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SHORT REPORT
Multilocus sequence types of invasive *Corynebacterium diphtheriae* isolated in the Rio de Janeiro urban area, Brazil

S. Z. VIGUETTI[1], L. G. C. PACHECO[1,2], L. S. SANTOS[3], S. C. SOARES[1], F. BOLT[4], A. BALDWIN[5], C. G. DOWSON[5], M. L. ROSSO[6], N. GUISO[6], A. MIYOSHI[1], R. HIRATA Jr.[3], A. L. MATTOS-GUARALDI[3] AND V. AZEVEDO[1]*

[1] *Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*
[2] *Institute of Health Sciences, Universidade Federal da Bahia, Salvador, Brazil*
[3] *Faculty of Medical Sciences, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*
[4] *Faculty of Medicine, Imperial College London, UK*
[5] *School of Life Sciences, University of Warwick, Coventry, UK*
[6] *National Reference Centre for Toxigenic Corynebacteria, Institut Pasteur, Paris, France*

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SUMMARY

Invasive infections caused by *Corynebacterium diphtheriae* in vaccinated and non-vaccinated individuals have been reported increasingly. In this study we used multilocus sequence typing (MLST) to study genetic relationships between six invasive strains of this bacterium isolated solely in the urban area of Rio de Janeiro, Brazil, during a 10-year period. Of note, all the strains rendered negative results in PCR reactions for the *tox* gene, and four strains presented an atypical sucrose-fermenting ability. Five strains represented new sequence types. MLST results did not support the hypothesis that invasive (sucrose-positive) strains of *C. diphtheriae* are part of a single clonal complex. Instead, one of the main findings of the study was that such strains can be normally found in clonal complexes with strains related to non-invasive disease. Comparative analyses with *C. diphtheriae* isolated in different countries provided further information on the geographical circulation of some sequence types.

Key words: *Corynebacterium diphtheriae*, endocarditis, invasive strains, multilocus sequence typing, sucrose fermenting.

Diphtheria, the classical disease caused by *Corynebacterium diphtheriae*, is an acute communicable infection of the upper respiratory tract that can be fatal [1]. Although the global incidence of this disease has reduced dramatically as a result of increased vaccine coverage, infections by the different biotypes of *C. diphtheriae* continue to be reported in many countries [2, 3]. Moreover, the emergence in recent years of non-toxigenic strains of this bacterium as causative agents of severe invasive infections in vaccinated and non-vaccinated individuals has raised major concerns [3, 4].

Most of the reports in the literature regarding invasive infections caused by *C. diphtheriae* are related to infective endocarditis [5]. Mortality rates are estimated to be high (43% in the cases reviewed by Mishra *et al.* [5]), and predisposing factors such as incompetent cardiac valves and intravenous drug use apparently contribute to most cases [5]. Nonetheless,

* Author for correspondence: Dr V. Azevedo, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte-MG, 31270-901 Brazil.
(Email: vasco@icb.ufmg.br) [V. Azevedo]
(Email: guaraldi@uerj.br) [A. L. Mattos-Guraldi]

Table 1. *Clinical and microbiological information on the strains of* Corynebacterium diphtheriae *used in the study and results of the MLST analysis*

Strain [ref.]	Clinical information: Patient age (yr)/sex/ vaccinated	Disease	Year/ location	Microbiological properties: Biotype	*tox*/*dtxR* PCR*	Suc	MLST result: Allelic profile†	ST‡
Invasive strains								
HC01 [6]	14/F/Yes	Endocarditis	1993/RJ	*mitis*	−/+	+	2-1-37-19-24-3-4	175
HC02 [6]	9/F/Yes	Endocarditis	1999/RJ	*mitis*	−/+	+	2-4-17-4-7-5-2	176
HC03 [6]	9/F/Yes	Endocarditis	2000/RJ	*mitis*	−/+	+	2-2-36-19-3-3-6	171
HC04 [6]	7/F/Yes	Endocarditis	2003/RJ	*gravis*	−/+	−	2-1-47-19-13-3-6	**128**
402	75/F/NK	Pneumonia	1998/RJ	*belfanti*	−/+	+	3-4-8-1-7-2-9	173
814	71/M/NK	Pneumonia	2000/RJ	*mitis*	−/+	−	3-2-2-4-3-32-6	172
Other strains								
TR241 [11]	NK	Respiratory diphtheria	1981/RJ	*mitis*	+/+	+	3-2-13-38-14-33-4	174
VA01 [11]	32/F/Yes	Respiratory diphtheria	1999/RJ	*gravis*	−/+	−	2-1-32-19-13-3-6	**80**
Control strains								
ATCC 27012	n.a.	Respiratory diphtheria	n.a.	*mitis*	+/+	−	8-2-16-1-3-3-12	**26**
CDC E8392	n.a.	Respiratory diphtheria	n.a.	*mitis*	+/+	−	2-2-4-1-3-3-2	**50**

