Improvement in Laboratory Diagnosis of Wound Botulism and Tetanus among Injecting Illicit-Drug Users by Use of Real-Time PCR Assays for Neurotoxin Gene Fragments

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An upsurge in wound infections due to *Clostridium botulinum* and *Clostridium tetani* among users of illegal injected drugs (IDUs) occurred in the United Kingdom during 2003 and 2004. A real-time PCR assay was developed to detect a fragment of the neurotoxin gene of *C. tetani* (TeNT) and was used in conjunction with previously described assays for *C. botulinum* neurotoxin types A, B, and E (BoNTA, -B, and -E). The assays were sensitive, specific, rapid to perform, and applicable to investigating infections among IDUs using DNA extracted directly from wound tissue, as well as bacteria growing among mixed microflora in enrichment cultures and in pure culture on solid media. A combination of bioassay and PCR test results confirmed the clinical diagnosis in 10 of 25 cases of suspected botulism and two of five suspected cases of tetanus among IDUs. The PCR assays were in almost complete agreement with the conventional bioassays when considering results from different samples collected from the same patient. The replacement of bioassays by real-time PCR for the isolation and identification of both *C. botulinum* and *C. tetani* demonstrates a sensitivity and specificity similar to those of conventional approaches. However, the real-time PCR assays substantially improves the diagnostic process in terms of the speed of results and by the replacement of experimental animals. Recommendations are given for an improved strategy for the laboratory investigation of suspected wound botulism and tetanus among IDUs.

Heroin, cocaine, and amphetamines are the most widely injected illegal drugs, and complications among users of these injected drug (IDUs) resulting in infections are the most frequent cause of hospitalization among this group of patients (28). Soft tissue infections caused by spore-forming bacteria among IDUs emerged as a serious disease problem in the United Kingdom during 2000, and cases caused by *Clostridium novyi* (23), *Clostridium botulinum* (8, 21), *Clostridium histolyticum* (7), and *Bacillus cereus* (12) were reported. During late 2003 and early 2004, an upsurge in cases of *Clostridium tetani* infections among IDUs also occurred (16). A similar increase in wound botulism among IDUs was described in the United States during the 1990s (33). The major risk factors for all of these infections was the availability of higher purity heroin, as well as skin or muscle “popping,” which tends to occur when access to veins is lost (8, 16, 23, 33).

Both botulism and tetanus are rare but potentially fatal diseases caused by *C. botulinum* neurotoxin (BoNT) and *C. tetani* neurotoxin (TeNT), respectively (18). BoNT specifically targets the peripheral nervous system, where it is internalized into the presynaptic nerve terminal of motor neurons and blocks release of the neurotransmitter acetylcholine. This prevents nerve impulses to the muscle resulting in the characteristic bilateral flaccid paralysis of botulism. TeNT specifically targets the central nervous system and is internalized into peripheral nerve terminals, where it is transported by retro-

grade axonal transport to nerve cells in the spinal cord and brain (22). Once here, TeNT is transcytosed to inhibitory interneurons, where it blocks the release of inhibitory neurotransmitters (glycine, gamma-amino butyric acid) that normally block the nerve impulses. Failure to block the nerve impulse results in generalized muscular spasms and the characteristic spastic paralysis of tetanus (18, 22).

In humans, tetanus and botulism can occur as a result of neurotoxin production by the bacteria growing in traumatic wounds. Botulism can also be food-borne due to ingestion of preformed BoNT in foods in which *C. botulinum* had grown, as well as caused by production of BoNT after intestinal colonization of infants and, more rarely, adults by *C. botulinum* (18). The most common current presentation of this disease in the United Kingdom is wound botulism among IDUs (8, 21).

C. botulinum is a diverse species of gram-positive, anaerobic spore-forming bacteria defined on the basis of the ability to produce neurotoxin. BoNTs can be divided into seven antigenically distinct types designated A to G. Almost all cases of botulism in humans are due to types A, B, E, and F, with E and F being least common. The detection of BoNT(s) production in vitro is, therefore, a cardinal feature for the identification of *C. botulinum*, including its distinction from genetically close relatives including *Clostridium sporogenes* and *Clostridium novyi* (11). *C. tetani* is also a gram-positive, anaerobic spore-forming bacterial species. The neurotoxin produced by *C. tetani* is similar to those produced by *C. botulinum* but occurs as a single antigenic type. Not all in vitro grown *C. tetani* are toxigenic and, hence, both the identification of *C. botulinum* and the assessment of *C. tetani* toxigenicity rely on the detection of neurotoxin (18).

