A PCR approach to determine the distribution of toxin genes in closely related *Clostridium* species: *Clostridium botulinum* type C and D neurotoxins and C2 toxin, and *Clostridium novyi* α toxin

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The closely related *Clostridium novyi* and *Clostridium botulinum* types C and D are of current interest because of their association with serious infections in injecting drug users (*C. novyi* type A) and equine and feline dysautonomias (*C. botulinum* types C/D). The species are defined by the major toxins they produce: the α toxin of *C. novyi*, and the type C and D neurotoxins of *C. botulinum* (BoNT/C and BoNT/D). The other major toxin produced by this group, and previously thought to be restricted to the botulinum types, is the chromosomally encoded C2 – a binary toxin consisting of two components, I and II. In the current study 44 of these clostridia from the authors' culture collection were investigated – most of which had been identified previously by conventional biochemical tests as '*C. novyi* type A'. The aim was to check the distribution of toxin genes by PCR to see if the identities were consistent with the genes carried, and to ascertain if the C2 gene was only found in authentic *C. botulinum* strains. Several combinations of the species-defining genes and the two components of the C2 genes were detected. Only the authentic BoNT/C- and BoNT/D-positive *C. botulinum* strains and one of two non-neurotoxic variants of type C carried genes for both components of the C2 toxin. Of the remaining 40 *C. novyi* type A-like strains, the gene for the α toxin was found in 22, with 19 of these also possessing the gene for component I (16) or component II (3) but not both. In the α toxin-negative strains (22), both of the C2 genes were detected in 5 strains (3 *C. botulinum*), with component I in 11 strains and neither gene in 6 strains.

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INTRODUCTION

Clostridium botulinum type C and D (group III *C. botulinum*) and the non-neurotoxic *Clostridium novyi* type A are phenotypically similar and cannot be distinguished by traditional methods of culture or biochemical properties – including GLC fatty acid profiles (Hunter & Poxton, 2002). Immunologically they share many cross-reactive surface antigens (Poxton & Byrne, 1984). Identification of this group of organisms to the species level is by the detection of the major toxin they produce: the botulinum neurotoxin C (BoNT/C) or D (BoNT/D) for *C. botulinum* and the α toxin for *C. novyi*. Traditionally this was done by observing their lethal effects in animal models – usually with specific neutralization by anti-toxin, and this is still the only standard method for definitive identification of *C. botulinum* types.

The genes encoding the major toxin of this group of species are carried on pseudolysogenic bacteriophages and the host-phage relationship is unstable. The phage is readily lost

during subculture and sporulation, and it is thought that a cycle of phage loss and reinfection occurs *in vivo*. Interconversion of toxin types also occurs between type C and type D strains, and *C. novyi* type A. Furthermore, non-toxicogenic strains can be converted to type C or D or *C. novyi* type A by infection with the converting phages. Infection with one phage confers immunity to the other phage suggesting that the two phages are closely related (Sunagawa & Inoue, 1991, 1992; Eklund *et al.*, 1974; Hunter & Poxton, 2002).

The group III *C. botulinum* members also produce a binary ADP-ribosylating cytotoxin, the C2 toxin. This consists of two separate components: the enzymic component, C2I, and the transport component, C2II (Ohishi *et al.* 1980). The genes for this toxin are located on the bacterial chromosome and are produced by the majority of type C and some type D strains (Fujii *et al.*, 1996; Kimura *et al.*, 1998). It is produced during sporulation and may form part of the spore coat (Nakamura *et al.*, 1978).

C. botulinum C and D are well known for causing botulism in animals, particularly in birds, but not in humans (Hatheway, 1995). *C. novyi* is recognized as a rare cause

Abbreviation: IDU, injecting drug user.

of wound and soft tissue infections in humans, classically war wounds, and in farm animals, but the species has been rarely encountered in clinical medicine over the past 50 years (Poxton, 2005). However, recently, awareness of these organisms has increased: *C. novyi* has been encountered in lethal infections in injecting drug users (IDUs) (McGuigan *et al.*, 2002), and *C. botulinum* C and possibly D has been identified as the probable aetiological agent of equine dysautonomia/equine grass sickness (Hunter *et al.*, 1999; McCarthy *et al.*, 2004) and of feline dysautonomia/Key-Gaskell syndrome (Nunn *et al.*, 2004) – both characterized by damage occurring to the autonomic nervous system – as revealed by histopathology.

