Emergence and molecular characterisation of non-toxigenic *tox* gene-bearing *Corynebacterium diphtheriae* biovar *mitis* in the United Kingdom, 2003–2012

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Non-toxigenic *Corynebacterium diphtheriae* have become increasingly recognised as emerging pathogens across Europe causing severe invasive disease. A subset of non-toxigenic *C. diphtheriae* are ‘non-toxigenic *tox* gene-bearing’ (NTTB) strains; these strains are genotypically *tox*positive, but do not express the protein. The circulation of NTTB strains was first observed during the 1990s upsurge of diphtheria in Eastern Europe but has not been reported in other European countries. Circulation of NTTB strains could be considered an increased risk for diphtheria and other related diseases, given their possible role as a *tox* gene reservoir with the theoretical risk of re-emerging toxin expression. Here we report the characterisation of 108 non-toxigenic *C. diphtheriae* biovar *mitis* isolates submitted to the World Health Organization (WHO) Global Reference Centre for Diphtheria at Public Health England, London, between 2003 and 2012, in order to determine the presence of NTTB strains. Using molecular methods, five NTTB isolates were identified; four human isolates (MLST type 212) and one isolate from a companion cat (MLST type 40). The emergence of these strains could indicate continuation of the circulation of potentially toxigenic strains and appropriate laboratory diagnostic methods should be used for detection. Given the complacency that currently exists in Europe awareness with regards to diphtheria diagnostics must be enhanced.

Introduction

Infections caused by toxigenic strains of *Corynebacterium diphtheriae* have become uncommon in Europe as a result of widespread immunisation, implemented during the first half of the 20th Century. In the European Union (EU)/European Economic Association (EEA) countries, the number of reported (toxigenic) diphtheria cases has declined over the past years to 46, 15 and 16 confirmed cases in 2008, 2009 and 2010, respectively [1]. However, diphtheria is still present in all six World Health Organization (WHO) regions and

new epidemics are regularly reported [2]. These and the Eastern European epidemic in the 1990s with more than 157,000 cases and 5,000 deaths between 1990 and 1998, clearly demonstrate the unbroken threat of the disease in the post-vaccine era [3].

The toxigenicity of *C. diphtheriae* strains is solely attributed to the expression of a very potent exotoxin (DTX) which inhibits protein synthesis in mammalian cells [4]. The structural gene (*tox*), consisting of subunits A and B, is carried by a corynebacteriophage and regulated by the chromosomally encoded regulator DtxR (diphtheria toxin repressor). Integration of *tox*-carrying bacteriophages into the bacterial genome can convert non-toxigenic strains into toxigenic and virulent strains. This transformation has been described for example in patients but is generally believed to occur rarely in nature [4,5].

Typical diphtheria is caused by infection with toxigenic strains of *C. diphtheriae*, leading to respiratory or cutaneous symptoms. The characteristic of severe respiratory diphtheria is the presence of a strongly adherent greyish-white pseudomembrane, typically progressing from the tonsils into the larynx and trachea and suffocation following aspiration of the membrane is a common cause of death in untreated cases [6]. Cutaneous diphtheria is more common in tropical regions with a usually mild non-systemic clinical presentation in the form of infected skin lesions and shallow ulcers often occurring in combination with poor hygienic conditions [6]. The most effective treatment against diphtheria is the diphtheria antitoxin (DAT) which binds and neutralises circulating toxin which has not yet bound to tissue. DAT is therefore only recommended for treatment of acute disease. The only way to induce long lasting immunity and to prevent the disease is vaccination; the diphtheria vaccine is one of the oldest vaccines and available as bi- (in combination with tetanus toxoid (DT/dT)) or trivalent vaccine (tetanus, diphtheria,

pertussis (DTaP)) or as combination vaccine with *Haemophilus influenzae* type b (Hib) vaccine) or inactivated polio vaccine (IPV), depending on national vaccination schedules [7].

Non-toxigenic strains have become increasingly recognised as causes of severe invasive disease causing e.g. endocarditis and bacteraemia [8-10]. Infections caused by non-toxigenic *C. diphtheriae* are not preventable by vaccination and pathogenic mechanisms are generally not well understood.

