Molecular epidemiology and characteristics of Corynebacterium diphtheriae and Corynebacterium ulcerans strains isolated in Italy during the 1990s

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INTRODUCTION

As a result of the widespread use of diphtheria toxoid for immunization (Galazka, 2000), diphtheria became increasingly uncommon in developed countries after the Second World War, until 1980, when only 623 cases were reported in the European Region of the World Health Organization (WHO). At that time, the elimination of diphtheria in the European Region seemed imminent and the WHO Regional Committee for Europe endorsed the target of eliminating indigenous cases by the year 2000 (WHO, 1996). In 1993, however, a large epidemic of diphtheria occurred in Russia and the Newly Independent States of the former Soviet Union (NIS) (Robertson & Oblapenko, 1995; Vitek & Warton, 1998). Several cases of diphtheria also occurred in other European countries, some of which were associated with the NIS epidemic (Eurosurveillance Editorial Board, 1997). In response to the situation in the NIS, the WHO encouraged all Western countries to increase their clinical, microbiological and epidemiological awareness of this disease. In 1993 a European Laboratory Working Group on Diphtheria (ELWGD) was established (Efstratiou et al., 2003).

Non-toxigenic strains of C. diphtheriae have been increasingly reported as causes of invasive disease, including endocarditis, bacteremia, septic arthritis and splenic abscesses (Alexander, 1984; Foilane et al., 1995; Patey et al., 1997; Belko et al., 2000; Mattos-Guaraldi et al., 2001; Funke et al., 1999). Nasopharyngeal carriage and disease caused by this organism have been documented amongst homeless intravenous drug users, alcoholics, Australian aborigines and the Northern Plains Indian Community (Gubler et al., 1998; Anonymous, 1997). The emergence of non-toxigenic C. diphtheriae has also been observed in England and Wales; in particular, clusters of cases of sore throats associated with the isolation of C. diphtheriae were observed during an enhanced surveillance study (Reacher et al., 2000).

In this study the microbiological and molecular characteristics of C. diphtheriae and C. ulcerans isolates received by the Reference Centre for Corynebacterium spp. of the Italian Public Health Institute during the 1990s are described. Italy became a member of the ELWCD in 1994 and participated as a partner within the European Commission BioMed 2 Diphtheria Project (1998–2001).

METHODS

Isolates. C. diphtheriae and C. ulcerans isolates were received by the Istituto Superiore di Sanita’, the Italian Public Health Institute, over a 9-year period (1993–2001).

Microbiological data

(i) Biotyping. Strains received were identified or confirmed as C. diphtheriae or C. ulcerans using API Coryne (bioMerieux).

(ii) Susceptibility to antibiotics. The sensitivities to nine antibiotics (penicillin, ampicillin, cefotaxime, erythromycin, clindamycin, ciprofloxacin, rifampicin, tetracycline and vancomycin) were determined (penicillin, ampicillin, cefotaxime, erythromycin, clindamycin, ciprofloxacin, rifampicin, tetracycline and vancomycin) using the E-test (AB Biodisk). The assay was performed on Mueller–Hinton II broth. An inoculum density of 5×10⁸ cfu ml⁻¹ was added to each tube and incubated for 1 h at 50 °C. GES reagent (50 μl) (5 M guanidium isothiocyanate, 100 mM EDTA, 1 M sodium chloride and 0·5 % lauroyl sarcosine) was added to each tube and incubated for 1 h prior to enumeration. All experiments were conducted in duplicate. Staphylococcus aureus ATCC 29213 was used as a control for the broth dilution and E-test. MIC and MBC values were calculated using cumulation and interpolation (Hamilton-Miller, 1991). MBC was defined as a ≥ 99·9 % reduction in the number of c.f.u. ml⁻¹ from the starting inoculum. In the absence of standardized breakpoints for C. diphtheriae, sensitivity to antimicrobials was assessed using the criteria for Streptococcus spp. other than Streptococcus pneumoniae (National Committee for Clinical Laboratory Standards, 2000).

(iii) Toxigenicity. Strains were tested for toxin production by direct immunodiffusion using a modified Elek test (Engler et al., 1997) and by the in vivo guinea pig subcutaneous virulence test performed at the Italian Reference Laboratory. Toxigenic (C. diphtheriae NCTC 10648), weakly toxigenic (C. diphtheriae NCTC 3984) and non-toxigenic (C. diphtheriae NCTC 10356) strains were used as reference strains in all tests.

