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Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period

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Clostridium difficile-associated disease continues to be a major problem in hospitals and long-term care facilities throughout the developed world. Administration of certain antibiotics such as amoxycillin, oral cephalosporins and clindamycin is associated with the greatest risk of developing *C. difficile* disease. The two antibiotics used for treatment of *C. difficile* disease are vancomycin and metronidazole, to which there is currently very little resistance. Randomly selected isolates (186) from 90 patients being investigated during an 18-month epidemiological study into the disease were tested for their susceptibility to vancomycin, metronidazole, amoxycillin, clindamycin, cefoxitin and ceftriaxone by the NCCLS agar dilution method. There was a narrow range of MIC for the two treatment agents (vancomycin and metronidazole), from 0·5 to 4 μg ml^{-1}, with no evidence of resistance. All strains were resistant to cefoxitin (MIC 64–256 μg ml^{-1}), the antibiotic used in most selective media. All strains were of similar sensitivity to amoxycillin (MIC_{90} = 4 μg ml^{-1}). Most strains were resistant to ceftriaxone (MIC ⩾ 64 μg ml^{-1}) or of intermediate resistance (MIC ⩾ 32 μg ml^{-1}), with only two sensitive strains (MIC 16 μg ml^{-1}). Clindamycin resistance was common, with 67 % of strains resistant (MIC ⩾ 8 μg ml^{-1}), 25 % with intermediate resistance (MIC ⩾ 4 μg ml^{-1}) and only 8 % sensitive (MIC ⩽ 2 μg ml^{-1}). Twelve isolates from six different patients had very high resistance to clindamycin (MIC ⩾ 128 μg ml^{-1}). Multiple isolates from the same patient, taken at different times, showed changes in susceptibility patterns over time. The only major change in susceptibility over the time-period was in clindamycin resistance; some strains appeared to become more resistant while others became less resistant. No differences were seen in the MIC_{50} and MIC_{90} of the different S-types of *C. difficile* identified, although some S-types were present in very small numbers. There was no correlation between the antibiotics prescribed and susceptibility.

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INTRODUCTION

Clostridium difficile is an important cause of nosocomial, antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis. Its clinical manifestations range from asymptomatic carriage to severe diarrhoea and pseudomembranous colitis with toxic megacolon. Although it was first described in 1935, as a commensal in the gut flora of infants, it was implicated in antibiotic-associated colitis in the 1970s (Tedesco *et al.*, 1974; Bartlett *et al.*, 1978). *C. difficile* is prevalent in hospitals and long-term care facilities and increases costs to health services for the care of infected patients as well as in isolation and infection-control procedures (Spencer, 1998).

Disease is generally thought to occur after depletion of the patient's normal protective bowel microbiota following use of broad-spectrum antibiotics (Borriello & Barclay, 1986; Larson & Welch, 1993). This state leaves the patient vulnerable to overgrowth by *C. difficile* that is already in the patient in small numbers (endogenous) or, more commonly, from another patient or the environment (exogenous). The third-generation cephalosporins, clindamycin and amoxycillin are associated with the greatest risk for developing AAD because of their widespread use in the hospital as well as the community, but almost all antibiotics can cause the disease (Mylonakis *et al.*, 2001).

The antibiotics used to treat *C. difficile* diarrhoea are vancomycin and metronidazole, with metronidazole being the drug of choice because it has fewer side-effects, is cheaper and is not associated with selection of vancomycin-resistant enterococci (Wilcox & Dave, 2001). Few reports have appeared of decreased susceptibility to these therapeutic

agents (Barbut *et al.*, 1999; Johnson *et al.*, 2000; Brazier *et al.*, 2001; Peláez *et al.*, 2002). The majority of strains with reported decreased susceptibility to metronidazole have been non-toxigenic and are therefore considered clinically insignificant (Barbut *et al.*, 1999; Johnson *et al.*, 2000; Brazier *et al.*, 2001).

The aims of this study were to obtain current information on the sensitivity (as MICs) of a sample of *C. difficile* isolates to a variety of precipitating and treatment antibiotics during the 18-month period of a major epidemiological study. It also investigated the patterns of susceptibility in relationship to phenotype (S-type) and antibiotics prescribed. This work was not meant to be a comprehensive study of susceptibility. When more than one isolate was taken from a patient, they were used to determine changes in sensitivity patterns over time. Another paper (to be published elsewhere) will contain the demographical information together with computer modelling.

