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

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Five cases of diphtheria were reported in Italy between January 1990 and June 2001. Three cases were confirmed microbiologically by the isolation of toxigenic Corynebacterium diphtheriae (two cases) and Corynebacterium ulcerans (one case). Over the same period, 11 cases of non-toxigenic C. diphtheriae infection were reported to the Italian Public Health Institute, from which the causative organism was isolated from a skin infection in one case and from the throat in the other ten. Seven of the throat isolates were associated with fever, severe pharyngitis and tonsillitis and were all biotype gravis.

Because there are no standardized breakpoints, the antimicrobial sensitivities of C. diphtheriae were determined in accordance with the National Committee for Clinical Laboratory Standards guidelines for Streptococcus spp. other than Streptococcus pneumoniae. MICs for penicillin ranged between 0·125 and 0·250 mg l\(^{-1}\) and 7 out of 11 strains had a minimal bactericidal concentration (MBC)/MIC ratio > 32. All strains were sensitive to clindamycin (MIC < 0·25 mg l\(^{-1}\) ), rifampicin (MIC < 1 mg l\(^{-1}\) ) and tetracycline (MIC < 2 mg l\(^{-1}\) ), and showed moderate susceptibility to cefotaxime (MIC 0·75 – 1·5 mg l\(^{-1}\) ).

Molecular typing (ribotyping) demonstrated the presence of several distinct ribotypes. The ribotype designated ‘D11’ has been documented amongst strains isolated in the UK, Russia, Germany, Romania and Sweden. Ribotype ‘D75’ has only been documented in the UK. The C. ulcerans strain had a ribotype pattern identical to that found in recent isolates from the UK.

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., 1994).
2000) and later, within the remit of the European Commission DGXII, BioMed 2 programme (1998–2001), a network of European Diphtheria Reference Centres was established (Efratiou et al., 1999).

Over the past 10 years, notifications of diphtheria in England and Wales have included several cases caused by Corynebacterium ulcerans as well as Corynebacterium diphtheriae (Engler, 2001). C. ulcerans was first described in 1926, when the organism was isolated from human throat lesions (Gilbert & Stewart, 1926). In addition to a dermonecrotic toxin, strains of C. ulcerans can produce a diphtheria toxin immunologically identical to that of C. diphtheriae (Wong & Groman, 1984; MacGregor, 1995).

Non-toxigenic strains of C. diphtheriae have been increasingly reported as causes of invasive disease, including endocarditis, bacteraemia, septic arthritis and splenic abscesses (Alexander, 1984; Poilane et al., 1995; Patey et al., 1997; Belko et al., 2000; Mattos-Guaralaldi et al., 2001; Funke et al., 1999). Nasopharyngeal carriage and disease caused by this organism have been documented amongst homeless people, 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 and respiratory tract infections have been reported 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 ELWGD 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 Sanità, 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 (bioMérieux).

(ii) Susceptibility to antibiotics. The sensitivities to nine antibiotics (penicillin, ampicillin, cefotaxime, erythromycin, clindamycin, ciprofloxacin, rifampicin, tetracycline and vancomycin) were determined by serial twofold dilutions (32–0·03 mg l\(^{-1}\)) of penicillin in Mueller–Hinton II broth. An inoculum density of 5\(^{10}\) c.f.u. ml\(^{-1}\) of exponentially growing cells was used. Tubes were incubated under agitation at 37 °C for 24 h. Following incubation, viable counts were determined by plating 100 µl samples from each tube with no visual growth on to Columbia blood agar (supplemented with 5 % v/v sheep blood; CBA). Plates were incubated at 37 °C in air for 24 to 48 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) Toxicity. Strains were tested for toxin production by direct immunodiffusion using a modified Eik 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 loopful of bacteria, grown on CBA, was resuspended in 0·5 ml lysosome (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 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 loopful 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 \(\times\) 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 was amplifed using the oligonucleotides 5′-TGTAGAGATTATCACATT 3′ and 5′-TGCCCACTGTCCTGAAAGA-3′ (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX (homologous to nt 1302–1322 and 1155–1175 of EMBL sequence NCCDTOX accession no. X00703, and nt 292 523–292 503 and 292 356–292 376 of Sanger’s sequencing project C. diphtheriae contig 185, respectively)). The reaction mixture (30 µl) contained 1 µl supermaster, 200 µl PCR buffer, 300 µl of 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 ml lysosome (50 mg ml\(^{-1}\) water) (1) 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 ml) (5 M guanidinium isothiocyanate, 100 mM EDTA, 1 M sodium chloride and 0·5 % Na-lauryl sarcosine) was added to each tube, and the tube was mixed gently for 15 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 left 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 vols of 2-propanol.