(iv) tox locus analysis. tox locus analysis was performed by PCR and Southern blotting.

For Southern blotting, genomic DNAs of C. diphtheriae strains were extracted as described by Rappuoli et al. (1988). In brief, a loofop of bacteria, grown on CBA, was resuspended in 0·5 ml lysomyce (10 mg ml⁻¹ of 10 mM Tris and 10 mM EDTA, pH 8) and DNA was extracted according to standard procedures. The DNA was digested with the restriction endonucleases BamHI and EcoRI (Boehringer Mannheim), separated by gel electrophoresis (1 % w/v agarose) and transferred onto nylon membranes. Blotted DNAs were hybridized with a digoxigenin (DIG)-labelled T21 probe, a DNA fragment encoding subunit A of the C. diphtheriae toxin gene (Rappuoli et al., 1988). The T21 probe was labelled using the DIG DNA labelling and detection kit according to the manufacturer’s instructions (Boehringer Mannheim). For PCR, a loofop of organisms, freshly grown on CBA, was transferred into 0·5 ml sterile distilled water. The suspension was boiled for 15 min and then centrifuged at 8000 ×g for 1 min. The oligonucleotide primers used for the amplification of toxin gene fragment A spanned a region of 248 nt as described by Pallen et al. (1994). The B fragment of the toxin gene fragment A was amplified using the oligonucleotides 5′-TGGTAGAGATTTATGAAATT3′ and 5′-TGCCCCCATCTCCCTTGAATAA-3′ (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX_A) and 5′-CAATTTGAAATT3′ and 5′-TGCCCCCATCTCCCTTGAATAA-3′ (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX_B).

For Southern blotting, genomic DNAs of C. diphtheriae strains were extracted as described by Rappuoli et al. (1988). In brief, a loofop of bacteria, grown on CBA, was resuspended in 0·5 ml lysomyce (10 mg ml⁻¹ of 10 mM Tris and 10 mM EDTA, pH 8) and DNA was extracted according to standard procedures. The DNA was digested with the restriction endonucleases BamHI and EcoRI (Boehringer Mannheim), separated by gel electrophoresis (1 % w/v agarose) and transferred onto nylon membranes. Blotted DNAs were hybridized with a digoxigenin (DIG)-labelled T21 probe, a DNA fragment encoding subunit A of the C. diphtheriae toxin gene (Rappuoli et al., 1988). The T21 probe was labelled using the DIG DNA labelling and detection kit according to the manufacturer’s instructions (Boehringer Mannheim). For PCR, a loofop of organisms, freshly grown on CBA, was transferred into 0·5 ml sterile distilled water. The suspension was boiled for 15 min and then centrifuged at 8000 ×g for 1 min. The oligonucleotide primers used for the amplification of toxin gene fragment A spanned a region of 248 nt as described by Pallen et al. (1994). The B fragment of the toxin gene fragment A was amplified using the oligonucleotides 5′-TGGTAGAGATTTATGAAATT3′ and 5′-TGCCCCCATCTCCCTTGAATAA-3′ (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX_A) and 5′-CAATTTGAAATT3′ and 5′-TGCCCCCATCTCCCTTGAATAA-3′ (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX_B). The reaction mixture (30 μl) contained 1 μM primers, 200 μM each of dATP, dCTP, dGTP and dTTP, 30 pmol of each primer, 2·5 U Taq DNA polymerase and PCR buffer. The genes were amplified by one denaturation cycle of 96 °C for 2 min and then 30 cycles of 94 °C for 15 s, 50 °C for 1 s and 72 °C for 30 s, followed by a final 10 min extension cycle at 72 °C.

