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INFECTION IN INJECTING DRUG USERS

Amplified fragment length polymorphism (AFLP) analysis of *Clostridium novyi, C. perfringens* and *Bacillus cereus* isolated from injecting drug users during 2000

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As part of the follow-up investigations associated with an outbreak of severe illness and death among illegal injecting drug users during 2000, 43 cultures of *Clostridium novyi* type A, 40 *C. perfringens* type A and 6 isolates of *Bacillus cereus* were characterised by amplified fragment length polymorphism (AFLP) analysis. Among the 43 *C. novyi* isolates, 23 different AFLP profiles were detected. The same AFLP profile was detected in isolates from 18 drug users investigated during 2000 from Scotland, England, the Republic of Ireland and Norway and a wound from a patient in 2000 who was not identified as a drug user. Unique AFLP profiles were obtained from four drug users from England and the Republic of Ireland, 10 historical isolates from culture collections, an isolate from food (1989) and three isolates from wounds (1995, 1991, 1988). The 40 *C. perfringens* isolates were from 13 drug users, the contents of one syringe and two samples of heroin. Sixteen AFLP types of *C. perfringens* were distinguished and there was little evidence for commonality among the isolates. The AFLP types of *C. perfringens* from heroin differed and were unique. Six isolates of *B. cereus* were from four drug users and two samples of heroin. Four different AFLP patterns were distinguished. Three AFLP types were isolated from four drug users. *B. cereus* isolates from an aspirate and a heroin sample collected from the same drug user were identical, and were also indistinguishable from an isolate from a groin infection in a second drug user. The AFLP type of the isolate from a second and unrelated heroin sample was unique. The AFLP results showed no or very limited evidence for commonality between the different isolates of *B. cereus* and *C. perfringens*. In marked contrast, the *C. novyi* isolates from the majority of the drug users during 2000 were homogeneous, suggesting a common source or clonal selection of a *C. novyi* type, or both, which either had an adaptive advantage in spore germination, survival or growth following the drug preparation and the injection procedure, or produced a more severe clinical presentation.

Introduction

A range of viral and bacterial infections is associated with illegal injecting drug use [1–3]. Endospore-forming bacteria, including *Clostridium botulinum* [4–6], *C. tetani* [7] and *Bacillus anthracis* [8] are increasingly recognised as a group of agents that infect injecting drug users. These agents are particularly problematic in this patient group because of their occurrence in the intestinal tract, widespread distribution in the environment, the robust nature of bacterial endosopores and their resistance to the process of drug preparation [9].

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In 2000, an unusual increase of morbidity and mortality was reported among illegal injecting drug users in the UK and Ireland. During the intensive investigation more than 108 cases (at least 43 deaths) were recognised between 1 April and 31 August 2000 [10–12]. *C. novyi* type A was the likely cause of the more serious illness [12–15], although other endospore-forming bacteria were also associated with some cases including *C. perfringens* and *B. cereus* [15–19]. In addition, six suspected cases of wound botulism were also reported in injecting drug users during 2000 and these represent the first recognition of this condition in the UK [16, 19, 20, PHLS unpublished data].

Amplified fragment length polymorphism (AFLP) has been applied successfully to bacteria from a wide variety of different genera [21–25]. The method involves restriction endonuclease digestion of total purified chromosomal DNA followed by ligation of the resulting fragments to a double-stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification by PCR of sets of these fragments is achieved with primers corresponding to the contiguous base sequences in the adapter, the restriction site plus one or more nucleotides in the original target DNA. The resulting PCR-amplified DNA fragments are then analysed by gel electrophoresis.

It was reported previously that AFLP could be applied successfully for epidemiological typing of *B. cereus* and *C. perfringens* cultures from cases of food poisoning [26, 27]. The purpose of the present study was to use AFLP for molecular epidemiological investigations of isolates of *B. cereus* and *C. perfringens* from injecting drug users during the 2000 outbreak, and to apply this technique to *C. novyi* isolates.

Materials and methods

Sources of cultures and case definitions

Isolates of *C. novyi*, *C. perfringens* and *B. cereus* were obtained as pure cultures or other material referred directly to the PHLS laboratories, from clinical microbiology laboratories, or were from culture collections; origins of isolates are shown in Tables 1–4 [28].