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Diagnosis of wound botulism and tetanus relies on clinical observations assisted by laboratory confirmation, including the detection of toxin in body fluids, usually serum or material from infected wounds, and/or isolation of *C. botulinum* or *C. tetani* from infected wounds (4). Detection of toxin and the isolation and identification of toxigenic *C. botulinum* or *C. tetani* traditionally relies on the use of mouse bioassays (18).

We previously described the use of sensitive and specific real-time PCR assays for BoNTA, -B, and -E gene fragments for the investigation of cases of food-borne and infant botulism (1). Here we describe the development of a real-time PCR assay for the *C. tetani* neurotoxin gene, together with the application of this and the BoNT gene assays to material from cases of wound infections among illegal IDUs.

MATERIALS AND METHODS

Bacterial cultures. For evaluation of the real-time PCR assay to detect TeNT gene fragment, cultures of *Clostridium* species were obtained from the National Collection of Type Cultures (NCTC; HPA Centre for Infections) and included nine *C. botulinum* type A (NCTC 887, 2012, 2916, 3805, 3806, 4587, 7272, 9837, and 11199), five *C. botulinum* type B (NCTC 751, 3807, 3815, 7273, and 12265), two *C. botulinum* type C (NCTC 3732 and 10914), one *C. botulinum* type D (NCTC 8265), one *C. botulinum* type E (NCTC 8266), one *C. botulinum* type F (NCTC 10281), one *C. absonum* (NCTC 10984), one *C. beijerinckii* (NCTC 11920), one *C. bifermentans* (NCTC 8780), two *C. butyricum* (NCTC 6084 and 7423), 1 *C. chauvoei* (NCTC 8070), one *C. difficile* (NCTC 11204), one *C. histolyticum* (NCTC 503), one *C. novyi* (NCTC 538), one *C. paraperfringens* (NCTC 10987), one *C. paraperfringens* (NCTC 11833), one *C. perfringens* (NCTC 8359), six *C. sporogenes* (NCTC 275, 276, 532, 533, 534, and 8594), one *C. tertium* (NCTC 541), and two *C. tetani* (NCTC 5405 and 9573) strains. The following wild-type cultures were obtained from culture collections of the HPA Anaerobe Reference Unit Cardiff and the HPA Food Safety Microbiology Laboratory: 11 *C. tetani*, 11 *C. histolyticum*, 1 *C. sporogenes*, 10 *Listeria monocytogenes*, and 10 *Bacillus* sp. strains. Bacteria were stored on glass beads in glycerol-nutrient broth (15% [vol/vol]) at -70°C (14).

For application to the investigation of BoNTs and TeNT gene fragments, cultures suspected to be *C. botulinum* and *C. tetani* from IDUs were either from HPA Food Safety Microbiology Laboratory, HPA Anaerobe Reference Laboratory Cardiff, or from hospital laboratories.

Primary samples. Clinical samples (serum, tissue, aspirates, wound swabs and pus) were collected from suspected cases of both botulism and tetanus as defined elsewhere (4). Serum, tissue, and wound samples were tested for the presence of BoNT or TeNT by mouse bioassay as described previously (2, 3, 5, 29). Appropriate United Kingdom Home Office Licenses were held for performance of mouse bioassays, and no additional mouse bioassays were performed for the completion of the present study above those for routine analysis.

