An investigation of a nosocomial outbreak of *Clostridium difficile* by pyrolysis mass spectrometry

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Summary. Isolates from a presumptive nosocomial outbreak of *Clostridium difficile* infection at a large teaching hospital were typed by pyrolysis mass spectrometry (PMS) and antibiograms. One isolate, from the putative index case, was dissimilar from the outbreak strain, but 24 isolates from 16 patients were indistinguishable by both methods. The outbreak centred on two wards for the acute care of the elderly, with a few cases elsewhere. Transfer of patients appeared to be the route of transmission between wards. There was a significant fall in the incidence of cases following intervention by the Infection Control Unit. This included ward inspection, advice on antibiotic usage and advice on prevention of faecal-oral transfer, particularly by proper handwashing. Subsequent monitoring of *C. difficile* infection showed a background of sporadic, dissimilar isolates with occasional apparent cross-infection incidents limited to a few patients. In suspected outbreaks, patterns of antibiotic susceptibility may be useful in initial screening, before referral for more sophisticated typing. There was excellent correlation between PMS results, antibiograms and epidemiological information.

Introduction

Toxigenic strains of *Clostridium difficile* cause pseudomembranous colitis (PMC) and antibiotic-associated diarrhoea (AAD).^{1,2} Cross-infection may occur,³ and outbreaks of nosocomial *C. difficile* AAD, sometimes with cases of PMC, in elderly hospitalised patients have become a major problem in some hospitals.^{4–7} The few methods for typing *C. difficile* are generally complex, expensive, low-throughput techniques limited to a few specialised laboratories. Methods include unmodified SDS-PAGE,⁸ SDS-PAGE of ³⁵S-methionine-labelled cultures,^{9,10} immunoblotting of outer-membrane proteins,¹¹ serogrouping,¹² bacteriocin and bacteriophage typing,¹³ and restriction endonuclease fragment length polymorphism analysis.¹⁴ Antibiograms may also be helpful in typing.^{13,15} Knowledge of the epidemiology of *C. difficile* outbreaks remains limited because of the restricted availability of typing services.

A recent study¹⁶ of pyrolysis mass spectrometry (PMS) typing for *C. difficile* illustrates the usefulness of this rapid high-throughput technique. Soon after PMS apparatus was installed at the University Hospital of Wales (UHW) Public Health Laboratory, a preliminary study of PMS typing for *C. difficile* was planned, involving isolates from putative UK outbreaks retained at the Anaerobe Reference Laboratory. An outbreak of *C. difficile*-associated AAD

occurred at UHW immediately before practical work commenced, and isolates from this outbreak were included in the experimental design. The initial PMS typing experiment, subsequent analyses of UHW isolates, and a blind comparison of PMS and antibiogram typing results for the outbreak isolates are described.

Materials and methods

Patients

Faecal samples from patients of the University Hospital of Wales and other Cardiff hospitals were submitted to the Department of Medical Microbiology and Public Health Laboratory, UHW. Clinical diagnosis of *C. difficile*-associated AAD or PMC was confirmed by isolation of *C. difficile* and detection of enterotoxin in the stools.

Isolation procedure

Faecal samples were cultured for *C. difficile* by the alcohol shock method.¹⁷ The deposit was inoculated on to cefoxitin-cycloserine egg yolk agar,¹⁸ and into cooked meat broth for enrichment. Isolates were identified as *C. difficile* by their characteristic pungent odour, fluorescence under UV light and lecithinase reaction,¹⁸ and with the Microscreen *C. difficile* latex agglutination diagnostic kit (Mercia Diagnostics, Guildford, Surrey).¹⁹ Toxin A (enterotoxin) was

Table I. Monthly incidence of patients yielding their first *C. difficile* positive culture during the outbreak and post-outbreak phases

Month for 1991-92	AG care unit				Other UHW wards	Other Cardiff wards	Total (affected)	Total (unaffected)	Requests*	Yield† (%)
	Affected wards		Unaffected wards							
	A	B	C	D						
October	1	0	1	0	0	0	1	1	...	...
November	8	3	0	0	0	1	11	1	90	13.3
December	0	3	1	2	3	3	3	9	77	15.6
January	2	5	3	0	3	1	7	7	129	9.3
February	1	4	1	2	3	2	5	8	108	9.3
March	5	0	0	1	1	3	5	5	131	6.9
April	0	1	0	1	1	3	1	5	114	7.0
May	1	0	0	0	0	4	1	4	100	5.0
June	1	2	0	0	0	4	3	4	85	8.2
July	1	2	2	0	2	1	3	5	111	6.3

* Total number of requests for *C. difficile* culture during month.