Clinical isolates of *C. novyi*, *C. perfringens* and *B. cereus* were from illegal injecting drug users in England, Scotland, the Republic of Ireland and Norway: each patient was designated with a number. All cases were in individuals who injected heroin extravascularly (i.e., subcutaneously or intramuscularly) and who presented to hospital with moderate to severe inflammation in soft tissue at an injection site unless otherwise stated. The cases were categorised as described elsewhere [15] as follows: definite, a case with an abscess or other significant inflammation at an injecting site, and both a severe inflammatory process (large abscess/extensive oedema) at or around the injecting site, and a severe systemic inflammatory reaction with evidence of multi-organ failure and a high white cell count; probable, a case with an abscess or other significant inflammation at an injecting site, and either a severe inflammatory process (large

Table 1. Origins and characterisation of *C. novyi* isolates from drug users

Patient no.	Sex	Country	Case status	Onset/ isolation date	Source of isolates	AFLP type
5	M	England	Possible	December 1999	Abscess aspirate	B
6	M	England	Definite	February 2000	Tissue	A
7	F	England	Probable	April 2000	Tissue	A
8*	F	Scotland	Definite	April 2000	PM tissue	A
					PM tissue	A
9	M	England	Definite	May 2000	PM injection site	A
10	M	Scotland	Definite	May 2000	PM tissue	A
11	M	Scotland	Definite	May 2000	PM kidney	A
12	M	Scotland	Definite	May 2000	AM tissue	A
13	F	Scotland	Probable	May 2000	AM tissue	A
14	F	Scotland	Probable	May 2000	AM tissue	A
3†	M	Scotland	Definite	May 2000	AM tissue	A
24	NK	Republic of Ireland	Definite	May 2000	PM tissue	A
15*	M	Scotland	Definite	June 2000	AM tissue	A
16	F	Scotland	Probable	June 2000	PM tissue	A
17*	M	Scotland	Probable	June 2000	PM tissue	A
18*	M	Scotland	Definite	June 2000	AM tissue	A
19	M	Scotland	Probable	June 2000	PM blood	A
20	M	England	Possible	July 2000	Calf skin	C
21	F	Scotland	Definite	August 2000	PM tissue	A
22	M	Norway‡	Definite	August 2000	Tissue	A
23	F	Republic of Ireland	Probable	October 2000	Calf abscess	D
34	M	England	Probable	January 2001	Tissue	K

NA, not applicable; NK, not known; M, male; F, female; AM, ante-mortem; PM, isolates collected at necropsy.
**C. perfringens* also isolated from these patients.
†*B. cereus* also isolated from this patient.
‡As described by Maagaard *et al.* [28].

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Table 2. Origins and characterisation of other *C. novyi* isolates

Culture no.	Year of isolation	Source of isolate	AFLP type
NCTC 6738	1944	NK	E
NCTC 538	1920	Gas gangrene	F
NCTC 13029	NK	Type strain (ATCC 17861)	G
CN 1376	1941	War wound (Germany)	M
CN 926	1944*	Human mild infection of thigh (Australia)	N
CN 902	1943*	Human gas gangrene (Middle East)	O
CN 930	1944*	'Fatal case' (Australia)	P
CN 1498	1946*	Human post-operation gas gangrene (UK)	J
CN 870	1918	War wound (South Africa)	Q
CN 925	1944*	Fatal human gas gangrene (New Guinea)	R
R 4502	1991	Wound, UK	H
R 3140	1988	Wound, UK	I
R 8711	1995	Blood, UK	L
R14386	2000	Human leg abrasion, UK†	A
ED 15485	NK	Horse, grass sickness	S
ED 15486	NK	Hare	T
ED 15487	NK	Horse, grass sickness	U
ED 15489	NK	Horse, grass sickness	V
ED 15490	NK	Horse, grass sickness	W
R3738	1989	Food, UK	J

NCTC, National Collection of Type Cultures, London; CN, Wellcome Bacterial Collection, held in the NCTC, London; R, PHLS Anaerobe Reference Unit, Cardiff PHL; ED, Department of Medical Microbiology, University of Edinburgh; NK, not known.
*Year of deposition.
†As described by McGuigan and Roworth [41].

Table 3. Origins and characterisation of *C. perfringens* isolates

Patient no.	Sex	Country	Case status	Onset/isolation date	Source of isolates	Number of culture tested	Serotype	AFLP type
Drug users								
8*	F	Scotland	Definite	April 2000	PM tissue	1	18,29	h
						8	TW8	b
						2	NT	b
						1	30,33	c
						3	NT	c
						1	TW8	c
25	M	Scotland	Definite	May 2000	AM tissue	1	NT	i
29	F	England	Definite	May 2000	AM fluid	1	NT	p
31	F	Scotland	Possible	May 2000	PM tissue	1	NT	g
17†	M	Scotland	Probable	June 2000	AM wound aspirate	1	NT	e
						1	61	e
15*	M	Scotland	Definite	June 2000	AM cavity swab	3	NT	a
18*	M	Scotland	Definite	June 2000	AM tissue	1	NT	l
						1	NT	o
26	F	Scotland	Probable	June 2000	Tissue	1	PS43	c
27	F	Scotland	Probable	June 2000	AM tissue	1	NT	a
						1	NT	c
28	F	Scotland	Probable	June 2000	Abscess swab	1	34	n
30	F	England	Definite	June 2000	Tissue	1	NT	m
32	NK	Scotland	NK	July 2000	PM blood	1	NT/43	j
33	NK	Scotland	NK	August 2000	PM blood	1	NT	k
						1	NT/PS30	k
Other isolates								
NA	NA	Scotland	NA	May 2000	Washings from syringe	2	NT	a
NA	NA	Scotland	NA	June 2000	Heroin	1	30,33	d
NA	NA	England	NA	August 2000	Heroin	2	25	f
						1	NT	f

NA, not applicable; NT, non-typable; NK, not known.
**C. novyi* also isolated from these patients.
†*C. novyi, C. scindens* and *C. perfringens* isolated from this patient.

abscess/extensive oedema) at or around the injecting site, or a severe systemic inflammatory reaction with evidence of multi-organ failure and a high white cell count; or possible, a case with an abscess or other significant inflammation at an injecting site.

