. Alternatively, toxins may not be produced if the patient is being treated for CDI with either metronidazole or vancomycin, or in the presence of inhibitory bowel flora. Theoretically, this effect could also be seen if the sample contained only *C. difficile* spores in small numbers.

It is also of interest to consider whether the level of toxin B present in a sample indicates the likelihood of detecting toxin A. Although not absolute, there does seem to be some correlation between the level of toxin B in a sample and the likelihood of toxin A being detected. As stated earlier, it is assumed that both toxins are produced in equimolar quantities originally by the organism. The greater stability of toxin A to several environmental factors (Borriello *et al.* 1992b); (Sullivan *et al.* 1982) may result in it remaining at higher levels in a stool sample than toxin B. Consequently, although the detection methods for toxin A are roughly 1000 times less sensitive than those for toxin B, it is possible that toxin A could be detected at the same time as very low levels of toxin B. Most of the time, however, this will not be the case and samples with low toxin B titres will not have detectable levels of toxin A present. The author's findings do support this theory, because no sample which had a toxin B titre of greater than 10^4 was toxin A negative.

Correlation between levels of toxin B in stool samples and detection of toxin A by ELISA have been demonstrated previously by some investigators (Lyerly *et al.* 1983), but others have found no such link (Borriello *et al.* 1992).

The tendency of toxin B levels to fall in samples that are frozen and then thawed (Lyerly *et al.* 1986b) is confirmed by the author's observations from this study. Toxin B in stool filtrates appears to deteriorate more readily than when it is in stool specimens. The fact that some samples had no detectable toxin but did contain toxigenic organisms suggests that culture of toxigenic organisms alone from a sample may not automatically justify the treatment of a patient for *C. difficile* infection.

4.4 EPIDEMIOLOGY OF *CLOSTRIDIUM DIFFICILE* INFECTION

C. difficile is known to be a ubiquitous environmental organism which is particularly associated with the hospital environment. Despite widespread exposure to this organism, it is not commonly found in the gastrointestinal tract of normal adults. However, at the extremes of age, or after normal bowel microflora is compromised, *C. difficile* can readily colonise the bowel and may lead to disease. Some strains of *C. difficile* are non-toxigenic and not thought to cause disease, whilst other strains are frequently associated with CDI in hospital patients. One clone in particular (PCR ribotype 1), accounts for the majority of isolates from hospital patients (57%) sent to the PHLS Anaerobe Reference Unit, Cardiff PHL (see section 1.5.4). The next most frequently isolated strain (ribotype 15) accounts for only 5% of isolates.

In addition to allowing the detection of outbreaks of CDI, epidemiological investigation also detects trends in the particular *C. difficile* strains causing disease. When strains such as ribotype 1 are identified, further research can proceed to determine what enables them to cause disease so readily. Ribotype 1 (p24) displays several characteristics which may distinguish it as more virulent than average. It is known to sporulate more readily on exposure to detergents (Wilcox and Fawley, 2000) and has been found to be more resistant to antibiotics than other strains tested (Freeman and Wilcox, 2001). These properties may imbue upon it a survival advantage, both in the bowel and in the environment. It is preferable to study the strains that cause most disease, comparing them with those which do not, to try to determine what factors are most important in the pathogenesis of CDI. Monitoring the level of environmental contamination in high-risk hospital areas is also valuable as it can indicate when additional cleaning programmes may be required.

A second epidemiological issue that is currently of increasing importance is the prevalence of A-B+ strains. Increases in the number of such strains are of great interest because of the potential for missing cases of CDI if tests that detect only toxin A are used for diagnosis. Currently these tests detect the terminal repeat region of the toxin A molecule, which is absent in the mutant strains. Redevelopment of toxin A only tests might be one way of addressing the problem, but otherwise, methods which include toxin B detection should be used for diagnosis of CDI. By monitoring the prevalence of these A-B+ strains, it is possible to determine how safe current toxin A only tests are in a particular area.

Although CDI in hospitals accounts for most cases recorded at present, there is currently increasing interest in the burden of disease in the community. This is not particularly well described (see section 1.5.3.2), despite the large quantities of antibiotics which are prescribed by General Practitioners. The level of undiagnosed CDI in UK

community patients is not known and research into this area remains to be done. Another interesting challenge is to discover whether CDI in the community is caused by the same *C. difficile* strains that affect hospital patients. Furthermore, a comparison of community strains that cause disease in different parts of the country could add an extra dimension to our understanding of this pathogen in the UK.

4.4.1 Toxin gene determination of *C. difficile* from Leeds and Bradford

No A-B+ strains were detected out of the 269 that were tested (which included 5 community isolates). All toxigenic strains tested were noted to produce a PCR product (Kato) and all non-toxigenic strains were found to produce no PCR product. As mentioned in section 1.5.4, the reported incidence of A-B+ in the UK is 3%, although from one hospital 10% of isolates typed were A-B+ strains. It would appear, therefore, that the level of A-B+ strains in Leeds and Bradford is below the national average. Based on results from SJUH, the incidence of non-toxigenic strain isolation amongst *C. difficile* positive stools was 7.8%. Because little is published about such rates comparison is difficult. One non-toxigenic strain was detected from a specimen that was toxin B positive and this was found to be due to a mixture of two strains in the sample. As the sample had been initially found to be toxin B positive when tested in the diagnostic laboratory, it seems unlikely that the toxigenic strain was a later contaminant. Furthermore, the non-toxigenic strain was the more prevalent in the sample and would also appear not to be a contaminant. Samples containing more than one strain of *C. difficile* are not thought to be common (O'Neill *et al.* 1991) but have been described (Sharp and Poxton, 1985).

One strain of *C. difficile* was found, in a reproducible fashion, to yield a PCR product of ~1400 bp rather than the expected 1200 bp. It produced toxin B when tested by CPE and toxin A was detected using the Oxoid toxin A test. Consequently, the product is likely to be genuine, because if the band seen was merely non-specific, a normal 1200 bp

band should still be found representing the presence of the normal toxin A gene. PCR for *tcdA* and *tcdB* as described by Cohen *et al.* (2000) demonstrated products of the expected size for each gene. Further investigation of this strain is warranted, as the larger product produced may indicate an insertion occurring in the terminal repeat region of the toxin A gene.