Non-toxigenic *C. diphtheriae* usually completely lack the *tox* gene; exceptionally some non-toxigenic strains also bear the *tox* gene. These strains are genotypically tox-positive, but do not express the protein. They are called 'non-toxigenic *tox* gene-bearing strains' (NTTB).

During and after the 1990s diphtheria epidemic in countries in the Eastern part of the WHO European region, circulation of NTTB strains was widely observed. In 2004, Melnikov et al. published a study on 828 *C. diphtheriae* non-toxigenic strains isolated in different regions of Russia between 1994 and 2002, and found approximately 10% to be NTTB strains [11,12]. All NTTB strains were found to belong to *C. diphtheriae* biovar mitis; some of the isolates were from patients with severe respiratory illnesses. Molecular analyses of the NTTB isolates suggested two mechanisms contributing to the blockage of *tox* gene expression: a single base deletion (52-55 bp), resulting in a frame shift, or the presence of an insertion element (38-46 bp), both localised in the A-subunit of the *tox* gene. Similar studies have, however, not been conducted in other parts of the European region.

At present, the prevalence and epidemiological significance of *C. diphtheriae* NTTB strains across Europe is unknown. The circulation of NTTB strains could be considered an increased risk for diphtheria, given the possible role of these strains as a *tox* gene reservoir in the population and the theoretical possibility of re-emerging toxin expression through spontaneous reversion into toxigenic strains or through homologous recombination between different corynebacteriophages [5].

A recent and unique European screening study comprising 10 European countries and coordinated by the Diphtheria Surveillance Network (DIPNET) (13), showed that toxigenic, non-toxigenic and NTTB strains of *C. diphtheriae* are circulating in Europe, despite a high vaccination coverage among children in many European countries and a presumed absence of clinical disease [14]. Several European countries reported an increase in non-toxigenic *C. diphtheriae* infections in recent years based both on surveillance data and also related to outbreaks [10,15].

The gold standard for laboratory diagnosis of diphtheria is the phenotypic Elek test for toxigenicity characterisation; strains that phenotypically express the

toxin always carry the gene. However, if Elek is the sole test used, the presence or absence of the *tox* gene cannot be determined in strains not expressing toxin. The aim of this study was therefore, to determine the presence, estimate the prevalence and characterise, using molecular methods, NTTB strains submitted to the WHO Global Reference Centre for Diphtheria at Public Health England, London, United Kingdom (UK) between 2003 and 2012 in order to support public health management of diphtheria.

Methods

One-hundred and twenty-two *C. diphtheriae* biovar mitis isolates were received during the period from 15 November 2003 to 16 July 2012, all were human isolates from the UK, except for one, which was originally isolated in Belgium from a cat's nasal swab (this was referred to the reference unit for confirmation of toxigenicity). In case of multiple specimens from one individual only the first isolate was considered. All specimens received in the laboratory were cultivated on blood agar and tellurite agar for initial screening. Typing and biochemical characterisation of all isolates was performed using the API® Coryne test (API bioMérieux, Marcy l'Etoile, France) in addition to the pyrazinimide, the cystinase and the toxigenicity tests (Elek test, polymerase chain reaction (PCR)) if applicable [16]. Five isolates had been previously identified as toxigenic (tox +) by Elek test [17,18] and were excluded from the analysis together with nine specimens that could not be recultivated. PCR analysis was performed for 108 specimens. Two strains, NTTB strain 99/CD/196 (ribotype St. Petersburg, isolated in 1999) and NCTC 13129 (GenBank: NC_002935.2) were included in the molecular analysis as reference strains.

For extraction of bacterial DNA, overnight cultures of *C. diphtheriae* strains cultivated on blood agar were incubated at 37°C (16). A loopful of bacterial culture was added to 500 µl sterile water and heat-treated for 30 minutes at 100°C. Cell debris were separated by centrifugation (12,000 rpm, 1 min) and the supernatant containing DNA was transferred to a sterile tube. For PCR reactions, 1-5 µl of supernatant was used.