Ribotyping. Genomic DNA was extracted using a modified method of Pitcher et al. (1989). Briefly, an overnight growth of isolates on CBA plates was harvested into 200 μl lysomyce (50 mg ml⁻¹ water) and incubated for 3 h at 37 °C. Ten microlitres of proteinase K (25 mg ml⁻¹) was added to each tube and incubated for 1 h at 50 °C. GES reagent (50 μl) (5 M guanidium isothiocyanate, 100 mM EDTA, 1 M sodium chloride and 0·5 % Na-lauroyl sarcosine) was added to each tube and the tube was mixed gently for 5 min, until the cells were fully lysed. Two hundred and fifty microlitres of 7·5 M ammonium acetate and 250 μl chloroform/isoamyl alcohol (24:1, v/v) were added to each tube, which was vortexed and kept on ice for 10 min. Tubes were centrifuged for 10 min at 13 000 ×g and the supernatant was removed to a fresh tube. DNA was precipitated using 0·5 μl of 3·0 M sodium acetate.
DNA was cleaved at 37 °C for 3 h with BstEII (Gibco). Restriction fragments were separated by electrophoresis on 0.7-2% (w/v) agarose gel (Gibco) in 0.5X Tris/borate-EDTA (TBE) at 120 V for 6 h. The restriction fragments were blotted onto a positively charged nylon membrane (Hybond-N; Amersham) and hybridized at 37 °C for 4 h using a 32P oligonucleotide probe (OligoMix5) labelled with DIG as described by Regnault et al. (1997). Colorimetric detection was performed using the DIG wash and blocking buffer set (Boehringer Mannheim), sheep anti-DIG-AP Fab fragments (Roche) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma) to produce a brown precipitate. BstEII profiles were analysed using the Taxotron software package (Taxolab, Institut Pasteur, Paris, France). The pattern obtained with Citrobacter koseri CIP 105177, restricted by MluI, served as a fragment size marker (Regnault et al., 1997).

RESULTS

Epidemiological characteristics

Five cases of diphtheria, two of which were fatal, were reported in Italy between January 1990 and June 2001. Toxigenic strains of Corynebacterium spp. were isolated from three of these cases, namely C. diphtheriae var. gravis, C. diphtheriae var. mitis and C. ulcerans (Table 1). This last strain was isolated from a woman aged 75 who was admitted to hospital as an emergency with pharyngitis, pseudomembranes, sinusitis, lymphadenopathy and palatal paralysis.

Several non-toxigenic strains of C. diphtheriae were isolated over the same period (Table 2). Eight strains were isolated from children and young adults (aged 4–28 years) who presented with fever, severe pharyngitis and tonsillitis; two patients also had a recurrent sore throat. Most (five of seven) strains associated with this type of infection were of biotype gravis, and Streptococcus pyogenes was isolated in association with biotype gravis or mitis from three of the patients. The screening of family contacts detected asymptomatic carriers who were associated with three cases (Table 2). None of these cases or carriers had travelled outside Italy.

The screening of 33 Rwandan children, who arrived in Italy through a humanitarian association, yielded two isolates of C. diphtheriae biotype mitis (Table 2), one from the throat and the other from a severe skin lesion. A strain of C. diphtheriae biotype belfanti was isolated from the throat of an immigrant in association with Klebsiella pneumoniae.

All C. diphtheriae strains were examined for toxin production by the Elek test, Southern blotting and PCR amplification of both the fragment A and B of the diphtheria toxin gene (tox). The in vivo guinea pig virulence test was performed only for the strains isolated from clinically typical cases of diphtheria. The toxin gene was detected in all strains derived from diphtheria cases and was biologically active, as demonstrated by the in vitro test. C. ulcerans produced not only diphtheria toxin, but also the dermonecrotic toxin (MacGregor, 1995). Analysis of the tox locus in strains 2786 and 2912 demonstrated the presence of a corynephage with endonuclease patterns similar to that of β phage only in strain 2912 (Fig. 1). All C. diphtheriae strains isolated from pharyngitis or from asymptomatic individuals were identified as non-toxigenic by the Elek test, PCR and Southern blotting.

Antibiotic susceptibility of non-toxigenic isolates

All non-toxigenic isolates were susceptible to erythromycin (MIC < 0.016 mg l⁻¹) (Table 3). MICs of penicillin ranged between 0.012 and 0.025 mg l⁻¹ and between 0.012 and 0.030 mg l⁻¹ for ampicillin. The majority of the strains therefore showed an intermediate susceptibility to these two antibiotics using Streptococcus spp. breakpoints. Furthermore, 7/12 (64%) had an MBC/MIC ratio ≥ 32 for penicillin, which has been suggested as indicating tolerance (Amsterdam, 1996). All the strains were sensitive to clindamycin (MIC ≤ 0.025 mg l⁻¹), rifampin (MIC ≤ 1 mg l⁻¹), tetracycline (MIC ≤ 2 mg l⁻¹), ciprofloxacin (MIC ≤ 0.049 mg l⁻¹). All strains had an intermediate susceptibility for cefotaxime (MIC 0.75–1 mg l⁻¹) and vancomycin (MIC 1–2 mg l⁻¹). Strains isolated from close contacts had similar antimicrobial susceptibility patterns to their relative index case (data not shown).