4.4.2 Typing of community *C. difficile* isolates

Currently, research into the prevalence of CDI in community patients has only been relatively limited (see section 1.5.3.2), although estimates of asymptomatic *C. difficile* colonisation rates in community patients have been made by some UK researchers (Samore *et al.* 1994); (Settle *et al.* 1999). Another large UK study suggested that there may be a large number of community cases who never even present to their GP's (Wheeler *et al.* 1999). One prospective Swedish study reported that 28% of CDI in hospitals was of community origin (Karlstrom *et al.* 1998). Analysis of whether strains causing CDI in community patients differ from those causing such illness in hospital patients forms another integral part of epidemiological research. In addition to this, knowledge of whether the strains that cause disease in the community differ depending on location is also of interest. The PHLS Anaerobe Reference Unit, Cardiff PHL has noted that amongst community strains they have tested, the relative frequencies of strains found are different to those amongst hospital strains (see section 1.5.4).

The comparison of *C. difficile* strains from community patients in Leeds, with strains from similar patients in Truro, was intended to demonstrate whether similarities exist between *C. difficile* isolates causing CDI in different communities. The opportunity to compare isolates from an urban setting with those from a rural one was ideal and the findings raise some interesting questions. The rate of colonisation with non-toxigenic strains of 15%

is similar to rates previously reported in elderly patients (see section 1.5.2.2.1.3). Of more interest, is the complete lack of strain p24 (described as ribotype 1 by Stubbs *et al.* 1999) amongst the toxigenic isolates from Truro, whilst it represents 60% of those from Leeds (p= <0.0001). Although the sample size of the Leeds cohort is small and therefore susceptible to bias, it is consistent with the high level of p24 which is thought to cause disease in Leeds hospitals (>80% of *C. difficile* isolates from patients with CDI on two care of the elderly wards). Nevertheless, this is a different picture to that noted by Brazier, (1998b) amongst 390 community isolates when very few ribotype 1 isolates were detected. In order to be more certain of whether the finding is genuine, a greater number of strains from Leeds require testing. If the finding is representative of the situation in Leeds, then it is perhaps merely a reflection of the exceedingly high burden of strain p24 within Leeds hospitals, which then disseminates out into the community. In one study, 82% of patients who were noted to have *C. difficile* in hospital were still positive for the organism at discharge (McFarland *et al.* 1989). Rather than having acquired it in the community, Leeds patients may have only recently been discharged from hospital and still be carrying the organism in their intestine. Ribotype 1 (p24) is also known to be the most common strain isolated from patients with CDI in the UK (see section 1.5.4). The complete absence of p24 amongst the strains from Truro suggests that it is an uncommon strain there, if present at all. Nevertheless, a similar and possibly related strain does account for the greatest quantity of a single strain found in Truro (13%, 5/39). This strain has the same RS-PCR pattern as p24, but is recognised as being distinct from it by RAPD PCR. It is noticeable that amongst Truro isolates there is no individual strain that predominates to the same degree that p24 does in Leeds. Perhaps the rural nature of the area has prevented the dissemination of this otherwise widespread and prevalent clone.

4.5 CONCLUSIONS AND FURTHER WORK

4.5.1 Aetiology of *C. difficile* infection

Antibiotics undoubtedly play an important contributory role in the development of CDI. Some antibiotics would seem to be more hazardous in this respect than others and 'third generation' cephalosporins, along with clindamicin appear to pose the greatest risk. As elderly patients are identified as one of the most susceptible patient populations for CDI, it is reasonable to consider alternative antibiotic policies for the management of serious infection, which minimise the use of such agents. More than one approach is valid in this respect, but the use of PT in this setting can be considered as one option, given the findings of this report. Despite greater expense, it is possible that a large reduction in the number of patients developing CDI, with consequent reduction in length of stay would go a long way to balancing this out. In addition, any reduction in morbidity and mortality would represent an increase in the quality of care delivered to the patient.

Further work is required to try and confirm the findings of this report and this might be accomplished by attempting to alter prescribing habits on elderly care units, whilst monitoring *C. difficile* colonisation and CDI rates, along with environmental contamination levels. Provided that the patient population being treated did not change over time and nor did prescribing patterns of other drugs, then a reduction in CDI and *C. difficile* colonisation with the new prescribing policy could confirm the findings of this study (see below). Levels of environmental contamination should also be similar when comparing historical records with the beginning of the new prescribing policy period. This would ensure that a lower infection or colonisation rate was not due to lower levels of environmental contamination.

If the environmental load of *C. difficile* was seen to fall after introduction of a new antibiotic policy, then this might represent several possibilities:

1. The new policy resulted in patients being less susceptible to developing CDI or *C. difficile* colonisation and so there was less environmental soiling due to fewer cases of disease. The environmental load of *C. difficile* would then diminish after the reduction in cases of disease.
2. The new policy resulted in lesser excretion of *C. difficile* in those individuals with disease, although they were not any less susceptible to disease and the number of cases was not lower. The reduction in the environmental burden would then be followed by a decline in the number of cases of disease.
3. An outbreak was in progress before the change of policy that resolved at the same time that the new policy was instituted.

One alternative way of testing whether the findings of this study are accurate would be to repeat a similar type of study, with larger numbers of patients in each arm. However, this type of study might be deemed unethical in the light of current beliefs regarding the relationship of 'third generation' cephalosporins with the development of CDI. Consequently gaining ethical approval may prove impossible.

If patients could be cared for in a standardised environment (probably a single room), with the same initial level of environmental *C. difficile*, then likelihood of developing CDI should only relate to the antibiotic received. Regular monitoring of the environmental burden of *C. difficile* could be used to assess the degree of *C. difficile* excretion. This would allow a direct comparison of environmental *C. difficile* levels when patients who were given either PT or CTX developed CDI. It may also be necessary to measure antibody responses

in patients , to try and get an indication of their ability to resist development of disease in the presence of *C. difficile* toxins.

