INFECTION IN INJECTING DRUG USERS

An investigation into the microflora of heroin

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In 2000, an unusual increase of morbidity and mortality among illegal injecting drug users in the UK and Ireland was reported and *Clostridium novyi* was identified as the likely source of the serious infection, although infections due to *C. botulinum* and *Bacillus cereus* were also reported. Because heroin was a possible source of infection, this study investigated the microflora of heroin samples seized in England during 2000 and 2002. Two methods were developed for the examination of the microflora of heroin. The first consisted of suspension of the drug in maximum recovery diluent (MRD) which was inoculated directly into Clostridium Botulinum Isolation Cooked Meat Broth (CBI). The second method rendered the heroin soluble in citric acid, concentrated particulate material (and bacterial cells) by filtration and removed heroin residues by washing with citric acid and phosphate-buffered saline before placing the filter in CBI broth. Duplicate CBI broths from both methods were incubated without heating and after heating at 60°C for 30 min. Subcultures were made after incubation for 7 and 14 days on to eight different solid media. The methods were evaluated with heroin samples spiked with either *C. botulinum* or *C. novyi* spore suspensions; recovery of 10 spores in the original sample was demonstrated. Fifty-eight heroin samples were tested by citric acid solubilisation and 34 by the MRD suspension technique. Fifteen different gram-positive species of four genera were recognised. No fungi were isolated. Aerobic endospore-forming bacteria (*Bacillus* spp. and *Paenibacillus macerans*) were the predominant microflora isolated and at least one species was isolated from each sample. *B. cereus* was the most common species and was isolated from 95% of all samples, with *B. licheniformis* isolated from 40%. Between one and five samples yielded cultures of *B. coagulans*, *B. laterosporus*, *B. pumilus*, *B. subtilis* and *P. macerans*. *Staphylococcus* spp. were isolated from 23 (40%) samples; *S. warneri* and *S. epidermidis* were the most common and were cultured from 13 (22%) and 6 (10%) samples respectively. One or two samples yielded cultures of *S. aureus*, *S. capitis* and *S. haemolyticus*. The remainder of the flora detected comprised two samples contaminated with *C. perfringens* and two samples with either *C. sordellii* or *C. tertium*. Multiple bacterial species were isolated from 43 (74%) samples, a single species from the remaining 15. In 13 samples *B. cereus* alone was isolated, in one *B. subtilis* alone and in one sample *B. pumilus* alone. *C. botulinum* and *C. novyi* were not isolated from any of the heroin samples. Recommendations for the optimal examination of the microflora of heroin are given.

Introduction

Several viral and bacterial infections are 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 infecting injecting drug users (IDUs). 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 endospores and their resistance to the process of drug preparation [9].

In 2000, an unusual increase of morbidity and mortality among IDUs in the UK and Ireland was reported. During the intensive investigation more than 108 cases with at least 43 deaths were recognised between April and Aug. 2000 [10–13]. *C. novyi* type A was the likely cause of the more serious illnesses [12,14–16], although other endospore-forming bacteria were also associated with some cases including *C. perfringens* [16] and *B. cereus* [17]. Cases of wound botulism were also reported in IDUs during 2000 and these represent the first recognition of this condition in the UK [18–21].

During the course of the investigations among IDUs during 2000, *B. cereus*, *C. botulinum* and *C. perfringens* were isolated from either heroin or drug injection equipment ([17] PHLS unpublished data). Because of the possibility that heroin was the source of infection, especially with *C. novyi*, investigations were established to develop methods for the analysis of the microbial flora of heroin. There are relatively few data on the microflora of heroin [22–25] and none available for drugs seized in the UK. During drug injection, heroin is solubilised in acidic solutions. Because heroin has been reported to have marked antimicrobial properties [26], methods were devised to culture from both suspensions of the drug as well as solutions made by dissolving the drug in citric acid and attempting to wash away antimicrobial residues by filtration. This report describes the results obtained from the characterisation of the microflora of heroin seized in England during 2000 and 2002.

Materials and methods

Microbiological media and reagents

A citric acid (C0706; Sigma-Aldrich) 10% w/v solution was prepared in distilled water and filter sterilised through a 0.2- μ m membrane filter into a sterile bottle. This solution was prepared fresh each time of use. Maximum Recovery Diluent (MRD; peptone saline diluent CM733; Oxoid), horse blood 5% in columbia blood agar base (CBA; Oxoid), Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA, Oxoid), Sabouraud dextrose agar (Sab; CM41; Oxoid), Neomycin Blood Agar (NeoBA; Oxoid) and phosphate-buffered saline (PBS; Dulbecco A phosphate-buffered saline; Oxoid) were prepared according to the manufacturers' instructions.