Ribotyping

Ribotyping results are shown in Tables 1 and 2. Ribotyping of the non-toxigenic strains produced several distinct ribotype patterns.

Table 1. Characteristics of toxigenic corynebacteria isolated in Italy

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>Patient from</th>
<th>Age (years)/sex</th>
<th>Source of isolate</th>
<th>Species (strain no.)</th>
<th>Ribotyping pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td>Peru</td>
<td>38/F</td>
<td>Pseudo-membrane</td>
<td>C. diphtheriae biotype gravis (2767)</td>
<td>D4S+</td>
</tr>
<tr>
<td>1995</td>
<td>Italy</td>
<td>3/F</td>
<td>Pharyngeal secretion</td>
<td>C. diphtheriae biotype mitis (2912)</td>
<td>ND</td>
</tr>
<tr>
<td>1998</td>
<td>Italy</td>
<td>75/F</td>
<td>Throat, nose</td>
<td>C. ulcerans (3910)</td>
<td>U1</td>
</tr>
</tbody>
</table>

*Reported in von Hunolstein et al. (1995) |
†ND, Not determined – the strain was not available for ribotyping.
patterns. The *gravis* isolates produced two distinct ribotypes provisionally designated as 'D11' and 'D75'. Isolates of *C. diphtheriae* biotype *mitis* from Italy produced two distinct patterns provisionally designated as 'D76' and 'D77', and the two isolates of biotype *mitis* from the Rwandan children also produced two distinct ribotype patterns designated as 'D54' and 'D55' (provisional nomenclature). The *C. diphtheriae* var. *belfanti* isolate produced a distinct ribotype ('D78').

Strains isolated from close contacts of the case produced ribotype patterns which were indistinguishable from that produced by the case.

### DISCUSSION

Following the introduction of routine immunization for diphtheria in the 1940s and the improvements in social conditions, the incidence of diphtheria in Italy declined to nil in the 1980s. The circulation of toxigenic strains also declined in other countries with good vaccination coverage against diphtheria (Saragea *et al.*, 1979). However, during the 1990s four cases of diphtheria caused by *C. diphtheriae* and the first case due to *C. ulcerans* were reported in Italy. Apart from the fatal imported case that occurred in a Peruvian immigrant, patients with fever, severe pharyngitis and tonsillitis. *Strains isolated from close contact (family) with 2989.*

†Strains isolated from close contact (family) with 4396.

§Strain isolated from close contact (family) with 4870.

*In association with group A streptococci.*

*In association with Klebsiella ozaenae.*
The isolation rate of non-toxigenic *C. diphtheriae* or indeed globally is not known. The isolation rate of non-toxigenic *C. ulcerans* to substan
ting the current pattern of immunity to diphtheria showed that there was a high proportion of adults with insufficient levels of protection in all countries (Edmunds et al., 2000). In Italy, approximately 26% of individuals over 40 years old and 35% of individuals between 40 and 49 years old had no protective antibody levels to diphtheria toxin (<0.01 IU ml⁻¹) (von Hunolstein et al., 2000). Immunity to diphtheria toxin protects not only against diphtheria caused by toxigene
c *C. diphtheriae*, but also from diphtheria caused by toxigenic *C. ulcerans* (MacGregor, 1995).

Diphtheria caused by *C. ulcerans* is very rare, but in recent years, several cases have been recorded in the UK (Engler, 2001). Interestingly, the strain isolated in Italy produced a ribotype indistinguishable to that produced by UK isolates of *C. ulcerans*. The significance of this is unclear, as there were no apparent epidemiological links with the UK. The dissemi
nation of *C. ulcerans* strains within the European Region or indeed globally is not known.