Polymerase chain reaction and sequencing of the *tox* gene

Amplification of the *tox* gene (subunit A) was performed as described previously [19,20] using the Novocastra *C. diphtheriae* Primer Set (Leica Microsystems) according to manufacturer's instructions; each reaction included an internal positive control as amplification control. For direct DNA sequencing, the *tox* gene was amplified using the HotStarTaq Master Mix (Qiagen) and selected primers as previously described [21] or primers specifically designed for this study (Table 1 and Figure 1). Sequencing was performed with a BigDye Terminator v1.1 or v3.1 Cycle Sequencing kit (Applied Biosystems) and sequencing reactions were set up using the PCR primers. Sequence data were analysed using the

TABLE 1

Primers for polymerase chain reaction analysis and sequencing of non-toxigenic *tox* gene-bearing *Corynebacterium diphtheriae*, United Kingdom 2012

Primer	Position ^a	Nucleotide sequence (5' -3')	length (bp)
Dipht 1F [21]	(-130) - (-110)	TTGCTAGTGAAGCTTAGCTAG	NA
Dipht 4R [21]	817-837	TGCCGTTTGATGAAATCTTC	973
Dipht 4F [21]	535-555	GAACAGGCGAAAGCGTTAAGCG	NA
Dipht 8R [21]	1731-1751	TCTACCTGTGCATACTATAGC	1217
NTTB 10F ^b	(-25)-(-4)	ATGAGTCCTGGTAAGGGGATACGTTGT	581 ^c
NTTB 11F ^b	(-694)-(-669)	AGCTGCATGAGTGTGTAGCTGCTT	840 ^d
NTTB 12F ^b	(-399)-(-372)	GGCCTGATGATATTGATCTAGATGAGA	545 ^d
NTTB 12R ^b	117 - 146	TTAGTCCCGTGGTACGAAGAAAAGTTTTC	NA

NA: not applicable

^a relative to the first nucleotide of the start codon (+1)

^b this study

^c with Dipht 4R

^d with Dipht 12R

Nucleic acid position indicated according to *Corynebacterium diphtheriae* NCTC 13129 (GenBank Reference Sequence: NC_002935.2)

softwares BioNumerics (Applied Maths) and BioEdit (<http://www.mbio.ncsu.edu/BioEdit/page2.html>).

Multilocus sequence typing (MLST) based on a sequencing scheme comprising seven housekeeping genes was performed as described previously [22].

All isolates were subjected to both the conventional and the modified Elek tests as described previously [16-18].

Antimicrobial sensitivity testing

Antimicrobial sensitivity testing of NTTB isolates was performed according to British Society for Antimicrobial Chemotherapy (BSAC) [23]/The European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2013 guidelines for coryneform organisms [24]. Using Etest strips (bioMérieux, Marcy l'Etoile, France) minimum inhibitory concentration (MIC) breakpoints in mg/L of

>0.12 (resistant (R)) and <0.12 (sensible (S)) were considered for Penicillin [25].

Results

Pure cultures for the 108 *C. diphtheriae* biovar mitis strains were mainly referred from throat swabs (n=87; 80.6%), wound swabs (n=10; 9.3%) and furthermore from blood culture (n=1), nasal swab (n=2) swab/other (n=1), pus (n=1), skin (n=2), ulcer (n=1), for three specimens, no information was provided; 62 specimens were from men (57.4%) and one from an animal (cat). The average age of the human patients was 27.3 years (range 1-72).

C. diphtheriae biovar mitis isolates analysed in this study were submitted between July 2003 and November 2012 with the highest number of isolates submitted (per calendar year) in 2005 (n=14) and 2004 (n=10) and

FIGURE 1

Scheme of the *tox* gene and location of sequencing primers used in this study

Primers were selected for sequencing of subunit A (grey) and subunit B (white) of the *tox* gene. Primers indicated in dotted lines were designed for sequencing and determination of the promoter and upstream region of the four human non-toxigenic *tox* gene-bearing *Corynebacterium diphtheriae* isolates.