Additional isolates from heroin (*B. cereus* and *C. perfringens*) and from used syringe residues (*C. perfringens*) from England or Scotland in 2000 were also included. Because of the lack of comparitors for characterising the *C. novyi* isolates, previously depos-

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Table 4. Origins and characterisation of *B. cereus* isolates

Patient no.	Sex	Country	Case status	Onset/isolation date	Source of isolates	Serotype	AFLP type
Drug users							
1	M	England	NA‡	June 2000	Blood	NT	I
2	M	Scotland	Probable	June 2000	Aspirate	NT	II
3*	M	Scotland	Definite	May 2000	Tissue	11	III
4	F	Scotland	Probable	June 2000	Groin tissue	NT	II^{-1}
Other isolates							
NA	NA	England	NA	June 2000	Heroin	NT	IV
NA†	NA	Scotland	NA	June 2000	Heroin	NT	II

NA, not applicable; NT, non-typable; NK, not known.
C. novyi was also isolated from this patient.
†Heroin collected from patient 2 [18].
‡No local inflammation around injection site.

ited cultures from other human or animal infections and one from food were analysed. These isolates were obtained from the culture collection of the PHLS Anaerobe Reference Unit; the National Collection of Type Cultures (NCTC), PHLS Central Public Health Laboratory, London; the Wellcome Bacterial Collection (also held at the NCTC); and from the Department of Medical Microbiology, University of Edinburgh (Table 2).

C. novyi cultures

The identity of all *C. novyi* type A isolates was confirmed according to conventional criteria [29] that included: spreading growth on blood agar, strong haemolysis on blood agar, lecithinase and lipase reactions positive on egg-yolk agar, and variable indole production. The identity of isolates was further confirmed by ⩾98% 16S rDNA sequence identity of a *c*. 1500-bp sequence of *C. novyi* type A strain NCTC 538 (EMBL database accession no. X68188) determined by methods described previously [30].

DNA was extracted from *C. novyi* cultures as described previously for *B. cereus* and *C. perfringens* [26, 27]. AFLP analysis was performed with either *Hin*dIII [24] or *Eco*R1 digestion [22]. For the *Hin*dIII digestion, ligation was as described previously [26, 27] and four separate PCR amplifications were performed with primers HI-A, HI-C, HI-G and HI-T (5′-GGTAT GCGACAGAGCTTA-3′, 5′-GGTATGCGACAGAGC TTC-3′, 5′-GGTATGCGACAGAGCTTG-3′ and 5′-GGTATGCGACAGAGCTTT-3′ respectively). Identical AFLP conditions were used for the *Eco*RI digestion, except the ligation adapters were 5′-CTCGTAGACT GCGTACC-3′ and 5′-AATTGGTACGCAGTCTAC-3′. Four separate PCR amplifications were performed on the ligated and digested DNA with primers EC-A, EC-C, EC-G and EC-T (5′-GACTGCGTACCAATTCA-3′, 5′-GACTGCGTACCAATTCC-3′, 5′-GACTGCGTACCA ATTCG-3′, and 5′-GACTGCGTACCAATTCT-3′, respectively).

Brief epidemiological details on the origins of the isolates are shown in Tables 1 and 2.

C. perfringens cultures

The identity of all *C. perfringens* type A cultures was confirmed by characteristic colonial morphology on Colombia blood agar and a positive Nagler reaction. All cultures were analysed by serotyping [31–34]. DNA was prepared as described previously and tested for amplification of fragments of the α-toxin and enterotoxin genes [35] and by AFLP analysis [26]. The latter procedure comprised digestion of DNA with *Hin*dIII, ligation with adapters ADH1 and ADH2 followed by amplification with primer HI-G. Brief epidemiological information on the origins of the isolates is shown in Table 3.

B. cereus cultures

The identity of *B. cereus* cultures was confirmed by: characteristic colonial morphology on Columbia blood agar and Kendall's medium (lecithinase production and no fermentation of mannitol); the production of acid and gas from glucose but not xylose, mannitol or arabinose; resistance to γ phage and penicillin; motility at 30°C; and characteristic Gram and spore stains [36]. All cultures were analysed by serotyping of flagellar antigens [37, 38] and characterised by AFLP analysis as described previously [27]. AFLP analysis comprised the digestion of total DNA with *Hin*dIII, ligation with adapters ADH1 and ADH2 followed by amplification with primer HI-A. Brief epidemiological details on the origins of the isolates are shown in Table 4.