In preliminary studies to develop PCR methods to detect the genes that encode the toxins one or both components of the C2 toxin genes were detected in some isolates previously identified as *C. novyi* type A. As this cytotoxin may have an important role in the pathogenesis of the conditions described above, it prompted us to examine our laboratory collection of *C. novyi* and related strains, previously identified by a range of phenotypic characteristics, for the presence of genes encoding the C2 toxin as well as the characteristic major toxins of the group: the neurotoxins BoNT/C and BoNT/D, and the *C. novyi* α toxin.

METHODS

Bacterial strains and culture. A total of 44 strains from our lyophilized culture collection were investigated in this study and are listed in Table 1. The strains included 40 labelled *C. novyi* type A or *C. novyi*-like, together with 1 BoNT/C-positive and 1 BoNT/D-positive *C. botulinum* strain and 2 BoNT/C-negative *C. botulinum* type C strains. These BoNT/C-negative strains had been considered neurotoxicogenic when originally deposited in the National Collection of Type Cultures. It is unclear how all the strains had been identified as they were collected over 40 years. All had been identified by R. Brown utilizing biochemical reactions, including the detection of fatty acid end products by GC, since about 1977 and the identification keys used are described in Collee *et al.* (1996).

All strains were cultured in 5 ml anaerobic identification medium broth (Brown *et al.*, 1996) with cooked meat particles and incubated under anaerobic conditions (Mark III or MACS Anaerobic Workstations, Don Whitley Scientific) at 37 °C for 24 h and subcultured onto Fastidious Anaerobe Agar (LabM) supplemented with 10% egg yolk emulsion (Oxoid) for overnight incubation.

Template preparation. Ten large colonies were picked from each plate and emulsified in 100 μ l 5% suspension Chelex 100 (Bio-Rad) following the method of de Lamballerie *et al.* (1992). Samples were heated at 100 °C for 10 min to release DNA and centrifuged at 1000 g for 5 min. A volume of 5 μ l each supernatant was used as the template in the PCR mix.

DNA amplification. PCR was performed in a reaction volume of 50 μ l containing 1 $\times$ REDTaq PCR buffer (Sigma), 1.5 mM MgCl₂ (3.75 mM for BoNT/C), 0.2 mM dNTP mixture, 2 U REDTaq DNA polymerase, 10 pmol each primer and 5 μ l each DNA template. All primers were synthesized by MWG Biotech (<http://www.mwg-biotech.com>) and their sequences and reference source are detailed in Table 2. All amplification reactions were carried out in a PCR thermocycler (Techne TC-412). Cycling conditions are given in

Table 3. The amplified products were separated by electrophoresis in 1% agarose gels and stained with ethidium bromide. DNA ladder (ReadyLoad 100 bp, Invitrogen) was used as a molecular mass marker to indicate the sizes of the amplified products.

RESULTS AND DISCUSSION

The type strains of *C. botulinum* type C and D, 4564 (NCTC 373) and 3923 (NCTC 8265), respectively, were the only strains in which the genes for the C and D neurotoxins were detected (Fig. 1a). These also carried the genes for both components of the C2 toxin (Fig. 1b), but note the size of the PCR product for the type D strain was larger than for that found in the type C or *C. novyi* strains. However, sequencing and a BLAST search identified it as the gene for C2 component I (results not shown). Two strains originally supplied by NCTC as type C cultures 2510 (NCTC 10914) and 4565 (NCTC 3732) had previously been shown to be BoNT/C-negative by use of a specific ELISA for the toxin and this was confirmed by PCR. Both of the neurotoxin-producing strains also yielded the expected amplification products for both genes of the components of C2 toxin, as did one of the BoNT/C-negative strains. However, the other, 4565, yielded the C2 component I gene but not component II. The loss of the neurotoxins has been acknowledged by NCTC.

