Changes in antibiotic susceptibility and ribotypes in *Clostridium difficile* isolates from southern Scotland, 1979–2004

Surabhi K. Taori,¹ Val Hall² and Ian R. Poxton¹

Correspondence

Ian R. Poxton

i.r.poxton@ed.ac.uk

¹Medical Microbiology, Centre for Infectious Diseases, University of Edinburgh College of Medicine and Veterinary Medicine, Edinburgh, UK

²Anaerobe Reference Laboratory, NPHS, Microbiology Cardiff, University Hospital of Wales, Cardiff, UK

An increase in the incidence of clinical cases of *Clostridium difficile* infection has been reported in recent years, but few studies have examined changes in molecular epidemiology and antibiotic resistance over a long period of time. A collection of 179 isolates of *C. difficile* obtained from symptomatic adult patients in southern Scotland between 1979 and 2004 was used to determine changes in the prevalence of epidemiological types and antibiotic susceptibilities to common antibiotics. PCR ribotyping and MIC determination were performed on all isolates. A total of 56 different ribotypes were identified, among which ribotype 002 was the commonest type overall (14.0%), followed by ribotypes 014 (7.3%), 012 (5.0%), 015 (5.0%), 020 (5.0%) and 001 (4.5%). Ribotype 078 was also identified. The 10 commonest ribotypes comprised 55% of the total isolates. Ribotype 001 increased in prevalence from 1.5 to 12.2% over the study years, whereas the prevalence of ribotype 012 decreased from 8.7 to 2.0%. Resistance to clindamycin, erythromycin and ceftriaxone was found in 95.5, 14.0 and 13.4% of isolates, respectively. Resistance to vancomycin or metronidazole was not detected. Thirty-two (17.9%) and 14 (7.8%) isolates were resistant to two and three or more antibiotics, respectively. Ribotype 001 displayed maximum resistance, with 50% of isolates resistant to erythromycin, moxifloxacin and ceftriaxone, and 100% resistant to clindamycin. Over the 26 years of the study, antibiotic resistance and ribotype prevalence have changed, and antibiotic pressures may have been the major driver of this change.

Received 28 July 2009

Accepted 22 November 2009

INTRODUCTION

Clostridium difficile has been recognized as a cause of antibiotic-associated diarrhoea since 1978, but has become an infection control emergency more recently as the incidence and severity of cases have increased (Barbut *et al.*, 2007; Pépin *et al.*, 2004). There are also reports of increased mortality due to some epidemiological strains such as ribotype 027 (Cookson, 2007).

The incidence of *C. difficile* infection in England and Wales increased sixfold in the period from 1990 to 1993 (Settle & Wilcox, 1996). In Scotland, the total number of reported cases increased from 214 to 2130 during the period from 1988 to 2000 (Lee *et al.*, 2001).

The major hypothesis towards the cause of the rising incidence of *C. difficile* infection is the increasing use of broad-spectrum antibiotics (Dallal *et al.*, 2002; Kyne *et al.*, 2002). It is postulated that antibiotics disrupt the normal flora of the colon and make the intestinal environment susceptible to *C. difficile* colonization. The evidence implicating antibiotics in the pathogenesis of *C. difficile* infection is

overwhelming, although some antibiotics such as penicillins, cephalosporins and clindamycin are more strongly associated than others (Schroeder, 2005; Wiström *et al.*, 2001).

Few studies (Ackermann *et al.*, 2003b; Climo *et al.*, 1998; McDonald *et al.*, 2005; Schmidt *et al.*, 2007) have looked into the long-term changes in epidemiology and susceptibility patterns of *C. difficile* in a defined geographical region, largely due to a paucity of isolates available for retrospective study. This is because direct toxin detection from stool samples, without microbiological culture, is sufficient for clinical diagnosis. The aim of this study was to utilize our extensive collection of *C. difficile* isolates to observe how the prevalence of epidemiological types has changed since 1979 and how their resistance to antibiotics may have influenced the changes.