4.5.2 Diagnosis of *C. difficile* infection

New diagnostic tools are always being developed in medicine, to try and allow easier and faster diagnosis of illness. It remains important for such new methods to be independently evaluated, to ensure that they are appropriate for the use that they have been designed for. The author's evaluation of the Oxoid toxin A test suggests that it is not sufficiently sensitive to be used as a single test for the diagnosis of CDI. Nevertheless, due to its relative ease of use and its self-contained design it may be useful in some laboratories where cell tissue culture is not possible or where very low numbers of samples require to be tested. However, if a negative result is obtained in a situation where clinical evidence strongly suggests the diagnosis of CDI, then it would be wise to treat the patient as such and retest a further specimen. If the local level of A-B+ strains is known to be very low then a further negative result would be highly suggestive of an alternative aetiology. If level of A-B+ strains is high, or unknown, then it would be sensible to select a test which includes the ability to detect toxin B as well as toxin A.

4.5.3 Epidemiology of *C. difficile* infection and colonisation

With the recent recognition of A-B+ strains of *C. difficile*, capable of causing disease, it is important to know what the local level of such a pathogen is. This will allow an informed decision about which tests should be used to diagnose CDI. In addition, screening for such isolates may identify strains with other mutations in the toxin A and B gene region, which could subsequently throw further light on the structure and function of these toxins. This investigation has detected a strain that may have a mutation in the toxin gene region as

it produced a product larger than expected by toxin gene PCR (Kato). Toxins A and B were detected by traditional methods, and PCR for *tcdA* and *tcdB*, described by Cohen *et al.*, demonstrated products of the correct size. Further evidence that the product is indeed specific to the toxin gene region being tested for, could be obtained by using a probe of a known section of the expected product to hybridise with it. The probe would be labelled to allow its subsequent detection. If the PCR product was confirmed as being from the correct region, then analysing its nucleotide sequence and comparing this with the corresponding sequence from a type strain could clarify whether an insertion was present and what its identity was.

The proportion of community p24 strains isolated from Leeds patients is interesting and suggests recent hospitalisation or a high degree of environmental contamination with this strain in the community. However, to be more certain that this observation is accurate, larger numbers of isolates need to be tested. The marked difference in strain types from the Truro community patients, with no highly prevalent single clone and p24 not represented at all, may indicate the natural state in this country if contamination from patients who have been hospitalised is avoided. In order to investigate this further it would be useful to use gel documentation systems to better identify exactly how many distinct individual strains are present, as it is not possible to test every strain against every other one manually. In addition it may be useful to allow a reference facility such as the PHLS Anaerobe Reference Unit to ribotype the strains to find out whether any are previously undocumented.

Obtaining strains from the local hospitals in the Truro area, both from the environment and from patients, would also be very valuable in trying to determine the relationship between disease and carriage in hospital and in the community.

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Prospective study of the risk of Clostridium difficile *diarrhoea in elderly patients following treatment with cefotaxime or piperacillin–tazobactam*

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SUMMARY

Background: Rates of *Clostridium difficile* diarrhoea have recently been rising, with the elderly being at highest risk.

Aim: To compare the incidence of *C. difficile* colonization and diarrhoea in elderly patients treated for presumed infection with either empirical cefotaxime (CTX) or piperacillin–tazobactam (PT).

Methods: A prospective, ward-based, crossover study was carried out on two well-matched care of the elderly wards at a UK tertiary care hospital, in patients requiring empirical broad-spectrum antibiotic treatment.

Results: There was a highly significant increased incidence of *C. difficile* colonization (26/34 vs. 3/14, $P = 0.001$) and diarrhoea (18/34 vs. 1/14, $P = 0.006$) in patients who received CTX as opposed to PT. DNA fingerprinting suggested that most infections arose from strains acquired from the hospital environment.

Conclusions: Elderly patients are significantly less likely to develop *C. difficile* diarrhoea after treatment with PT than after CTX. The source of *C. difficile* appears to be predominantly from the ward environment.

INTRODUCTION

Clostridium difficile has been recognized as the cause of pseudomembranous colitis for 20 years, and is currently the principal infective cause of hospital-acquired diarrhoea.[1, 2] Laboratory reports of *C. difficile* continue to increase, with a 32% increase in numbers in England and Wales between 1996 and 1997.[3] Infection usually occurs following antibiotic therapy, particularly in elderly patients.[2] It is thought that broad-spectrum antibiotics alter the balance of normal aerobic and anaerobic intestinal flora, thereby reducing 'colonization resistance' and allowing *C. difficile* to proliferate.[4]

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In order to develop *C. difficile* diarrhoea (CDD), patients need to be susceptible to infection, and also to be colonized by or to acquire *C. difficile*. The source of *C. difficile* in sporadic cases of infection is unclear, being possibly patient (endogenous) strains, or exogenous bacteria acquired either directly from the hospital environment or via healthcare staff.[5–7] In some hospital settings 'endemic' strains of *C. difficile* exist, which account for the great majority of both sporadic and outbreak infections.[8] These may occur due to differences in the environmental distribution, transmissibility or virulence of *C. difficile* strains.

Retrospective studies have shown that third-generation cephalosporins such as cefotaxime (CTX) or ceftriaxone are particularly associated with CDD.[9–11] Some data indicate that anti-pseudomonal penicillins, with or without beta-lactamase inhibitors such as clavulanic

acid or tazobactam, may have a reduced propensity to induce CDD compared with third-generation cephalosporins.[9, 10, 12, 13] However, to date there have been no prospective studies, controlled for exposure to *C. difficile*, which directly compare two broad-spectrum antibiotic agents, in order to determine their influence on the risk of developing CDD. A recent survey of UK hospitals identified that the percentage of units which had ward closures or a change of antibiotic policy due to *C. difficile* infection increased by 3-fold and > 5-fold, respectively, between 1993 and 1996.[14] Preventative measures which are proven to be effective are required to reverse the increasing incidence of nosocomial *C. difficile* infection. In the present study we compared colonization with *C. difficile* or development of CDD in elderly patients treated with either CTX or piperacillin–tazobactam (PT), using a ward crossover design.