Of the 40 strains originally named as *C. novyi* type A or *C. novyi* type A-like, only 22 (55%) tested positive for the *C. novyi* α toxin gene by PCR. All negative strains were tested on at least two further occasions with consistently negative results for the α toxin gene. Whether this result is real or due to sequence mismatch at the site of primers is not yet known. Further work involving sequence determination of several different α toxin genes and the design of new primers would clarify this point. Unexpectedly, however, 28 of the *C. novyi* strains (77%) contained the gene for the C2CI toxin (these included 16 of the 22 α toxin-positive strains). Five '*C. novyi*' strains (12.5%) contained the gene for the C2CII toxin (three α toxin-positive and two negative) and nine (22.5%) were negative for both components. No α toxin-positive isolates contained both of the C2 components. The overall distribution of gene profiles is indicated in Table 1 and is summarized in Table 4.

As far as we can ascertain few, if any, of the so-called *C. novyi* strains had been tested in our laboratory specifically for the α toxin when originally identified, although some guinea pig inoculations had been performed in the 1970s but records are sketchy. However, all gave biochemical profiles – including volatile fatty acid patterns typical of *C. novyi* type A, but the gene encoding the α toxin was detected in only just over half of our collection.

It is noteworthy that all of the strains in our collection that had been isolated from cases of infection in IDUs, with characteristic pathology, were found to contain the gene for the α toxin. In 2000 there were 60 cases of extremely serious infection in Scotland with 23 deaths, with *C. novyi* definitely

Table 1. List of clostridial strains used and toxins identified

Lab no.	Original identity	Source	Toxin profile (C1 D C2I C2II α)*
141	<i>C. novyi</i> type A-like	Horse ileum, subacute EGS	- - + - -
465	<i>C. novyi</i> type A	Wound site, common bile duct drain	- - + - -
2306	<i>C. novyi</i> type A	?	- - + - -
2307	<i>C. novyi</i> type A	?	- - + + -
2510	<i>C. botulinum</i> type C†	NCTC 3732	- - + + -
2530	<i>C. novyi</i> type A	NCTC 538	- - + - +
2531	<i>C. novyi</i> type A	Glaxo collection	- - + - +
2533	<i>C. novyi</i> type A	NCTC 6735	- - + - -
2534	<i>C. novyi</i> type A	?	- - + + -
2535	<i>C. novyi</i> type A	?	- - + - -
2536	<i>C. novyi</i> type A	?	- - + - +
3209	<i>C. novyi</i> type A	PHLS 2680	- - + - +
3341	<i>C. novyi</i> type A	Soil (St Kilda)	- - - - -
3923	<i>C. botulinum</i> type D	NCTC 8265	- + + + -
4540	<i>C. novyi</i> type A	Injection site (Glasgow)	- - + - +
4541	<i>C. novyi</i> type A	Injection site (Glasgow)	- - + - +
4545	<i>C. novyi</i> type A	Crush injury wound swab	- - + - +
4547	<i>C. novyi</i> type A	Injection site (PHLS Cardiff)	- - + - +
4548	<i>C. novyi</i> type A	Injection site (PHLS Cardiff)	- - + - +
4549	<i>C. novyi</i> type A	Horse ileum, AGS	- - + - -
4550	<i>C. novyi</i> type A	Horse ileum, AGS	- - - - -
4551	<i>C. novyi</i> type A	Horse ileum, AGS	- - + - -
4552	<i>C. novyi</i> type A	Hare with dysautonomia, small intestine	- - - - +
4553	<i>C. novyi</i> type A	Hare with dysautonomia, small intestine	- - - - -
4554	<i>C. novyi</i> type A	Hare with dysautonomia, small intestine	- - + - -
4556	<i>C. novyi</i> type A	Horse faeces, CGS	- - - - -
4557	<i>C. novyi</i> type A	Horse faeces, recovered CGS	- - - - -
4558	<i>C. novyi</i> type A	Horse faeces, contact CGS	- - + - -
4559	<i>C. novyi</i> type A	Horse faeces, contact CGS	- - - - -
4561	<i>C. novyi</i> type A	Horse ileum, AGS	- - + - -
4562	<i>C. novyi</i> type A	Horse ileum, CGS	- - + - +
4563	<i>C. novyi</i> type A	Horse faeces, CGS	- - + - +
4564	<i>C. botulinum</i> type C	NCTC 8548	+ - + + -
4565	<i>C. botulinum</i> type C†	NCTC 10914	- - + - -
4566	<i>C. novyi</i> type A	Injection site (Glasgow)	- - + - +
4567	<i>C. novyi</i> type A	Injection site (Glasgow)	- - + - +
4568	<i>C. novyi</i> type A	NCTC 6738	- - + - +
4569	<i>C. novyi</i> type A	Injection site (Dublin)	- - + - +
4570	<i>C. novyi</i> type A	ARU collection	- - - - +
4571	<i>C. novyi</i> type A	NCTC 538	- - + - +
4572	<i>C. novyi</i> type A	Injection site (Bournemouth)	- - - - +
4573	<i>C. novyi</i> type A	Injection site (Glasgow)	- - - + +
4574	<i>C. novyi</i> type A	Injection site (Glasgow)	- - - + +
4575	<i>C. novyi</i> type A	Injection site (Stockton)	- - - + +