Clostridium Botulinum Isolation (CBI) Cooked Meat Broth was prepared as described previously [27]. On the day of use, the broths were steamed for 15 min (with loose lids), cooled to room temperature (with tightened lids) and sterile lysozyme was added to give a final concentration of 10 μ g/ml.

All agar plates used for anaerobic growth were incubated in either a gas jar with an anaerobic gas generation system (Oxoid) or in an anaerobic cabinet (Don Whitley Scientific) with an atmosphere of N₂ 80%, H₂ 10%, CO₂ 10%. All agar plates for anaerobic culture were pre-reduced in either an anaerobic jar or an anaerobic cabinet overnight. CBI agar containing egg-yolk emulsion and antibiotic (ACBI) agar containing cycloserine sulphamethoxazole trimethoprim egg-yolk emulsion were prepared as described previously [27]. BMRL medium with horse blood (BMBH) was prepared as described elsewhere [28].

Heroin samples

Samples of heroin seized by the Merseyside Police during 2000 and 2002 were made available for analysis. All samples were received at the Central Public Health Laboratory (CPHL) under police escort and stored securely during the course of this investigation. The samples were taken from items seized as exhibits in criminal prosecutions in the Merseyside area during and after the period of the heroin-related deaths. Heroin analysis was performed for the preservation of evidential integrity. With support from local and regional public health officials and advice from the Crown Prosecution Service, Chief Police Officers for the area agreed to provide samples, and CPHL obtained Home Office authority to be in possession of drugs scheduled as class 1, 2, 3 and 4 under the Misuse of Drugs Act 1971. At the conclusion of this investigation, all material was disposed of under Home Office supervision.

Method 1, suspension in MRD

A 0.1-g sample of heroin was suspended in 10 ml of MRD and 0.1 ml was inoculated directly on to two CBA, one PEMBA and one Sab agar plates. Three plates were incubated aerobically at 37°C and one of the CBA plates was incubated anaerobically at 30°C. Two 1-ml samples of the suspensions of heroin in MRD were each filtered through a sterile 0.2- μ m membrane filter and inoculated into a CBI cooked meat broth. One broth was heated at 60°C for 30 min in a water bath and both were incubated at 30°C. Heated and unheated broths were subcultured after 7 and 14 days on to pre-reduced ACBI, CBI, NeoBA, BMBH and CBA plates (incubated anaerobically) and CBA incubated aerobically. All plates were incubated at 30°C, except the NeoBA which was incubated anaerobically at 37°C. Plates were examined daily for 4 days except for Sab which was examined after 7 days.

Method 2, citric acid solubilisation

A 0.1-g sample of heroin was dissolved in citric acid. A minimum of 50 ml was used, although larger volumes were required to dissolve all solid material in some samples. From this solution, 0.1 ml was spread plated on to each of ACBI, CBI, NeoBA, BMBH and CBA plates (which were pre-reduced and incubated anaerobically at 30°C, except for NeoBA which was incubated at 37°C), and CBA, PEMBA and Sab agar plates which were incubated aerobically at 37°C. All plates were examined daily for 4 days except Sab agar, which was examined for growth up to 7 days.

Volumes of heroin dissolved in citric acid equivalent to 0.02 g were filtered through a sterile 0.2- μ m membrane filter. The filter was rinsed with 20 ml of citric acid, then with 40 ml of PBS. One inoculated filter was placed in each of two pre-reduced CBI cooked meat broths with lysozyme; one of these was heated to 60°C for 30 min in a water bath. Both broths were incubated at 30°C and subcultured at 7 and 14 days as described above for the MRD method.

Identification of isolates

Because of the large numbers of isolates obtained, representatives of each different colonial type from the different treatments were identified.

Bacillus isolates were initially identified on the basis of Gram's and spore stains, colonial morphology and growth on blood agar incubated anaerobically and aerobically. Isolates were identified to species level by methods outlined previously [29–31] in the PHLS Food Safety Microbiology Laboratory.

Lipase and lecithinase reactions of suspected clostridia on CBI plates were recorded. Clostridia were identified as gram-positive (variable) rods, catalase negative, sensitive to metronidazole and growing anaerobically but not aerobically. Isolates suspected to be *C. perfringens* (typical morphology on blood agar and positive Nagler test) were identified in the PHLS Food Safety Microbiology Laboratory. All other anaerobes were identified by the PHLS Anaerobe Reference Unit, Cardiff, as described elsewhere [31].