METHODS

The study took place between June 1996 and August 1997 on two care of the elderly wards at the General Infirmary at Leeds. Ethics committee approval was originally obtained for a randomized, double-blind pilot study. As it was found that most eligible patients were unable to give informed consent, recruitment was extremely poor (one patient in 6 months). Therefore, with ethics committee approval, the protocol was changed to a ward crossover design without randomization, so that individual patient consent would not be required. Patients on one ward (A) received intravenous (i.v.) CTX 1 g t.d.s. when broad-spectrum antibiotic therapy was required, as had been normal practice before the study. On the second ward (B), i.v. PT 4.5 g t.d.s. was prescribed instead. No other changes were made to antibiotic prescribing protocols. Patients who had a history of penicillin allergy were given CTX instead of PT. The two wards were of similar size (ward A, 32 beds, ward B, 28 beds) and had comparable admission policies and patient mix. Screening in the 8 months prior to the start of the study indicated that ward B had twice the level of environmental *C. difficile* contamination and that the incidence of CDD was 47% greater than on ward A. Ward B was therefore selected for initial PT use in order to minimize bias in favour of PT. Study end-points were discharge or death. CDD was defined as documented loose stools (once or more per day for at least 2 days), which was not attributable to another cause, in patients with concurrent *C. difficile* cytotoxin-positive faeces. After 10 months, ward-based antibiotic therapy crossed over so that PT was used on ward A and CTX on ward B. After a further 4 months the study wards were due to be re-sited as part of a major hospital building development. We were unaware that this move was to take place until late in the study. At this point an analysis of the results was performed and the study was terminated due to ethical considerations (see below).

A faecal specimen was obtained from each patient as soon as possible after the prescription of the study antibiotic, and then weekly during the patient's hospital stay, where feasible. Records were kept of the patients' maximum daily temperature and the nature and frequency of stools, biochemistry, haematology and microbiology results, and all drugs received. Culture of stool samples for *C. difficile* was performed by plating onto cycloserine–cefoxitin-egg yolk (CCEY) agar (Lab M, Bury, UK) and incubating anaerobically at 37 °C for 48 h. Suspect *C. difficile* colonies were identified by characteristic colonial morphology and odour before being tested for toxin B production by a microtitre tray cytotoxin assay using HEp-2 cells with *C. sordellii* antitoxin control wells. Suspect non-toxigenic isolates were identified using the RapID ANAII identification kit (Innovative Diagnostics Systems, Norcross, GA).

Environmental contamination with bacterial spores was also monitored in order to assess the relative risk of *C. difficile* exposure of patients on each ward. This was achieved by monthly, standardized swabbing of pre-selected sites, incubating the samples in Robertson's cooked meat broth at 37 °C for 48 h, before sub-culture to CCEY agar and re-incubation at 37 °C for 48 h. All environmental and patient strains were fingerprinted using PCR amplification of 16S-23S ribosomal spacer DNA. Briefly, target DNA was extracted using lysozyme and proteinase K.[15] Oligonucleotide primers and reaction conditions were based on those reported by Jensen *et al.*[16] Two-tailed Fisher's exact probability tests and Mann–Whitney *U*-tests were used for statistical analyses.

RESULTS

Forty-eight patients were enrolled; 34 received CTX and 14 PT (Table 1). The two groups were well matched for age (median 82 and 84.5 years for CTX and PT groups, respectively) and primary diagnosis. The two most frequent diagnoses in both groups were chest infection and stroke. The number of females in each group was

higher than the number of males, but did not differ significantly between groups (23/34 vs. 13/14, $P = 0.13$). The patients receiving CTX had a significantly shorter total hospital stay compared with the PT patients (median 33 vs. 69 days, $P = 0.04$), but this was mostly accounted for by the length of stay before study entry (median 1 vs. 11 days, $P = 0.07$). Duration of hospital stay after study entry was similar in each group (median 27.5 vs. 34.5 days, $P = 0.51$). Total antibiotic days before study admission did not differ significantly [117 ($n = 34$) vs. 35 ($n = 14$), $P = 1.00$], and days of antibiotics in the 72 h immediately prior to study entry were also similar [20 ($n = 34$) vs. 8 ($n = 14$), $P = 0.93$]. (When patients were treated concurrently with more than one antibiotic, one day was recorded for each complete day per antibiotic.) The antibiotics most frequently administered in the CTX group before study entry were ciprofloxacin, ampicillin and erythromycin, compared with ampicillin, trimethoprim and cephradine in the PT patients. Mortality rates in the two groups did not differ significantly (11/34 CTX vs. 3/14 PT, $P = 0.44$). Response to therapy was also similar (2/34 CTX vs. 1/14 PT had bacteriological failure of therapy, $P = 1.00$).

Of 34 CTX patients, 26 were colonized with *C. difficile*, of whom 18 developed CDD (Table 1). In the PT group, three of 14 patients were colonized with *C. difficile*, and one of these developed CDD. There was a significant difference between the groups for development of CDD (18/34 vs. 1/14, $P = 0.006$) and for *C. difficile* colonization (26/34 vs. 3/14, $P = 0.001$). Of the 18 CTX patients who developed CDD, 14 were treated, two died of other causes (with diarrhoea), and two recovered spontaneously. The patient who developed CDD after receiving PT did not receive specific treatment and the symptoms resolved after 2 days. Before the crossover, 77% (17/22) of study patients on ward A (CTX) were colonized by *C. difficile* (of whom 11 developed CDD), whilst on ward B (PT) 20% (2/10) were colonized by *C. difficile* (of whom one patient developed CDD). This represents a CDD incidence of 50% in patients who received CTX vs. 10% in patients who received PT. After crossover, on ward A (now PT), the incidence of CDD remained high (75%) in the patients who still received CTX (due to penicillin allergy), but was 0% in the patients given PT. On ward B (now CTX), the *C. difficile* colonization rate increased from 20% to 71% with a CDD incidence of 43% in patients who received CTX (Table 1). During the study there was greater consumption of oral cephalosporins (cephradine, 78 days and cefaclor, 19 days) in the CTX group, usually as follow-on therapy, compared with the PT patients (0 days). However, of the 18 patients who developed CDD in the CTX group, seven received one of these cephalosporins while 11 did not ($P = 0.76$). Overall, comparing patients who received another cephalosporin before or during the study with those who did not, there was no significant difference between the two groups in incidence of CDD ($P = 0.91$).

