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Potential pathogenic role of aggregative-adhering *Corynebacterium diphtheriae* of different clonal groups in endocarditis

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Invasive diseases caused by *Corynebacterium diphtheriae* have been described increasingly. Several reports indicate the destructive feature of endocarditis attributable to nontoxigenic strains. However, few reports have dealt with the pathogenicity of invasive strains. The present investigation demonstrates a phenotypic trait that may be used to identify potentially invasive strains. The study also draws attention to clinical and microbiological aspects observed in 5 cases of endocarditis due to *C. diphtheriae* that occurred outside Europe. Four cases occurred in female school-age children (7-14 years) treated at different hospitals in Rio de Janeiro, Brazil. All patients developed other complications including septicemia, renal failure and/or arthritis. Surgical treatment was performed on 2 patients for valve replacement. Lethality was observed in 40% of the cases. Microorganisms isolated from 5 blood samples and identified as *C. diphtheriae* subsp *mitis* (N = 4) and *C. diphtheriae* subsp *gravis* (N = 1) displayed an aggregative adherence pattern to HEp-2 cells and identical one-dimensional SDS-PAGE protein profiles. Aggregative-adhering invasive strains of *C. diphtheriae* showed 5 distinct RAPD profiles. Despite the clonal diversity, all 5 *C. diphtheriae* invasive isolates seemed to display special bacterial adhesive properties that may favor blood-barrier disruption and systemic dissemination of bacteria. In conclusion, blood isolates from patients with endocarditis exhibited a unique adhering pattern, suggesting a pathogenic role of aggregative-adhering *C. diphtheriae* of different clones in endocarditis. Accordingly, the aggregative-adherence pattern may be used as an indication of some invasive potential of *C. diphtheriae* strains.

Key words: Aggregative adherence; *Corynebacterium diphtheriae*; Endocarditis; HEp-2 cells; Random amplified polymorphic DNA

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Introduction

Invasive diseases add new aspects to the infectious processes caused by *Corynebacterium diphtheriae*, and identify this species as an emerging pathogen. In the early years of the new millennium, infective endocarditis still proves to be difficult to diagnose and is associated with a high death rate (21-41%) worldwide (1,2). Infective endocarditis due to *C. diphtheriae* is perhaps more common than supposed and on occasion may be an aggressive fatal disease (3). Few reports have dealt with the microbiological aspects of community-acquired endocarditis caused

by *C. diphtheriae* (4-6). Entry of *C. diphtheriae* by invasive processes was described as being caused by percutaneous trauma, skin (7,8) and throat colonization (9). Unlike classical diphtheria, invasive disease caused by *C. diphtheriae* affects both vaccinated and non-vaccinated persons, and is mostly induced by nontoxigenic isolates (10). Diphtheria epidemics are likely to spread with a clonal character (11). Likewise, systemic diseases caused by *C. diphtheriae* have been related to invasive clones (7,12,13).

In addition to host factors, bacterial virulence determinants may contribute to the outcome of invasive infections. Microbial adhesive properties may contribute to the spread and outcome of invasive processes (14). Despite the destructive feature of invasive infections, the pathogenicity aspects that favor the invasive ability of certain *C. diphtheriae* strains remain unclear (10,15). The ability to survive within cultured epithelial cells was observed for *C. diphtheriae* strains isolated from throat and blood. The blood isolate showed a higher percentage of adherence and internalization compared with throat isolates (5). Further study showed that all sucrose fermenting and non-fermenting strains isolated from throat and skin lesions exhibited localized and diffuse adherence patterns, respectively (16). However, the binding properties of *C. diphtheriae* strains related to invasive disease have not yet been investigated. Moreover, identifying organisms belonging to invasive clones, including locations where diphtheria is rarely encountered, may provide valuable information and allow timely preventive measures.

In addition to describing clinical and microbiological characteristics that illustrate the destructive features of endocarditis due to *C. diphtheriae*, this study was undertaken to assess the patterns of adherence to HEp-2 cells of endocarditis-associated *C. diphtheriae* strains representative of five different clones.

Material and Methods

Bacterial strains and growth conditions

Five *C. diphtheriae* strains isolated from blood samples of patients with endocarditis were used to illustrate specific biological aspects of invasive disease and to evaluate phenotypic methods that could be used to discriminate potentially invasive strains (Table 1). Microorganisms were recovered from clinical specimens routinely submitted for culturing to the Bacteriology Laboratory of Hospital Universitário Pedro Ernesto, HUPE (LABAC) and the Laboratory of Diphtheria and Non-diphtheria Corynebacterial Infections at Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro (UERJ), Rio de Janeiro, RJ, Brazil.

