INFECTION IN INJECTING DRUG USERS

Isolation and identification of *Clostridium* spp. from infections associated with the injection of drugs: experiences of a microbiological investigation team

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Pathogenic species of the genus *Clostridium* may contaminate the materials used in the injection of drugs and under the right conditions may cause serious or life-threatening disease. *C. novyi* type A was implicated in an outbreak of severe infection with high mortality in injecting drug users who injected heroin extravascularly. The isolation of such highly oxygen-sensitive clostridia from clinical material may require adherence to enhanced methods and, once isolated, commercially available anaerobe identification kits alone may not give an accurate identification. Additional phenotypic tests that are useful in recognising the main pathogenic species are described. Differentiation of *C. novyi* type A from *C. botulinum* type C in reference laboratories was based on 16S rDNA sequence data and specific neutralisation of cytopathic effects in tissue culture.

Introduction

Anaerobic spore-forming bacilli of the genus *Clostridium* are ubiquitous in the environment, existing in the form of exo-spores that can remain viable indefinitely. If illicit substances used by injecting drug users (IDU) become contaminated with these spores, their injection, particularly non-intravenous injection, may result in severe illness or death such as occurred in parts of the UK during 2000. Between April and August 2000, a total of 108 cases of IDU-associated infections was identified, 60 in Scotland (including 50 in Glasgow), 22 in Dublin and 26 in England, and there were 43 deaths [1, 2]. Typically, the illness was characterised by the development of moderate to severe inflammation at or near an injection site several days after extravascular injection of heroin that had been dissolved in heated acid. In the most severe cases this was followed by hypotension, multi-organ failure and death, despite intensive treatment. The experiences gained by microbiologists involved in the investigations into this outbreak are reviewed here.

Infections among recreational drug users account for 31–58% of their hospital admissions and 19% of their deaths associated with intravenous, intramuscular or subcutaneous injection [3–5]. Bacterial infections at the site of injection can be of a minor nature with local abscess formation; however, if injected material or paraphernalia are contaminated with certain clostridial spores, severe illness may result. Once introduced into a suitably oxygen-reduced environment within live human or animal tissue, these spores can germinate and, in the right conditions, certain pathogenic species may liberate powerful exotoxins that can have severe and life-threatening effects.

The genus *Clostridium* contains several species of known potential pathogenicity for man and animals. The pathogenic clostridia include the gas gangrene group – *C. perfringens*, *C. septicum*, *C. sordellii*, *C. novyi* and *C. histolyticum* – and the causal organisms of tetanus (*C. tetani*), botulism (*C. botulinum*), clostridial food poisoning (*C. perfringens*) and antibiotic-associated diarrhoea and colitis (*C. difficile*). The contamination of wounds with clostridial species and the development of severe infection has hitherto been associated mostly with injuries sustained as a result of warfare. Gas gangrene (clostridial myonecrosis) was a

major problem in the trench warfare during World War I, when deep artillery (shrapnel) wounds were commonly contaminated with soil and faeces, two prime sources of clostridial spores [6]. However, the practice of injecting substances into muscle tissue mimics the risk factors of infection associated with the trauma of warfare and, if the injectate contains clostridial spores, serious infections akin to those of wartime may result.

The practice of injecting morphine under the skin was described as far back as the 1830s [7], and the first reports of infections in people addicted to morphine injection were of tetanus in 1876 [8]. By the 1950s in New York in the USA, drug addiction accounted for the majority of cases of tetanus [9, 10]. This was thought to have been associated with the 'cutting' of the street heroin with quinine that enhanced abscess formation, thus creating conditions suitable for the germination of *C. tetani* spores. Laboratory investigation of suspected clostridial disease often involves isolation of these organisms from clinical material such as tissues or exudates by anaerobic culture methods. However, the aero-tolerance of the pathogenic clostridial species varies considerably and attention to strict handling protocols is essential for the isolation of the more oxygen-sensitive species.

Investigations for *Clostridium* species in human tissue and heroin

In the early investigations into sudden deaths and serious illness during 2000 in IDUs who injected heroin intramuscularly or subcutaneously, there was clinical evidence of oedema, necrotising fasciitis or myonecrosis (without gas production) related to the injection site, coupled with rapid systemic deterioration and death [11]. At the outset it was suspected that spore-forming bacteria could be of potential importance because it was common practice to heat the heroin in citric acid or lemon juice before injection. This combination of low pH (estimated to be about pH 2.1) and heat (70–80°C) would almost certainly destroy most vegetative bacterial cells but probably not damage bacterial spores.