Position numbers correspond to *Corynebacterium diphtheriae* NCTC 13129 (GenBank Reference Sequence: NC_002935.2)

TABLE 2

Identified non-toxigenic *tox* gene-bearing and toxigenic *Corynebacterium diphtheriae* biovar mitis strains among isolates submitted to the Diphtheria Reference Unit, United Kingdom, 2003–2012 (n=10)

Isolate	Sex	Age ^a	Specimen	Characteristics
2011/1	M	27	Throat swab	NTTB (PCR+, tox–)
2011/2	M	24	Throat swab	NTTB (PCR+, tox–)
2011/3	M	24	Throat swab	NTTB (PCR+, tox–)
2012/1	M	27	Throat swab	NTTB (PCR+, tox–)
2012/cat	Cat		Nasal swab	NTTB (PCR+, tox–)
2012/1	M	67	Wound swab	Toxigenic (PCR+, tox+)
2010/1	F	57	Skin swab	Toxigenic (PCR+, tox+)
2009/1	M	72	Wound swab	Toxigenic (PCR+, tox+)
2008/1	M	17	Throat swab	Toxigenic (PCR+, tox+)
2008/2	F	7	Bronchoalveolar lavage	Toxigenic (PCR+, tox+)

F: female; M: male; NTTB: non-toxigenic *tox* gene-bearing; PCR: polymerase chain reaction; PCR+: positive; tox: result from conventional and modified tox+/tox–: tox-positive/tox-negative in conventional and modified Elek test.

^a Age refers to age at time point when specimen was taken.

Toxigenicity was determined for all strains using the conventional and modified Elek test and molecular methods (PCR). Full antibiotic sensitivity to penicillin was determined for all non-toxigenic *tox* gene-bearing isolates.

a consistent number of isolates submitted between 2006 and 2011 (n= 5 to 8 isolates per year).

Overall, five *C. diphtheriae* biovar mitis NTTB strains (4.6 %) were identified through PCR analysis and the phenotypic non-toxigenicity for all five NTTB strains was subsequently reconfirmed, using the conventional and modified Elek tests. All five *C. diphtheriae* biovar mitis NTTB strains were referred between 2011 and 2012. One of the NTTB strains originated from a nasal swab of a cat. All four human strains were isolated from throat swabs taken from men (Table 2).

Molecular characterisation of *tox* gene-bearing and toxigenic *Corynebacterium diphtheriae* biovar mitis strains

The five identified *C. diphtheriae* biovar mitis NTTB isolates were subjected to further molecular analysis which included whole *tox* gene sequencing (Figure 2) and molecular typing using MLST [22]. For control and reference purposes, three of the toxigenic strains (*C. diphtheriae* biovar mitis, 2010/1, 2008/1 and 2008/2 (Table 1)) and one previously described NTTB strain from the 1990s Eastern European diphtheria epidemic (St. Petersburg 1999/196) were also included.

Analysis of the sequencing data revealed the presence of the whole *tox* gene, including subunits A and B for all five NTTB isolates. In contrast, the NTTB strain St. Petersburg 1999/196 contained only subunit A of the *tox* gene. Subsequent sequence analysis revealed identical sequences for all isolates subjected to sequencing for the *tox* subunit B (data not shown) and sequence variations for toxsubunit A and within the respective promoter regions (Figure 2).

All four human NTTB strains showed identical sequences for the *tox* subunit A which included a one base pair (bp) deletion at position 25 (Figure 2). The region upstream from the start codon could not be sequenced in the four human NTTB isolates, which included the promoter region, approximately 690 bp, from position -25 to -694, strains 2011/1-3, 2012/1 (Figure 2).

Interestingly, *tox* subunit A in the animal NTTB isolate (2012/cat) was identical to the Russian NTTB strain (St. Petersburg 1999/196) and included an identical promoter region, a one base pair deletion (bp 55) and a one base pair substitution (bp 60) in comparison to the toxigenic strains (Figure 2).

Molecular typing, using MLST, revealed similar MLST sequence types (ST) for the four human NTTB strains (ST 212) and different MLST sequence types for the NTTB isolate from the cat (ST 40) and the toxigenic isolates (STs 500 and 67).