METHODS

***C. difficile* isolates and characterization of S-types.** Isolates were obtained through an 18-month epidemiological study (July 1999–December 2000) during which over 1000 faecal samples were taken from 390 patients, of whom 100 were culture-positive. The study was unable to determine whether *C. difficile* was acquired from the hospital or the community environment, as it was difficult to guarantee that a stool sample was taken immediately on admission to the ward. Stool samples were taken at least once during a patient's stay and weekly if possible. Patient data and stool samples were collected by a research nurse in wards 5 and 6 (care-for-the-elderly wards) of the Royal Victoria Hospital in Edinburgh. Full demographical details including patient age, antibiotic usage and health are to be published elsewhere. However, briefly, 1003 specimens were taken from 390 patients: mean age 82·5, SD 7·3, 65 % female, with 44 % being transferred from another hospital ward.

The stool samples were processed on cefoxitin/cycloserine/egg-yolk (CCEY) selective agar (Brazier, 1993). The isolates were identified by characteristic colony morphology, smell and appearance on a Gram film. From over 500 isolates (with up to six from a sample), a subset of 186 from 90 patients was randomly selected for study of antibiotic-sensitivity patterns. These included multiple isolates from 38 patients. Subcultures were stored in cooked meat+anaerobic investigation medium (AIM; Brown *et al.*, 1996) for maintenance.

One isolate from each sample was S-typed using guanidine hydrochloride to extract their S-layer proteins followed by analysis on SDS-PAGE (McCoubrey & Poxton, 2001). Where multiple isolates were available, differences were observed in the S-types present. Isolates were also tested for toxin production using Techlab Tox A+B ELISA kits. All the data collected were stored in a Microsoft Access database. Only one isolate from each sample was used for MIC determinations.

Antibiotics and MIC determinations. Details of all antibiotics used in the treatment of these patients were available in the database. The antibiotics selected for this study (all from Sigma) were not meant to be extensive, but representative: the two agents used for treatment of *C. difficile*-associated disease, vancomycin (concentrations used 8–0·125 μg ml^{-1}) and metronidazole (8–0·125 μg ml^{-1}), and four of the agents with known association with *C. difficile* disease, amoxycillin (64–1 μg ml^{-1}), clindamycin (128–2 μg ml^{-1}), ceftriaxone (256–8 μg ml^{-1}) and cefoxitin (256–8 μg ml^{-1}); the latter is also used in the CCEY selective medium, at 8 μg ml^{-1}. The non-treatment agents were chosen because they are the most common precipitating agents of *C. difficile* diarrhoea –they have poor *in vitro* activity against *C. difficile*.

MICs were determined using the agar dilution protocol in the NCCLS guidelines (NCCLS, 1997). The isolates were subcultured from spores in cooked-meat broth into pre-reduced (80 % N_2, 10 % H_2, 10 % CO_2 at 37 °C) thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K_1 and 1 mg $NaHCO_3$ ml^{-1} and incubated overnight anaerobically at 37 °C. This yielded approximately 1×10^8 bacteria ml^{-1}. Purity of the cultures was checked by Gram stain and was checked retrospectively by anaerobic and aerobic incubation for 48 h on Columbia blood agar (Oxoid; supplemented with 5 % horse blood). Aliquots (1–2 μl) of the cultures were spotted onto Brucella agar (Oxoid) supplemented with 5 % defibrinated sheep blood, 5 μg haemin ml^{-1} and 1 μg vitamin K_1 ml^{-1} using a multi-point inoculator.

MIC_{50} and MIC_{90} were calculated by pasting all the MICs for each strain into an Excel spreadsheet and sorting into ascending order. The MIC_{50} was taken as the MIC that was the median value. Of 186 isolates, this was cell 93. Similarly, the MIC_{90} was the value found in row 168 (90 % of 186) and represented the concentration of antibiotic that would inhibit 90 % of the isolates tested.