Little is known about the microbial content of heroin, but Tuazon *et al.* [12] found that spore-forming aerobes (*Bacillus* spp.) and the anaerobe *C. perfringens* ranked as first and third, respectively, of all the organisms isolated from 'street' heroin and associated paraphernalia. To help determine the cause of these episodes of severe illness and deaths during 2000, tissue samples were examined by enhanced anaerobic methods and anaerobic isolates were identified by a combination of phenotypic and genotypic methods. In the investigation for the presence of clostridial spores in ante- or post-mortem tissues from IDU patients who had fulfilled the case definition, the following protocol was drawn up to maximise their recovery.

Protocol adopted to maximise the growth of clostridia from spores in clinical tissues

- Perform all manipulations inside an anaerobic chamber (N₂ 80%, H₂ 10%, CO₂ 10%).
- Ante- or post-mortem tissue for culture should be examined visually for any areas with evidence of necrosis and a portion of such material should be excised aseptically. This tissue should be divided in two and each half placed in a sterile glass or heat-stable plastic tube of suitable size. An equal volume of Fastidious Anaerobe Broth (Lab M) should be added to both containers and homogenised as much as possible by vortex mixing. Hard tissue may need grinding. One tube of the homogenised tissue/broth suspension should be removed to a heating block set at 80°C for 10 min. After heating, the container should be returned to the anaerobic chamber, and both the heat-shocked and non-heat-shocked suspensions should be inoculated on to Fastidious Anaerobe Agar (FAA Lab M) supplemented with horse blood 6% and also into a cooked meat broth; both should be incubated anaerobically. Material should also be inoculated on to blood agar and incubated aerobically and in air with CO₂ 5% in parallel. All plates and broths should be incubated at 37°C. Any materials left should be mixed with an equal volume of a cryo-preserved such as glycerol and frozen at –80°C.
- All anaerobic, aerobic and aerobic + CO₂ cultures should be examined daily for up to 10 days. Any isolates on aerobic or aerobic + CO₂ plates should be identified by standard laboratory protocols.
- If no anaerobic growth appears after that time, both heated and non-heated tissue in cooked meat broths should be subcultured to FAA blood agar and incubated as described above.

Recognition of pathogenic clostridial species associated with wound infection

C. novyi type A

C. novyi type A may be widely distributed in soil and, in one study, was present in 88% of samples examined [13]. When an anaerobic chamber is used for isolation, anaerobic cultures for *C. novyi* type A should be examined after overnight incubation within the anaerobic chamber for any small, flat, rough or rhizoidal, translucent, haemolytic colonies with a spreading edge. It is advised that plates should not be removed from the anaerobic chamber at this stage, as exposure to air would be toxic to any micro-colonies that have not begun sporulation. If anaerobic jars are used, these should be left unopened for at least 48 h before the plates are examined. After incubation for 48–72 h, colonies will often coalesce to give a fine spreading growth that may cover the entire plate, often with a marked β -haemolysis so as to make the blood agar plate completely transparent. Gram's staining shows

gram-variable rods with occasional sub-terminal spores. A characteristic of *C. novyi* type A is the production of both lecithinase C and lipase on egg-yolk agar. The indole reaction can be variable and it can be difficult to differentiate *C. novyi* type A from *C. botulinum* type C. It may require specific neutralisation of the cytopathic effect of alpha toxin in bioassay, or 16S rDNA sequence analysis to differentiate between these species. *C. novyi* type A is usually unreactive in commercial anaerobe identification kits and commonly is not identified by this approach. *C. novyi* type B has different phenotypic characteristics and can be distinguished by its biochemical reactions. The *C. novyi* type A isolates in the IDU outbreak were variable indole producers and could not be differentiated from *C. botulinum* type C by phenotypic tests alone. Definitive identification was obtained by 16S rDNA gene sequence analysis [14] and detection of alpha toxin in bioassay. Cell-free culture supernates of *C. novyi* isolates from IDUs produced identical cytopathic effects to the type strain of *C. novyi* (NCTC 538) in Vero cells and these effects were completely neutralised by the international standard for *C. novyi*, i.e., Gas Gangrene (*Cl. oedematiens*) Antitoxin (3rd British Standard Established 1966) (64/24). 16S rDNA gene sequence analysis gave 100% homology with *C. novyi* type A accession no. X68188 (NCTC 538) listed in the EMBL database but only 98% homology with that listed for *C. botulinum* type C.