Basic epidemiological features

Only basic epidemiological information for the NTTB isolates could be extracted from the laboratory referral forms. All four human NTTB isolates were throat swabs and collected from young men (Table 1) temporally and/or spatially dispersed. Specimens 2011/1 and 2011/2 were both collected during the same week in April 2011 but in different cities in the UK (distance > 300 km). Isolate 2011/3 was collected November 2011 and in the same city as isolate 2011/2. Subsequently, isolate 2012/1 was collected in January 2012 in the same city as isolate 2011/1. No clinical or further epidemiological information was provided on the referral

FIGURE 2

tox gene sequence alignment (-75 to +62 bp) of non-toxicogenic *tox* gene-bearing *Corynebacterium diphtheriae* biovar mitis strains identified and selected reference strains, United Kingdom 2012

NTTB: non-toxicogenic *tox* gene-bearing; Tox: toxicogenic strains.

Sequencing analysis revealed identical sequences for the four human isolates (NTTB 2011/1-3, 2012/1) including a one base pair deletion (bp 25) and a C>T replacement (bp 244, data not shown). Sequence similarity was revealed for the animal NTTB isolate (NTTB 2012/cat) and the reference Russian isolates (NTTB 199/196) including a one base pair deletion (bp 55) and base pair replacements (bp 60 (A>G) and 431 (G>A, not shown)). All deletions and replacements were located within subunit-A of the *tox* gene.

forms. The animal isolate was from a nasal swab taken from a cat whose owner was diagnosed with a *C. ulcerans* leg ulcer infection.

Discussion

Non-toxicogenic *C. diphtheriae* are increasingly recognised as pathogens across Europe (26). However, in contrast to the toxicogenic *C. diphtheriae* strains, disease and epidemiology is less well understood and monitored and currently, non-toxicogenic *C. diphtheriae* are not included in the case-definitions for surveillance of diphtheria in Europe [27] and no public health action beyond antibiotic treatment is implemented [28].

In the 1990s and during the upsurge of epidemic diphtheria in Eastern Europe, a subset of non-toxicogenic *C. diphtheriae* were described which were *tox* gene-positive, but did not express the protein, the so called NTTB. In this study, we describe, to our knowledge, for the first time after the epidemic upsurge of diphtheria in the 1990s, systematic screening for NTTB strains, conducted among non-toxicogenic *C. diphtheriae* biovar mitis isolates submitted from the UK (plus one animal isolate from Belgium) to the WHO Global Reference Centre at Public Health England, between 2003 and 2012. Overall, five NTTB isolates (4.6 %) were identified among 108 specimens, isolated from four human patients and one animal (cat) carrier. Unfortunately, no information could be obtained on the nature of symptoms or the severity of disease amongst the patients.

MLST of the four human NTTB isolates revealed sequence type (ST) 212 for all four isolates. This ST has until now only been documented twice in the published literature (29, 30). Interestingly, both reports described men; one symptomatically infected with a toxicogenic *C.*

diphtheriae biovar mitis (ST 212) strain and one asymptomatic carrier of *C. diphtheriae* biovar mitis (ST 212), retrospectively identified as an NTTB strain [29,30]. Furthermore, all six ST 212 *C. diphtheriae* biovar mitis isolates described thus far were collected between March 2011 and January 2012; however, they were widely dispersed across Europe (France, Germany, UK).

This study shows that *C. diphtheriae* biovar mitis NTTB strains are circulating in the UK. The temporal pattern, the limited information about patients and the basic molecular analysis of the four human isolates suggests a recent clonal introduction and spread rather than a wide circulation of NTTB strains in the UK. Sequence type 212 described in the German case report (30) was associated with sexual transmission and interestingly, all six ST 212 isolates described to date were isolated from men. Sexually transmitted or -associated diphtheria infections have been described rarely in the literature thus far. A prospective screening study from the 1990s performed at a Genitourinary Medicine (GUM) Clinic in the UK found six (1%) mildly symptomatic pharyngeal carriers of *C. diphtheriae* in a cohort of 578 homosexual men and one (0.05%) asymptomatic carrier in a cohort of 653 heterosexual men and no carriers among 1,043 women [31], but similar and more recent studies are lacking. However, a more detailed and discriminatory molecular analysis (e.g. ribotyping) in addition to detailed epidemiological information is required to allow definite conclusions about the route of transmission.