Environmental screening in the 8 months prior to the start of the study showed that ward B was more heavily contaminated with *C. difficile* (26% vs. 13% of sites positive). In the first 10 months of the study, contamination rates increased on both wards, reaching 56% of sites (from 13%) on ward A (CTX) and 40% of sites (from 26%) on ward B (PT). In the last 4-month period environmental *C. difficile* rates were 31% on ward A (PT) and 38% on ward B (CTX). Despite the general increase in environmental contamination on both wards during the first study period, the difference was highly significant on the ward using CTX (13% to 56%, $P < 0.0001$, ward A) but not significant on the ward using PT (26% to 40%, $P = 0.17$, ward B). Also, there was a significant decrease in environmental

Table 1. *C. difficile* colonization and CDD before and after the crossover on each ward

	Before crossover			After crossover			Totals	
Ward*	A	B		A		B	A + B	A + B
Number of patients†	22 (CTX)	10 (PT)	1 (CTX)	4 (PT)	4 (CTX)	7 (CTX)	34 (CTX)	14 (PT)
C. difficile colonization‡	17 (77%)	2 (20%)	1 (100%)	1 (25%)	3 (75%)	5 (71%)	26§ (76%)	3§ (21%)
CDD	11 (50%)	1 (10%)	1 (100%)	0 (0%)	3 (75%)	3 (43%)	18¶ (53%)	1¶ (7%)

*Ward A used cefotaxime and ward B used piperacillin–tazobactam (PT) initially, and vice versa afterwards.
†CTX, cefotaxime; PT, piperacillin–tazobactam.
‡Figures for *C. difficile* colonization include patients with CDD.
Using Fisher's exact probability test, §$P = 0.001$; ¶$P = 0.006$.

Table 2. Environmental contamination with *C. difficile* on the two wards before and during the study

% colonized sites	Before study (8 months)	First study period (10 months)	Second study period (4 months)
Ward A	13%*	56%*†	31%†
Ward B	26%‡	40%‡§	38%§
Difference in colonization rates between wards	$P = 0.16$	$P = 0.08$	$P = 0.84$

All probabilities in table calculated using two-tailed Fisher's exact probability test.
*Change in colonization rate from before study to first study period, $P < 0.0001$.
†Change in colonization rate from first to second study period, $P = 0.03$.
‡Change in colonization rate from before study to first study period, $P = 0.17$.
§Change in colonization rate from first to second study period, $P = 1.00$.

contamination on ward A after changing from CTX to PT, from 56% (over 10 months) to 31% (over 4 months) of sites positive ($P = 0.03$). There was no significant difference in environmental contamination levels between the two wards either before ($P = 0.16$), during the first 10 months ($P = 0.08$), or the last 4 months of the study ($P = 0.84$) (Table 2).

Of the strains isolated from the environments of wards A and B, seven different DNA fingerprints were identified, with an endemic strain accounting for 87% of isolates (Figure 1). This strain accounts for most sporadic cases of CDD at this hospital. *C. difficile* strains with the endemic DNA fingerprint were isolated from 22/29 patients. In 10 patients, non-endemic strains were recovered during the study, although in three cases this was following previous isolation of the endemic strain. In 34% (10/29) of the patients colonized with *C. difficile*, a culture- and toxin-negative faecal sample had been obtained before any positive sample, implying that the infecting strains were hospital-acquired. Eight of these strains (80%) were the endemic strain. For the remaining patients, molecular typing methods showed that the strain isolated initially was the endemic strain in 74% (14/19), and was a non-endemic strain in 26% (5/19) of cases.

DISCUSSION

In the present study we observed a highly significant difference in the rates of colonization with *C. difficile* and

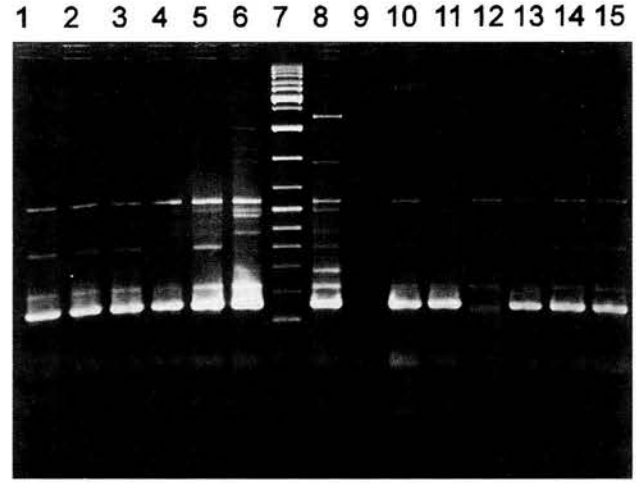

Figure 1. Fingerprints of environmental and patient *C. difficile* isolates obtained by PCR amplification of 16S-23S ribosomal spacer DNA. Lanes 1–5: endemic *C. difficile* strain isolated from CTX-treated patients; lanes 10, 11, 13–15: endemic *C. difficile* strain isolated from ward environments; lane 6, 8: non-endemic patient strains; lane 12: non-endemic environmental strain; lane 7: 100 base pairs DNA ladder; and lane 9: negative control.

development of CDD between the two antibiotic groups. The relative risk of CDD in CTX-treated patients was 7.4 (95% CI: 1.7–33) compared with those who received PT. Although CTX-treated patients received more non-study cephalosporins, usually as oral follow-on therapy, this factor was not associated with an increase in the incidence of CDD. The patient groups were well matched in all respects, except for the duration of hospital stay, primarily before study entry, which was greater in the PT group. This factor could be expected to make it more likely for patients treated with PT to acquire *C. difficile*, because of their prolonged exposure to the contaminated hospital environment, which is at odds with our findings. Therefore, our observation of a relative sparing of CDD in PT-treated patients is strengthened. Secondly, these patients may have been less well in general, perhaps explaining the lack of difference in length of hospital stay between the groups, despite fewer cases of CDD in those receiving PT.

We attempted to determine the main source of the *C. difficile* strains causing symptomatic infection. Rates of colonization with *C. difficile* in elderly patients on admission to hospital are ≈13% at our institution (unpublished data). Recent work shows that asymptomatic, colonized patients are at lower risk of developing CDD than non-colonized patients.[7] It therefore seems unlikely that the *C. difficile* isolation rate (76%)

in patients receiving CTX therapy can be accounted for by endogenous strains. A more likely hypothesis is that *C. difficile* is acquired from the ward environment either directly or via healthcare professionals. This theory is supported by the observation that the proportion of environmental isolates found to be the endemic strain (87%) is similar to the proportion of patient isolates shown to be the same strain in this study (76%). In this hospital the majority of cases of CDD in elderly patients (> 80%) are caused by one 'endemic' strain. The overall incidence of CDD in this hospital has been falling for the last 2.5 years, in contrast with national experience,[3] and the cases which we continue to experience are not time clustered. We are confident therefore that patients who developed CDD on the study wards represented sporadic cases and were not part of an outbreak *per se*.