Isolates of *Staphylococcus* spp. were identified in the Laboratory of Hospital Infection (CPHL) as described elsewhere [32].

Method validation

For validation of the methods, spore suspensions of *C. botulinum* and *C. novyi* were prepared. Two *C. novyi* strains were used in these experiments; one was from a case of gas gangrene in 1920 (NCTC 538, National Collection of Type Cultures, CPHL, London) and the second (R14386, Anaerobe Reference Unit, PHLS,

Cardiff) was from the thigh tissue of an IDU case in 2000 [33] which was indistinguishable from other isolates from IDUs during 2000 [34]. A *C. botulinum* culture from an infected wound of an IDU (R254/02, PHLS FSML, London) isolated in 2002 was also included in this analysis. Spore suspensions were prepared by harvesting the growth from blood agar plates after incubation for 4 weeks under anaerobic conditions. Numbers of viable spores were estimated by colony counts after heat shock at 60°C for 30 min of a decimal dilution series in MRD from which 0.1-ml volumes were spread plated on to pre-reduced blood agar and incubated for 3 days anaerobically at 30°C.

To ensure the recovery of *C. botulinum* and *C. novyi* from heroin, a heroin sample in MRD or citric acid was 'spiked' with a decimal dilution series of either *C. botulinum* or *C. novyi* spore suspensions. The spiked heroin samples were filtered as described above and inoculated into CBI broths. Spore suspensions were added to give final concentrations of 10–100 000 spores in each CBI broth (both heated and unheated) and subcultured on to CBA (incubated aerobically and anaerobically), CBI and ACBI agars. Both *C. botulinum* and *C. novyi* were recognised by characteristic colonial morphology and positive lipase reaction.

To simulate the possible inhibition of *C. botulinum* or *C. novyi* growth in the liquid enrichment broths resulting from co-culture with *Bacillus* spp., vegetative cells of *B. licheniformis* (growth from two colonies suspended in MRD) and spores of either *C. botulinum* or *C. novyi* were added to heroin in MRD or citric acid. The original heroin sample was selected to already contain *B. cereus* as part of its microflora. The heroin suspensions or solutions, which had a final *C. botulinum* or *C. novyi* concentration of 10–100 spores, were filtered and inoculated into CBI broths, heated or unheated, and then subcultured after 7 and 14 days on to CBA (incubated aerobically and anaerobically) and CBI, ACBI, BMBH and NeoBA agars incubated anaerobically.

Results

Method validation

With the first experimental protocol and spiked samples of heroin, viable *C. botulinum* (strain R254/02) and *C. novyi* (R14286) were recovered from enrichment broths with 10–100 000 spores (both with and without heating) after either 7 or 14 days. Neither organism was recovered on direct subculture. A similar experiment was performed with *C. novyi* strain NCTC 538, which was recovered after incubation for 7 days in enrichment broth with both the MRD and citric acid protocols, with and without heating, when 1000 spores were spiked into the original sample.

C. botulinum (strain R254/02) and *C. novyi* (R14286)

were detected in the spiking experiment when both *B. cereus* and *B. licheniformis* were present, and were recovered from both heated and unheated broths after 7 and 14 days on all media except the CBA incubated aerobically.

In both experiments, *C. botulinum* and *C. novyi* were not recovered from uninoculated controls.

Microflora of heroin

A total of 58 heroin samples was analysed. All samples were tested by the citric acid solubilisation technique and 34 by the MRD suspension technique.

On direct inoculation, growth was recorded with only seven samples. Growth was detected only from the MRD suspension; no growth was observed following citric acid solubilisation. Among the seven samples in which bacterial growth was detected after direct inoculation, growth was observed on CBA incubated aerobically only where 1–27 colonies were recorded; *B. cereus* alone was identified in six samples and *B. licheniformis* alone in one sample.

The list of all bacterial species isolated from the 58 samples by all treatments is shown in Table 1. Fifteen different gram-positive species of four genera were identified. Two isolates (one *Bacillus* sp. and one *Staphylococcus* sp.) were non-viable before full identification could be performed and were identified to genus level only. No yeasts or moulds were isolated. Aerobic endospore-forming bacteria (*Bacillus* spp. and *Paenibacillus macerans*) were the predominant microflora isolated and at least one of these species was isolated from each sample. *B. cereus* was the most common bacterium and was isolated from 95% of samples, followed by *B. licheniformis* (40%). The next most common components of the microflora detected were *Staphylococcus* spp. which were isolated from 23

(40%) samples. *S. warneri* and *S. epidermidis* were the most commonly isolated and were detected in 13 (22%) and 6 (10%) samples, respectively. The remainder of the flora isolated comprised three species of *Clostridium* (*C. perfringens*, *C. sordellii* and *C. tertium*) which were isolated from four different samples of heroin. Multiple bacterial species were isolated from 42 (74%) samples; a single species was isolated from the other 16. *B. cereus* alone was isolated from 14 of the samples, *B. subtilis* alone from one and *B. pumilus* alone from one sample.