METHODS

Isolates. Almost 700 *C. difficile* isolates were collected and stored lyophilized by the Microbial Pathogenicity Research Laboratory,

University of Edinburgh, UK, between 1979 and 2004. Most of these strains were referred from laboratories throughout Scotland as well as other parts of the UK, as our laboratory was recognized as the Scottish Anaerobe Reference Laboratory for much of this period. The isolates submitted included those from sporadic cases and from small and large outbreaks, and therefore represented an unbiased strain collection. A set of 179 of these isolates was selected for this study, and the criteria for choosing these were that they were derived from stool samples of clinically ill adult patients admitted to hospitals around southern and central Scotland, mainly in the Lothian, Strathclyde and Fife regions. As information available for these strains included date and place of isolation and a brief clinical history of the patient from whom they were isolated, any duplicate isolates from the same patient or isolates that appeared to be part of an outbreak from which an isolate had already been selected were excluded.

Toxigenesis. Prior to this study, isolates had been tested for toxin production by a cytotoxin assay or toxin A + B ELISA (Tech Lab) on pure cultures before being stored in a lyophilized form. For this study, toxigenesis was confirmed using the toxin A + B ELISA.

Ribotyping. The PCR ribotyping technique described by O'Neill *et al.* (1996) was used in this investigation with the modifications suggested by the NPHS Anaerobe Reference Laboratory, Cardiff, Wales, UK (Hastings, 2006). Briefly, the changes included a reduction in the reaction volume from 100 to 50 µl. The new amplification protocol involved initial denaturation at 95 °C for 2 min, followed by 30 cycles of amplification with denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. An additional amplification cycle consisted of 95 °C for 1 min, 55 °C for 45 s and 72 °C for 5 min. PCR products were concentrated at 75 °C for 45 min. Gels were run at 60 V and 200 A for 3 h. Ribotypes of the isolates were identified using GelCompar II software (Applied Maths).

Antibiotic susceptibility testing. Antibiotic susceptibility tests were performed by the MIC method following the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) reference agar dilution method (NCCLS, 2004). *C. difficile* NCTC 11223, strain 338a (a locally endemic strain), strain 630 and a known ribotype 027 from Amsterdam (resistant control for moxifloxacin) were used as controls for every batch. The MICs of these strains were known from previous studies (Drummond *et al.*, 2003; Mutlu *et al.*, 2007).

MICs were determined against the following antibiotics: metronidazole (0.5–32 µg ml⁻¹), vancomycin (0.125–16 µg ml⁻¹), ceftriaxone (4–256 µg ml⁻¹), clindamycin (0.5–32 µg ml⁻¹), tetracycline (1–64 µg ml⁻¹), erythromycin (0.5–32 µg ml⁻¹) and moxifloxacin (0.25–32 µg ml⁻¹). As the manufacturers (Bayer) could not supply moxifloxacin powder, we used parenteral moxifloxacin. This method, although not recommended by the CLSI, has been compared in our laboratory against moxifloxacin E-test strips (AB Biodisk) and the results found to be comparable (E. Mutlu, unpublished data). The breakpoints used (see Table 2) were adapted from CLSI criteria for anaerobes and previously published data (Mutlu *et al.*, 2007; NCCLS, 2004).

Analysis. Fisher's exact test was used to determine statistical significance, using Microsoft Office Excel 2003 and Minitab 15.1.0.0 statistical software.

RESULTS AND DISCUSSION

Ribotype prevalence

A total of 179 selected isolates were studied, of which 147 were toxigenic and 32 non-toxigenic. Among these, 56

different PCR ribotypes were identified, of which 44 were toxigenic, 11 were non-toxigenic and one comprised both toxigenic and non-toxigenic ribotypes (Table 1). Ten of the commonest ribotypes comprised 55 % of the total. Both toxigenic and non-toxigenic variants were found for ribotype 026. To be able to observe changes in the distribution of ribotypes over time and subsequently antibiotic resistance patterns, the isolates were divided into groups covering three time periods: A, 1979–1986 (69 isolates); B, 1987–1995 (61 isolates); and C, 1996–2004 (49 isolates). These are summarized in Table 1.

The commonest isolate overall was ribotype 002 with a total of 25 isolates, and this remained the commonest isolate in all three time periods individually at around 14 %. A study from the same region in 2005 (Mutlu *et al.*, 2007) reported that the incidence of ribotype 002 had fallen to 2.0 %. Ribotype 014 was the next most common type overall, but it decreased in incidence from time period A (8.7 %) to C (6.1 %). Mutlu *et al.* (2007) reported a further decrease to 2.7 % in 2005.