In vitro culture methods. Samples of tissue, aspirates, wound swabs and pus were inoculated into prereduced *Clostridium* Botulinum Isolation Cooked Meat Broth with added starch and glucose (CBIB [5]). After inoculation, enrichment broths were either untreated or heat shocked at 60°C for 30 min and incubated for 5 days either at 30°C for *C. botulinum* (5) or at 37°C for *C. tetani* (17). Enrichment broths were tested by mouse bioassay after 5 days of incubation for BoNT and/or tested after 1, 3, and 5 days incubation by using real-time PCR assay. Broths were subcultured onto *Clostridium* botulinum isolation agar with or without added antibiotics (CBIA and CBI [13, 25]) and 5% horse blood in a Columbia blood agar base (ColBA; Oxoid). For isolation of *C. botulinum*, CBIA, CBI, and ColBA were incubated in an anaerobic cabinet (Don Whitley Scientific) with an atmosphere of 80% N_2 , 10% H_2 , and 10% CO_2 or in anaerobic jars by using an anaerobic gas generating kit (Oxoid, Basingstoke, United Kingdom) at 30°C . CBI and ColBA were incubated at 37°C in anaerobic jars as described above for the isolation of *C. tetani*.

DNA extraction. Different DNA extraction strategies were used for direct analysis of clinical material, inoculated broths, bacterial growth harvested from agar plates, and individual bacterial colonies, as described previously (1).

For assay development and for preparation of positive control material, DNA was extracted from pure bacterial cultures grown on agar plates. A 10- μl loopful of bacterial growth was subjected to MagNAPure LC automated nucleic acid extraction by using a Bacterial and Fungal DNA Isolation Kit III (Roche). For sensitivity tests, purified DNA was further treated with 1 mg of RNase A (Sig-

ma)/ml at 37°C for 2 h, followed by 95°C for 10 min. DNA was quantified spectrophotometrically at 260 and 280 nm.

DNA from tissues, aspirates, wound swabs, and pus was extracted by using a modification of the Boom method as described previously (24).

DNA was extracted from broth cultures by using an Instagene matrix (Bio-Rad Laboratories). The biomass of 1 ml of broth culture was harvested by centrifugation at $13,000 \times g$ for 3 min, washed once in 1 ml of nuclease-free water (Sigma), and resuspended in 200 μl of premixed Instagene matrix by vortexing. Samples were then heated for 30 min at 56°C , followed by 8 min at 100°C , and DNA present in the supernatant was recovered by centrifugation.

For testing individual bacterial colonies from agar plates, a crude DNA extract was prepared on the day of use by emulsifying approximately a 1- μl loopful of bacterial cells into 100 μl of nuclease-free water (Sigma). The suspension was heated at 100°C for 10 min and microcentrifuged for 3 min at full speed, and the supernatant was used in subsequent PCR assays on the day of preparation.

For each PCR assay, 5 μl of DNA, prepared as described above, was used as a template. All tests were performed in duplicate, and DNA was tested undiluted and diluted 1:10 in nuclease-free water (Sigma).

Real-time PCR assays. Real-time PCR assays for BoNTA, -B, and -E gene fragments were performed as described by Akbulut et al. (1). For development of the real-time PCR assay for the TeNT gene fragment, nucleotide sequences GenBank X06214 and X04436 were retrieved from the Entrez Nucleotide database and aligned by using the BioEdit Sequence Alignment Editor v.5.0.9 program (Tom Hall, Department of Microbiology, North Carolina State University). Primers and probes were designed by using Primer Express software version 1.5 (Applied Biosystems) suitable for PCR detection assays using the Applied Biosystems 7700 Sequence Detector System under default conditions. BLAST searches were performed on all primer and probe sequences to ensure the desired specificities. The oligonucleotide probe for the TeNT gene fragment was 5' labeled with 6-carboxy-fluorescein (FAM) and 3' labeled with a minor groove binder ligand and a nonfluorescent quencher (MGB; Applied Biosystems). Details of primer and probe nucleotide sequences for TeNT and BoNT gene PCR assays are given in Table 1.

The PCR analysis was performed as a duplex for detection of BoNTA and BoNTB gene fragments and as a monoplex for BoNTC and TeNT. To determine the presence of PCR inhibition from DNA extracts, an internal positive control was performed by using the TaqMan exogenous internal positive control kit (IPC; Applied Biosystems, Ltd.) according to the manufacturer's instructions.

All assays were performed in 96-well MicroAmp optical plates with MicroAmp optical plate seals (Applied Biosystems). Each reaction included 25 μl of $1 \times$ qPCR Master Mix (Eurogentec), 0.1 μM concentrations of fluorescent probe(s), 0.3 μM concentrations each of the appropriate reverse and forward oligonucleotide primers, and 5 μl of DNA template. Nontemplate controls of sterile distilled water and positive controls containing 5 μl of MagNAPure extracted DNA from each target were included in each batch of PCR assays.

