Antimicrobial resistance among Brazilian *Corynebacterium diphtheriae* strains

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The increasing problems with multidrug resistance in relation to Corynebacterium, *including* C. diphtheriae, *are examples of challenges confronting many countries. For this reason, Brazilian* C. diphtheriae *strains were evaluated by the E-Test for their susceptibility to nine antibacterial drugs used in therapy. Resistance (MIC < 0.002; 0.38 µg/ml) to penicillin G was found in 14.8% of the strains tested. Although erythromycin (MIC_{90} 0.75 µg/ml) and azithromycin (MIC_{90} 0.064 µg/ml) were active against* C. diphtheriae *in this study, 4.2% of the strains showed decreased susceptibility (MIC 1.0 µg/ml) to erythromycin. Multiple resistance profiles were determined by the disk diffusion method using 31 antibiotics. Most* C. diphtheriae *strains (95.74%) showed resistance to mupirocin, aztreonam, ceftazidime, and/or oxacillin, ampicillin, penicillin, tetracycline, clindamycin, lincomycin, and erythromycin. This study presents the antimicrobial susceptibility profiles of Brazilian* C. diphtheriae *isolates. The data are of value to practitioners, and suggest that some concern exists regarding the use of penicillin.*

Key words: *Corynebacterium diphtheriae* - multiresistance - penicillin - antibiotics

Penicillin and erythromycin have long been the drugs of choice for the eradication of toxin-producing strains of *Corynebacterium diphtheriae* from air passages. However, resistance to penicillin G, oxacillin, erythromycin, and other drugs including rifampicin, tetracycline, and clindamycin used in therapy of *C. diphtheriae* infections have been reported (Formiga et al. 1971, Rockhill et al. 1982, Gruner et al. 1992, Maple et al. 1994, Patey et al. 1995, Gladin et al. 1999, Von Hunolstein et al. 2002). Resistance to ß-lactams should also be considered in systemic infections, since failure to eliminate *C. diphtheriae* in cases of endocarditis treated with penicillin have been reported. In the near future, antimicrobial susceptibility tests for both toxigenic and non-toxigenic *C. diphtheriae* strains may become practical. The increasing problems with multidrug resistance in *C. diphtheriae* are examples of challenges confronting tropical countries (Kneen et al. 1998, Gladin et al. 1999). In Brazil, as in much of the developing world, antibiotics are available freely without prescription and the emergence of multidrug-resistant *C. diphtheriae* is possibly another problem of concern. Thus, we report herein the susceptibility to antibacterial drugs used in therapy and the diverse multiresistance phenotypes of Brazilian *C. diphtheriae* strains.

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Forty-seven (32 sucrose fermenting and 15 non sucrose fermenting; 42 toxigenic and 5 non-toxigenic) Brazilian *C. diphtheriae* strains isolated over a 21-year period (1981-2002) were examined. Phenotypic characteristics of microorganisms were confirmed by API Coryne System (bioMérieux, France) according to the manufacturer's instructions (Freney et al. 1991). Toxin production was evaluated by the Elek test (Efstratiou et al. 1998). *C. diphtheriae* subsp. *mitis* - CDC E-8392 and ATCC 27012 (toxigenic), ATCC 27010 (non-toxigenic), *Staphylococcus aureus* - ATCC 29213 (β-lactamase positive), and ATCC 25923 (β-lactamase negative) strains were used as controls.