In contrast, ribotype 001, which was the sixth most common isolate overall, increased in incidence from period A (1.5 %) to C (12.2 %). Mutlu *et al.* (2007) reported a 75.8 % incidence of ribotype 001 in 2005, suggesting that the epidemiology of this region had been changing in recent years and that ribotype 001 had emerged as the most common isolate. This may indicate a sudden clonal expansion of this ribotype in the patient population studied in 2005, although the authors suggested that this was not the case. An earlier study in the same area of Scotland by McCoubrey *et al.* (2003), where isolates were collected between 1999 and 2000 and which utilized S-layer typing, revealed that a single S-type (5236) was the endemic strain and accounted for 73 % of the isolates. S-type 5236 is the equivalent of ribotype 001 (McCoubrey, 2002). Recent data from Health Protection Scotland suggest that ribotype 001 decreased in prevalence to 18.5 and 24.5 % in 2006–2007 and 2007–2008, respectively. Ribotype 106, although not identified in our study, was found to comprise 8.1 % of the isolates studied by Mutlu *et al.* (2007) in 2005, and the overall incidence in Scotland (Health Protection Scotland, 2009) was reported as 64 % in 2006–2007 and 37.6 % in 2007–2008, making it the commonest ribotype in Scotland today (Health Protection Scotland, 2007, 2009).

In our study, there were similar numbers of ribotypes in all periods: 36 different types in the first period ($n=69$), 31 in the second ($n=61$) and 24 in the third ($n=49$), out of a total of 56 in all periods. Mutlu *et al.* (2007) reported only 15 different ribotypes among the 116 isolates they tested, but that study was from a more restricted geographical area. When the numbers of the most prevalent ribotypes (defined as a ribotype to which ≥ 4 % isolates in the respective study period belonged) were compared over the different periods, only seven and six ribotypes were found in the earlier two periods, respectively, compared with 12

Table 1. Distribution of PCR ribotypes and toxin production in the *C. difficile* collection 1979–2004 ($n=179$)

Ribotype	Toxin status*	No. of ribotypes in time period (%)			
		1979–1986	1987–1995	1996–2004	Total 1979–2004
002	T	9 (13.0)	9 (14.8)	7 (14.3)	25 (14.0)
014	T	6 (8.7)	4 (6.6)	3 (6.1)	13 (7.3)
012	T	6 (8.7)	2 (3.3)	1 (2.0)	9 (5.0)
015	T	1 (1.5)	5 (8.2)	3 (6.1)	9 (5.0)
020	T	0	6 (9.8)	3 (6.1)	9 (5.0)
001	T	1 (1.5)	1 (1.6)	6 (12.2)	8 (4.5)
010	N	3 (4.3)	1 (1.6)	3 (6.1)	7 (3.9)
013	T	2 (2.9)	2 (3.3)	2 (4.1)	6 (3.4)
026	5N/1T	2 (2.9)	2 (3.3)	2 (4.1)	6 (3.4)
078	T	0	4 (6.6)	2 (4.1)	6 (3.4)
023	T	2 (2.9)	1 (1.6)	2 (4.1)	5 (2.8)
103	T	1 (1.5)	3 (4.9)	1 (2.0)	5 (2.8)
039	N	3 (4.3)	1 (1.6)	1 (2.0)	5 (2.8)
059	T	3 (4.3)	0	1 (2.0)	4 (2.2)
029	T	1 (1.5)	1 (1.6)	1 (2.0)	3 (1.7)
032	N	2 (2.9)	1 (1.6)	0	3 (1.7)
071	N	3 (4.3)	0	0	3 (1.7)
176	T	1 (1.5)	0	2 (4.1)	3 (1.7)
003	T	0	2 (3.3)	0	2 (1.1)
005	T	1 (1.5)	1 (1.6)	0	2 (1.1)
009	N	1 (1.5)	1 (1.6)	0	2 (1.1)
011	T	1 (1.5)	1 (1.6)	0	2 (1.1)
035	N	2 (2.9)	0	0	2 (1.1)
046	T	1 (1.5)	1 (1.6)	0	2 (1.1)
054	T	0	0	2 (4.1)	2 (1.1)
056	T	0	1 (1.6)	1 (2.0)	2 (1.1)
064	T	0	1 (1.6)	1 (2.0)	2 (1.1)
087	T	2 (2.9)	0	0	2 (1.1)
116	T	2 (2.9)	0	0	2 (1.1)
154	T	0	2 (3.3)	0	2 (1.1)
018	T	0	1 (1.6)	0	1 (0.6)
030	N	1 (1.5)	0	0	1 (0.6)
038	N	0	0	1 (2.0)	1 (0.6)
042	T	0	1 (1.6)	0	1 (0.6)
050	T	1 (1.5)	0	0	1 (0.6)
053	T	0	0	1 (2.0)	1 (0.6)
057	T	0	0	1 (2.0)	1 (0.6)
070	T	1 (1.5)	0	0	1 (0.6)
072	T	0	0	1 (2.0)	1 (0.6)
081	T	0	1 (1.6)	0	1 (0.6)
083	T	1 (1.5)	0	0	1 (0.6)
092	T	0	1 (1.6)	0	1 (0.6)
100	N	0	1 (1.6)	0	1 (0.6)
111	T	0	1 (1.6)	0	1 (0.6)
119	N	1 (1.5)	0	0	1 (0.6)
120	T	1 (1.5)	0	0	1 (0.6)
137	T	0	1 (1.6)	0	1 (0.6)
142	T	1 (1.5)	0	0	1 (0.6)
153	T	1 (1.5)	0	0	1 (0.6)
165	T	1 (1.5)	0	0	1 (0.6)
179	T	1 (1.5)	0	0	1 (0.6)
184	N	0	0	1 (2.0)	1 (0.6)
186	T	0	1 (1.6)	0	1 (0.6)
189	T	1 (1.5)	0	0	1 (0.6)
192	T	1 (1.5)	0	0	1 (0.6)
210	T	1 (1.5)	0	0	1 (0.6)
All		69	61	49	179