Amplification and detection were performed under default conditions on an ABI Prism 7700 Sequence Detection System that consisted of 2 min at 55°C and 10 min at 95°C , followed by 40 cycles of 95°C for 15 s and finally by 60°C for 1 min. PCR products were detected by monitoring the increase in fluorescence due to release of reporter dyes from the probes due to the thermodependent 5' exonuclease activity of Taq polymerase. Assay results were calculated by using the manufacturers software as signal threshold cycle (C_T) values, where C_T is the PCR cycle number at which fluorescence of the released reporter dye reaches a threshold level. Samples were designated as positive when C_T values of <35 were obtained and negative when C_T values ≥ 35 were obtained.

RESULTS

Development and evaluation of a real-time PCR assay for TeNT. A real-time PCR assay to detect a fragment of the TeNT gene was developed that was specific for this target in an in silico analysis. The sensitivity of the PCR assay was determined empirically using dilutions of purified DNA from *C. tetani* NCTC 5405. The limit of detection was determined to be equivalent to 92 genomes, assuming an approximate *C. tetani* genome size of 2.79 Mbp as determined for the single whole-genome sequence of *C. tetani* E88 (10).

The specificity of the assay was further evaluated by empirical tests against DNA extracted from 87 pure bacteria cultures of 16 different bacterial species. The assay detected the TeNT

TABLE 1. Primers and probes used for real-time PCR detection of *C. tetani* and *C. botulinum* neurotoxin gene fragments

Assay and target	Oligonucleotide sequence	Position in GenBank sequence no.
TeNT gene fragment assay		
Primers		
TQ Tet1	5'-CCT AGT TTC AAA ACT TAT TGG CTT ATG TAA-3'	1314–1343 in X06214
TQ Tet 2	5'-CAT AAT TCT CCT CCT AAA TCT GTT AAT GAT G-3'	1394–1424 in X06214
Probe (label)		
Tet (FAM)	5'-TAT ACC ACC AAC AAA TAT AAG AG-3' (FAM)	1350–1372 in X06214
BoNT gene fragment assay		
Primers		
TQ A1	5'-CGAAATGGTTATGGCTCTACTCAA-3'	529–553 in M30196
TQ A2	5'-TTGCCTGCACCTAAAAGAGGAT-3'	614–635 in M30196
TQ B1	5'-GATTATAAATGGTATACCTTATCTTGGAGATAGAC-3'	333–367 in X71343
TQ B2	5'-CGCTCCACTTCTCCTGGATTAC-3'	428–449 in X71343
TQ E1	5'-AATATTGTTTCTGTAAAAGGCATAAGGAA-3'	1240–1268 in X62683
TQ E2	5'-AAGTTACTGTATCGTCAATTTCTTTAGGAG-3'	1349–1378 in X62683
Probes (label)		
A (FAM)	5'-ACTTCAAGTGACTCCTCAAACCAAATGTAATCTG-3'	569–605 in M30196
B (VIC)	5'-TGTTCCACTCGAAGAGTTTAACACAAACATTGC-3'	369–401 in X71343
E (FAM)	5'-TTCTCGGAAGCCACAAAAATAACTCACCAT-3'	1292–1322 in X62683

gene fragment in all 13 *C. tetani* strains but not in any of the other bacterial species. Thus, this assay was found to be 100% specific for *C. tetani*.

Real-time PCR assays were performed in duplicate for every DNA sample tested (including those from clinical material, enrichment cultures, and bacterial colonies for TeNT and BoNT gene fragments as described below), and the same result was obtained for duplicates.

Application of real-time PCR assays to clinical samples from suspect tetanus among IDUs. Two serum samples, one tissue sample, and four cultures suspected to be *C. tetani* were obtained during 2004 from five patients with a clinical diagnosis of tetanus. Three of the patients were female and two were male, and the mean age was 37 years (range, 21 to 49 years). The two serum samples from two of the patients were tested by bioassay for TeNT, and toxin was not detected. The tissue sample was subjected to anaerobic enrichment, both with and without heat treatment, and the TeNT gene fragment was not amplified by PCR from any broths, cultures, or subsequent bacterial colonies. However, the TeNT gene fragment was amplified from both broths and colonies from two of the four cultures suspected to be *C. tetani*.