Analysis of DNA fragments

DNA fragments produced by PCR were separated on 1% (α-toxin and enterotoxin PCR for *C. perfringens*), 1.5% (AFLP of *C. novyi* and *C. perfringens*) or 2% (AFLP of *B. cereus*) agarose-ethidium bromide electrophoresis gels, observed with UV transillumination and fluorescent bands were recorded with type 667 film

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(Polaroid, St Albans, UK). Images of the ethidium bromide-stained gels were analysed with Bionumerics version 2.0 (Applied Maths, Kortrijk, Belgium), by the unweighted pair group method using arithmetic means (UPGMA) with the Dice coefficient. Capital letters (*C. novyi*), lower case letters (*C. perfringens*) or roman numerals (*B. cereus*) arbitrarily designated different AFLP patterns. Each AFLP banding pattern was defined as having more than two bands different from any other profiles for *C. perfringens* and *B. cereus* as described previously [26, 27]. The analysis of the *C. novyi* banding patterns is described later.

Results

C. novyi cultures

Forty-three isolates of *C. novyi* were analysed; 23 cultures were from 22 injecting drug users with *C. novyi* infection presenting during 1999 (one case), 2000 (20 cases) and 2001 (one case) (Table 1). Because AFLP typing of *C. novyi* had not been attempted previously, other unrelated cultures were also included for comparison and these comprised three isolates from the NCTC; seven cultures from the Wellcome Bacterial Collection; two wound, one blood and one food isolate deposited in the PHLS Anaerobe Reference Unit in 1988, 1989, 1991 and 1995; five isolates from animals; and one further isolate from a leg abrasion of a patient during 2000 who was not known to be a drug user (Table 2).

AFLP analysis was performed on DNA from the 43 cultures and amplified by PCR with eight primers (primers HI-A, HI-C, HI-G and HI-T for the *Hin*dIII digestion, and EC-A, EC-C, EC-G and EC-T with the *Eco*RI digestion). Satisfactory banding patterns were obtained with primers HI-C, HI-G, EC-A, EC-C and EC-T. Banding patterns of the amplified DNA obtained with HI-A, HI-T and EC-G were poor and indistinct and are not considered further. The numbers of DNA fragments per pattern obtained with the remaining five primers were: primers HI-C, HI-G and EC-T three-to-five fragments; primer EC-A, three-to-eight fragments; primer EC-C, four-to-six fragments. The numbers of different banding patterns differing by more than one band and obtained with these five primers were: 10 patterns (HI-C), 10 (HI-G), 18 (EC-A), 12 (EC-C) and 6 patterns (EC-T). Representative banding patterns for each of the primers are shown in Fig. 1.

The reproducibility of the system was assessed initially by testing duplicate DNA extractions from three cultures with all five primers; identical banding patterns were obtained from each isolate. Two separate isolates were obtained from patient 8 (Table 1) and both gave identical banding patterns with all five primers. All cultures were tested with primer HI-G on two or more occasions and eight cultures were tested with primers HI-C, EC-A, EC-C and EC-T on two or more occasions; identical banding patterns were obtained for each culture in all instances.

An overall similarity was calculated based on the five sets of AFLP results and all isolates were assigned to one of 23 AFLP types based on >95% UPGMA/Dice similarity (Tables 1 and 2). A multi-dimensional scaling diagram showing a combined representation of the five sets of UPGMA/Dice similarity matrices is shown in Fig. 2. Of the 22 infected drug users from whom *C. novyi* was isolated, 18 cases occurred within the outbreak period (1 April–31 August 2000) previously defined [15]. Of these, eight definite and five probable cases occurred in Scotland; one definite, one probable and one possible case occurred in England; one definite case occurred in the Republic of Ireland; and a further definite case occurred in Norway during the outbreak period [28]. Of the remaining four injection-related infections, one possible and one definite case occurred in England in December 1999 and February 2000 respectively, a probable case occurred in the Republic of Ireland in October 2000, and a probable case in England in January 2001 (Table 1).

Among *C. novyi* isolates from the 22 drug users, cultures from 17 of the 18 patients (from Scotland, England, the Republic of Ireland and Norway) during the April–August 'outbreak period' yielded AFLP pattern A: 11 of these cases had a definite and six a probable status. The *C. novyi* from the remaining case during the outbreak period (patient number 20) was of possible status and yielded a unique AFLP profile designated as type C. *C. novyi* cultures from three of the four patients outside the outbreak period (patients 5, 23 and 34; December 1999, October 2000 and January 2001, respectively) who were of possible or probable case status gave unique AFLP patterns designated as B, D and K. The *C. novyi* from the remaining patient, who was of definite status, and whose infection occurred in England in February 2000, yielded AFLP pattern A (Table 1).

Other patterns were generated by 19 of the 20 additional isolates (designated as types E–J and L–W), which were not related to drug users (Table 2). The remaining *C. novyi* isolate from a leg abrasion of a patient in the UK who died during 2000 yielded AFLP pattern A (Table 2).