*T, Toxigenic; N, non-toxigenic.

Table 2. MIC₅₀ and MIC₉₀ values for the seven antibiotics tested during the three periods of the study

Antibiotic resistance (breakpoint, µg ml ⁻¹)	MIC ₅₀ (µg ml ⁻¹)					MIC ₉₀ (µg ml ⁻¹)				
	1979–1986	1987–1995	1996–2004	Total	2005*	1979–1986	1987–1995	1996–2004	Total	2005*
	1979–2004					1979–2004				
Metronidazole (≥32)	<0.5	<0.5	<0.5	<0.5	1	<0.5	<0.5	<0.5	<0.5	2
Vancomycin (≥32)	1	1	1	1	2	2	2	2	2	4
Erythromycin (≥8)	2	4	2	2	≥32	>32	4	>32	>32	≥32
Tetracycline (≥16)	<1	<1	<1	<1	≤1	8	<1	4	<1	2
Moxifloxacin (≥8)	2	2	2	2	≥32	2	2	8	2	≥32
Ceftriaxone (≥64)	32	32	32	32	64	64	32	64	64	64
Clindamycin (≥8)	16	16	8	16	8	>32	16	8	16	16

*Results from Mutlu *et al.* (2007).

in the latter-most period. Conversely, when ribotypes not appearing in the period were counted, there were 32 'missing' from the later period compared with 20 and 25 in the earlier periods, respectively. This suggested that some ribotypes have become predominant over time, whilst the less common ones have disappeared.

Ribotype 012, rarely reported from other areas of the world, comprised 5 % of our total isolates. Ribotype 078, thought to be an emerging epidemiological type with evidence to support zoonotic transmission (Jhung *et al.*, 2008), comprised 3.4 % of our isolates. However, due to the absence of clinical data, a history of animal contact could not be established. An initially unidentified ribotype was confirmed by the Anaerobe Reference Laboratory at Cardiff to be a novel ribotype, now designated ribotype 210. Ribotype 027 was not found in our study, although subsequent surveillance from all of Scotland suggests that the presence of this epidemiological type is now established and has risen from 1 % in 2006–2007 to 12 % in 2007–2008 (Health Protection Scotland, 2007, 2009).

Antibiotic susceptibility

For all isolates, the MICs for the five tested antibiotics are given in Table 2 and resistance levels are summarized in Tables 3 and 4. The majority of isolates were resistant to clindamycin (95.5 %); the next most prevalent resistances were erythromycin (14.0 %) and ceftriaxone (13.4 %). In keeping with previous reports (Drummond *et al.*, 2003), no resistance to vancomycin or metronidazole was detected. Between the toxigenic and non-toxigenic isolates, the differences in observed resistances were not statistically significant for any of the antibiotics except erythromycin (10.9 % toxigenic isolates resistant compared with 28.1 % non-toxigenic isolates resistant, $P=0.002$).