The isolation rate of non-toxigenic *C. diphtheriae* from throat swabs of children and young adults with sore throats and fever within Italy is similar to that reported from England and Wales (Reacher et al., 2000; Wilson, 1995; Bonnet & Begg, 1999). It is difficult to be certain that the non-toxigenic strains were the cause of the pharyngitis and responsible for the clinical severity, but *C. diphtheriae* was isolated in association with group A streptococci in only three cases. Non-toxigenic strains in the throat were therefore probably capable of a pathogenic as well as a colonizing role; further evidence for this pathogenic role is provided in other reports (Patey et al., 1997; Belko et al., 2000; Gubler et al., 1998) which describe severe invasive disease caused by non-toxigenic strains. In several instances, the infection was fatal, but the virulence factors of these strains are still unknown. Gianciotto & Groman (1997) demonstrated that a subset of non-toxigenic corynebacteria carried the diphtheria toxin gene in a cryptic form. Non-toxigenic tox-bearing *C. diphtheriae* strains currently represent 20–30% of the non-
toxigenic *C. diphtheriae* biotype *mitis* from the Russian Federation (Melnikov et al., 2000). By Southern hybridiza
tion analysis all our non-toxigenic isolates were shown to lack the toxin gene.

*Table 3. In vitro activity (MIC mg l⁻¹) by E-test of various antimicrobial agents against non-toxigenic *C. diphtheriae***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biotype</th>
<th>CEF</th>
<th>PEN</th>
<th>AMP</th>
<th>ERY</th>
<th>CLI</th>
<th>CIP</th>
<th>RIF</th>
<th>TET</th>
<th>VAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2831</td>
<td><em>mitis</em></td>
<td>1·00</td>
<td>0·125</td>
<td>0·125</td>
<td>&lt;0·016</td>
<td>0·125</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>ND</td>
<td>1·0</td>
</tr>
<tr>
<td>2834</td>
<td><em>mitis</em></td>
<td>1·00</td>
<td>0·125*</td>
<td>0·190</td>
<td>&lt;0·016</td>
<td>0·190</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>0·50</td>
<td>1·5</td>
</tr>
<tr>
<td>2989</td>
<td><em>gravis</em></td>
<td>1·00</td>
<td>0·125*</td>
<td>0·190</td>
<td>&lt;0·016</td>
<td>0·125</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>0·50</td>
<td>1·0</td>
</tr>
<tr>
<td>3319</td>
<td><em>mitis</em></td>
<td>1·00</td>
<td>0·190*</td>
<td>0·125</td>
<td>&lt;0·016</td>
<td>0·250</td>
<td>0·047</td>
<td>&lt;0·016</td>
<td>0·19</td>
<td>1·0</td>
</tr>
<tr>
<td>4060</td>
<td><em>gravis</em></td>
<td>1·00</td>
<td>0·190</td>
<td>0·380</td>
<td>&lt;0·016</td>
<td>0·190</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>0·50</td>
<td>1·0</td>
</tr>
<tr>
<td>4112</td>
<td><em>mitis</em></td>
<td>1·00</td>
<td>0·125</td>
<td>0·125</td>
<td>&lt;0·016</td>
<td>0·250</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>0·19</td>
<td>1·0</td>
</tr>
<tr>
<td>4129</td>
<td><em>gravis</em></td>
<td>1·50</td>
<td>0·250*</td>
<td>0·250</td>
<td>&lt;0·016</td>
<td>0·250</td>
<td>0·064</td>
<td>&lt;0·016</td>
<td>0·50</td>
<td>1·0</td>
</tr>
<tr>
<td>4252</td>
<td><em>belfanti</em></td>
<td>0·75</td>
<td>0·190</td>
<td>0·250</td>
<td>&lt;0·016</td>
<td>0·125</td>
<td>0·047</td>
<td>&lt;0·02</td>
<td>0·50</td>
<td>1·5</td>
</tr>
<tr>
<td>4366</td>
<td><em>gravis</em></td>
<td>1·50</td>
<td>0·250*</td>
<td>0·380</td>
<td>&lt;0·016</td>
<td>0·190</td>
<td>0·094</td>
<td>&lt;0·016</td>
<td>0·50</td>
<td>2·0</td>
</tr>
<tr>
<td>4396</td>
<td><em>gravis</em></td>
<td>ND</td>
<td>0·190*</td>
<td>0·380</td>
<td>&lt;0·016</td>
<td>0·125</td>
<td>0·094</td>
<td>ND</td>
<td>ND</td>
<td>1·0</td>
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<tr>
<td>4870</td>
<td><em>gravis</em></td>
<td>1·50</td>
<td>0·190*</td>
<td>0·250</td>
<td>&lt;0·016</td>
<td>0·190</td>
<td>0·047</td>
<td>&lt;0·002</td>
<td>0·38</td>
<td>1·0</td>
</tr>
</tbody>
</table>

*Tolerant for penicillin.*
C. von Hunolstein and others

sample is therefore 0.07%. If the two strains from the family members of the index case are also considered, the percentage increases to 0.14%. A surveillance study within the general population may have yielded a higher carriage rate of C. diphtheriae.