Suc, Sucrose fermentation; NK, not known; n.a., not applicable (additional information available from the American Type Culture Collection, USA, or the Centers for Disease Control and Prevention, USA).

* PCR reactions targeting the *tox* gene of *C. diphtheriae* were performed according to Pimenta *et al.* [8].

† Allelic order: *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, *rpoB*.

‡ Novel sequence types (ST) are underlined. Numbers in bold indicate a match with previously identified STs.

endocarditis caused by *C. diphtheriae* has also been reported in individuals without recognized risk factors [6].

Microbiological studies indicate that *C. diphtheriae* biotypes *gravis* and *mitis* are attributed to invasive diseases at higher rates (97·5% of the cases reviewed by Mishra *et al.* [5]), while infections by *intermedius* and *belfanti* biotypes are much less common [5]. Regardless of the biotype, most infections for which information is available were apparently caused by non-toxigenic strains of *C. diphtheriae* [5]; molecular-typing methods were used to identify specific clones of this bacterium as the causative agents of such invasive infections in only a few examples [4, 7].

In the present work, we investigated the genetic relationships between invasive *C. diphtheriae* strains isolated in the urban area of Rio de Janeiro, Brazil, during a 10-year period (from 1993 to 2003).

Clinical and microbiological data on the *C. diphtheriae* invasive strains studied are shown in Table 1. All the strains ($n=6$) rendered negative results in PCR reactions with previously published primers [8] which target the *tox* gene (Table 1); amplification of the *dtxR* gene was used as a species-specific marker, as described previously [8]. Another characteristic of some of these *C. diphtheriae* invasive isolates ($n=4$) is the atypical sucrose-fermenting ability (Table 1) [9]. The sucrose-positive phenotype has also been observed in a strain causing classical diphtheria in Brazil (strain TR241) [9]; therefore, this strain was included in the study along with a sucrose-negative strain (VA01) which also caused typical disease and was isolated in the same geographical region (Table 1). The sucrose-negative *C. diphtheriae* strains ATCC 27012 and CDC E8392 were used as controls.

We used a recently introduced multilocus sequence typing (MLST) scheme [10] for molecular characterization of *C. diphtheriae* strains. This method types strains by indexing nucleotide variation within seven housekeeping genes: *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA* and *rpoB*. After amplification, the respective genes' sequencing reactions were performed using a nested approach, according to a standardized protocol [10]. Allelic profiles and sequence type (ST) designations for each studied strain were obtained by depositing the generated DNA sequences in the PubMLST database (http://pubmlst.org/cdiphtheriae/).

Of the six invasive *C. diphtheriae* strains studied five were new STs (Table 1). Only the sucrose-negative

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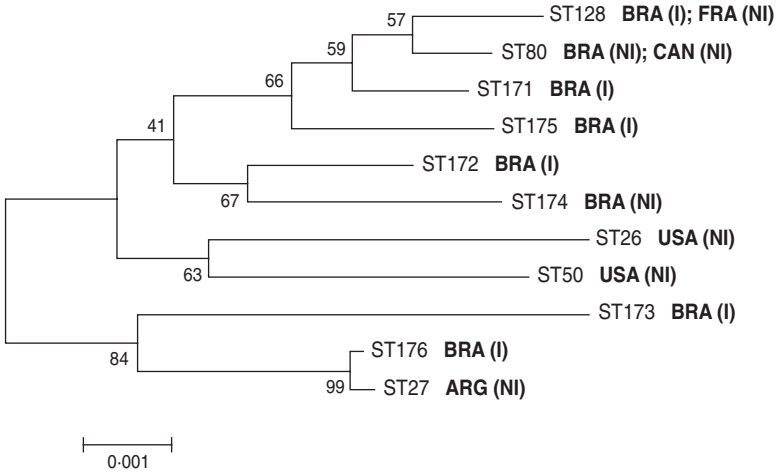