Those cultures with the closest similarities to AFLP type A in the multi-dimensional scaling diagram (and therefore of most similar patterns) were the Wellcome Bacterial Collection cultures CN 930 and CN 902, plus NCTC 6738 and NCTC 13029.

C. perfringens cultures

Forty *C. perfringens* cultures from 13 drug users, the residue from one syringe and two samples of heroin

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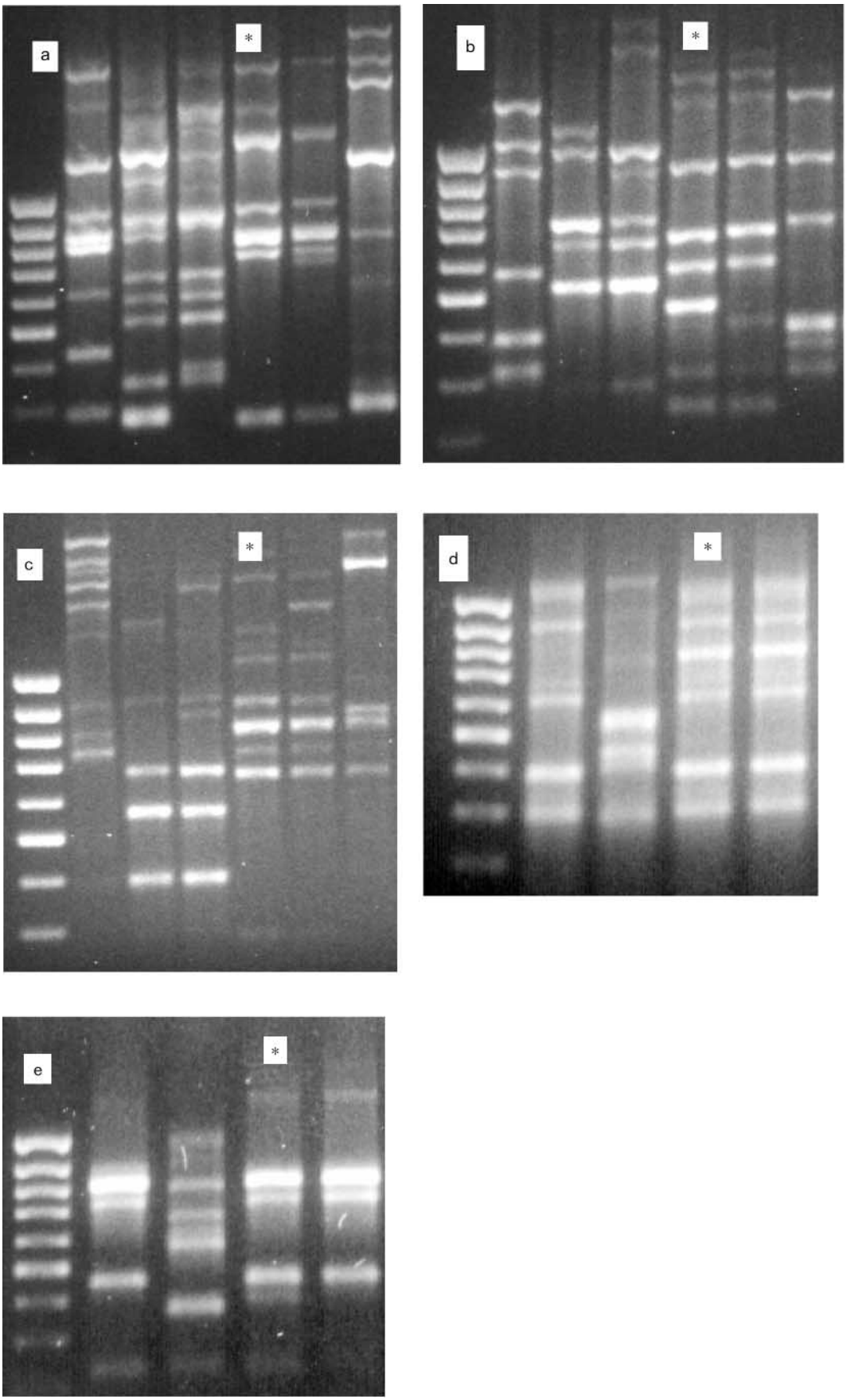

Fig. 1. Electrophoresis gels of representative *C. novyi* AFLP banding patterns. Primers used were: **a**, EC-A; **b**, EC-C; **c**, EC-T; **d**, HI-C; **e**, HI-G. *Isolates with AFLP type A. Molecular weight markers are shown on the left side of each gel.