† Total number of new patients diagnosed as a percentage of requests.

sought directly in faecal samples and in culture supernates by ELISA (Premier; Launch Diagnostics, Longfield, Kent).^{20, 21}

The first batch analysed in PMS included blind coded duplicate cultures of *C. difficile* NCTC 11207, and 13 isolates from elsewhere in the UK stored by the Anaerobe Reference Laboratory. Ten of these isolates had been received from single units over a short time span, with some suggestion that they were from outbreaks: one set of five from Manchester, another set of two from Manchester received a year earlier and one set of three from Romford. A further three were single isolates of epidemiologically distinct origin, one from Kettering, one from St Albans and one veterinary isolate from an orang utan in Jersey. These had been stored in glycerol broth at -70°C after submission for identification.

PMS typing

Single colony subcultures of *C. difficile* isolates were grown for 24 h at 37°C on Fastidious Anaerobe Agar (Lab M) in an atmosphere of H_2 10%, CO_2 10%, N_2 80%. Methods were as previously published.²² Briefly, colony material was sampled on a straight wire and smeared on each of four replicate pyrolysis foils. The foils were heated to 100°C for 10 min within 10 min of sampling and held in a vacuum desiccator before processing. Automated processing on the PYMS 200X (Horizon Instruments, Heathfield, E. Sussex) involved evacuation, Curie point pyrolysis for 4 s at 530°C and mass spectrometry of the pyrolysis products. The instrument had been upgraded since previous publications, with improvements in the stability of the electronics, the data acquisition and analysis programs, and the addition of a ring sampler, allowing uninterrupted automated processing of up to 150 foils. Data analysis was performed as previously,²² but with additional checking of the homogeneity of groups by the teaching-challenge set strategy.²³ Isolates were tested in three batches: (1) to validate the method and

to confirm that there was an outbreak caused by a single strain; (2) to investigate the extent of the outbreak; and (3) to assess the effectiveness of control measures and monitor post-outbreak epidemiology.

Antimicrobial susceptibility patterns

Disk susceptibility tests with chloramphenicol (Chl; 10 μg), tetracycline (Tet; 10 μg), erythromycin (Ery; 5 μg), vancomycin (30 μg), clindamycin (2 μg), rifampicin (2 μg) and penicillin (1.5 IU) disks were performed on DST (Oxoid) containing lysed horse blood 5%, with *C. perfringens* NCTC 8237 as a control organism. Intermediate results were regarded as non-comparable, and significant inter-strain differences were found for only three antibiotics. All strains were susceptible to vancomycin and most showed intermediate susceptibility or resistance to penicillin, clindamycin and to rifampicin. Therefore, antibiograms and toxin detection results are shown in the form SIR + -, indicating in this case susceptibility to Chl, intermediate susceptibility to Tet, resistance to Ery, and toxin production in cultures but no toxin detected on direct examination of the faeces (Tox); this notation is maintained throughout the tables.

Results

Description of the outbreak

The University Hospital of Wales is an 856-bed teaching hospital with, after re-organisation of care for the elderly in September 1991, an acute geriatric (AG) care unit comprising one shared ward (A; 19 AG beds) and three full wards (B, C and D; 33, 33 and 29 beds, respectively). Isolation rates for *C. difficile* in the hospital were *c.* 1/month before October 1991. Three AG patients on the shared ward (A) developed diarrhoea during 2 weeks of October–November 1991; each had been hospitalised for between 18 and 34 days. Two further patients developed AAD during the subsequent week, one on ward A and another on ward

Fig. 1. A representation of the extent of statistically significant differences between isolates in PMS analyses of batch 1 as a scattergram of isolate points. The distance between strain points indicates the level of dissimilarity between the strains. The diagram illustrates *c.* 52% of the differences on the first two canonical discriminant functions (CDFs). Further information on individual Cardiff isolates is presented in table II. Isolate origins are: 1, Cardiff; 2, Manchester; 3, Manchester (outlier); 4, duplicate spectra of NCTC 11207; 5, Manchester (earlier outbreak); 6, Romford (outlier); 7, Romford; 8, Kettering; 9, St Albans; 0, Jersey (veterinary).

Table II. PMS and antibiogram results for the local isolates of batch 1

Lab no.	Date	Patient	Ward	Susceptibility to†			PMS result	
				Chl	Tet	Ery	Tox‡	
34138*	3/11/91	a1	A					
34355	11/11/91	a2	A					
34505	13/11/91	a3	A	s	s	r	++	Indistinguishable
34114	5/11/91	a4	A					
34487	13/11/91	b1	B					
31889	31/10/91	a5	A	...	...	...	++	Distinct
34514*	14/11/91	a1	A	s	s	s	+-	Distinct
34654	9/11/91	g1	G	s	s	s	++	Distinct

* Isolate series from a single patient (a1) containing distinct strains.