Application of real-time PCR assays to clinical samples from suspect botulism among IDUs. To evaluate the usefulness of the BoNT gene fragment PCR assays for the investigation of IDUs, material was collected from 25 suspect botulism cases, 5 of which occurred in 2003, and the remaining 20 occurred in 2004. Eighteen of the patients were male, seven were female, and the mean age was 29 years (range, 19 to 43). Primary material was collected from all cases and included 16 serum samples from 16 patients, 40 wound samples (tissue, wound swabs, pus, and aspirates) from 21 patients, and 9 anaerobic broths with pure cultures suspected to be *C. botulinum* from 8 patients.

Sixteen serum samples from sixteen patients, five tissue samples from three patients, and one wound swab from one patient

were tested by mouse bioassay. BoNT was detected in the sera from nine patients, BoNTA was detected in seven patients, and a mixture of BoNTA and B was detected in two patients. BoNTA was detected directly in a tissue sample from one patient (Table 2).

DNA was extracted directly from 12 tissue samples, one pus swab and one aspirate from six patients (seven tissues and one pus swab from one patient) and tested for the presence of BoNT gene fragments by using the real-time PCR assays, as well as for PCR inhibition using the IPC. BoNTA gene fragments were detected at low levels in three of the tissue samples from two patients; BoNTA gene fragments were also detected in enrichment broths and from pure cultures on solid media isolated from the same samples (Table 2). No PCR inhibition was detected in these samples. BoNT gene fragments were not detected directly in two tissue samples, one pus swab, and one aspirate where PCR inhibition was not detected. Two of these samples (one tissue sample and one pus swab from patient 13) were determined to be positive by PCR for BoNT gene fragments when enrichment cultures were subsequently tested (Table 2). BoNT gene fragments were not detected in the remaining seven tissue samples but since PCR inhibition was detected by using the IPC, BoNT gene fragments may not have been amplified due to PCR inhibition. However, BoNT gene fragments were not detected in any of these tissue samples on enrichment culture.

Three tissue samples, one pus sample, and one wound swab from three patients were inoculated into anaerobic broths by the original hospital laboratories and were sent to this laboratory by conventional post. DNA was extracted from all five broths on the day of receipt, and BoNTA gene fragments were detected by PCR in four, three of which were inoculated with tissue and one with pus (data not shown). Nine pure cultures suspected to be *C. botulinum* were received from hospital microbiology laboratories. The BoNTA gene fragment was amplified from four of these cultures (Table 2). All four were

TABLE 2. Bioassay and PCR results for individual specimens from 25 cases of suspected botulism in IDUs^a