The treatment (MRD, citric acid, heated or unheated) and day of isolation for all the bacterial species is shown in Table 2. No growth was detected after either MRD or citric acid treatment in the heated broths from 20–47% of the samples, but was detected in all except one broth without heating. Seven different bacterial species were isolated after the MRD treatment and 14 after the citric acid treatment. No major differences were detected between the MRD or citric acid treatments for the isolation of *Bacillus* spp. However, with both the MRD and citrate treatments, *B. cereus* was more likely to be grown from unheated than heated broths at both 7 and 14 days, and *B. licheniformis* was more likely to be grown from heated broths at day 7 but not at day 14. *C. perfringens* and *C. sordellii* were isolated only from heated broths after both MRD and citric acid treatment. *C. tertium* was grown from one sample from an unheated broth only after citric acid treatment. Except for two isolates of *S. warneri*, the remainder of the *Staphylococcus* isolates were detected only after citric acid treatment in the unheated CBI broths, although *S. epidermidis* and *S. warneri* were isolated from one sample each after heat treatment.

The media with which the different species were isolated are shown in Table 3. *B. cereus* was isolated on all media, although less often on the anaerobically incubated antibiotic-containing media (i.e., ACBI and NeoBA). The remaining *Bacillus* spp. and the single *P. macerans* isolate were isolated only from the non-selective media incubated both anaerobically and aerobically, and did not grow on PEMBA, ACBI or NeoBA. The three *Clostridium* spp. were isolated on all anaerobically incubated media, except *C. sordellii* which was not isolated on CBA or NeoBA, and *C. tertium* which was not isolated on ACBI agar. *Staphylococcus* spp. were isolated from all media except ACBI and NeoBA.

Discussion

At the onset of this study, there were limited data on the optimal method for the examination and characterisation of the microflora of heroin. Tuazon and colleagues [22] examined 100 samples of heroin collected in Washington during 1972, Shamsuddin *et al.* [23] examined 49 samples again from Washington

Table 1. Species of bacteria isolated from 58 samples of heroin

Species of bacteria	Number of samples (%)
<i>B. cereus</i>	55 (95)
<i>B. coagulans</i>	1 (2)
<i>B. licheniformis</i>	23 (40)
<i>B. laterosporus</i>	1 (2)
<i>B. pumilus</i>	5 (9)
<i>B. subtilis</i>	1 (2)
<i>Bacillus</i> sp.*	1 (2)
<i>C. perfringens</i>	2 (3)
<i>C. sordellii</i>	1 (2)
<i>C. tertium</i>	1 (2)
<i>P. macerans</i>	1 (2)
<i>S. aureus</i>	1 (2)
<i>S. capitis</i>	2 (3)
<i>S. epidermidis</i>	6 (10)
<i>S. haemolyticus</i>	1 (2)
<i>S. warneri</i>	13 (22)
<i>Staphylococcus</i> sp.*	1 (2)

*Isolates not fully identified.

Table 2. Treatment and day of isolation of individual bacterial species from 58 samples of heroin

Species isolated	Number of isolates after					
	7 days			14 days		
	Heated	Unheated	Total	Heated	Unheated	Total
MRD suspension method (n = 34)						
<i>B. cereus</i>	13	30	30	13	31	33
<i>B. coagulans</i>	0	0	0	0	0	0
<i>B. licheniformis</i>	4	2	6	5	9	14
<i>B. laterosporus</i>	0	0	0	0	0	0
<i>B. subtilis</i>	0	0	0	0	1	1
<i>B. pumilus</i>	0	1	1	1	1	2
<i>C. perfringens</i>	1	0	1	1	0	1
<i>C. sordellii</i>	1	0	1	1	0	1
<i>C. tertium</i>	0	0	0	0	0	0
<i>P. macerans</i>	0	0	0	0	0	0
<i>S. aureus</i>	0	0	0	0	0	0
<i>S. capitis</i>	0	0	0	0	0	0
<i>S. epidermidis</i>	0	0	0	0	0	0
<i>S. haemolyticus</i>	0	0	0	0	0	0
<i>S. warneri</i>	0	2	2	0	2	2
No growth	16	0	0	13	1	1
Citric acid solubilisation method (n = 58)						
<i>B. cereus</i>	36	54	54	35	52	55
<i>B. coagulans</i>	1	1	1	0	0	0
<i>B. licheniformis</i>	10	5	15	13	12	25
<i>B. laterosporus</i>	0	0	0	1	1	1
<i>B. subtilis</i>	0	0	0	0	0	0
<i>B. pumilus</i>	1	1	2	2	5	5
<i>C. perfringens</i>	1	0	1	1	0	1
<i>C. sordellii</i>	1	0	1	1	0	1
<i>C. tertium</i>	0	1	1	0	1	1
<i>P. macerans</i>	1	0	1	0	0	0
<i>S. aureus</i>	0	1	1	0	1	1
<i>S. capitis</i>	0	1	1	0	2	2
<i>S. epidermidis</i>	1	2	3	1	6	6
<i>S. haemolyticus</i>	0	1	1	0	1	1
<i>S. warneri</i>	2	8	10	4	9	13
No growth	12	0	0	13	0	0