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DNA was cleaved at 37°C for 3 h with BseII (Gibco). Restriction fragments were separated by electrophoresis on 0.7% (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 (37°C for 4 h) using a five 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. BseII profiles were analysed using the Taxotron software package (Taxolab, Institut Pasteur, Paris, France). The pattern obtained with \(C. diphtheriae\) biotype gravis, or \(C. diphtheriae\) 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 53 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 \(Klebsiella\) \(ozaenae\).

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 \((\text{tox})\). 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 vivo test. \(C. ulcerans\) produced not only diphtheria toxin, but also the dermonecrotic toxin (MacGregor, 1995). Analysis of the tox locus in strains 2767 and 2912 demonstrated the presence of a coryneplasm with endonuclease patterns similar to that of \(\beta\) 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.

### 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.

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### 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 0·125 and 0·25 mg l\(^{-1}\) and between 0·125 and 0·380 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 ≤ 0·25 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 0·75–1·5 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

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**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>&quot;D45&quot;*</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>&quot;U1&quot;</td>
</tr>
</tbody>
</table>

*Reported in von Hunolstein et al. (1995)
†ND, Not determined — the strain was not available for ribotyping.
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, 8 patients with fever, severe pharyngitis and tonsillitis.

![Fig. 1. Southern blot of chromosomal *C. diphtheriae* DNA. DNA (5 μg) extracted from reference lysogenic strain C7(197), non-lyso- genic parental strain C7 and toxigenic clinical isolates 2912 and 2767 was digested with BamHI (lanes 1) and EcoRI (lanes 2). Nylon membrane was hybridized with DIG-labelled T21 tox probe.](image)

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>Isolate no.</th>
<th>Source of isolate</th>
<th>Patient from: Age (years)/sex</th>
<th><em>C. diphtheriae</em> biotype</th>
<th>Ribotyping pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994</td>
<td>2831</td>
<td>Skin lesion</td>
<td>Rwanda</td>
<td><em>mitis</em></td>
<td>'D54'</td>
</tr>
<tr>
<td></td>
<td>2834</td>
<td>Throat</td>
<td>Rwanda</td>
<td><em>mitis</em></td>
<td>'D55'</td>
</tr>
<tr>
<td>1995</td>
<td>2989</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td></td>
<td>2992†</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td></td>
<td>2993†</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td></td>
<td>2994†</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td>1997</td>
<td>3319</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>mitis</em></td>
<td>'D76'</td>
</tr>
<tr>
<td>1999</td>
<td>4060</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
<tr>
<td></td>
<td>4112</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>mitis</em></td>
<td>'D77'</td>
</tr>
<tr>
<td></td>
<td>4129</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
<tr>
<td></td>
<td>4252</td>
<td>Nose</td>
<td>Italy</td>
<td><em>belfanti</em></td>
<td>'D78'</td>
</tr>
<tr>
<td></td>
<td>4366</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
<tr>
<td>2000</td>
<td>4396</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td></td>
<td>4402†</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D11'</td>
</tr>
<tr>
<td></td>
<td>4403†</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
<tr>
<td>2001</td>
<td>4870</td>
<td>Throat*</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
<tr>
<td></td>
<td>4899§</td>
<td>Throat</td>
<td>Italy</td>
<td><em>gravis</em></td>
<td>'D75'</td>
</tr>
</tbody>
</table>

*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*.  