Patient no.	Bioassay result for:		Specimen type received (no. of specimens)	Detection of BoNT gene fragments:		
	Serum sample	Wound sample		Directly from wound samples	In enrichment cultures by PCR	From pure cultures
1	BoNTA and BoNTB	ND	Tissue (3)	NT	ND*	ND
2	BoNTA	BoNTA	Tissue (1)	NT	BoNTA*	BoNTA
			Pure culture (2)	NA	NA	BoNTA
3	NA	ND	Swab (1)	NT	ND*	ND
4	ND	ND	Tissue (1)	NT	ND*	ND
5	BoNTA	NT	Pure culture (1)	NA	NA	BoNTA
	BoNTA	NT	Pus (1)	NT	BoNTA	BoNTA
	BoNTA	NT	Pus (1)	NT	BoNTA	BoNTA
6			Pure culture (1)	NA	NA	BoNT
7	BoNTA	NT	Tissue (3)	NT	BoNTA	BoNTA
8	ND	NT	Aspirate (1)	ND	ND	ND
			Pure culture (1)	NA	NA	ND
9	NA	NT	Aspirate (1)	NT	ND	ND
10	ND	NT	Tissue groin skin, right (2)	NT	ND	ND
		NT	Pus (1)	NT	ND	ND
		NT	Tissue, wrist skin, left (1)	NT	ND	ND
11	ND	NT	Tissue (1)	ND	ND	ND
12	NA	NT	Wound swab (1)	NT	ND	ND
13	BoNTA and BoNTB	NT	Tissue, left arm (2)	BoNTA	BoNTA	BoNTA
		NT	Tissue, left arm (1)	I	ND	ND
		NT	Pus swab, left arm (1)	ND	BoNTA	BoNTA
		NT	Tissue, right forearm (2)	I	ND	ND
		NT	Tissue, right arm (2)	NT	ND	ND
		NT	Tissue, right arm (1)	ND	BoNTB	BoNTB†
		NT	Tissue, left leg (1)	I	ND	ND
14	BoNTA	NT	Pure culture (1)	NA	NA	BoNTA
15	ND	NT	Wound swab (2)	NT	ND	ND
16	NA	NT	Pus (1)	NT	BoNTB	BoNTB
17	NA		Pure culture (1)	NA	NA	ND
18	BoNTA	NT	Tissue, right leg (1)	NT	BoNTA	BoNTA
		NT	Pus, right leg (1)	NT	BoNTA	BoNTA
19	ND	NT	Wound swab (1)	NT	ND	ND
20	NA	NT	Tissue (1)	BoNTA	BoNTA	BoNTA
			Pure culture (1)	NA	NA	ND
21	NA	NT	Tissue (1)	I	ND	ND
22	NA	NT	Tissue (2)	I	ND	ND
23	ND	NT	Tissue (1)	NT	ND	ND
24	NA	NT	Wound swab (1)	NT	ND	ND
25	BoNTA		Pure culture (1)	NA	NA	BoNTA

^a ND, not detected; NT, not tested; NA, not applicable since no sample was received or the test was not relevant; I, PCR inhibited. *, Filtered supernatant was tested by mouse bioassay and results in agreement with the PCR result; †, BoNTB was detected only from mixed bacterial growth and not from isolated pure cultures.

consistent phenotypically with *C. botulinum* being lipase-positive, lecithinase-negative, gram-positive, spore-bearing rods that grew on ACBI agar plates.

Forty wound samples including tissue, wound swabs, pus, and aspirates from 21 patients were enriched in CBIB and incubated at 30°C. There was insufficient material to inoculate broths for both heated and unheated treatments. Therefore, 40 wound samples were inoculated into broths that were heated, but only 23 wound samples from six patients were inoculated into broths that were not heat treated.

Filtered supernatants from six CBIB (five inoculated with tissue samples and one with a wound swab) from four patients were tested by mouse bioassay after 5 days of incubation. BoNTA was detected in one broth inoculated with a tissue sample (Table 2) but BoNTs were not detected in the other five broths. The remaining inoculated broth samples were tested by PCR only.

Sixty-three CBIB inoculated in this laboratory were tested by

PCR for BoNTA, -B, and -E gene fragments after 1 day of incubation. If BoNT gene fragments were not detected, these broths were retested after 3 days incubation, and if negative they were tested again after 5 days of incubation. Among the 22 broths from which BoNT gene fragments were amplified, 36% were detected after 1 day, 41% were detected after 3 days, and the remaining 23% were detected at 5 days. There was no significant difference to the time of BoNT gene fragment detection between the unheated and heat-treated CBIBs (χ^2 test for trend = 0.38; $P = 0.54$). BoNT gene fragments were detected in 22 broths from eight of the patients: BoNTA was amplified from six patients, BoNTB from was amplified one, and BoNTA and BoNTB from different broths were amplified from the same patient (Table 2). Of the 22 PCR-positive broths, 15 were heat treated and 7 were unheated. There was no significant difference between detection in heated and unheated broths (χ^2 test = 0.32; $P = 0.57$). The BoNTB gene fragments amplified from two of the patients (Table 2) were

both detected after 5 days: one from both heated and unheated broths and one in an unheated broth only.

All inoculated CBIBs were subcultured on CBI, CBIA, and ColBA agars and incubated at 30°C. DNA was extracted from suspect colonies by using the rapid-boiling method and tested by real-time PCR assay for BoNT gene fragments. BoNT gene fragments were detected only from lipase-positive colonies growing on CBI and CBIA with a typical *C. botulinum* phenotype as described above. BoNT gene fragments were detected from all CBIBs, which had already been shown to be positive by PCR for BoNT gene fragments except for one CBIB broth in which BoNTB was detected by PCR (patient 13) but *C. botulinum* was not isolated (Table 2). All CBIB broths that were negative by PCR were also negative for *C. botulinum* on subculturing.