Although the characteristics of our sample may be open to question, this was the only way to perform a surveillance study in a country where diphtheria is not a public health problem. The data indicated that non-toxigenic C. diphtheriae strains may be circulating; considering the few cases of diphtheria that occurred during the 1990s, toxigenic strains could also be in circulation albeit at very low levels. Therefore, it is extremely important that laboratory diagnostic expertise and accountability is maintained and, moreover, the immune status of the population for diphtheria must be preserved by routine boosting of adults.

The susceptibility of the non-toxigenic strains to penicillin, ampicillin, clindamycin, vancomycin was similar to that reported by other authors (Funke et al., 1999; Patey et al., 1995). In contrast to other studies, no isolates were found to be resistant to rifampin, tetracycline, erythromycin and clindamycin. Isolates from invasive infections have been previously shown to be resistant to rifampin and tetracycline (Funke et al., 1999; Patey et al., 1995) and resistance to erythromycin and clindamycin has been documented amongst non-toxigenic isolates from skin infections (Coyle et al., 1979) and diphtheria cases in Vietnam (Kneen, 1998).

Laboratory results showed that, in five out of eight cases, treatment of the patient with penicillin for up to 10 days did not eradicate C. diphtheriae from the throat. A second course of antibiotic therapy, usually with a macrolide, was found to be necessary. The treatment failure could be related to the fact that the MBCs of penicillin for these non-toxigenic strains are high, often above the penicillin serum level of about 2–4 mg ml⁻¹ (Chamber & Neu, 1995), and that the majority of C. diphtheriae isolates are tolerant to this antibiotic. Seventy percent of sore throat isolates of non-toxigenic C. diphtheriae biotype gravis were recently reported to be tolerant to penicillin (von Hunolstein et al., 2002). It is therefore important to be aware that where C. diphtheriae is the only pathogen to be isolated from cases of pharyngitis/C226lactams should not eradicate C. diphtheriae from the throat. A second course of antibiotic therapy, usually with a macrolide, is therefore recommended for the treatment of these infections. Resistance of C. diphtheriae to β-lactams should also be considered in systemic infections, as C. diphtheriae tolerant to amoxicillin has been isolated from a case of endocarditis (Dupont et al., 1995).

To gain some insight into the epidemiology of toxigenic and non-toxigenic strains, all strains were ribotyped. Ribotyping is currently the ‘gold standard’ for typing C. diphtheriae. The technique is reproducible, highly discriminatory and appeared to be a powerful means of differentiating the strains in the diphtheria epidemic in the NIS (De Zoya et al., 1995). A unique band pattern, not previously seen amongst isolates of biotype gravis was observed for toxigenic strain 2767. This pattern has not been identified amongst European isolates previously examined (Grimont et al., 2000) and therefore it was assumed that the strain was imported from South America. The ribotype ‘D11’, which was produced by the non-toxigenic biotype gravis isolates, has been documented amongst non-toxigenic biotype gravis strains isolated in the UK, Russia, Germany, Romania and Sweden. Ribotype ‘D75’ was also seen amongst non-toxigenic biotype mitis UK isolates whilst the other ribotypes had not been documented anywhere else (A. De Zoya, unpublished observation).

In the absence of archival isolates, and thus only on the basis of the few strains that have been collected and studied during the 1990s, it can be inferred that some ribotypes (‘D11’ and ‘D75’) persist in Italy. It is also interesting that some ribotypes are widely distributed, even if, in the case of the non-toxigenic strains, the epidemiological significance of this phenomenon is difficult to explain. These data are important in view of the still ongoing circulation of toxigenic strains as demonstrated by the four cases of diphtheria, and in the fact that toxigenic C. diphtheriae strains can be imported from areas where diphtheria is endemic and that non-toxigenic strains may be responsible for severe infection. Although the risk of a resurgence of diphtheria in Italy is low, the capacity to control and to prevent this disease must be maintained, as well as awareness and laboratory capabilities in this specialized area of microbiology.

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C. diphtheriae and C. ulcerans in Italy


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