Table 3. Media used to recover different species of bacteria from 58 samples of heroin

Species	Number of samples with isolation on						
	Total	CBA	PEMBA	CBI	ACBI	BMBH	NeoBA
<i>B. cereus</i>	55	55	33	55	28	55	10
<i>B. coagulans</i>	1	1	0	1	0	1	0
<i>B. licheniformis</i>	23	23	0	23	0	23	0
<i>B. laterosporus</i>	1	1	0	1	0	1	0
<i>B. pumilus</i>	5	5	0	4	0	0	0
<i>B. subtilis</i>	1*	0	0	0	0	0	0
<i>C. perfringens</i>	2	2	0	2	2	2	2
<i>C. sordellii</i>	1	0	0	1	1	1	0
<i>C. tertium</i>	1	1	0	1	0	1	1
<i>P. macerans</i>	1	1	0	1	0	1	0
<i>S. aureus</i>	1	1	0	0	0	1	0
<i>S. capitis</i>	2	2	1	2	0	0	0
<i>S. epidermidis</i>	6	6	0	5	0	4	0
<i>S. haemolyticus</i>	1	1	0	0	0	0	0
<i>S. warneri</i>	13	13	4	9	0	13	0

PEMBA, polymyxin pyruvate egg-yolk mannitol bromothymol blue agar (aerobic); CBI, Clostridium Botulinum Isolation Agar; ACBI, Antibiotic Clostridium Botulinum Isolation Agar; BMBH, BMRL agar with horse blood; NeoBA, neomycin blood agar (all anaerobic).

*Isolated from aerobically incubated CBA only.

in 1981 and Moustoukas *et al.* [25] examined 31 samples obtained in New Orleans during 1979–1980. The methods used were direct plating together with adding heroin directly to thioglycolate [22], trypticase

soy [23] or chopped meat glucose [25] broths and subculture on to solid selective media after incubation for 4–14 days. Antimicrobial activity of heroin was noted, especially against staphylococci [25, 26]. Two

methods were developed in the present study for analysis of the microflora of heroin. One consisted of suspending heroin in MRD broth, which was inoculated directly into a CBI broth. The second method consisted of solubilising the drug in citric acid and recovering insoluble residues (including bacterial cells) free of heroin by filtration and washing with further volumes of citric acid and then PBS. The results presented here are consistent with antibacterial activities of heroin in that more bacterial species (especially the staphylococci) were isolated by the citric acid solubilisation technique than by suspension of the drug in MRD. This is despite the fact that the MRD procedure described here used five times more heroin from each sample than the citric acid method. None of the previous studies [22, 23, 25] used heating as a selective procedure, which also has the effect of heat-shocking (activating) bacterial endospores. The present study demonstrated the usefulness of this technique by the isolation of the small numbers of clostridia, although treatment to higher temperatures (70–80°C) has been advocated by others for the isolation of this group of organisms [35].

Among the 100 heroin samples examined in the USA in 1972 [22], at least one micro-organism was isolated from 89% of samples, 44% as a single organism only. The four most common components of the microflora of the heroin examined in the USA were *Bacillus* spp. from 29% of the samples, *Aspergillus* spp. from 24%, coagulase-negative staphylococci from 17% and *C. perfringens* from 10% [22]. Similar results were obtained in the two other studies in the USA [23, 25], in which no organisms were recovered from 39% and 69% of samples and bacteria (*Bacillus* spp. plus a small number of gram-positive cocci) and fungi (*Aspergillus* and *Penicillium* spp.) were the only organisms isolated. This is similar to the data presented here for the range of organisms isolated, with the exception of fungi, which were not isolated from any samples examined here. However, in this study, all samples were shown to be contaminated with at least one species of *Bacillus* (*B. cereus* followed by *B. licheniformis* were the most common), 40% of samples contained a *Staphylococcus* sp. (almost all of which were coagulase-negative) and a small number of other species (*P. macerans*, *C. perfringens*, *C. sordellii* and *C. tertium*) were also isolated. More than one bacterial species was isolated from 74% of the samples examined here. Although information is not available to categorise the type of heroin previously examined, a similar but more extensive microflora was detected here than described previously [22], suggesting advantages of the methods described here.