RESULTS

MICs

In total, 186 representative isolates were investigated. Table 1 shows the ranges of MICs among the isolates for the six antibiotics used, together with MIC_{50} and MIC_{90} values and, where known, the break-points for the antibiotics. The two antibiotics used for treatment (vancomycin and metronidazole) both showed a narrow range, between 0·5 and 4 μg ml^{-1}. Cefoxitin, the antibiotic used in the selective medium (at 8 μg ml^{-1}), showed a range of MICs from 64 to 256 μg ml^{-1}. The other three precipitating antibiotics all showed a wider range of MICs.

The MIC_{50} and MIC_{90} for the six antibiotics used were either the same or twofold different. This highlights the closeness in sensitivity of the majority of isolates. The MIC_{50} and MIC_{90} for vancomycin and metronidazole were low (2 μg ml^{-1}), and only five strains (2·7 %) had an MIC of 4 μg ml^{-1} to vancomycin and two (1·1 %) had an MIC of 4 μg ml^{-1} to metronidazole. None of the isolates tested was resistant to the two treatment agents for *C. difficile* diarrhoea. Both the MIC_{50} and MIC_{90} values for amoxycillin were 4 μg ml^{-1}. This shows that, even though the range of MICs to this antibiotic was relatively broad ($\leqslant$ 1–16 μg ml^{-1}), the majority of the isolates had very similar sensitivity. Clindamycin produced a range of sensitivities within the tested isolates ($\leqslant$ 2 to $>$ 128 μg ml^{-1}). For this antibiotic, the MIC_{50} and MIC_{90} values were respectively 8 and 16 μg ml^{-1}. The NCCLS break-point for clindamycin resistance is $\geqslant$ 8 μg ml^{-1}; therefore, 66·7 % ($n = 124$) of the isolates were resistant to clindamycin, 24·7 % ($n = 46$) had intermediate resistance (MIC 4 μg ml^{-1}) and the rest were sensitive. Twelve *C. difficile* isolates with MICs to clindamycin of $\geqslant$ 128 μg ml^{-1} from six patients were found. The MIC_{50} and MIC_{90} of cefoxitin were the same, at 256 μg ml^{-1}. The NCCLS guidelines state that MICs of $\geqslant$ 64 μg ml^{-1} indicate

Table 1. Range of MIC values from 186 isolates

Antibiotic	MIC range ($\mu g\ ml^{-1}$)	MIC_{50}	MIC_{90}	Break-point
Vancomycin	0·5–4	1	2	8
Metronidazole	0·5–4	1	2	8
Amoxycillin	⩽ 1–16	4	4	?
Clindamycin	⩽ 2–> 128	8	16	8
Cefoxitin	64–256	256	256	64
Ceftriaxone	16–256	64	64	64

resistance to cefoxitin (NCCLS, 1997); therefore, none of the 186 isolates tested was sensitive. According to the NCCLS guidelines, MICs of ⩾ 64 $\mu g\ ml^{-1}$ indicate resistance to ceftriaxone. Isolates had MIC_{50} and MIC_{90} values of 64 $\mu g\ ml^{-1}$ to ceftriaxone. Thirty-three strains (17·7 %) had intermediate resistance to ceftriaxone, with MICs of 32 $\mu g\ ml^{-1}$ (NCCLS, 1997). Only two strains (1·1 %) were sensitive, with MICs of 16 $\mu g\ ml^{-1}$.

Relationship of S-layer types to MICs

Of the 186 strains included in the collection for MIC determinations, 145 were phenotyped by analysis of their S-layer proteins on SDS-PAGE. Most strains (76·5 %; $n = 111$) belonged to the common S-type 5236, with most of the others being S-type 5242 (14·5 %; $n = 21$). Of the remainder, 3·4 % ($n = 5$) were S-type 5140 and 2·8 % ($n = 4$) were S-type 5438, with single isolates of S-types 5941 and 5144. Two strains collected were non-typable: they did not show the typical two major S-layer bands on SDS-PAGE.

There was a degree of variation in sensitivity to antibiotics depending on the S-type of the isolate (Table 2). The common S-type 5236 had a large range of MICs, and there was no difference in overall pattern between this and the total population. However, the less common S-types did show some variations, in particular in respect to clindamycin sensitivity. Both of the non-typable strains were extremely sensitive to clindamycin, with MICs of ⩽ 2 $\mu g\ ml^{-1}$, and had below-average MICs to cefoxitin and ceftriaxone, respectively 64 and 32 $\mu g\ ml^{-1}$.