Fig. 1. Neighbour-joining phylogenetic tree showing the genetic relationships between the sequence types (STs) of *C. diphtheriae* studied. Concatenated MLST sequences were used for this analysis. The countries of isolation of the different *C. diphtheriae* strains are indicated. I, Isolate from invasive disease; NI, isolate from non-invasive disease (typical diphtheria).

strain HC04, isolated from a case of endocarditis in 2003, generated a MLST profile that had been previously deposited in the database, ST128. Interestingly, this was a single locus variant (SLV) of ST80 at the *dnaK* locus, indicating that these two STs are clonally related; this latter ST was assigned in this study to a sucrose-negative strain (VA01) previously recovered from a case of typical diphtheria that occurred in a patient that had regular contact with European travellers during the last 5 days before onset of symptoms [11]. The fact that both strains (HC04 and VA01) were assigned to already known STs means that they are part of a clonal complex that comprises strains isolated in different countries including France (ST128) and Canada (ST80) (Fig. 1). The *C. diphtheriae* control strains used in this study, both from North America, were also assigned to known MLST profiles (Table 1).

In order to study further the clonal relationships between invasive *C. diphtheriae* strains, we performed analyses using the eBURST V3 program (http://eburst.mlst.net/). The analyses also included 12 previously identified STs [10], which were representative of different *C. diphtheriae* biotypes circulating during different years in The Americas, Europe, Africa and Asia (data not shown). Two STs were considered as being part of a clonal complex if they shared at least six of the seven MLST alleles studied, as described previously [10].

eBURST analysis demonstrated that invasive *C. diphtheriae* strains isolated in the metropolitan area of Rio de Janeiro, Brazil are part of distinct clonal complexes. This result is different from results reported in other countries, where invasive infections were apparently related to specific clones [4, 7]. In addition, the analysis was also not indicative of clonal relationships between all the sucrose-positive isolates of *C. diphtheriae*. This may be due to some recombinogenic potential of the strains, as suggested by the index of association (I_A) between alleles at the different MLST loci, calculated according to Bolt *et al.* [10]: I_A sucrose-positive strains = 0·0283; I_A all invasive strains = 0·1508.

Of note, ST176 (strain HC02; biotype *mitis* isolated in 1999) formed a clonal complex with ST27, which was previously assigned to a toxigenic biotype *mitis* strain isolated in Argentina in 1995 causing classical diphtheria (Fig. 1). This result may be indicative of a specific *C. diphtheriae* biotype *mitis* clonal complex with circulation associated with South America, as has also been suggested in previous studies [2], although this warrants further investigation. Most importantly, MLST results obtained for strains HC02 (ST176) and HC04 (ST128) demonstrate that *C. diphtheriae* strains causing invasive infections in Brazil can be normally found in clonal complexes with strains related to non-invasive disease (Fig. 1). Genetic and microbiological factors leading to the invasive phenotype also need further study [4, 6].

Hirata and co-workers evaluated microbiological aspects that might be related to endocarditis caused by some of the *C. diphtheriae* strains used in the present study [6]. They found a characteristic adherence phenotype of the invasive strains, which apparently correlated with specific protein profiles in SDS–PAGE gels. Moreover, this same group showed

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that the capacity to bind to Fbn and convert Fbn to fibrin may play a role in pseudomembrane formation and act as a virulence determinant of both non-toxigenic and toxigenic strains [12]. Further studies are underway to evaluate the contribution of these factors in development of invasive infections. A preliminary analysis of genetic diversity of five Brazilian *C. diphtheriae* invasive strains was performed using random amplification of polymorphic DNA (RAPD) [6], which has been suggested recently to be as discriminatory as ribotyping, previously regarded as the 'gold standard' method for analysis of *C. diphtheriae*, until the emergence of MLST. Our MLST results were in accordance with the previous RAPD results [6], in which four of the invasive strains studied had already been proposed to be part of different clonal groups. Besides extending this analysis to novel strains, the intrinsic advantages of MLST over other molecular-typing methods allowed for a better understanding of the genetic relationships between invasive and non-invasive strains of *C. diphtheriae* isolated in Brazil.

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DECLARATION OF INTEREST

None.

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