Of 10 wound samples from patient 13, BoNT gene fragments were detected in enrichment broths inoculated with two tissue samples and one pus swab from the left arm, and BoNTB was detected in an enrichment broth inoculated with tissue from the right arm only. *C. botulinum* type A was isolated from the left arm wounds, but type B was not recovered in pure culture from tissue from the right arm, although the BoNTB gene fragment was detected in the mixed bacterial growth recovered from solid media. Both BoNTA and BoNTB were detected in serum from this patient (Tables 2).

There was almost complete agreement between the results from real-time PCR assays and mouse bioassays. BoNT was detected in the sera from nine of the patients, and gene fragments of the same BoNT type were detected either directly in tissue, in enrichment cultures, or from colonies growing on solid media in all instances except for samples from patient 1 (Table 2). BoNT gene fragments were detected in a further two patients (patients 16 and 20) whose serum samples were not available for analysis (Table 2). In the remaining 14 patients, BoNT or BoNT gene fragments were not detected in any of the samples tested. The same type of BoNT gene fragments were detected from individual samples where the DNA was extracted at different stages in the isolation process. However, in samples from patient 13, the BoNTA gene fragment was detected directly in tissue and in pure cultures, but BoNTA and BoNTB toxins and gene fragments were detected in serum and DNA from separate enrichment broths.

DISCUSSION

Infections due to endospore-forming bacteria of the genus *Clostridium* became recognized as an emerging problem in IDUs in the United Kingdom in 2000 and have continued up to the present time. *C. botulinum* (8, 21) and *C. tetani* (16) infections were a particular problem in this patient group during 2004, and because of difficulties in diagnosing these infections PCR-based assays were developed for the rapid detection of the neurotoxin gene fragments produced by both of these bacterial species.

The conventional diagnostic assays to detect clostridial neurotoxins involve the use of mouse bioassays. These tests, although of high sensitivity, are slow, labor-intensive, and require the use of live experimental animals. In vitro assays, including enzyme-linked immunosorbent and functional tests for the detection of the zinc-dependent peptidase activity of

the BoNT (but not for TeNT), have been described as alternatives to the animal assays (15, 26, 27, 34), although the sensitivity of these assays is lower than that for the mouse bioassay. Recent data have shown that the BoNTA, -B, -C, -D, -E, and -F can be unequivocally identified by matrix-assisted laser desorption ionization and electrospray mass spectrometry (30, 31). However, the improved immunoassays, functional in vitro assays, and mass spectrometric approaches have yet to be proven as alternatives to mouse bioassays for the detection of these toxins in complex matrices such as serum, feces, wound tissue, and food.

We previously reported rapid, reproducible, sensitive, and specific real-time PCR assays for BoNTA, -B, and -E gene fragments for the investigation of human food-borne and infant botulism (1). This is the first report of real-time PCR assay to detect *C. tetani* neurotoxin gene fragments, as well as detection of *C. botulinum* neurotoxin gene fragments, in clinical material from users of illegal injected drugs. As in our previous report (1), results from these BoNT gene fragment PCR assays show a very high level of agreement with the mouse bioassay and show a similar sensitivity with respect to confirming the clinical diagnosis. The PCR assays proved successful for the direct detection of neurotoxin gene fragments in patient's tissue, as well as for the identification of cultures growing in vitro. Although TeNT gene fragments were not detected in tissue from suspect cases of tetanus, these gene fragments were detected in two cultures isolated from suspect cases of tetanus which were later confirmed as *C. tetani* by 16S sequencing by the HPA Anaerobic Reference Laboratory (Cardiff, United Kingdom).

For laboratory diagnosis of both botulism and tetanus, there may be insufficient clinical material available or the numbers of organisms present may be too low for direct PCR detection. Enrichment culture allows low numbers of organisms to recover and grow, and the PCR assays can be used as a rapid screen for the presence of *C. botulinum* and *C. tetani*. Importantly, the use of the real-time PCR assays described here has provided faster presumptive diagnoses, with results being available, in some instances, within one working day of the samples being received at this laboratory or after 1 day of enrichment.