Microorganisms were stored in 10% skim milk with 20% glycerol at -70ºC until further use. The procedures for susceptibility testing and the range of interpretative categories of susceptibility for each antimicrobial agent were similar to those recommended previously (Martínez-Martínez et al. 1995, Funke et al. 1996, Von Hunolstein et al. 2002). Antimicrobial susceptibility assays were determined by using Mueller Hinton base supplemented with 5% sheep blood and inoculums of ~10^5 CFU\ml. Because of the absence of accepted breakpoints obtained by the disk diffusion method for coryneform bacteria CLSI (2006), guidelines for organisms other than *Haemophilus* spp. and *Neisseria gonorrhoeae* were used. The breakpoints for *S. aureus* were considered in the cases of penicillin, ampicillin, amoxicillin/clavulanic acid, oxacillin (Martínez-Martínez et al. 1995, Funke et al. 1996), methicillin, mupirocin (Salmenlinna et al. 2000), and linezolid (Goldstein et al. 1999). The E-Test (Biodisk, Solna, Sweden) was used to assay penicillin G, erythromycin, azithromycin, imipenem, tetracycline, gentamicin, clindamycin, rifampicin, and

vancomycin in accordance with the guidelines provided by the manufacturers. Since the E-test previously presented an accordance of 94.9% with results obtained by the disk diffusion method to study antimicrobial susceptibly of corynebacteria (Martínez-Martínez et al. 1995), we used the disk diffusion test for determining the multiresistance profiles of *C. diphtheriae* strains for the following antibiotics (all from Oxoid, Hampshire, England): penicillin G (10U), ampicillin (10 µg), oxacillin (1 µg), methicillin (5 µg), amoxicillin/clavulanate (20/10 µg), cephalothin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), gentamicin (10 µg), amikacin (30 µg), netilmicin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), perfloxacin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), vancomycin (30 µg), rifampicin (5 µg), erythromycin (15 µg), lincomycin (2 µg), clindamycin (2 µg), chloramphenicol (30 µg), tetracycline (30 µg), aztreonam (30 µg), imipenem (10 µg), mupirocin (200 µg and 5 µg), and linezolid (30 µg). The β-lactamase production was evaluated by the nitrocefin disk method (Cefinase; Becton Dickinson Microbiology Systems, Cocksville, USA) (Riegel et al. 1994).

Antibiotics have been used to prevent further diphtheria toxin production and dissemination in symptomatic patients, clinical disease, spread from asymptomatic carriers, and colonization of close contacts. The presence of diphtheria bacilli resistant to drugs frequently used in the treatment of infections from different sources was noticed in some European countries (Von Hunolstein et al. 2002). Likewise, Brazilian *C. diphtheriae* strains exhibited resistance to different drugs used in antimicrobial therapy. The MIC range, MIC_{50} and MIC_{90} values determined by E-Test are shown in Table I. In our study, the evaluation of bacterial susceptibility to all drugs used in antimicrobial therapy exhibited 94.3% correlation among the results obtained by the disk diffusion method and E-Test, similar to data observed for coryneforms (94.9%) by Martínez-Martínez et al. (1995) (data not shown). Even considering the breakpoints for *S. aureus,* we observed a decreased susceptibility to penicillin among 14.8% of the *C. diphtheriae* strains. Penicillin tolerance has been hypothesized to be a cause of treatment failure of *C. diphtheriae* infections (Patey et al. 1997, Von Hunolstein et al. 2002). All Brazilian strains tested were β-lactamase negative by the nitrocefin disk method. Penicillin represents the first antibiotic used to treat systemic *C. diphtheriae* infections, usually in combination with an aminoglycoside (Patey et al. 1997). A case of endocarditis in Rio de Janeiro proved to be fatal despite therapy with penicillin (Mattos-Guaraldi & Formiga 1998). Presently, blood isolates showed susceptibility to all antimicrobial agents by E-test with MIC range from < 0.0016 to 1.5.