Over the three time periods, resistance to moxifloxacin has increased progressively (Table 5). It is postulated that, although moxifloxacin was not in extensive use until the late 1990s, the widespread use of ciprofloxacin in the 1980s might

have driven the increase in resistance to this antibiotic. Similarly, Ackermann *et al.* (2003b) showed a progressive increase in antibiotic resistance to erythromycin, clindamycin and moxifloxacin in 192 isolates from 1986 to 2001, although the differences did not reach statistical significance. Ackermann *et al.* (2003a) studied the genome of 63 strains of *C. difficile* and demonstrated mutations in the codon for aa 83 of the *gyrA* gene in all moxifloxacin-resistant strains. The stability of this region of the genome of the moxifloxacin-resistant strains compared with that of other ribotypes should thus be an area for further investigation.

In contrast to the moxifloxacin results, resistance to tetracycline has decreased progressively over the years (Table 5), leading us to believe that the decline in the use of tetracyclines may have influenced this phenomenon. However, Schmidt *et al.* (2007) studied 317 *C. difficile* isolates (obtained from symptomatic patients from Leipzig, Germany, from 2002–2004) and found that resistance to erythromycin, clindamycin, moxifloxacin and doxycycline increased during the short study period.

Ribotypes, antibiotic susceptibility and virulence

Thirty-two (17.9 %) and 14 (7.8 %) of the 179 isolates were resistant to two and three or more antibiotics, respectively.

Table 3. Differences in antibiotic resistance between toxigenic and non-toxigenic strains

Antibiotic	No. resistant (%)			P value
	Toxigenic	Non-toxigenic	Overall	
Erythromycin	16 (10.9)	9 (28.1)	25 (14.0)	0.002
Tetracycline	9 (6.1)	3 (9.4)	12 (6.7)	0.45
Moxifloxacin	8 (5.4)	0	8 (4.5)	0.35
Ceftriaxone	19 (12.9)	5 (15.6)	24 (13.4)	0.77
Clindamycin	139 (94.6)	32 (100)	171 (95.5)	0.35

Table 4. Resistance patterns of selected ribotypes

Ery, Erythromycin; Tet, tetracycline; Moxi, moxifloxacin; Ceft, ceftriaxone; Clin, clindamycin.

Ribotype (n)	Resistance to antibiotic (%)				
	Ery	Tet	Moxi	Ceft	Clin
001 (8)	50.0	0	50.0	50.0	100.0
002 (25)	4.0	0.0	4.0	8.0	92.0
012 (9)	33.3	88.9	0.0	66.7	88.9
014 (13)	0.0	7.7	0.0	15.4	100.0
015 (9)	0.0	0.0	0.0	0.0	88.9
020 (9)	0.0	0.0	0.0	11.1	100.0
078 (6)	50.0	0.0	0.0	0.0	100.0

Of the latter, six were ribotype 012 (66.7 % of all ribotype 012) and four were ribotype 001 (50 % of all ribotype 001). Thus, these two types are frequently associated with multidrug resistance and this may explain in part their widespread prevalence in some of the time periods. Mutlu *et al.* (2007) reported that 95.4 % of their ribotype 001 isolates were resistant to three or more antibiotics. This may explain the higher prevalence of this type in their study. However, as both are now on the decline in this region (Health Protection Scotland, 2007), they are either losing the advantage gained by multidrug resistance or other ribotypes have acquired increased virulence. No strain from our collection was found to be resistant to all of the antibiotics tested, although Mutlu *et al.* (2007) reported one isolate of ribotype 001 to be resistant to five antibiotics. Various studies have suggested that the virulence of *C. difficile* may depend on factors such as cytotoxin production (Rupnik *et al.*, 2009), the surface layer protein (Drudy *et al.*, 2004; Merrigan *et al.*, 2006), the presence of binary toxin and the ability to sporulate (Freeman *et al.*, 2005; Saxton *et al.*, 2009). Unfortunately, no reliable tests for virulence of *C. difficile* exist and thus were not performed in this study.