Table 2. Characteristics of non-toxigenic *C. diphtheriae* isolated in Italy
The isolation rate of non-toxigenic *C. diphtheriae* toxigenic *C. ulcerans* genic toxin protects not only against diphtheria caused by toxigenic strains, 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. Giancotti & Groman (1997) demonstrated that a subset of non-toxigenic corynebacteria carried the diphtheria toxin gene in a cryptic form. Non-toxigenic *C. diphtheriae* strains currently represent 20–30 % of the non-toxigenic *C. corynebacteria mitis* (MacGregor, 1995). Immunity to diphtheria toxin protects not only against diphtheria caused by toxigenic *C. corynebacteria mitis*, but also from diphtheria caused by toxigenic *C. corynebacteria 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 strains within the European Region toxigenic *C. corynebacteria ulcerans* biotype *mitis* from the Russian Federation (Melnikov et al., 2000). By Southern hybridization 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*

Abbreviations: CEF, cefotaxime; PEN, penicillin; AMP, ampicillin; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; RIF, rifampicin; TET, tetracycline; VAN, vancomycin; ND, not determined.

<table>
<thead>
<tr>
<th>Strain/Year</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>1999</td>
<td>mitis</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>1994</td>
<td>mitis</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>
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<tr>
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<td>gravis</td>
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<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>1-0</td>
</tr>
<tr>
<td>1999</td>
<td>mitis</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>
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<tr>
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<td>gravis</td>
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<td>0-190</td>
<td>0-380</td>
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<td>0-190</td>
<td>0-064</td>
<td>&lt; 0-016</td>
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<td>1-0</td>
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<td>1999</td>
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<td>&lt; 0-016</td>
<td>0-250</td>
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<td>&lt; 0-016</td>
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</table>

*Tolerant for penicillin.*

Described previously (von Hunolstein et al., 1995), the other cases were all notified as occurring in Italians, one of whom was an unvaccinated child, who died. In contrast to reports from some Western countries (PHLS, 1979; Lumio et al., 1993; Anonymous, 1995; Hart et al., 1996), the diphtheria cases caused by *C. diphtheriae* were not linked to the epidemic in Eastern Europe or contracted during travel to endemic diphtheria areas (Africa, Eastern Mediterranean, South East Asia, South America).

The risk of contracting diphtheria is usually associated with the epidemiological situation within a country and the immune status of individuals. Results from a serological survey undertaken by seven European countries to document 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 toxigenic *C. corynebacteria*, but also from diphtheria caused by toxigenic *C. corynebacteria 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 dissemination 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. Giancotti & 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 hybridization analysis all our non-toxigenic isolates were shown to lack the toxin gene.

*C. diphtheriae* biotype *belfanti* was isolated from the nose of a patient affected by a chronic atrophic rhinitis, oxena, in association with *Klebsiella ozaenae*. The presence of non-toxigenic *C. diphtheriae*, and in particular of the biotype *belfanti* from this disease has been reported, but the role of this organism has not yet been established (Patey et al., 1998; Hennebicq-Reig et al., 1995).

Within the remit of the European Commission DG XII Biomed 2 project, a regional surveillance study to determine the occurrence of *C. diphtheriae* in throat swabs from patients presenting to a selected clinical laboratory in three different cities in Italy (Palermo, Rome and Perugia) was undertaken for 9 months from October 1999 to June 2000. Amongst a total of 2821 swabs that were screened, two non-toxigenic strains were isolated; toxigenic strains of *C. diphtheriae* were not found. Screening of family members of the index cases led to the isolation of two additional strains. The carriage percentage of *C. diphtheriae* in this selected, non-random
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 could 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 one 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/tonsillitis, this organism could be the cause of that infection and that penicillin is not the most appropriate antibiotic. In such cases, the symptoms always responded to erythromycin, which is therefore recommended for the treatment of these infections. Tolerance of *C. diphtheriae* to ß-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 among 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.

**ACKNOWLEDGEMENTS**

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C. diphtheriae and C. ulcerans in Italy


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