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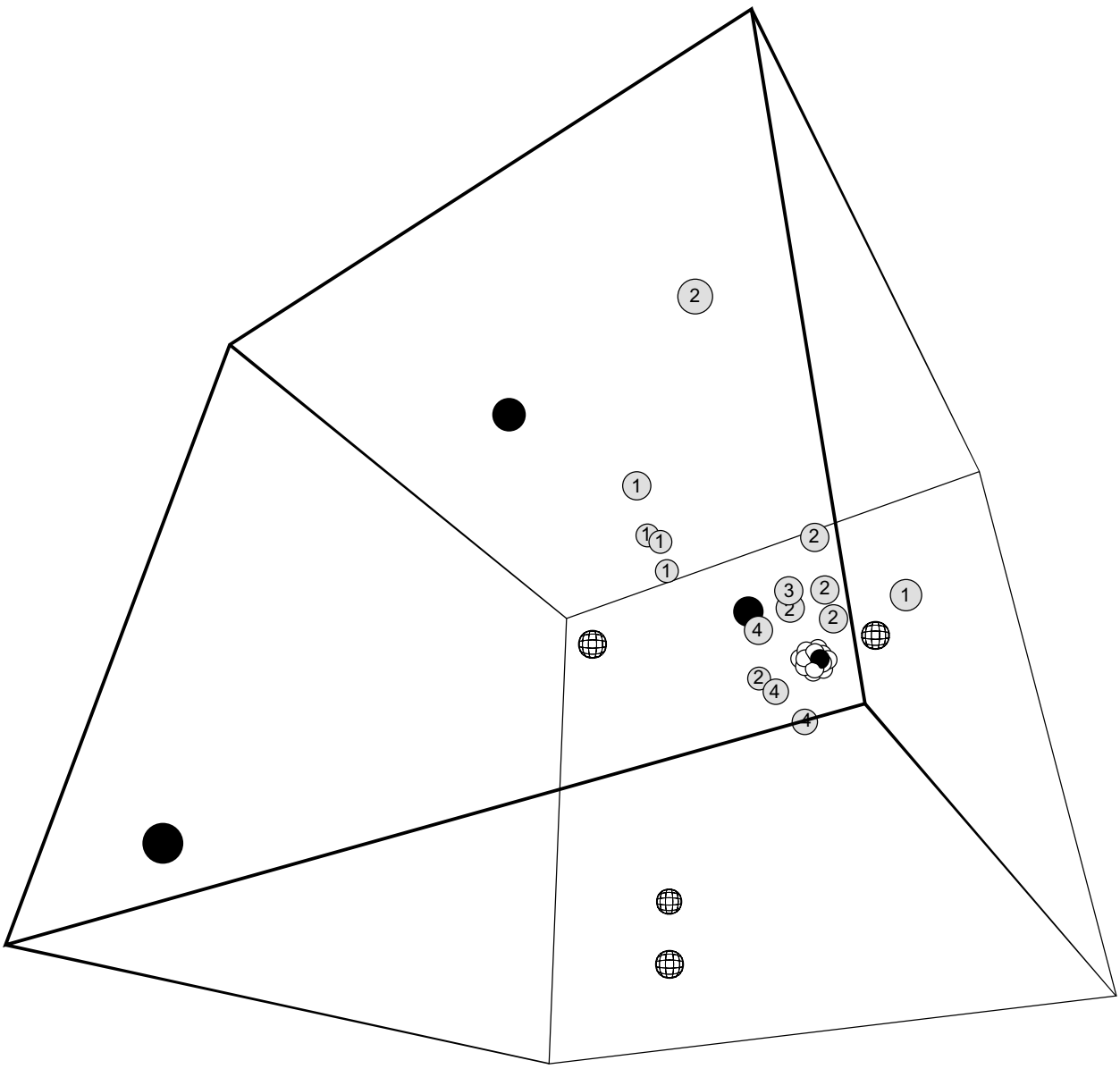

Fig. 2. Multi-dimensional scaling diagram of *C. novyi* AFLP based on a combined set of five similarity matrices obtained from primers HI-C, HI-G, EC-A, EC-C and EC-T. Sources of isolates: ○, drug users, AFLP type A; ⊕, drug users, other AFLP types; ●, other human infections; ①, animals; ②, historical isolates from human injections (Wellcome collection); ③, food; ④, NCTC cultures. One of the historical isolates is hidden from view.

were analysed. Among the cultures from the 13 drug users, 6 were collected during life, 4 at necropsy, and the remainder could not be classified. One of the heroin samples was obtained in Scotland as part of the investigations of the April–August outbreak [15]. The isolates from the syringe contents and the second heroin sample were associated with deaths in drug users but were not known to be associated with this outbreak.

A fragment of the α-toxin was amplified from DNA isolated from all 40 cultures (further confirming their identity as *C. perfringens*), and all were negative for the enterotoxin gene fragment except for one of the two isolates from patient 27.

Nineteen (48%) of the 40 isolates were serotypable and nine different serotypes were detected among these (Table 3). Multiple isolates were available from eight sources: three of the sources (cases 15, 18 and 27 plus the syringe residue) all yielded non-serotypable cultures, and the remaining four sources (cases 1, 17 and 33 plus the heroin from England) yielded typable as well as non-typable isolates.