† Chl, chloramphenicol; Tet, tetracycline; Ery, erythromycin; ..., no susceptibility results were available for isolate 31889.

‡ Toxin production, in culture and in faeces.

B. One infected patient had been transferred temporarily from ward A to ward B early after the onset of AAD. *C. difficile* was isolated from, and toxin A was detected in, faecal specimens.

Faecal specimens were obtained from all AG patients on the unit, whether symptomatic or not, and *C. difficile* was found in specimens from 11 patients during November (eight in ward A, three in ward B; table I), only half of whom were recorded as having diarrhoeal symptoms. Advice on the need for strict handwashing was given to the staff on wards A and B, and the clinicians were advised to restrict antibiotic usage throughout the AG care unit. Standards of ward cleaning were improved.

The number of new patients yielding *C. difficile* fell during December, and no further patients were found to be *C. difficile*-positive on ward A (table I). During

January 1992, the general rate of isolation in the AG care unit increased again; this was paralleled by increased rates of isolation in other wards and units elsewhere in the city. Further inspections revealed that standards of cleaning had fallen over the Christmas period, and it was notable that the ward worst affected (B) was that where rates of isolation of *C. difficile* were highest. Rates remained high in February, but returned to pre-outbreak levels in April.

PMS typing

The findings of Batch 1 (fig. 1) demonstrated that: (i) isolates from purported earlier outbreaks in the UK tended to cluster as distinct source-related groups; (ii) there was a clear cluster of five isolates from the AG care unit, indistinguishable in PMS; and (iii) three

Fig. 2. A representation of the extent of statistically significant differences between isolates in PMS analysis of batch 2. The diagram illustrates c. 74% of significant differences. Points representing isolates that were indistinguishable in PMS are circled. Further information on individual isolates is presented in table III.

Table III. PMS and antibiogram results for the isolates of batch 2

Lab no.	Date	Patient	Ward	Susceptibility to				PMS result
				Chl	Tet	Ery	Tox	
*34138†	3/11/91	a1	A					+
34355†	11/11/91	a2	A					+
34505†	13/11/91	a3	A					+
34707	17/11/91	a3	A					+
36077	21/11/91	a3	A					—
36085	22/11/91	a3	A					—
34114†	5/11/91	a4	A					+
34666	15/11/91	a6	A					+
34853	18/11/91	a6	A					+
36086	22/11/91	a6	A					+
34667	15/11/91	a7	A					+
34834	18/11/91	a7	A					+
34885	18/11/91	a8	A	s	s	r	+	Indistinguishable
36047	20/11/91	a9	A					—
34487†	13/11/91	b1	B					+
34692	14/11/91	b1	B					+
36386	28/11/91	b2	B					—
36916	9/12/91	b3	B					—
39192	13/12/91	b3	B					+
39497	20/12/91	b4	B					—
36885	9/12/91	d1	D					—
36603	3/12/91	d2	D					—
36920	9/12/91	f1	F					+
39511	23/12/91	i1	I					—
*34514†	14/11/91	a1	A	s	s	s	+	— Distinct
36047	21/11/91	b5	B	i	r	r	—	— Distinct
39169	13/12/91	b6	B	r	s	r	+	+
39590	28/12/91	d3	D	s	s	r	+	— Distinct
39050	11/12/91	d4	D	s	s	s	+	+
‡34603	14/11/91	e1	E	s	s	s	+	+
‡34631	15/11/91	e1	E	r	s	i	+	+
34654†	9/11/91	g1	G	s	s	s	+	+
39089	10/12/91	h1	H	s	s	s	—	— Distinct
39554	23/12/91	i2	I	s	s	r	+	+

Abbreviations as for table II.

† Isolate series from single patients (, a1; ‡, e1) containing distinct strains.

† Isolates were included in batch 1.

further isolates from the AG care unit and elsewhere in the city were clearly distinct from one another and from all other isolates examined.

The results of this blind PMS trial were supported by antibiogram typing of the UHW isolates (table II). One isolate (31889) had been lost on subculture, and

antibiotic susceptibility tests could not be performed. This was particularly unfortunate as this isolate was from the supposed index case, but it had been found to be distinct in PMS. Two isolates from a single patient (a1) were distinct in PMS and antibiogram typing. The combined data of PMS, antibiogram typing and the

Fig. 3. A representation of the extent of statistically significant differences between isolates in PMS analysis of batch 3. The diagram illustrates *c.* 49% of significant differences. Points representing indistinguishable serial isolates from the same patient are joined by lines. Further information on individual isolates is presented in table IV.