Repeat samples and changes in antibiotic-sensitivity patterns over time

Thirty-eight patients were sampled more than once, with some being sampled up to 10 times. Of those S-typed, at least 50 % (19/38) retained the same S-type throughout the study, while 13 % (5/38) definitely harboured different S-types over time, with one patient having three different types at different times. Isolates from 19 patients exhibited changing patterns of sensitivity to one or more of the six antibiotics. While some of these changes related to changes of S-type, others did not. Typical changes in isolates that were all of the same S-type were no more than two- to fourfold differences in MIC. However, some major changes occurred in sensitivity to clindamycin. One noteworthy example of this was an isolate with an MIC to clindamycin of 8 $\mu g\ ml^{-1}$. Two subsequent samples taken from the same patient 13 and 15 days later each produced a highly clindamycin-resistant strain, with an MIC of > 128 $\mu g\ ml^{-1}$. The isolates from these samples were all S-type 5236. Another example of changing clindamycin sensitivity was in a patient who also harboured isolates of S-type 5236. The first sample produced an isolate with an MIC of > 128 $\mu g\ ml^{-1}$. A month later, another sample contained a

Table 2. Variation in MICs among different S-types

Abbreviations: Van, vancomycin; Met, metronidazole; Amox, amoxycillin; Clin, clindamycin; Cefo, cefoxitin; Ceft, ceftriaxone. NT, Not typable.

S-type	Isolates [% (*n*)]	MIC [range (MIC_{90})] ($\mu g\ ml^{-1}$)					
		Van	Met	Amox	Clin	Cefo	Ceft
All	100 (145)	0·5–4 (2)	0·25–4 (2)	⩽ 1–16 (4)	⩽ 2–> 128 (16)	64–256 (256)	16–256 (64)
5236	76·5 (111)	1–4 (2)	0·5–2 (2)	⩽ 1–16 (4)	⩽ 2–> 128 (16)	64–256 (256)	16–64 (64)
5242	14·5 (21)	1–4 (2)	0·5–4 (2)	⩽ 1–8 (8)	⩽2–16 (8)	64–128 (128)	32–64 (64)
5140	3·4 (5)	1–2 (2)	0·25–1 (1)	1–4 (4)	4–16 (16)	64–128 (128)	32–64 (64)
5438	2·8 (4)	1–2 (2)	0·5–1 (1)	2–4 (4)	4–16 (16)	64–256 (256)	32–64 (64)
5144	0·7 (1)	2	1	2	> 128	64	16
5941	0·7 (1)	1	1	2	8	128	32
NT	1·4 (2)	2	1	2–4 (4)	⩽ 2	64	32

strain with an MIC of 16 μg ml^{-1} followed, 3 days later, by one with an MIC to clindamycin of 8 μg ml^{-1}. Neither of these patients was on clindamycin or any other macrolide. No significant changes in the MIC of the patients' isolates were found to the other five antibiotics.

No clear patterns emerged from the data to suggest any link between prescribed antibiotics and specific sensitivities. For example, patients on amoxycillin showed no propensity to produce isolates more resistant to that agent.

Overall, 97 % of the total isolates produced toxin (by the Techlab A+B kit). However, there was no correlation between toxin production and antibiotic susceptibility or, indeed, S-type.

DISCUSSION

This 18-month study has investigated the susceptibility of *C. difficile* isolates to a range of antibiotics associated with development of *C. difficile*-associated diarrhoea, together with the two antibiotics used in therapy of the disease. There was no evidence of any resistance to vancomycin or metronidazole, the treatment agents. However, such strains, especially human isolates, are still extremely rare (Brazier *et al.*, 2001). Five strains (2·7 %) had slightly reduced susceptibility to vancomycin, with MICs of 4 μg ml^{-1}. This low level of reduced susceptibility has also been reported by others (Peláez *et al.*, 2002), also in small numbers. There was general resistance to the cephalosporin and cephamycin antibiotics, but not to the other beta-lactam amoxycillin. Resistance to clindamycin was common, despite its infrequent use. In summary, this shows that increased colonization with *C. difficile* and subsequent disease may well be due to acquisition of resistant strains of the bacterium, but, as has been shown frequently in the past, other mechanisms must also be operating, as demonstrated by the apparent sensitivity to amoxycillin. Amoxycillin is used widely, both in the hospital environment and in the community, and is one of the most common precipitating antibiotics (Freeman & Wilcox, 1999).