Besides diphtheria and/or prevention of infection through close contacts, macrolides have also been used in treatment of systemic *C. diphtheriae* infections. Azithromycin was described to have similar in vitro activity to erythromycin against *C. diphtheriae* (MIC 0.16; 0.3 µg/ml) (Patey et al. 1997). Resistance to erythromycin has been described in different regions,

TABLE I

Antimicrobial susceptibilities of 47 Brazilian *Corynebacterium diphtheriae* strains evaluated by E-Test[a]

Antimicrobial agent	E-Test (µg ml^{-1})			
	MIC_{50}	MIC_{90}	MIC Range	% Resistance
Penicillin	0.023	0.19	< 0.002 - 0.38	14.8
Imipenem	0.016	0.19	< 0.002 - 1.5	0
Erythromycin	0.094	0.75	< 0.016 - 1.0	4.2
Azithromycin	0.016	0.064	< 0.016 - 1.0	0
Vancomycin	0.50	0.75	< 0.016 - 1.5	0
Clindamycin	0.047	4	< 0.016 - 32	17
Rifampicin	< 0.016	0.016	< 0.016 - 0.016	0
Gentamicin	0.023	0.19	< 0.016 - 0.75	0
Tetracycline	0.094	6	< 0.016 - 48	12.8

a: E-Test was assayed in accordance with the guidelines provided by Martínez-Martínez et al. (1995).

TABLE II

Antimicrobial multiresistance patterns presented by Brazilian *Corynebacterium diphtheriae* strains

Multiple resistance phenotypes	Number of strains (n = 47)	
	Sucrose-positive	Sucrose-negative
MUP, ATM, CAZ, OXA, AMP, PEN, TET	0	1
MUP, ATM, CAZ, OXA, AMP, PEN, LIN	1	0
MUP, ATM, CAZ, OXA, AMP, CLI, ERY	1	0
MUP, ATM, CAZ, OXA, PEN, CLI, TET	0	1
MUP, ATM, CAZ, OXA, AMP, PEN[a]	1	2
MUP, ATM, CAZ,OXA, AMP, CLI	1	0
MUP, ATM, CAZ,OXA, AMP, TET	1	0
MUP, ATM, CAZ, OXA, PEN, CLI	1	0
MUP, ATM, CAZ, OXA, PEN, TET	1	0
MUP, ATM, CAZ, OXA, LIN, ERY	1	0
MUP, ATM, OXA, AMP, PEN, TET	0	1
MUP, ATM, CAZ, OXA, AMP[b]	7	5
MUP, ATM, CAZ, OXA, PEN	1	1
MUP, ATM, CAZ, OXA, CLI	1	1
MUP, ATM, CAZ, OXA, TET	1	0
MUP, ATM, OXA, AMP, PEN	0	1
MUP, ATM, CAZ, OXA	7	2
MUP, ATM, CAZ, AMP	3	0
MUP, ATM, CAZ, LIN	1	0
MUP, ATM, OXA, AMP	1	0
Total %	95.74	

a: *C. diphtheriae* ATCC 27012 (toxigenic) and ATCC 27010 (non-toxigenic) strains used as controls; *b*: Blood isolates from patients with endocarditis and *C. diphtheriae* subsp. *mitis* - CDC E-8392 used as control; AMP: ampicillin; ATM: aztreonam; CAZ: ceftazidime; CLI: clindamycin; ERY: erythromycin; LIN: lincomycin; MUP: mupirocin; OXA: oxacillin; PEN: penicillin; TET: tetracycline.

including industrialized and tropical countries (Vietnam) (Kneen et al. 1998, Gladin et al. 1999). Our results showed that erythromycin and azithromycin were active in vitro against *C. diphtheriae* isolates at different levels with MIC_{90} values of 0.75 µg/ml and 0.064 µg/ml, respectively. However, we observed a decreased susceptibility to erythromycin (4.2%; MIC 1.0 µg/ml) for two sucrose fermenting *C. diphtheriae* strains.

The presence of a plasmid among erythromycin-resistant (Em^r) *C. diphtheriae* has been previously reported (Schiller et al. 1983). All microorganisms tested here were preliminarily screened for plasmids. Negative results (data not shown) confirmed earlier findings that plasmid carriage by *C. diphtheriae* is uncommon (Mattos-Guaraldi et al. 2000).