Based on the data presented here, subculturing at 7 and 14 days is necessary because some organisms were isolated only on one of these occasions, and no additional advantages were found from cultivation on BMBH or NeoBA agars. No additional advantage was

observed over the incubation temperature of 37°C used for the NeoBA.

A limited range of both genera and species was isolated from the heroin samples described here. Although this may reflect a limited microflora that survives in heroin, it may also reflect interactions between organisms during in-vitro cultivation which prevent recovery. The ubiquitous presence of *Bacillus* spp. in all samples makes recovery of anaerobic organisms more difficult, even on solid media with antibiotics. Because, with the exception of the effect of heating broths, there are no selective media for isolation of *C. botulinum* or *C. novyi*, growth of the heroin microflora in the non-selective broths may inhibit the growth of these organisms, although this was not demonstrated in 'spiking' experiments. The presence of a single *Bacillus* sp. in 26% of the samples is probably indicative of interactions within the non-selective broths. The possible inhibitory effects of the heroin are discussed above.

Although no direct quantitative data were obtained, the results from direct examination of the heroin described here suggest that small numbers of bacteria are present in the heroin. Heroin is prepared from opium, which is a dried latex obtained from the opium poppy. Morphine base is extracted from opium and, as this is insoluble it cannot be injected and is converted to heroin by a chemical process involving heat and treatment with acetic anhydride. Adulterants are added to heroin during processing and cutting. A limited range of microflora was detected both here and previously [22], comprising both aerobic and anaerobic endospore-forming bacteria (i.e., those belonging to the genera *Bacillus*, *Clostridium* and *Paenibacillus*) and staphylococci. Endospore-forming bacteria are all present in soil in the environment; some of them also occur in the intestinal tracts of man and animals. Hence, these are likely to be present in the original plant material, as well as in the heroin manufacturing environment and in any adulterants. It is not known if the endospore-forming bacteria survive the heroin manufacturing process, but as manufacture is performed under non-sterile conditions, post process contact with the environment is just as likely a source of contamination as the original starting material. Although robust, staphylococci do not produce endospores and are generally not as environmentally resistant as members of the genera *Bacillus* and *Clostridium*. All five species of *Staphylococcus* detected (i.e., *S. aureus*, *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. warneri*) are primarily resident on human skin (and sometimes that of other primates), with more temporary residence on domestic artiodactyls (pigs and horses) reported for *S. haemolyticus* and *S. warneri* [32]. Therefore, one likely source of contamination of this heroin is direct contact with the skin flora of the producers or processors.

During the course of the investigation of illness among

IDUs in 2000, 16 samples of heroin were collected in England and Scotland (two of which were not identified as part of the outbreak) and examined by various techniques [16, 17, 34, 35]. One of the samples was obtained from an IDU and indistinguishable isolates of *B. cereus* were obtained from both the aspirate from an injection site wound and from a heroin sample used by the patient [17, 34]. A summary of the results from these 16 samples is given in Table 4. Examination of syringe residues during 2000 also yielded cultures of *C. perfringens* [16, 34] and *C. botulinum* types A and B [35]. Results from that investigation (Table 4), together with the 58 samples described in this study did not identify any samples of heroin contaminated with either *C. novyi* or *C. botulinum* despite the fact that spiking experiments demonstrated that the procedures described here were capable of recovering these bacteria. Because only very low levels of all bacteria were detected, the possibility that *C. novyi* and *C. botulinum* were both present but were below the detection limit of the method used here cannot be excluded. However, these studies did identify contamination of heroin by *B. cereus*, *C. perfringens* and *S. aureus*, all of which were isolated from clinical samples collected from infected IDUs during the 2000 outbreak [16, 17]. *B. cereus* endocarditis and ophthalmitis from contaminated heroin or injection paraphernalia have been reported elsewhere [23, 36]. Staphylococcal endocarditis is a well recognised complication of injecting drug use [37]. The sources of *C. perfringens* and *S. aureus* for infection in IDUs are probably more likely to be the patients' own gut and skin flora. However, one case of *B. cereus* infection, described above, was associated with a contaminated sample of heroin [17]. Other sources of *C. novyi* or *C. botulinum*, such as injection paraphernalia or the acidulants used during drug injection, were not investigated. Lemon juice (also used as an acidulant in heroin abuse) has been implicated as a source of *Candida albicans* in outbreaks of systemic candidosis among IDUs [38, 39].