Isolates showed a wide range of sensitivities to clindamycin, with MICs varying from $\leqslant 2$ to > 128 μg ml^{-1}. Strains resistant to clindamycin have been widely reported, and some have been involved in epidemics (Johnson *et al.*, 1999). Clindamycin usage has decreased dramatically because of its involvement in the precipitation of *C. difficile* diarrhoea. There is now little selective pressure for clindamycin resistance. The usual mechanisms by which clindamycin resistance is conferred also mediate resistance to other macrolide, lincosamide and streptogramin B antibiotics: this is known as the MLS resistance determinant (Mullany *et al.*, 1996; Farrow *et al.*, 2001). The MLS determinant is the major mechanism of multiple resistance among Gram-positive anaerobes (Noren *et al.*, 2002). The gene responsible (*ermB* in *C. difficile* 630) encodes a 23S rRNA methylase that modifies the target site for the antibiotic. The sequence is 99 % similar to that of the *ermB* gene from *Clostridium perfringens* but, unlike this gene, it is not located on a plasmid (pIP402) but on a mobilizable, non-conjugative transposon, Tn*5398* (Farrow *et al.*, 2001). Whether the *C. difficile* isolates tested in this study have this gene has not yet been confirmed through PCR, though no other mechanism for such high-level resistance has been described in this species. *C. difficile* appears to be inherently resistant to the cephalosporin and cephamycin antibiotics, as the majority of isolates had MICs to these agents of $\geqslant 32$ μg ml^{-1} (NCCLS, 1997).

C. difficile possesses an outer cell coat, termed the S-layer, consisting of two polypeptides that form a regular crystalline array over the surface of the cell (Kawata *et al.*, 1984). The most common S-type in this study was 5236 (the number corresponds to the molecular masses in kDa of the two major polypeptides found on the cell surface). Of all strains tested so far, the molecular mass of the larger of the two proteins varies from 45 to 64 kDa, with the smaller ranging from 25 to 40 kDa (Poxton *et al.*, 1999). S-layer typing is a quick and easy method of phenotyping and appears to correspond well with other typing techniques, including ribotyping and serotyping (McCoubrey & Poxton, 2001). Toxigenic S-type 5236 is the same as ribotype 001 (McCoubrey, 2002), which is the most common ribotype in the UK (Stubbs *et al.*, 1999). The S-layer is a putative virulence factor that may have a role in adhesion of the bacterium to the host mucosal surface. It may also have a role in immune evasion or impermeability to certain compounds, including antibiotics. The two non-typable strains appeared to be more sensitive to clindamycin, cefoxitin and ceftriaxone. Though no firm conclusions can be made, especially when this pattern was rare, it may be speculated that, as they appear to lack a typical S-layer, they are more sensitive to some antibiotics. However, overall, there were no obvious correlations between S-type and resistance to antibiotics.

Multiple isolates were obtained from 37 patients and, for some patients, as many as 10 were available. These isolates permitted assessment of sensitivity patterns over time and within and between S-types. In the majority of cases, isolates did not change either in S-type or in sensitivity pattern. The isolates from some patients did change in antibiotic sensitivity and/or in S-type, suggesting that there had been reinfection with a different strain or, possibly, the emergence of a minor strain from an initially mixed infection. In patients whose isolate did not change in S-type, resistance to clindamycin was the only significant difference observed. Resistance to clindamycin typically resides on a transposon, Tn*5398* (Mullany *et al.*, 1996; Farrow *et al.*, 2001), which could transfer between strains. It is feasible that the strain acquired this resistance determinant or that the patient was reinfected with a clindamycin-resistant strain of the same, predominant S-type. In the patient whose strain appeared to lose clindamycin resistance, it is possible that the resistance determinant was lost. More likely is the explanation that the patient had picked up another S-type 5236 strain that lacked the clindamycin-resistance determinant. In the patients who produced same-type isolates with changing resistance, it would be interesting to use another typing method (sero- or

ribotyping) in order to try to identify subtypes, which may explain the sensitivity changes. There was no direct evidence that resistance to clindamycin was selected in strains, despite the use of macrolides in many patients during the study.

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