Brazilian *C. diphtheriae* strains were also observed to have 17% resistance to clindamycin. Data presented in Table II shows associated resistance to erythromycin and clindamycin (Patey et al. 1995, Gladin et al. 1999) for one *C. diphtheriae* strain. However, associated resistance to penicillin and erythromycin (Gladin et al. 1999) was not observed.

Resistance to tetracycline, although common in some countries, is very rare in others (Formiga et al. 1971, Patey et al. 1995, Gladin et al. 1999, Von Hunolstein et al. 2002). Tetracycline resistance was typical for isolates from Western Europe, Swiss intravenous drug users (Gruner et al. 1992), and hospitalized diphtheria patients in Jakarta, Indonesia (Rockhill et al. 1982). In previous studies, *C. diphtheriae* isolates from invasive infections were shown to be resistant to tetracycline and rifampicin (Patey et al. 1995, Von Hunolstein et al. 2002). Herein, Brazilian strains showed 12.8% resistance to tetracycline (MIC 16-48 µg/ml) and 100% susceptibility to rifampicin. The invasive isolates were susceptible to tetracycline and rifampicin.

Bacteremia and endocarditis caused by both non-toxigenic and toxigenic *C. diphtheriae* strains have been reported with increased frequency (Mattos-Guaraldi & Formiga 1998, Mishra et al. 2005). Only a few non-toxigenic strains were included in the study population. The toxigenicity seemed unrelated to drug resistance since resistance to different antimicrobial agents was also observed in all five non-toxigenic strains tested.

Since the 1970s, *C. diphtheriae* subsp. *mitis* of the sucrose fermenting biotype, uncommonly found in most industrialized countries, has been related with diphtheria outbreaks in different regions of Brazil (Mattos-Guaraldi et al. 2003). Here, multiple resistance phenotypes were observed among *C. diphtheriae* strains of both sucrose fermenting and non sucrose fermenting biotypes (Table II). Nearly all strains (95.74%) were resistant to between four and seven drugs tested. Four isolates (8.5%) had multiple resistance to seven drugs: mupirocin, penicillin and/or ampicillin, oxacillin, ceftazidime, aztreonam, tetracycline and/or lincomycin, clindamycin, erythromycin. Microorganisms were all susceptible to methicillin, amoxicillin/clavulanic acid, imipenem, and cephalosporines tested, except ceftazidime; and to linezolid, azithromycin, vancomycin, rifampicin, gentamicin, amikacin, netilmicin, chloramphenicol, trimethoprim/sulphamethoxazole, and quinolones evaluated in this study, except nalidixic acid. In contrast to data previously collected in St. Petersburg, Russian Federation, where 2.3% of the isolates showed multiple resistance to eight drugs (Gladin et al. 1999), multiresistant Brazilian isolates showed susceptibility to chloramphenicol and trimethoprim. Present data were also different from those previously observed in Vietnam (Kneen et al. 1998), where 20% of *C. diphtheriae* isolates were found multiresistant to drugs, but 27% presented increased resistance to erythromycin and 100% susceptibility to penicillin.

Only two strains did not show multiresistance to the antimicrobial agents tested. All strains, except one, showed resistance to oxacillin and/or ampicillin. However, 11 (5 sucrose-positive; 6 sucrose-negative) strains showed resistance to penicillin, indicating that resistance to penicillin was independent of sucrose fermentation. We did not observe microorganisms concomitantly resistant to penicillin and erythromycin. As shown in Table II, the expression of multiresistance phenotypes was independent of sucrose fermenting biotypes.

The expression of mupirocin resistance warrants further investigation of *C. diphtheriae* strains from different geographical areas. Data lead to the hypothesis that the control of the spread of methicillin-resistant staphylococci (Talon et al. 1995) may favor human colonization by diphtheria bacilli, including toxigenic and/or invasive strains in a nosocomial environment (Mattos-Guaraldi et al. 2003). Present data emphasize the need for a continuous survey of antibiotic susceptibility of *C. diphtheriae*, especially in developing countries where diphtheria is endemic and invasive infections may occur.

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