AFLP analysis was performed and the banding patterns were assigned to 16 groups based on >70% UPGMA/Dice similarity. The same AFLP types were detected among isolates from five of the eight sources where multiple isolates were tested, i.e., those from patients 15, 17 and 33, as well as the syringe contents and the

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heroin isolates in England. Of the remaining three patients from whom multiple isolates were tested (cases 8, 18 and 27), different AFLP types were obtained. The two isolates from patient 27 were already recognised as genetically different because a fragment of the enterotoxin gene was identified from one of them. Isolates from patient 8 were collected at necropsy, but the isolates from patients 18 and 27 were collected during life. Among the 16 *C. perfringens* isolates from patient 8, four different types were recognised, i.e., AFLP type h serotype 18,29 (one isolate), AFLP type b serotypes TW8 and non-typable (10 isolates), AFLP type c serotypes 30,33 and non-typable (four isolates) and AFLP type c serotype TW8 (one isolate). There was almost complete agreement in the groups of cultures identified by serotyping and AFLP analysis, e.g., eight of the nine isolates from patient 8 identified as serotype TW8 were classified as AFLP type b and both the heroin serotype 25 isolates were of AFLP type f.

Among the 13 drug users, six were classified as definite, four as probable and one as possible cases; there was no information on the remaining two cases. Among the 16 *C. perfringens* AFLP types, 14 were unique to their individual sources; this included the AFLP type d which was recognised only in the *C. perfringens* isolate from heroin examined in Scotland during the investigation of drug-related infections. The remaining two AFLP types were common to two groups: AFLP type a was found in isolates from patients 15 and 27 and the syringe residues, and AFLP type c in patients 8, 26 and 27. Despite the similar clinical presentation in these patients, overall results from both AFLP and serotyping indicated that these *C. perfringens* isolates were diverse. This was in marked contrast to the data with respect to the lack of diversity among the *C. novyi* cultures from drug users.

B. cereus cultures

Six isolates of *B. cereus* were tested, four from drug users and two from heroin (Table 4). One of the heroin samples was collected from patient 2 [18]. The second isolate from heroin was obtained from a sample collected in England during 2000 but was not known to be associated with infections in England or Scotland. All cultures except one (patient 3) were non-typable by serotyping, and four distinct AFLP patterns were identified (Table 4). Identical AFLP patterns were obtained from the isolates from the blood and heroin of patient 2 [18], and this pattern differed by only one band from that obtained from patient 4 (Table 4). Previous analysis has indicated that a single band difference is within the limit of reproducibility of this system for *B. cereus* [27] and hence the pattern from patient 2 is designated II^{-1} to indicate this difference. No other common AFLP patterns were recognised among the six isolates.

Discussion

C. novyi, C. perfringens and *B. cereus* are widespread in soil and in the environment [39]. Furthermore, *C. perfringens* is common as part of the intestinal microflora of animals and man, indeed this bacterium is probably the most widely occurring bacterial pathogen in the environment [39]. Hence, reliable typing techniques for these three bacterial species are essential for the tracking of strains in the environment and recognising their occurrence in isolates from infected patients. The purpose of this study was to apply molecular typing techniques and, where available, conventional phenotypic characterisation tests to *C. novyi, C. perfringens* and *B. cereus* isolates obtained during 2000 from cases of infection among illegal drug injectors. There have been no previous reports of the characterisation of endospore-forming bacteria associated with infections in injecting drug users; hence, baseline date for comparison with this study are not available.

Although serotyping schemes are available for *B. cereus* and *C. perfringens,* these systems were both developed originally for analysis of isolates from incidents of food poisoning [31–34, 37, 38]. In the present study, both *B. cereus* and *C. perfringens* generated a high proportion of non-serotypable results, hence these schemes are not ideally suited for analysis of cultures from the sources described here. No typing systems have been described previously for *C. novyi* type A.

A previous report described the application of AFLP analysis to cultures of *B. cereus* and *C. perfringens* [26, 27] and it was encouraging that the method was equally suitable for AFLP analysis of a previously untested species, i.e., *C. novyi*.

The characterisation of two *B. cereus* isolates from the blood and heroin of an individual patient in Scotland during 2000 by serotyping and AFLP analysis together with sequence information on three separate genes has been reported previously [18], and these two isolates were found to be indistinguishable by AFLP. In the present study, these isolates were found to differ from those recovered from two other cases of infection among injecting drug users and another sample of heroin, strengthening the causal association between the heroin and the blood culture isolates described previously [18]. Previous analysis by AFLP of *B. cereus* from incidents of food poisoning suggested that considerable variation in this bacterium can be detected by this technique [27]. Hence, it is intriguing that *B. cereus* isolates of indistinguishable AFLP profiles were obtained from two cases of infection among drug users, both of which occurred in the same geographical region of Scotland in June 2000. A common causal link by exposure to the same contaminated source is possible for these two patients.