We previously reported the sensitivity of the BoNT assays using purified DNA as between 16 and 141 genome equivalents (1), and this was comparable to the 92 genome equivalents reported here for TeNT assay. However, these estimates are based on the sizes of complete genome sequences of the *C. botulinum* Hall A strain (6) and *C. tetani* E88 strain (10), neither of which were used to generate quantified DNA in the experiments described here. Interspecies variation in genome size has been reported within clostridial species (20), and such variation does not allow an accurate estimation of the sensitivities of these assays.

The detection of both BoNTA and BoNTB in two patients is intriguing. Although two types of *C. botulinum* were not isolated from both patients, BoNTA was recovered in pure culture and BoNTB gene fragments were detected by PCR in enrichment broth and in mixed growth on agar plates for one patient. IDUs can accumulate multiple infected wounds, and the infecting microflora of separate lesions may differ. The observation here suggests for the first time that concurrent infection due to two different BoNT-producing organisms can

occur in the wounds of a single patient. Mixed *C. botulinum* infections have been reported in cases of infant botulism (9, 19). We are currently investigating typing strategies to establish both inter- and intrastain heterogeneities between *C. botulinum* isolates from these patients.

C. botulinum produces seven distinct neurotoxins (18); however, only BoNTA and BoNTB have, to date, has been associated with human wound infections (8, 32, 33). The PCR strategy described here is only able to detect BoNTA, -B, and -E gene fragments. However, because of data presented elsewhere (8, 32, 33) in addition to that from bioassay results described here, we believe it unlikely that the inability to confirm the diagnosis was due to the more unusual botulinum neurotoxins. However, we are developing and evaluating additional assays for the detection of the other neurotoxin genes, including BoNTC, -D, and -F, for application to laboratory diagnosis of all presentations of botulism including that in other animals.

The laboratory diagnosis of wound botulism is problematic. Appropriate samples are not always collected, i.e., samples are too small for toxin tests, the serum is not drawn before the administration of antitoxin, and wound samples are not collected before antibiotic therapy; the levels of toxin in serum can be below the level of detection, and the appropriate wound is not always sampled (8, 32, 33). We report here methods for the improvement in diagnosis by detection of neurotoxin genes in tissue and during in vitro culture. The detection of neurotoxin in patient body fluids remains the gold standard for diagnosis; however, the results presented here show that, where appropriate wound samples are collected, similar diagnostic sensitivity can be achieved more rapidly by the detection of neurotoxin gene fragments by PCR. Although we report the successful detection of the BoNT gene fragments in wound tissue (albeit at low levels), some of the DNA extracts from tissue were shown to be inhibitory to the PCR. Improved extraction strategies are currently being investigated to further optimize this stage in the procedure. We also demonstrate that some hospital laboratories were able to isolate both *C. botulinum* and *C. tetani* from wound tissue and that anaerobic culture broths inoculated by the original source laboratory provide a suitable sample for analyses in a national reference center after transport. We therefore recommend that laboratories collect suitable serum, pus, and tissue samples for bioassay and culture and that anaerobic broth culture media are also directly inoculated with tissue, swabs, or pus by the original investigating laboratory. The use of a combination of PCR and conventional culture with reduced numbers of bioassays provides a more rapid confirmation of clinical diagnosis in a similar proportion of cases than when mouse bioassay and conventional culture are used alone (8, 32, 33). The real-time PCR assays for both *C. botulinum* and *C. tetani* have sensitivities and specificities similar to those of conventional approaches but substantially improve the diagnostic process in terms of speed and by replacement of experimental animals.

In summary, we report here applications of real-time PCR assays for the detection of *C. botulinum* and *C. tetani* neurotoxin gene fragments for the investigation of soft tissue infections among IDUs. The assays are both sensitive and specific and are in almost complete agreement with results from detecting these toxins using mouse bioassays. The use of these

assays in our laboratory has resulted in a reduction of the numbers of animal assays performed and provided more rapid diagnoses of botulism and tetanus among IDUs.

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