EGS, Equine grass sickness; AGS, acute form of EGS; CGS, chronic form of EGS; PHSL, public health service laboratory; ARU, Anaerobe Reference Unit at Cardiff; ?, source unknown.

*Toxin profile: C1, BoNT/C; D, BoNT/D; C2I, C2 component I; C2II, C2 component II; α , *C. novyi* α toxin; +, positive; -, negative.

†Non-neurotoxin producer.

implicated in 23 cases and probably implicated in 37 cases as the cause. Spores of clostridia can contaminate the materials used by IDUs and when introduced into tissue in an anaerobic environment the spores can germinate and

produce exotoxins. All of the cases in Scotland involved IDUs injecting a solution of street heroin and citric acid into muscle tissue (McGuigan *et al.*, 2002). The few samples of heroin cultured from this outbreak were negative for *C.*

Table 2. Primer sequences used for the detection of *C. botulinum* types C and D, *C. novyi* α and C2 components

Gene	Primer	Sequence	Reference
Type C neurotoxin	Tox-384	5'-AAACCTCCTCGAGTTACAAGCCC-3'	Williamson <i>et al.</i> (1999)
	Tox-850	5'-GAAAATCTACCCTCTCCTACATCA-3'	
Type D neurotoxin	ToxD-F	5'-GTGATCCTGTTAATGACAATG-3'	Sunagawa <i>et al.</i> (1992)
	ToxD-R	5'-TCCTTGCAATGTAAGGGATGC-3'	
<i>C. novyi</i> α toxin	Nov-F	5'-GGTGCGATTCAAGAGGCCACA-3'	Hofmann <i>et al.</i> (1995)
	Nov-R	5'-CGCTCCTAGCAGTCCCGAAAT-3'	
C2 component I	C2CI-F	5'-AAGGAAGATAAAACAAAAAT-3'	Fujii <i>et al.</i> (1996)
	C2CI-R	5'-CCTAATGATACAAATGAAAA-3'	
C2 component II	C2CII-F	5'-GCAGAAGTTTCAGGTAGTTTACAAC-3'	Based on sequence Kimura <i>et al.</i> (1998)
	C2CII-R	5'-CGCATTCTATAACGACCTTCTGGA-3'	

novyi (Jones *et al.*, 2002) but the circumstantial evidence was compelling that *C. novyi* type A was the main if not only agent involved in the majority of cases.

The isolates obtained from environmental and animal sources do not appear to have the same distribution of toxin genes. Only 3/14 of these animal isolates contained the α toxin gene though all were phenotypically similar.

None of the isolates obtained from IDUs contained both components of the C2 toxin, but the majority (62 %) contain only the gene for the enzymic component (C2CI). It is possible that the C2CII transport component is not needed for transport into an already-damaged cell with the active C2CI component alone contributing to the pathology. The animal and environmental isolates show a similar pattern with 43 % containing the enzyme component of the C2 toxin and no isolates containing the gene for the binding component. None of the strains in our collection contained both of the C2 component genes together with the α toxin genes. In fact the only strains containing genes for both

components of C2 were the two type strains of neurotoxin producing *C. botulinum* (C and D) together with one of the BoNT/C-negative strains, and two *C. novyi* strains in which the α toxin genes were not detected (strains 2307 and 2534). Unfortunately the origin of these strains was not recorded but they could well represent *C. botulinum* strains that had lost their toxin-encoding phages.

Of the six strains in which none of the toxin genes was detected, one was from soil, and the others were from the gastrointestinal tracts of a hare with dysautonomia and four horses (two with active dysautonomia).