For phenotypic and genotypic analysis, the microorganisms were grown in trypticase soy broth (Difco Lab., USA) or agar medium for 48 h at 37°C. Two toxigenic strains (CDC-E8392 from Centers for Disease Control, USA, and 241 from Rio de Janeiro, Brazil, isolated from the respiratory tract of patients with diphtheria) were used for comparison.

Biochemical identification and toxigenicity test

Identification, biotype, and toxigenicity determination were performed by conventional microbiological methods as described elsewhere and by the API Coryne System (bioMérieux, France). The toxigenicity test was performed by the Elek assay (6,15,17,18).

HEp-2 adherence assays

Adherence patterns to HEp-2 of *C. diphtheriae* blood isolates were assayed by methods previously described (16). Adherence assays were performed within semi-confluent HEp-2 cells grown on circular coverslips in DMEM (Sigma, USA) supplemented with 5% fetal calf serum (Gibco-BRL, USA), 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B and 0.5% L-glutamine at 37°C in a 5% CO_2 atmosphere. Microorganisms were washed twice in 10 mM phosphate-buffered saline (PBS, pH 7.2) and resuspended in DMEM to a concentration of 10^7 colony-forming units per mL, and used in adherence assays (3-h incubation). Giemsa-stained coverslips were examined by bright field microscopy.

SDS-PAGE analysis

Protein profiles of crude extracts of bacterial strains were obtained after bacterial cell lysis by treatment with 5 mg/mL lysozyme (Sigma) in PBS for 2 h at 37°C. Thereafter, protein samples were submitted to treatment with sample buffer [0.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% ß-mercaptoethanol, and 0.001% bromophenol blue] and heating at 100°C for 15 min. Total crude cell lysates were cleared by centrifugation at 14,000 *g* for 5 min, and submitted to electrophoresis on 10% polyacrylamide gels in the presence of SDS (SDS-PAGE). The protein profiles were observed after staining with Coomassie brilliant blue R250 (Sigma) (19).

RAPD analysis

RAPD was performed using the Ready-to-go RAPD Kit (Pharmacia Biotech, USA), according to manufacturer instructions on a Minicycler PTC-100 Programmable Thermal Controller (MJ Research, USA) (20). Amplified products were electrophoresed on a 2% agarose gel for 3 h at 120 V. Gels were stained with ethidium bromide and the

amplicons were analyzed based on the unweighted pair group method with arithmetic mean (UPGMA) (21).

Results and Discussion

Clinical and microbiological features related to *C. diphtheriae* strains isolated from patients with native-valve endocarditis are presented in Table 1. Four strains (HC01, HC02, HC03, HC05) were identified as *C. diphtheriae* subsp *mitis* and one (HC04) as *C. diphtheriae* subsp *gravis.* All strains were toxigenic by the Elek assay.

We have found the incidence of infective endocarditis in the literature to be generally higher in males than in females (2:1), and the average age group affected is in the fifth decade (1). In contrast, the highest number of *C. diphtheriae* endocarditis in our community was found in children less than 18 years of age rather than in adults over 50 years of age (4:1), and in females more than in males (4:1). No cases were reported among children less than 5 years of age. The source of bacteremia was not precisely determined for all 5 cases. However, predisposing conditions to an increased risk of acquiring endocarditis (22), such as congenital heart disease and major dental treatment, were observed for patients HC03 and HC05, respectively. Patient HC02 experienced an episode of non-exudative pharyngitis 2 weeks before the onset of infection. Nevertheless, skin lesions were not seen in any patient. The preferred sites of attack of *C. diphtheriae* endocarditis isolates were mitral valves (80%). Complications observed in all five episodes of endocarditis included septicemia (N = 5), intracardiac abscess (N = 1), mycotic aneurysm (N = 1), peripheral (N = 2) and CNS emboli (N = 1), microaneurysm with brain hemorrhage (N = 2), arthritis (N = 2), and acute renal failure (N = 3). Surgical treatment was performed for valve replacement for patients HC02 and HC05. Patients HC03, HC04, and HC05 were submitted to antibiotic and serum therapy. The mortality rate (40%) due to infective endocarditis caused by *C. diphtheriae* was higher than that previously reported in the literature (27%) (23).

C. diphtheriae endocarditis isolates exhibited an aggregative-like adherence pattern, characterized by clumps of bacteria with a “stacked-brick” appearance that were attached to the surfaces of cultured epithelial cells and exposed areas of the glass slide among epithelial

Table 1. Clinical aspects of patients with endocarditis and microbiological properties of *Corynebacterium diphtheriae* blood isolates.