Molecular analysis of the four human NTTB isolates revealed a new genetic pattern, including a potential deletion of approximately 600 bp's upstream of the start codon which could not be sequenced and a one

base pair deletion at position 25 within *tox* subunit A. Previously and during the 1990s diphtheria epidemic characterised NTTB strains were described with a single base pair deletion (bp 52-55) or the presence of an insertion element (bp 38-46) [11,12]. Despite various attempts using PCR-based methods, the nature of genetic rearrangements in the promoter region of the four human NTTB isolates could not be determined. Further studies including e.g. whole-genome sequencing are needed to analyse the genetic rearrangements and understand the phylogenetic evolution of these strains. Ideally this study could be aimed at a more comprehensive strain collection of NTTB strains circulating in different countries in Europe and beyond.

Interestingly, sequencing analysis of subunit A of the *tox* gene of the animal NTTB isolate (2012/cat) revealed identical sequences with the Russian NTTB isolate (1999/96), representing the 'old' NTTB strains circulating during the 1990 Eastern European epidemic. Thus far, only one similar case has been reported in the literature; in 2010 a non-toxigenic *C. diphtheriae*, biovar belfanti though, was isolated from a domestic cat and retrospectively identified as an NTTB strain [32].

In a recent study on 103 non-toxigenic *C. diphtheriae* isolates collected between 1977 and 2011 in France, the authors described an increasing resistance to selected antibiotics and the potential risk of non-toxigenic strains expressing the diphtheria toxin after being lysogenised by a corynephage harbouring the *tox* gene [33]. However, the role and function of NTTB strains within this context and from a public health point of view has yet to be determined. Firstly, the emergence of these potentially 'toxigenic' strains indicates a potential reservoir for *tox* sequences among circulating strains in the population, which, together with effects of waning immunity [34] and decreasing laboratory capacity for detection of diphtheria in many countries, poses an increased risk for disease and outbreaks. Secondly, the public health management of these patients is unclear; no general guidelines are available on the evaluation of cases and contacts including therapeutic measures such as antibiotic eradication therapy.

Here we report that *C. diphtheriae* NTTB strains are circulating in the UK, associated with a very distinct molecular pattern among the four human isolates, and interestingly, also associated with animal carriage (cat). The presence of *C. diphtheriae* NTTB strains has also been reported from other European countries but only associated with individual cases and systematic data are not available.

Our data together with data from other European countries provides evidence that non-toxigenic, toxigenic and NTTB strains of *C. diphtheriae* are still circulating in Europe. The occurrence of diphtheria has always been characterised by periodicity and epidemic waves and while disease caused by toxigenic strains

of *C. diphtheriae* has become rare in the majority of countries in the WHO European region, non-toxigenic strains of *C. diphtheriae* have been increasingly recognised as emerging pathogens in several European countries. This together with a changing epidemiological pattern (e.g. shift in the age of patients) [35], the detection of multidrug-resistant (MDR) strains [36], reports of zoonotic transmission of *C. ulcerans* [37] and the global circulation of toxigenic strains [2,38], demonstrates the continuous threat posed by this ancient disease.

Complacency that currently exists in Europe with regards to diphtheria circulation and diagnostics should be addressed through regular snapshot or screening studies across European countries, together with both laboratory and epidemiological vigilance and the maintenance of high vaccination coverage. Our study also highlights the importance of close collaboration between clinicians, public health experts and microbiologists to ensure timely data exchange and information flow as our study was hampered in parts by the unavailability of supporting clinical data. Complex public health situations, such as diphtheria management require multidisciplinary approaches and rely on effective communication both on national and international levels.

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Conflict of interest

None declared.

Authors' contributions

Conceived the project: AE, SN, performed the experiments: KZ, analysed the data: KZ, SN, wrote the paper: KZ, contributed to paper writing/editing: AE, SN.

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