Table IV. PMS and antibiogram results for the isolates of batch 3

Lab no.	Date	Patient	Ward	Susceptibility to			Tox	PMS result
				Chl	Tet	Ery		
*54	3/11/92	d3	D	s	s	r	+ -	Indistinguishable
*458	15/01/92	d3	D	s	s	r	+ -	
349	12/01/92	a10	A	s	s	s	+ -	Indistinguishable
591	20/01/92	a10	A	s	s	s	+ -	
523	17/01/92	b7	B	s	s	s	++	Indistinguishable
789	27/01/92	a11	A	s	s	r	++	
841	28/01/92	a11	A	s	s	r	+ -	Indistinguishable
927	30/01/92	a11	A	s	s	r	+ -	
953	31/01/92	a11	A	s	s	r	+ -	Indistinguishable
446	14/01/92	b8	B	s	i	r	++	
‡159	6/01/92	b9	B	s	i	r	++	Indistinguishable
‡335	11/01/92	b9	C	s	s	r	+ -	
720	23/01/92	e2	E	s	s	r	++	Indistinguishable
334	11/01/92	b10	B	s	s	r	++	
762	24/01/92	e3	E	s	s	r	+ -	Indistinguishable
421	14/01/92	c1	C	s	s	r	++	
696	23/01/92	c1	C	s	s	r	++	Indistinguishable
794	24/01/92	c1	C	s	s	r	++	
36077†	21/11/91	a4	A	s	s	r	+ -	Indistinguishable
338	11/01/92	b11	B	s	s	r	+ -	
§93	6/01/92	c2	C	s	s	r	++	Indistinguishable
§470	15/01/92	c2	C	s	s	r	+ -	
628	21/01/92	g2	G	s	s	r	+	Indistinguishable
864	29/01/92	a11	G	s	s	r	+	
478	14/01/92	i3	I	s	s	r	- -	Distinct
*539	17/01/92	d3	D	s	r	r	++	Distinct
*976	1/01/92	d3	D	s	s	r	+ -	Distinct
321	11/01/92	c3	C	s	s	s	+ -	Distinct
144	6/01/92	g2	G	s	r	s	++	Distinct
627	21/01/92	g3	G	s	s	s	+	Distinct
986	4/02/92	b12	B	s	s	r	++	Distinct
§320	11/01/92	c2	C	s	s	r	++	Extreme outlier

Abbreviations as for table II.

‡§ Isolate series from single patients (, d3; ‡, b9; §, c2) containing distinct strains.

† Strain 36077 was a representative isolate of the outbreak strain from batch 2.

epidemiology provided support for the hypothesis that a single strain outbreak of cross-infection was occurring, accompanied by a few independent sporadic infections.

Batch 2, analysed in early January, included the surviving seven local isolates examined in batch 1 and a further 27 local isolates. In blinded trials, PMS results and antibiograms agreed once more (fig. 2,

table III), showing a core of indistinguishable isolates, with a minority of distinct strains, mostly from wards other than those of the AG care unit. The group membership for the seven re-examined isolates was identical to that found previously. One notable feature was that two patients (a1 and e1) gave serial isolates that were distinct in PMS and antibiograms; for one patient (a1) these were isolated within a 36-h period. Only one of these isolates (from a1) was indistinguishable from the outbreak strain. These results confirmed those of batch 1.

Again the evidence was of a single strain outbreak centred on two wards (A and B). Only four isolates from other wards or units were indistinguishable from the outbreak strain. For three of these, the patients had been transferred from, or re-admitted after, a stay on one of the affected wards. Of the 10 isolates found to be distinct from the outbreak strain, only three were isolated from patients on wards A and B. These appeared to be part of a background incidence of independent sporadic infections. This retrospective survey confirmed the conclusions of the first trial. The outbreak had clearly subsided by early December, with minimal incidence on the two affected wards.

The rising incidence of *C. difficile* isolation in January 1992 suggested that the outbreak had become re-established, although awareness of the AAD problem had prompted an increased rate of requests for *C. difficile* culture (table I). Therefore, a third batch of 32 local isolates was typed in early February 1992. This included one isolate (36077), representative of the outbreak strain, from batch 2. The PMS results (fig. 3, table IV) were in marked contrast to those of the previous batches. There was no major group of indistinguishable isolates, but rather a scatter of distinct single strains and small groups of two-to-five isolates, mainly comprising serial isolates from single patients. Equally striking was the failure of antibiogram typing to distinguish between many of the isolates found to be distinct in PMS. Only one isolate (338) appeared indistinguishable from the outbreak strain (represented by isolate 36077) in PMS.