Although we measured significant changes in the prevalence of environmental *C. difficile* at different stages during the study, the differences in contamination levels between the two wards during each phase did not reach statistical significance. There is compelling evidence that CTX use affects environmental contamination. In the first study period, which included winter, there was a prominent increase in CDD cases and ensuing environmental contamination by *C. difficile* on ward A (CTX, $P < 0.0001$), but not on ward B (PT, $P = 0.17$). In the second study period, which included summer, the decrease in CDD cases and environmental contamination was marked on ward A (PT, $P = 0.03$), but not on ward B (CTX, $P = 1.00$). Higher rates of CDD in patients receiving CTX compared with PT, when patients are assumed to have had equivalent exposure to *C. difficile*, also indicates an effect of antibiotic rather than of environment. Although it is difficult to be certain whether it is a highly contaminated ward which leads to greater acquisition rates of *C. difficile* or vice versa, our observations suggest the latter.

Antibiotics are accepted as the main causal factors for CDD, with elderly patients being recognized as the most susceptible. It is expected that PT is more likely than CTX to induce *C. difficile* colonization or CDD, given its broad-spectrum activity, particularly against anaerobes. This observation may cast doubt on the theory that the anaerobic gut flora are a critical determinant of 'colonization resistance'.[4] Alternatively, the relative *C. difficile* sparing effect of PT may reflect the limited penetration of this antibiotic into the gut lumen in some patients. Of 20 patients given PT, when sampled on one occasion during treatment, six had detectable faecal concentrations of piperacillin, of whom four had measurable levels of tazobactam, using a relatively insensitive antibiotic detection method.[17] Conversely, as *C. difficile* is more susceptible to PT than CTX, it may be more likely to be killed in PT-treated patients. Preliminary *in vitro* data using *C. difficile* inoculated faeces either spiked with PT or CTX, or taken from antibiotic-treated patients, do not demonstrate a difference in the growth of *C. difficile* (unpublished data). Further work is under way to determine the effects of antibiotic exposure on spore formation and toxin production by *C. difficile*.

The decision to end the study was taken because the wards were due to be relocated, and because data analysis at that time showed a marked difference in *C. difficile* colonization and, more importantly, infection rates. It was therefore considered to be unethical to continue using cefotaxime in elderly patients. We were anxious to exclude a confounding effect due to differences in environmental exposure to *C. difficile* spores, and therefore surveillance sampling was performed throughout the study. This factor has not previously been addressed in studies investigating antibiotic causality of *C. difficile* diarrhoea.[9–11] The fact that this study did not demonstrate a shorter hospital stay amongst patients receiving PT, secondary to fewer cases of CDD, may be due to differences in severity of illness in each group. Controlling for length of hospital stay before study entry is vital when comparing subsequent duration of admission in different antibiotic groups. In studies where this has been done, CDD patients stay significantly longer in hospital than controls.[18] Response to treatment with CTX or PT was similar, as might be expected in such patients, considering the spectra of the two antibiotics. Although the study was neither blinded nor randomized, we have confidence in the accuracy of our findings, given the absence of identifiable confounding factors and the objective measurements of *C. difficile* colonization and infection used. Furthermore, randomization was found to be impractical because of the confused elderly patient cohort under investigation.

Starr and colleagues recently speculated that the selective pressure resulting from cephalosporin prescribing may increase the proportion of *C. difficile* susceptible patients in a ward or unit.[19] In this setting administration of narrow spectrum antibiotics with otherwise relatively low propensities to select for *C. difficile* may subsequently induce symptomatic infection. Alternatives to cephalosporins, for example the combinations of penicillin and either trimethoprim[20] or ciprofloxacin[21]

for the empirical antibiotic treatment of infection have been shown in uncontrolled studies to be associated with a reduced incidence of *C. difficile* infection. Guidelines for the treatment of community-acquired pneumonia, a common cause of hospital admission in the elderly, cite cephalosporins, including cefotaxime, as the antibiotics of choice for severe, as opposed to mild to moderate, infections.[22] These guidelines were recently implicated in the increased incidence of *C. difficile* infection in a department of medicine for the elderly.[11]

We believe that if treatment of elderly patients with broad-spectrum antibiotics is required, PT is a better choice than CTX, due to lower morbidity secondary to CDD. These findings are most pertinent to units with endemic *C. difficile* infection, but although savings are likely to be greater on units with higher rates of CDD, the chance of developing CDD after CTX will probably be higher than after PT on any unit (ward B had a low rate of *C. difficile* colonization and disease before crossover to CTX, after which the rates quadrupled). CDD has been shown to be expensive to manage,[18, 23] with additional costs of up to £4000 per case.[18] The extra savings which could be expected if the 34 patients who received CTX had been given PT would be approximately £60 000, corresponding to 15.5 fewer cases of CDD. The supplementary antibiotic costs would be approximately £6000 (£40/day, PT vs. £15/day, CTX, per patient, British National Formulary prices), or 10% of the amount saved by the change. It remains unclear why reports of *C. difficile* continue to increase so markedly. Until such answers are forthcoming, judicious antibiotic prescribing and standard infection control containment measures are the best approaches.

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Comparison of the Oxoid *Clostridium difficile* toxin A detection kit with cytotoxin detection by a cytopathic effect method examined at 4, 6, 24 and 48 h

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Objective: To evaluate the Oxoid Toxin A test in comparison with a rapid cytotoxin method for the diagnosis of *Clostridium difficile* diarrhea in a UK tertiary referral hospital.

Methods: One hundred previously tested samples were examined using a cytopathic effect (CPE) method and the Oxoid Toxin A test. Culture and toxin B titer measurement of the samples were performed to evaluate discrepancies between the tests.