Studies that have looked into the differences between dominant and non-dominant ribotypes have reported a

Table 5. Changes in resistance to antibiotics over the study period

Antibiotic	No. of resistant isolates (%)		
	1979–1986 (n=69)	1987–1995 (n=61)	1996–2004 (n=49)
Erythromycin	12 (17.4)	5 (8.2)	10 (20.4)
Tetracycline	9 (13.0)	2 (3.3)	1 (2.0)
Moxifloxacin	0	2 (3.3)	5 (10.2)
Ceftriaxone	12 (17.4)	6 (9.8)	6 (12.2)
Clindamycin	67 (97.1)	59 (96.7)	45 (91.8)

higher resistance in the dominant ribotypes as opposed to the non-dominant ones for some antibiotics. We compared antibiotic susceptibilities among the six commonest ribotypes (defined as those that had a prevalence of ≥ 4 % in the entire collection studied, i.e. ribotypes 002, 014, 012, 015, 020 and 001; Table 1). Resistance was found to be higher in the dominant ribotypes for tetracycline, moxifloxacin and ceftriaxone (*P* values of 0.016, 0.123 and 0.026, respectively; Table 6). However, although not statistically significant, resistance to erythromycin was higher among the non-dominant ribotypes (*P*=0.29). Brazier *et al.* (2008) compared the differences between their commonest ribotypes (027, 106 and 001, considered to be epidemic strains) and non-epidemic strains and found that, although none were clinically resistant, there was a statistical difference between the mean MICs for metronidazole, which were higher for the epidemic strains. In addition, the epidemic ribotypes were more resistant to erythromycin and moxifloxacin. Mutlu *et al.* (2007) also found a higher resistance to ceftriaxone and moxifloxacin among their dominant ribotypes (001 and 106). However, as our isolates were selected to avoid duplication of strains from outbreaks, and included a different set of dominant ribotypes, the samples are not directly comparable with those from other studies.

A comparison between studies is also difficult due to the absence of universally accepted breakpoints to determine susceptibility among *C. difficile* isolates. The presence of the organism in the gut precludes the accurate interpretation of susceptibility to antibiotics whose criteria have been established for serum concentrations. In addition, most guidelines do not include susceptibility criteria for *C. difficile*. The breakpoints for moxifloxacin are not established, even for other anaerobes (NCCLS, 2004).

Wüst & Hardegger (1983) found that genetic transfer of antimicrobial resistance to clindamycin, tetracycline and erythromycin occurs via a non-plasmid-mediated mechanism. We attempted to determine whether the non-toxicogenic isolates, presently considered to be non-pathogenic, played a role as a pool of antibiotic resistance.

Table 6. Differences in antibiotic resistance between dominant and non-dominant strains

Dominant ribotypes were defined as those with an overall prevalence of ≥ 4 % among the collection studied. These were ribotypes 002, 014, 012, 015, 020 and 001.

Antibiotic	No. resistant (%)		<i>P</i> value
	Dominant (n=73)	Non-dominant (n=106)	
Erythromycin	8 (11.0)	19 (17.9)	0.29
Tetracycline	9 (12.3)	3 (2.8)	0.016
Moxifloxacin	5 (6.8)	2 (1.9)	0.123
Ceftriaxone	15 (20.5)	9 (8.5)	0.026
Clindamycin	69 (94.5)	102 (96.2)	0.71

However, antibiotic susceptibilities of toxigenic versus non-toxigenic isolates did not show statistical differences for any antibiotic except erythromycin (10.9 % toxigenic isolates resistant compared with 28.1 % non-toxigenic isolates resistant, $P=0.002$). This suggests that non-toxigenic isolates may undertake genetic exchanges with toxigenic isolates and act as a pool of antibiotic resistance whilst colonizing the normal gut, even though not causing active disease. The presence of non-toxigenic isolates in this collection, which was derived exclusively from symptomatic patients, also raises the query of whether non-toxigenic strains may have some pathogenic potential. The paucity of clinical data prevented us from investigating this hypothesis further.

An attempt was made to analyse the changes in antibiotic resistance over time for individual ribotypes. However, as there were only a few isolates of each ribotype in each time period, such trends could not be evaluated. The overall sensitivities of the common ribotypes (Table 4) did reveal some noteworthy observations. Ribotype 001 displayed maximum resistance, with 50 % of the isolates resistant to erythromycin, moxifloxacin and ceftriaxone, and 100 % resistant to clindamycin. Ribotype 012, which has remained prevalent throughout the years but with progressively decreasing numbers, also displayed substantial resistance to the tested antibiotics with 33.3, 88.9, 66.7 and 100 % of isolates resistant to erythromycin, tetracycline, ceftriaxone and clindamycin, respectively. Ribotype 014, although the second commonest ribotype overall, showed only 7.7 % of isolates resistant to tetracycline, 15.4 % to ceftriaxone and 100 % to clindamycin. The prevalence of ribotype 002 in our study was almost constant throughout the year groups (between 13.0 and 14.8 %), with resistance levels of 4, 4, 8 and 92 % to erythromycin, moxifloxacin, ceftriaxone and clindamycin, respectively.