C. perfringens

Although *C. perfringens* was isolated from several cases of infection in IDUs, this organism was not believed to be of general significance in the outbreak investigation as this organism is frequently found as a post-mortem contaminant, being part of the normal gut flora. This fast-growing species will usually produce quite large discrete colonies after overnight incubation. They may be flat and rough-edged or smooth and domed, and either non-haemolytic or with a narrow zone of complete haemolysis inside a larger zone of partial haemolysis. This 'target' haemolysis is more pronounced on sheep blood agar than on horse blood agar. Gram's staining shows straight-sided gram-variable rods with no spores. The classical method of identifying *C. perfringens* is the Nagler plate which is a half anti-alpha toxin egg-yolk plate that is streaked at

90° diametrically with the test organism. After incubation, the lecithinase C (alpha toxin) produces an opaque zone on the half plate without antitoxin that is inhibited on the half plate with the antitoxin. It has been difficult recently in the UK to obtain good quality anti-alpha toxin to *C. perfringens*. Poorer quality antisera may give a partial inhibition of lecithinase that is not species specific and may lead to misidentification of *C. bifermentans* as *C. perfringens*, for example. However, colony morphologies are distinct and Gram's stain will aid differentiation of these two species, as *C. bifermentans* is usually a prolific producer of sub-terminal spores whereas *C. perfringens* does not sporulate *in vitro* on normal agar media. Also, the result of the indole test differentiates between the two species (Table 1).

C. septicum

This organism is another rapidly growing *Clostridium* species that usually produces a thick swarming growth that is quite haemolytic. In culture, it has no characteristic odour, and Gram's staining reveals gram-variable rods with numerous sub-terminal spores. It should be recognised by use of commercially available identification kits. The most common source of *C. septicum* isolates seen in recent years has been from blood cultures from patients with malignancies of the colon or caecum for which this organism is a marker [15]. It can also be isolated from cases of spontaneous gas gangrene of the abdomen that are usually fatal.

C. botulinum

The proteolytic types A, B and F initially produce discrete rhizoidal colonies that spread and coalesce. Haemolysis is variable, but the odour is strong and redolent of rotten eggs due to production of H₂S. Gram's staining shows profuse sub-terminal and free spores and gram-variable bacilli. Phenotypically, they are identical to *C. sporogenes* and differentiation requires demonstration and specific neutralisation of botulinum toxin in a mouse assay. The non-proteolytic types of *C. botulinum* vary in their phenotypic characteristics but require similar toxin assays. In addition to food-borne illness, *C. botulinum* may be

Table 1. Simple tests to differentiate the pathogenic species of clostridia associated with wound infection

Species	Spores	Lecithinase	Lipase	Indole	Urease
<i>C. novyi</i> A	ST	+	+	V	—
<i>C. perfringens</i>	—	+	—	—	—
<i>C. septicum</i>	ST	—	—	—	—
<i>C. botulinum</i> (types A, B, F proteolytic)	ST	—	+	—	—
<i>C. tetani</i>	T	—	—	V	—
<i>C. sordellii/bifermentans</i>	C/ST	+	—	+	+/-

Spore position: ST, sub-terminal; T, terminal; C, central; V, variable.

implicated in cases of wound botulism and such cases have occurred in IDUs [16].

C. tetani

No cases of tetanus associated with IDU have been reported recently, probably as a result of the success of the immunisation programme. Colonies of *C. tetani* may produce a fine swarming growth that can be easily missed on cursory examination. Gram's staining of overnight cultures often reveals readily over-decoloured long bacilli without spores. Further incubation yields the classical 'drum stick' appearance of cells with terminal, round spores. Phenotypically it is quite unreactive although some strains produce indole, and attempts at identification by commercial kits often require supplementation to confirm its identification. However, not all strains of *C. tetani* are toxigenic and referral for toxin bioassay may be required.

C. sordellii

This organism has been reported in other IDU-associated infections [17] and recently has been implicated in a fatal post-knee transplant infection the USA [18]. Colonies are large, grey-white and irregular, sometimes with a 'fern-leaf' edge. They produce indole and lecithinase but are urease negative, which differentiates them from *C. bifermentans*, generally regarded as a non-pathogen.