Results: The sensitivity and specificity of the Oxoid Toxin A test were 72% and 94%, respectively. This was similar to the CPE method read at 6 h: 67% and 94% in comparison. At 48 h, the sensitivity and specificity of the CPE method reached 98% and 100%. Toxigenic strains of *C. difficile* were cultured from 58 of 100 samples, and toxin was detected in 48 of 58. Following 4 weeks of storage at −20°C, seven of 47 previously toxin B-positive stool filtrates had no detectable toxin.

Conclusions: The Oxoid Toxin A test does not demonstrate a high enough sensitivity and specificity to be used as a primary test for *C. difficile* in hospitals where CPE testing is possible. Toxigenic strains of *C. difficile* can be cultured from a significant number of samples where no toxins are detected. Toxin B titers in fecal samples and especially in stool filtrates, stored at −20°C, diminish after thawing.

Key words: *Clostridium difficile*, toxin testing, diagnosis

INTRODUCTION

The growing recognition of *Clostridium difficile* as a nosocomial pathogen [1–4] has led to a proliferation of commercial kits for the detection of the bacterium or its toxins. Antigen-detection kits for *C. difficile* toxin A or toxins A and B obviate the need for cell-culture facilities. The kits also aim to provide a more rapid method for diagnosing *C. difficile* infection than the standard cytopathic effect (CPE) tests using cell monolayers, which conventionally require at least overnight incubation. In theory, obtaining a same-day test result may help in preventing the unnecessary use of specific antibiotic treatment for patients with diarrhea, and may reduce the spread of *C. difficile* by ensuring that infected patients are isolated. We aimed to determine the accuracy of one of these new rapid methods compared with a CPE method that was read at 4, 6, 24 and 48 h. We then sought to determine the reasons for any observed differences in results, using both *C. difficile* culture and measurement of toxin B titers in stool supernatants.

MATERIALS AND METHODS

We tested 100 stool samples that had been stored at −20°C since original CPE testing, immediately after thawing. All samples were from patients with diarrheal illness where no other infective cause was identified, and 50 were previously cytotoxin positive while 50 were previously negative. *C. difficile* toxin detection was performed using both a CPE method and the Oxoid Toxin A test (Unipath, Basingstoke, UK). The principle of the test is that monoclonal toxin A antibody labeled with blue latex particles binds to any toxin A in the specimen when it is added to the sample well. The complex diffuses along the test strip and is bound to an immobilized line of toxin A monoclonal antibody, forming a blue line in a positive result. Immobilization

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of unbound latex particles occurs in a second window to indicate that diffusion past the test window has occurred. The 100 samples were randomly arranged so that positives were indistinguishable from negatives. They were then thawed in batches of 10, and roughly 0.5 g was suspended in the kit diluent or in 2 mL of phosphate-buffered saline (for the CPE test) (to a dilution of 1 in 5). The well-mixed suspensions were then centrifuged for 10 min at 1200 *g*, before being used for the kit test (according to the instructions), or kept in a refrigerator at 4°C (for <30 min) until the CPE test was set up. If the supernatants were cloudy following centrifugation, they were filtered through a 0.45 millipore filter (Nalge, Rochester, NY, USA).

C. difficile cytotoxin was detected by a modified 96-well microtiter tray (Life Technologies, Glasgow, UK) method [5], using HEp-2 cells with *C. sordellii* antitoxin (Pro-Lab Diagnostics, Bromborough, UK) protected controls, involving a further 1 in 10 dilution (20 μL of supernatant to 180 μL of culture medium). Each batch of tests was controlled using a known positive supernatant (with and without *C. sordellii* antitoxin), a well without any additions and a well with antitoxin only. The tests were incubated aerobically, in a wet box, at 37°C, conditions under which no problems with the cell line have been encountered. Wells were examined for CPE at 4, 6, 24 and 48 h independently by two individuals. CPE consisted of a discernible, neutralizable rounding-up of cells, often quite subtle at 4 h and sometimes 6 h, but always affecting >10% of cells initially and >50% of cells by 48 h.

The stool samples were prepared for the Oxoid Toxin A test according to the manufacturer's instructions. One hundred and twenty-five microliters of supernatant was added to the sample window of the test strip. This was left for 30 min before being examined for any evidence of a blue line in the result window. Tests where the control window had a blue line and there was any sign of a blue line in the result window were classified as positive.

All 100 specimens were cultured on cycloserine–cefoxitin egg yolk (CCEY) agar (Lab M, Bury, UK) [6] for 48 h in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2, before being examined for *C. difficile*. Isolates were recognized by typical colony appearance and characteristic odor. The toxigenicity of isolates cultured from fecal samples that were toxin negative by CPE was determined by CPE testing. Toxin-negative strains were then identified using the RapID ANAII kit (Innovative Diagnostics Systems, Norcross, Ga, USA). The supernatants used for CPE testing in this study were frozen at −20°C immediately after the cytotoxin test was set up. Cytotoxin titers were determined in these samples after 4 weeks, due to time constraints at the time of initial cytotoxin testing. They were tested for CPE neat (1 in 50 dilution) and at seven further 10-fold dilutions. The toxin titer was designated as the highest dilution which caused a readily discernible, classical CPE at 48 h (although in a few cases, at the highest dilution, these changes did not quite affect 50% of the total cells).

RESULTS

We found 53 samples to be CPE negative and 47 to be CPE positive (Table 1). Sensitivities and specificities are calculated using the CPE result at 48 h as the standard (with the addition of a sample which was Oxoid toxin A test positive, CPE negative, but culture positive for toxigenic *C. difficile* as a true positive). By comparison, the Oxoid Toxin A kit produced 13 false-negative results (sensitivity 72%) and three false positives (specificity 94%), although it did detect toxin A in one specimen which was CPE test negative. A strain of *C. difficile* was cultured from this specimen and found to be cytotoxin positive (Table 1 and 2).

There was a high degree of correlation (100% agreement at 48 h) between the two individuals who read the CPE test. At 6 h, one investigator (the more experienced of the two) identified three additional positive specimens, one of which proved to be a false positive.