Antibiotic pressures are likely to be driving the epidemiology of *C. difficile* in this region of Scotland. In the early years of this study, a variety of different epidemiological types existed. However, with the increasing use of antibiotics, some resistant ribotypes are emerging as dominant, whilst others are decreasing in incidence.

ACKNOWLEDGEMENTS

We are grateful to Robert Brown for carefully maintaining our culture collection during the period of the study.

REFERENCES

- Ackermann, G., Tang-Feldman, Y. J., Schaumann, R., Henderson, J. P., Rodloff, A. C., Silva, J. & Cohen, S. H. (2003a). Antecedent use of fluoroquinolones is associated with resistance to moxifloxacin in *Clostridium difficile*. *Clin Microbiol Infect* **9**, 526–530.
- Ackermann, G., Degner, A., Cohen, S. H., Silva, J. & Rodloff, A. C. (2003b). Prevalence and association of macrolide-lincosamide-

streptogramin B (MLS_B) resistance with resistance to moxifloxacin in *Clostridium difficile*. *J Antimicrob Chemother* **51**, 599–603.

Barbut, F., Gariazzo, B., Bonné, L., Lalande, V., Burghoffer, B., Luiuz, R. & Petit, J. (2007). Clinical features of *Clostridium difficile*-associated infections and molecular characterization of strains: results of a retrospective study, 2000–2004. *Infect Control Hosp Epidemiol* **28**, 131–139.

Brazier, J. S., Raybould, R., Patel, B., Duckworth, G., Pearson, A., Charlett, A., Duerden, B. I. & HPA Regional Microbiology Network (2008). Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English hospitals, 2007–08. *Euro Surveill* **13**, PPii=19000. <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19000>

Climo, M. W., Israel, D. S., Wong, E. S., Williams, D., Coudron, P. & Markowitz, S. M. (1998). Hospital-wide restriction of clindamycin: effect on the incidence of *Clostridium difficile*-associated diarrhoea and cost. *Ann Intern Med* **128**, 989–995.

Cookson, B. (2007). Hypervirulent strains of *Clostridium difficile*. *Postgrad Med J* **83**, 291–295.

Dallal, R. M., Harbrecht, B. G., Boujoukas, A. J., Sirio, C. A., Farkas, L. M., Lee, K. K. & Simmons, R. L. (2002). Fulminant *Clostridium difficile*: an underappreciated and increasing cause of death and complications. *Ann Surg* **235**, 363–372.

Drudy, D., Calabi, E., Kyne, L., Sougioultzis, S., Kelly, E., Fairweather, N. & Kelly, C. P. (2004). Human antibody response to surface layer proteins in *Clostridium difficile* infection. *FEMS Immunol Med Microbiol* **41**, 237–242.

Drummond, L. J., McCoubrey, J., Smith, D. G., Starr, J. M. & Poxton, I. R. (2003). Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period. *J Med Microbiol* **52**, 259–263.

Freeman, J., Baines, S. D. & Wilcox, M. H. (2005). Comparison of the efficacy of ramoplanin and vancomycin in both *in vitro* and *in vivo* models of clindamycin-induced *Clostridium difficile* infection. *J Antimicrob Chemother* **56**, 717–725.

Hastings, M. (2006). Anaerobe Reference Unit Operating Procedure Manual ASOP 39: PCR Ribotyping of *Clostridium difficile*. NPHS Microbiology Cardiff, issue 1, issue date 13/11/06.