It is evident that the complement of toxin genes that is carried by isolates of this group of clostridia is extremely variable. To cause classical botulism, the specific neurotoxins must be produced. Similarly, typical clinical *C. novyi*-mediated disease almost certainly requires the α toxin to be produced to induce the classical oedematous lesions. However, depending on the type of clinical situation, it is possible that the C2 toxin alone, or in combination with α

Table 3. Cycling conditions used for the detection of *C. botulinum* types C and D, *C. novyi* α and C2 components

Primer	Initial denaturation	No. of cycles	Cycling condition	Final extension
Tox-384/Tox850	5 min at 80 °C	30	1 min at 95 °C 1 min at 55 °C 1 min at 72 °C	5 min at 72 °C
ToxD-F/ToxD-R	10 min at 95 °C	25	1 min at 94 °C 1 min at 55 °C 1 min at 72 °C	3 min at 72 °C
Nov-F/Nov-R	3 min at 94 °C	30	1 min at 95 °C 1 min at 48 °C 1 min at 72 °C	5 min at 72 °C
C2CI-F/C2CI-R	3 min at 94 °C	35	45 s at 94 °C 2 min at 45 °C 1 min at 72 °C	5 min at 72 °C
C2CII-F/C2CII-R	3 min at 94 °C	40	45 s at 94 °C 1 min at 53 °C 3 min at 72 °C	5 min at 72 °C

Fig. 1. (a) PCR for the genes encoding the two neurotoxins and *C. novyi* α toxin. Lanes 1 and 2, BoNT/C, strain 4564 and negative control; lanes 3 and 4, BoNT/D, strain 3923 and negative control; lanes 5 and 6, *C. novyi* α toxin, strain 2307 and negative control. Side lanes 100 bp DNA ladder. (b) PCR investigating the same three strains as in (a) for the genes encoding the two components of the C2 toxin. C2CI, lanes 1–4, strains 4564, 3923, 2307 and negative control; C2CII, lanes 5–8, strains 4564, 3923, 2307 and negative control. Note that the PCR product for the *C. botulinum* type D strain is larger than the *C. botulinum* type C and *C. novyi* type A strains.

toxin or neurotoxin, may contribute towards the pathology. If α toxin is present there does not seem to be a major requirement for the C2CII component. However, when in combination with the botulinum neurotoxin it seems that both components may be required.

As the various toxins – both neurotoxins and lethal toxins – are likely to affect the pathogenic potential of individual strains it is useful to know which ones they produce. However, this may be difficult because of the inherent instability of the pseudolysogenic bacteriophages that encode the two neurotoxins and the α toxin. In nature this does not matter as constant cycles of loss and reacquisition of the bacteriophages will presumably occur. Additionally, the process of sporulation may result in the bacteriophage being lost from the cell. It is therefore important to try to prevent loss during the initial isolation and early subculture of toxigenic strains. It is recommended that several colonies are selected and they are cultured in liquid medium to allow the infection–reinfection to take place and thereby prevent this.

In conclusion, this preliminary investigation by PCR – using previously published sequences for all but the C2 component

Table 4. Distribution of toxin genes in Group III clostridia

Profile of toxin genes	Number
<i>C. novyi</i> α^+ , C2CI $^+$, C2CII $^+$	0
<i>C. novyi</i> α^+ , C2CI $^+$, C2CII $^-$	16
<i>C. novyi</i> α^+ , C2CI $^-$, C2CII $^-$	3
<i>C. novyi</i> α^+ , C2CI $^-$, C2CII $^+$	3
<i>C. novyi</i> α^- , C2CI $^+$, C2CII $^+$	5*
<i>C. novyi</i> α^- , C2CI $^+$, C2CII $^-$	11†
<i>C. novyi</i> α^- , C2CI $^-$, C2CII $^-$	6

*Includes two *C. botulinum* type C (one BoNT/C+ and one BoNT/C–) and one *C. botulinum* type D (BoNT/D+).

†Includes one *C. botulinum* type C (BoNT/C–).

II gene – has shown the variety of combinations of toxin genes found in this group of related clostridia. The detection of at least one of the genes encoding the components of the C2 toxin in strains that appear to be *C. novyi* is novel and suggests a role for one or both of the components in the pathogenesis of these organisms. The failure to detect certain genes – especially the α toxin genes in strains previously identified as *C. novyi* – may be real, or could be due to polymorphisms in the genes for these toxins with the primers we have selected not matching adequately. This must be investigated in any future studies.

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