Table 1 Sensitivity and specificity of CPE test for toxin B at various time intervals and for Oxoid toxin A test

	False test negatives	False test positives	Sensitivity	Specificity
CPE at incubation times (h)				
4	21	5	54	90
6	15	3	67	94
24	3	0	92	100
48	1	0	98	100
Oxoid toxin A test	13	3	72	94

Table 2 Concordance table for Oxoid Toxin A test and Toxin B detection by cytopathic effect

Kit	CPE +ve		CPE −ve
Positive	34		4
Negative	13		49
Sensitivity		72%	
Specificity		94%	
Correlation		83%	

In this study, all 47 CPE-positive samples were *C. difficile* culture positive, while 12 of the CPE-negative feces were also culture positive. Of these 12 strains, all but one was toxigenic by CPE testing. After storage of fecal supernatants at −20°C for 4 weeks, *C. difficile* cytotoxin titers were found to range between 50 and at least 5×10^8. In seven supernatants that were previously CPE positive, no cytotoxin was detected at this time. There were 11 samples with cytotoxin titers $>10^3$, and all were toxin A kit-test positive. One toxin A kit positive sample had a titer of only 1. No toxin A kit-negative samples had titers $>10^3$.

DISCUSSION

The study demonstrates that the rapid kit test studied had a similar accuracy to our CPE method at 6 h, with a sensitivity and specificity of 72% and 94% versus 67% and 94%, respectively. These results differ from another assessment of the Oxoid toxin A kit by Bentley et al in 1998 [7]. By 24 h, the sensitivity and specificity of the CPE method reach 92% and 100% respectively. The interesting observation that one sample was toxin A kit test positive but CPE test negative could be due to unequal rates of breakdown of the two toxins. The great majority of *C. difficile* strains produce equimolar quantities of toxins A and B [8]. The *C. difficile* culture results indicate that it is possible for a toxigenic strain of *C. difficile* to be present in a sample and yet for toxins A and B to remain undetectable. This could be due to proteolytic degradation of toxins in the fecal sample, or lack of production of toxins in the bowel, possibly secondary to antimicrobial therapy, or toxin inhibitory effects of bowel flora. Theoretically, this effect could also be seen if the sample contained only *C. difficile* spores in small numbers.

Some toxin A-positive samples had low cytotoxin titers, which may be because the greater stability of toxin A [9,10] resulted in more of it remaining in the sample than cytotoxin. There was an apparent correlation between cytotoxin titer and toxin A kit test positivity, as previously described for an ELISA method [11]. Other workers have reported no correlation between cytotoxin titer in samples and EIA readings [12]. The reliable threshold for toxin A detection in the present study appeared to be a titer of $\geq10^4$. Cytotoxin in stool specimens and particularly in stool filtrates may deteriorate when these are thawed, after being stored at −20°C, as previously noted [13]. The fact that some samples had no detectable toxin but did contain toxigenic organisms suggests that culture of toxigenic organisms alone from a sample may not automatically justify the treatment of a patient for *C. difficile* infection.

In this study, the sensitivity and specificity claimed in the kit insert by the manufacturer were not achieved (72% versus 90% and 94% versus 98%, respectively). However, the manufacturer's sensitivity figures were erroneously adjusted to classify cytotoxin-positive but culture-negative fecal samples as true kit negatives. The fact that detection of toxin A is not as sensitive as CPE methods that detect toxin B (and toxin A) is not surprising. Toxin B is extremely potent and can cause a cytopathic effect at concentrations as low as a few pg/mL, whereas the threshold of toxin A detection kits is in the ng/mL range [7].

It should also be emphasized that there are increasing reports of toxin A-negative, toxin B-positive (A−B+) *C. difficile* clinical isolates [10,14], and these will not be detected by toxin A kits [15–17]. Brazier reported that *C. difficile* A-B+ isolates account for 3% of the total number of strains received from laboratories (but 10% of the strains submitted from one laboratory) in England and Wales by the Anaerobe Reference Laboratory [16]. While such strains appear to be uncommon in the USA [15], 33% of isolates collected in one hospital in Japan were of this phenotype [18]. As *C. difficile* A-B+ isolates have been recovered from symptomatic patients [16,17], previously held beliefs that toxin A is the most important toxin in the pathogenesis of human antibiotic-associated diarrhea must be called into question.

The CPE test is achievable by most large clinical microbiology laboratories when several samples per day require examination for *C. difficile* cytotoxin. Although the CPE method at 6 h performs slightly less well than the Oxoid kit, it is noticeably superior by 24 h. This leaves the decision as to whether an immediate result is preferable to a more accurate one after 24 h. While in severe cases an immediate result may be argued to be of value, in practice most of these patients will be isolated and treated empirically. In addition, with the use of a test with lower sensitivity and specificity, some patients will be misidentified as being negative and others will receive unnecessary therapy. It should be remembered that a significant number of patients with *C. difficile* toxin-positive diarrhea will respond to withdrawal of antibiotics alone [19], and reacting to a very rapid test result may result in action before these patients declare themselves. The sensitivity of the CPE method may be improved if VERO cell lines are used. In our laboratory, testing around 5000 samples/year by CPE, the cost per test (including technician time) is around 25p. This compares to a list price of £4 per test for the Oxoid toxin A test, excluding technician time. Furthermore, an increased number of repeat tests would be required if the Oxoid toxin A test was used. The Oxoid toxin A kit does not appear to be accurate

enough for use in primary diagnosis of *C. difficile* disease. It is interesting to note that the sensitivity of CPE testing for cytotoxin can reach 67% at 6 h. The CPE method can also be employed to test the toxigenicity of *C. difficile* isolates directly from the culture plate. One or two colonies of *C. difficile* are emulsified in 0.5 mL of phosphate-buffered saline, which is then used in the same way as a stool filtrate in the CPE test, with results typically being readable after 3-4 h of incubation (Settle and Wilcox, unpublished data). Rapid kit tests are more suited to situations where tests are infrequent, or cell-culture facilities are not available (although a virology laboratory is not essential for cell culture to be performed), but greater sensitivity than that demonstrated by this kit is required.

In summary, the accuracy of the Oxoid toxin A test is not high enough for routine use in practice. Reduced sensitivity of the toxin A method may be due to specimens with low toxin titers being present. Toxin A levels may not decrease as quickly as toxin B levels, and specimens may be *C. difficile* culture positive but yet contain no detectable toxin A or B, even though the strain is toxigenic. Cytotoxin levels fall in samples, and particularly supernatants, that are frozen and thawed.

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