Discussion

The outbreak of a clostridial infection among IDUs in 2000 [1, 2] served to remind practising clinical microbiologists of some salient lessons. The fact that serious infections and deaths occurred as a result of injecting material containing clostridial spores into human tissue reminds us that so-called 'old' clostridial diseases are not consigned to history. New lifestyle practices can provide new opportunities for old diseases to re-emerge in a new setting. Hence, it is not just bullet, shrapnel and blast wounds that are of high risk of infection with clostridia. Although bacterial infections associated with drug injection are not a new phenomenon, the outbreak in 2000 was the first reported outbreak of infection amongst IDUs to involve *C. novyi* type A. Since the two World Wars, human infections with *C. novyi* type A have been reported rarely in the literature, and the PHLS Anaerobe Reference Unit for England and Wales has seen very few referrals of this organism in the last 20 years. Before the IDU outbreak, the last referral of *C. novyi* A type A was from a blood culture in a 23-year-old woman from Ireland in 1995, and before that from an infected crush injury to a thumb in an agricultural worker in 1990. Therefore, it is not surprising that diagnostic laboratories are unfamiliar with this organism and the fastidious conditions required for culture,

especially on primary isolation. This outbreak also demonstrated the need for strict anaerobic techniques in order to isolate these very oxygen-sensitive bacteria. Laboratories used to isolating *C. perfringens* from clinical material may be lulled into thinking their anaerobic techniques are adequate, whereas this very aero-tolerant species is not a good marker for optimal anaerobic technique.

Even after clostridia had been isolated from IDU infections, most diagnostic laboratories encountered difficulties in attempting definitive identification to species level. Some commercially produced anaerobe identification kits could not recognise *C. novyi* type A. This over-reliance on kit-based identification demonstrates the move by clinical microbiology laboratories away from 'traditional' methods of bacterial identification based on in-house phenotypic tests coupled with the use of a good identification manual or textbook. In the case of *C. novyi* type A, a strong clue to its recognition could be obtained by inoculation on to egg-yolk agar. This organism is unusual in that it usually produces both lecithinase and lipase. The difficulties of identifying this organism were highlighted more generally when it was issued by the UK National External Quality Assessment Scheme (NEQAS) for microbiology on four occasions during the period 1989–1999. Over the four issues, an average of only 16% of reporting laboratories correctly identified *C. novyi* type A, and an average of 15% reported the wrong species; 89% of laboratories did not go further than reporting 'Anaerobes isolated'.

In summary, colonies suspected of being clostridia isolated from wounds (especially those associated with drug abuse) usually require identification to species level to assess their potential significance. However, this often proves to be difficult for those clinical diagnostic laboratories that rarely encounter the pathogenic species. For confirmation of *C. perfringens*, many laboratories still rely solely on the Nagler reaction, sometimes with erroneous results. Although anaerobe identification kits may be of some assistance, they require careful interpretation and often require augmentation with further biochemical tests such as analysis of end-products of metabolism by gas-liquid chromatography and other simple tests as listed in Table 1. Molecular methods of identifying clostridia by 16S rDNA sequence data analysis are feasible, but in practice not attainable by most clinical diagnostic microbiology laboratories. Therefore, any putative clostridia should be referred to a specialist laboratory for identification of anaerobic bacteria.

From the reference laboratory's point of view, this outbreak clearly demonstrated the value of full 16S rDNA sequence analysis (c.1400 bp) for definitive identification of some very closely related clostridial species. There is much phenotypic similarity between some of the species and they may differ from each

other only by the production of certain large clostridial toxins that can be mediated by variable extra-chromosomal genetic elements such as transposons, insertion sequences or bacteriophages. In addition to bioassay for specific toxin neutralisation tests, the 16S rDNA database is an invaluable tool in distinguishing between these species and, in the outbreak investigation, the 16S rDNA data completely matched the *C. novyi* alpha toxin neutralisation tests. Furthermore, the 16S rDNA sequence data of the various *C. novyi* isolates from different towns and cities involved in this outbreak, hinted at differences that were largely borne out by the typing investigations of McLauchlin *et al.* [19].

Diagnostic microbiology laboratories should remain alert for clinical histories suggestive of injecting drug use and should employ optimal anaerobic methods to isolate any exacting clostridial species that may be present. They may also need to address the improvement of their clostridial identification skills to assess the potential significance of their findings.

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