Table 4. Results on the examination of 18 samples of heroin collected during 2000 and examined outside this study

Species of bacteria	Number of samples
<i>B. cereus</i> *	4
<i>B. firmus</i>	1
<i>B. licheniformis</i>	7
<i>Bacillus</i> spp. [†]	2
<i>C. bifermentans</i>	1
<i>C. perfringens</i>	2
<i>C. saccharolyticum</i>	1
<i>C. sporogenes</i>	1
<i>Staphylococcus</i> sp. [†]	1

Sixteen of the samples were collected in England and Scotland during the course of the investigation of illness among IDUs during 2000 [16, 34, 35], two samples (plus wrapping for one sample) were unrelated to this outbreak.

*One *B. cereus* isolate directly linked to infection in a heroin user [17].

[†]Isolates not fully identified.

In summary, an optimised and evaluated protocol for the examination of the microflora of heroin is described. The method involves the use of citric acid solubilisation followed by filtration, washing with citric acid and PBS then inoculation of the filters into CBI broths with and without heat treatment. Broths should be subcultured after 7 and 14 days on to CBA (incubated aerobically and anaerobically), PEMBA (incubated aerobically), CBI and ACBI (incubated anaerobically). All media should be incubated at 30°C.

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References

- Horsburgh CR, Anderson JR, Boyko EJ. Increased incidence of infections in intravenous drug users. *Infect Control Hosp Epidemiol* 1989; **10**: 211–215.
- Brettell RP. Infection and injection drug use. *J Infect* 1992; **25**: 121–131.
- Peat M, Budd J, Burns SM, Robertson R. Audit of blood borne virus infections in injecting drug users in general practice. *Commun Dis Public Health* 2000; **3**: 244–246.
- Martin C, Schaller MD, Lepori M, Liaudet L. Cranial nerve palsies and descending paralysis in a drug abuser resulting from wound botulism. *Intensive Care Med* 1999; **25**: 765–769.
- Werner SB, Passaro D, McGee J, Schechter R, Vugia DJ. Wound botulism in California, 1951–1998: recent epidemic in heroin injectors. *Clin Infect Dis* 2000; **31**: 1018–1024.
- Jenselius M, Lovstad RZ, Dhaenens G, Rorvik LM. A heroin user with a wobbly head. *Lancet* 2000; **356**: 1160.
- Talan DA, Moran GJ. Tetanus among injecting drug users: California 1997. *Ann Emerg Med* 1998; **32**: 385–386.
- Ringertz SH, Hoiby EA, Jenselius M *et al.* Injectional anthrax in a heroin skin-popper. *Lancet* 2000; **354**: 1574–1575.
- Bloomfield SF, Arthur M. Mechanisms of inactivation and resistance of spores to chemical biocides. *Soc Appl Bacteriol Symp Suppl* 1994; **23**: 91S–104S.
- Christie B. Gangrene bug “killed 35 heroin users”. *BMJ* 2000; **320**: 1690.
- Anon. Serious unexplained illness among drug injectors. *Commun Dis Rep CDR Wkly* 2000; **24**: 195.
- Anon. *Clostridium novyi* is likely cause of ‘serious unexplained illness’ as cases continue to be reported. *Commun Dis Rep CDR Wkly* 2000; **24**: 213, 216.
- Bellis MA, Beynon C, Millar T *et al.* Unexplained illness and deaths among injecting drug users in England: a case control study using Regional Drug Misuse Databases. *J Epidemiol Community Health* 2001; **55**: 843–844.
- Anon. Update: *Clostridium novyi* and unexplained illness among injecting drug users: Scotland, Ireland and England April–June 2000. *MMWR Morb Mortal Wkly Rep* 2000; **49**: 543–544.
- Ryan JM, Paul J, Curtis S, Patel NK. *Clostridium novyi* infection: a fatal association with injecting drug users. *Emerg Med J* 2001; **18**: 138–139.
- McGuigan CC, Penrice GM, Black M *et al.* Outbreak of infection with *Clostridium novyi* and other sporulating bacteria in Scottish injecting drug users. *J Med Microbiol* 2002; **51**: 971–977.
- Dancer SJ, McNair D, Finn P, Kolstot AB. *Bacillus cereus* cellulitis from contaminated heroin. *J Med Microbiol* 2002; **51**: 278–281.
- Athwal BS, Gale AN, Brett MM, Youl BD. Wound botulism in UK. *Lancet* 2000; **356**: 2001–2002.