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Previous analysis of *C. perfringens* from cases of food poisoning by AFLP suggested that genetic diversity in this species could be detected by this technique [26]. The present report supports the use of this technique to analyse *C. perfringens* from other sources. Among the 40 *C. perfringens* isolates tested, variation in type was detected by both serotyping and AFLP analysis. However, serotyping has the considerable drawback that non-typable *C. perfringens* cultures occur and this can be associated with 'roughness' resulting from loss of the capsular antigen [33]. Both serotypable and non-serotypable cultures can be analysed by AFLP and, in this study where multiple cultures were obtained from a common source, the same strains can be identified by AFLP. However, there was almost complete agreement in the groups of cultures identified by serotyping and AFLP techniques, albeit that an incomplete analysis was obtained by serotyping because of the high proportion of non-serotypable isolates.

A diverse range of *C. perfringens* types were recognised from the drug users examined here and there was very limited commonality between the patients' isolates despite having a similar clinical presentation. Because of the widespread distribution of this species, several strains of *C. perfringens* may be present simultaneously in both environmental samples and an individual patient's faeces [39]. Contamination of post-mortem tissue by *C. perfringens* from the patient's own gut contents is well recognised [40]. Some of the cultures were obtained at necropsy, so this finding may reflect that of the patient's own flora and not that in the infected lesion. However, as isolates were also obtained from samples taken during life, these may have derived from a contaminated source associated with drug injection as well as from the patient's own microflora. The AFLP data, and to a lesser extent the results from serotyping, did not suggest that either a single strain of *C. perfringens* was involved with the clinical presentation or that there was contamination of a source common to the drug users investigated here.

In contrast to the AFLP results for *C. perfringens*, AFLP analysis of *C. novyi* indicated that all except one of the isolates from injecting drug users during the outbreak period comprised a homogeneous group. Because other typing systems are not available for *C. novyi*, confirmation of this observation by an independent method was not possible. However, it is probable that these results reflect a truly homogeneous group because identical AFLP results were obtained on replicate testing and with two different restriction endonucleases together with five different PCR primers. Furthermore, partial confirmation of this grouping was obtained by 16S rDNA sequencing in that all of the AFLP type A were 100% identical to EMBL X68188 (NCTC 538). However, among 19 cultures of other AFLP types, 5 showed differences from the EMBL sequence (Brazier *et al*., unpublished data).

Of the *C. novyi* isolates cultured from cases defined as definite, all yielded AFLP profiles of type A, and this finding raises the possibility that the April–August outbreak may have started earlier than previously suspected, because one of these cases occurred in England in February 2000. The classification of all cases due to AFLP type A as probable or definite may be complicated by early medical and surgical intervention and increased awareness of the clinical presentation during the course of the outbreak, resulting in the curtailment of subsequent toxin-mediated clinical deterioration. However, with the exception of a single clinical isolate from a non-drug user in 2000, all of the patients infected with *C. novyi* AFLP type A presented with severe illness (classified as definite or probable). In contrast, the infections in the four other drug users whose *C. novyi* isolates were of four different and distinct AFLP types were classified as possible or probable, suggesting that *C. novyi* of AFLP type A may have biological differences when compared with other strains described here. Information was available for two of the four *C. novyi* cultures which were identified as having the closest similarities to AFLP type A, and both of these (CN 930 and CN 902) were from cases of severe infection.

The considerable interest of both the clinical and lay communities may have led to ascertainment bias in the examination of soft tissue infection (particularly for the isolation of strict anaerobic bacteria), especially among injecting drug users during the outbreak period. The increased awareness may have led to more detailed identification of *Clostridium* spp. than would otherwise have been performed. However, despite the increased awareness of soft tissue infections in drug users due to *Clostridium* spp., the consistent finding among definite cases of a single *C. novyi* AFLP type A adds to clinical and epidemiological observations about this outbreak. Investigation of the single non-drug injector who died and was infected with *C. novyi* AFLP type A did not reveal additional risk factors, although this individual was a keen gardener and had frequent contact with horses and domestic animals [41]. It is intriguing that this patient yielded the only other source of this AFLP type, and was resident within the UK at the same time as infections in drug users occurred. This observation highlights the need for intensive microbiological investigation (including strict anaerobic culture) for the investigation of patients with unexplained morbidity and mortality.

The majority of cases in this outbreak may have been linked by a common source contaminated by *C. novyi* AFLP type A. However, *C. novyi* was not isolated from any sources associated with drug injection, including heroin seized during 2000 [15, 42]. Alternatively, contamination of multiple *C. novyi* strains may have occurred, followed by clonal selection of a single *C. novyi* AFLP type which either had an adaptive advantage in spore germination, survival or growth

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following the drug preparation and injection procedure, or produced a more severe clinical presentation. The molecular epidemiological analysis reported here cannot distinguish between either a common source outbreak or clonal selection of *C. novyi* with specific virulence or survival properties; both possibilities are consistent with the data presented here.

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