Health Protection Scotland (2007). *Annual Report on the Surveillance of Clostridium difficile Associated Disease (CDAD) in Scotland, October 2006–September 2007*. The HPS *C. difficile* Working Group. <http://www.documents.hps.scot.nhs.uk/hai/sshaip/publications/cdad/2007-12-20-ar-cdad.pdf>

Health Protection Scotland (2009). *Annual Report on the Surveillance of Clostridium difficile Associated Disease (CDAD) in Scotland, October 2007–September 2008*. HPS Weekly Report **43**, no. 2009/02. The HPS *C. difficile* Working Group. <http://www.documents.hps.scot.nhs.uk/ewr/pdf2009/0902.pdf>

Jhung, M. A., Thompson, A. D., Killgore, G. E., Zukowski, W. E., Songer, G., Warny, M., Johnson, S., Gerding, D. N., McDonald, L. C. & Limbago, B. M. (2008). Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* **14**, 1039–1045.

Kyne, L., Hamel, M. B., Polavaram, R. & Kelly, C. P. (2002). Health care costs and mortality associated with nosocomial diarrhoea due to *Clostridium difficile*. *Clin Infect Dis* **34**, 346–353.

Lee, K., Locking, M. E. & Pavek, V. (2001). Surveillance of *Clostridium difficile* infection in Scotland 1988–2000. Centre for Infection and Environmental Health (SCIEH). <http://www.documents.hps.scot.nhs.uk/posters/2001/surveillance-clostridium-difficile-hai.pdf>

McCoubrey, J. (2002). *The epidemiology of Clostridium difficile in a geriatric unit*. PhD thesis, University of Edinburgh, UK.

- McCoubrey, J., Starr, J., Martin, H. & Poxton, I. R. (2003).** *Clostridium difficile* in a geriatric unit: a prospective epidemiological study employing a novel S-layer typing method. *J Med Microbiol* **52**, 573–578.
- McDonald, L. C., Killgore, G. E., Thompson, A., Owens, R. C., Kazakova, S. V., Sambol, S. P., Johnson, S. & Gerding, D. N. (2005).** An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med* **353**, 2433–2441.
- Merrigan, M. M., Gerding, D. N. & Vedantam, G. (2006).** Hypervirulent *Clostridium difficile* BI strains have altered protein expression and host cell adherence. Poster PI-12. In *Eighth Biennial Conference of the Anaerobe Society of America*, 25–28 July, Boise, ID, USA.
- Mutlu, E., Wroe, A. J., Sanchez-Hurtado, K., Brazier, J. S. & Poxton, I. R. (2007).** Molecular characterization and antimicrobial susceptibility patterns of *Clostridium difficile* strains isolated from hospitals in south-east Scotland. *J Med Microbiol* **56**, 921–929.
- NCCLS (2004).** *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*. Approved Standard, 6th edn. NCCLS document M11-A6. Wayne, PA: Clinical and Laboratory Standards Institute.
- O'Neill, G. L., Ogunsola, F. T., Brazier, J. S. & Duerden, B. I. (1996).** Modification of a PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. *Anaerobe* **2**, 205–209.
- Pépin, J., Valiquette, L., Alary, M. E., Villemure, P., Pelletier, A., Forget, K., Pépin, K. & Chouinard, D. (2004).** *Clostridium difficile*-associated diarrhoea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* **171**, 466–472.
- Rupnik, M., Wilcox, M. H. & Gerding, D. N. (2009).** *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* **7**, 526–536.
- Saxton, K., Baines, S. D., Freeman, J., O'Connor, R. & Wilcox, M. H. (2009).** Effects of exposure of *Clostridium difficile* PCR ribotypes 027 and 001 to fluoroquinolones in a human gut model. *Antimicrob Agents Chemother* **53**, 412–420.
- Schmidt, C., Löffler, B. & Ackermann, G. (2007).** Antimicrobial phenotypes and molecular basis in clinical strains of *Clostridium difficile*. *Diagn Microbiol Infect Dis* **59**, 1–5.
- Schroeder, M. S. (2005).** *Clostridium difficile*-associated diarrhoea. *Am Fam Physician* **71**, 921–928.
- Settle, C. D. & Wilcox, M. H. (1996).** Review article: antibiotic-induced *Clostridium difficile* infection. *Aliment Pharmacol Ther* **10**, 835–841.
- Wiström, J., Norrby, S. R., Myhre, E. B., Eriksson, S., Granström, G., Lagergren, L., Englund, G., Nord, C. E. & Svenungsson, B. J. (2001).** Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *J Antimicrob Chemother* **47**, 43–50.
- Wüst, J. & Hardegger, U. (1983).** Transferable resistance to clindamycin, erythromycin, and tetracycline in *Clostridium difficile*. *Antimicrob Agents Chemother* **23**, 784–786.