19. Khan M, Chay S, Bonner S, Brett M. Wound botulism in injecting drug users: second case in England. *Eurosurveillance Weekly* 2000; 22 June.
20. Hood J, Horne G, Sweeney G, Baird K. Third case of wound botulism in an injecting drug user. *SCIEH Weekly Report* 2000; **34**: 200.
21. Mulleague L, Bonner SM, Samuel A *et al.* Wound botulism in drug addicts in the United Kingdom. *Anaesthesia* 2001; **56**: 120–123.
22. Tuazon CU, Hill R, Sheagren JN. Microbiological study of street heroin and injection paraphernalia. *J Infect Dis* 1974; **129**: 327–329.
23. Shamsuddin D, Tuazon CU, Levy C, Curtin J. *Bacillus cereus* panophthalmitis: source of organism. *Rev Infect Dis* 1982; **4**: 97–103.
24. Alvarez M, Barturen B, Regulez P, Ponton J, Ezquerro JL, Cisterna R. Microbiological study of 30 samples of heroin. *Enferm Infecc Microbiol Clin* 1990; **8**: 231–233.
25. Moustoukas NM, Nichols RL, Smith JW, Garey RE, Egan RR. Contaminated street heroin. Relationship to clinical infections. *Arch Surg* 1983; **118**: 746–749.
26. Tuazon CU, Miller H, Shamsuddin D. Antimicrobial activity of street heroin. *J Infect Dis* 1980; **142**: 944.
27. Mills DC, Midura TF, Aron SS. Improved selective medium for the isolation of lipase positive *Clostridium botulinum* from feces of human infants. *J Clin Microbiol* 1985; **21**: 947–950.
28. Barr GD, Hudson MJ, Priddle JD, Jewell DP. Colonic bacterial proteases to IgA1 and sIgA in patients with ulcerative colitis. *Gut* 1987; **28**: 186–189.
29. Parry JM, Turnbull PCB, Gibson JR. A colour atlas of *Bacillus* species. London, Wolfe Medical Publications. 1983.
30. Gordon RE, Haynes WC, Pang CHN. The genus *Bacillus*. Agricultural Research Station Agriculture Handbook 427. Washington, DC, US Department of Agriculture. 1973.
31. Barrow GI, Feltham RKA. Cowan and Steel's manual for the identification of medical bacteria, 3rd edn. Cambridge, Cambridge University Press. 1993.
32. Kloos W. Taxonomy and systematics of *Staphylococci* indigenous to humans. In: Crossley KB, Archer GL (eds) *The staphylococci in human disease*. New York, Churchill Livingstone. 1997: 113–137.
33. McGuigan C, Roworth M. *Clostridium novyi* type A infection: a sporadic fatal case. *Scand J Infect Dis* 2002; **34**: 209–211.
34. McLauchlin J, Salmon J, Ahmed S *et al.* Amplified fragment length polymorphism (AFLP) analysis of *Clostridium novyi*, *C. perfringens* and *Bacillus cereus* isolated from injecting drug users during 2000. *J Med Microbiol* 2002; **51**: 990–1000.
35. Brazier JS, Duerden BI, Hall V *et al.* Isolation and identification of *Clostridium* spp. from infections associated with the injection of drugs: experiences of a microbiological investigation team. *J Med Microbiol* 2002; **51**: 985–989.
36. Tuazon CU, Murray HW, Levy C, Solny MN, Curtin JA, Sheagren JN. Serious infections from *Bacillus* spp. *JAMA* 1979; **241**: 1137–1140.
37. Ing MB, Baddour LM, Bayer AS. Bacteremia and infective endocarditis: pathogenesis, diagnosis and complications. In: Crossley KB, Archer GL (eds) *The staphylococci in human disease*. New York, Churchill Livingstone. 1997: 331–354.
38. Newton John HF, Wise K, Looke DFM. Role of lemon in disseminated candidiasis of heroin abusers. *Med J Aust* 1984; **140**: 780–781.
39. Podzamczek D, Ventin M, Sauca G, Fernandez-Viladrich P, Martin R, Gudiol F. Contaminated lemons as possible source of infection in heroin abusers with disseminated candidiasis. *Eur J Clin Microbiol* 1986; **5**: 477.