One patient (d3) yielded four sequential isolates, three of which were distinct in PMS, with two distinct antibiogram patterns; one patient (b9) yielded two sequential isolates distinct in PMS, with slight evidence of differences in antibiograms; and one patient (c2) yielded three sequential isolates, one of which was distinct in PMS, but not in antibiograms.

Although the antibiogram results did not distinguish many of the isolates of this batch judged distinct in PMS, there was little epidemiological evidence of a continuing single strain outbreak. There was no clear focus of increased incidence on a single ward (table I), and the parallel rise in incidence on other units seemed to indicate a common background trend, that had begun to rise in December in other units. Our conclusion was that the outbreak had not recurred. It seemed likely that the increased isolation rate was due to a combination of two factors—increased awareness

and investigation, and a possible seasonal change in the number of AG patients rendered susceptible to AAD by antibiotic treatment for respiratory complaints. No action was taken, and the ensuing fall in isolation rates supported our conclusions.

Discussion

Antibiograms were informative initially, indicating the possibility of a developing outbreak, but they gave less resolution than PMS, as was evident in the post-outbreak phase, when they provided little strain discrimination. However, this is a simple technique, available to any laboratory, whose use is indicated whenever an outbreak is suspected. It is an appropriate preliminary screen to filter out clearly unrelated strains when isolates are referred for typing. PMS provided a simple, rapid and inexpensive method of typing these isolates. The hasty shift from method development to successful routine use, forced by circumstances, emphasises the ease with which PMS can be adapted to new species as noted elsewhere.²² The conclusions from the PMS analyses were clearly supported by epidemiological evidence.

The results for the stored UK isolates of batch 1, and for the comparison of PMS and antibiogram results in all three batches of UHW isolates, offered strong evidence for the reliability of PMS discrimination. Further evidence is now available from other PMS studies.^{16,24} Results for isolates from another AAD outbreak investigated in PMS and in two independent SDS-PAGE studies showed broad agreement.²⁵

However, for five patients of the 40 investigated in typing, multiple distinct strains were isolated from a single patient over short time spans, indicating that a patient might be concurrently infected with multiple strains of *C. difficile*. This has been observed elsewhere,²⁶ and could present problems in typing. *C. difficile* has a pleomorphic colonial morphology that removes any possibility of detecting mixed strains, other than by typing multiple picks from the original isolation plate. Strain mixtures could also be a source of confusion in comparative trials of *C. difficile* typing methods.

Laboratories where diagnosis rests solely on detection of toxin in faeces, with no attempt to isolate the organism, are clearly at a disadvantage in a possible outbreak situation. With no isolates to submit for typing, by whatever method, it is difficult to distinguish between a simple rise in the incidence of independent sporadic infections, and a single strain outbreak. We strongly recommend that laboratories employ isolation and toxin detection methods in routine investigation of AAD.

The results also indicate that it is unnecessary to type large numbers of isolates in an outbreak. The key finding in this outbreak was that five of the eight isolates analysed in batch 1 were indistinguishable,

and all active measures flowed from this early result. Our conclusions from batch 3 would have been similar had we typed 10 isolates from any set of 10 patients. Considered selection of small numbers of isolates for typing, rather than every isolate found, has many merits, not least that reference laboratories are likely to return an early result for a manageable workload.

Many key questions about *C. difficile* have yet to be answered. The initial sources of outbreak strains are unknown and there is little data on carriage frequency in the population at risk. The background incidence of *C. difficile* in the hospital environment is unknown, although it appears to increase during outbreaks.^{27, 28} Our results hint at an underlying seasonal variation in incidence, which may correlate with the increased rates of admission for respiratory infections during the autumn and winter.

Our tentative conclusions from analysis of this and

other outbreaks is that initial sporadic cases occur in antibiotic-treated patients, presumably due to auto-infection in carriers. A break in hygienic precautions then allows spread among susceptible hosts, in which the most infective of the sporadic strains rapidly becomes predominant. Antibiotic control and measures to prevent faecal-environmental-oral spread appeared effective in this incident as they have in others.^{28, 29}

C. difficile is an opportunist pathogen that requires extraneous influences to cause overt infection, but can spread rapidly in a susceptible population. It is an enteric pathogen, but there is little evidence that it is food-borne, and its ability to sporulate opens potential pathways for spread that the common enteric pathogens cannot exploit. The availability of high-throughput typing methods is essential for the investigation of the epidemiology of this organism.

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