Case/year	Clinical aspects[a]						
	Gender/age/ childhood immunization*	Presumptive/ conclusive diagnosis	Complications	Prior heart defect	Heart valve destruction	Outcome	Microbiological properties[b]
HC01[c]/1993	Female/ 14 years old/ yes	Sepsis/ endocarditis	Peripheral emboli of legs and arms; microaneurism with brain hemorrhage	No	Mitral	Dead	Toxigenic *C. diphtheriae* subsp *mitis*
HC02[d]/1999	Female/ 9 years old/ yes	Rheumatic fever/ endocarditis	Arthritis; myositis; valvar abscess; acute renal failure	No	Mitral	Valve replacement; alive	Toxigenic *C. diphtheriae* subsp *mitis*
HC03/2000	Female/ 9 years old/ yes	Meningitis/ endocarditis	Mycotic aneurism; acute renal failure	Congenital	Mitral, tricuspid	Alive	Toxigenic *C. diphtheriae* subsp *mitis*
HC04/2003	Female/ 7 years old/ yes	Septic shock/ endocarditis	Arthritis; myositis; peripheral and CNS emboli; microaneurism with brain hemorrhage	Congenital	Mitral, aortic	Dead	Toxigenic *C. diphtheriae* subsp *gravis*
HC05[e]/2005	Male/ 58 years old/ yes	Sepsis/ endocarditis	Weight loss; acute renal failure	No	Aortic	Valve replacement; alive	Toxigenic *C. diphtheriae* subsp *mitis*

[a]Absence of skin lesions. [b]Nose and throat cultures were not performed. Toxin production evaluated by the Elek test. [c]Microbiological description of bacterial strain published by Mattos-Guaraldi et al. (15). [d]Pharyngitis without pseudomembrane formation two weeks before the onset of infection. [e]Patient submitted to prior dental surgery. *DTP, diphtheria, tetanus and pertussis.

cells. Figure 1 illustrates the aggregative pattern of the endocarditis isolates, independent of *C. diphtheriae* subspecies. Adhesion to HEp-2 cells has provided a useful model for the study of toxigenic (5,16) and non-toxigenic *C. diphtheriae* strains (4). Similar to some enteric pathogens (24), differing degrees of attachment to HEp-2 monolayers with predominance of localized and diffuse adherence patterns have been reported for *C. diphtheriae* strains isolated from throat and skin lesions, respectively (16). In the present study, we have identified a third adherence phenotype expressed by the *C. diphtheriae* blood isolates, distinct from those exhibited by strains isolated from throat and skin lesions. More information is necessary to correlate the aggregative adhesive properties with the pathogenicity of *C. diphtheriae* strains as observed for enteroaggregative *Escherichia coli* (25).

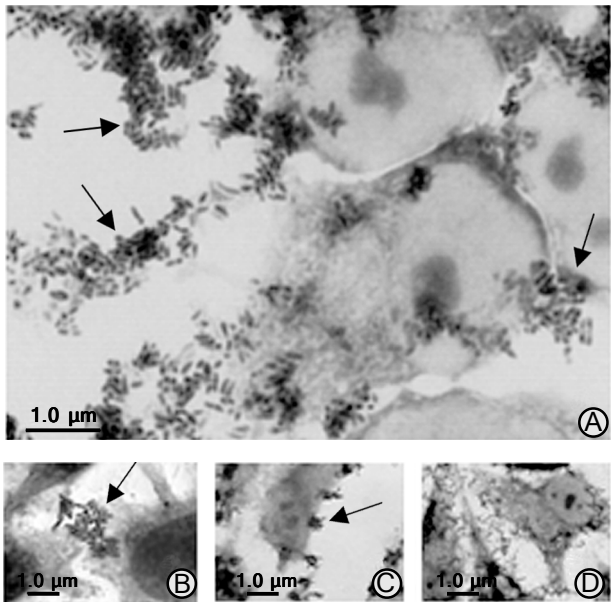

Figure 1. Light micrographs illustrating different adherence patterns to HEp-2 cells of *Corynebacterium diphtheriae* strains. *A* and *B*, Aggregative adherence pattern characterized by clumps of bacteria with a "stacked-brick" appearance expressed by strains isolated from blood of patients with endocarditis. *C*, Localized and *D*, diffuse adherence patterns expressed by control strains of sucrose-fermenting (241 strain) and non-sucrose fermenting (CDC-E8392 strain) biotypes isolated from patients with classical respiratory diphtheria, respectively. Magnification: 1000X.

C. diphtheriae	Adherence patterns	Protein profiles
HC01 strain	Aggregative	
241 strain	Localized	
CDC-E8392 strain	Diffuse	

Figure 2. HEp-2 cells adherence patterns and SDS-PAGE protein profiles of *Corynebacterium diphtheriae* strains.

Phenotypic and molecular typing methods have been used with varying success to distinguish between *C. diphtheriae* strains (26,27). Despite the fact that SDS-PAGE profiles are not considered to provide consistent results for the evaluation of clonality among bacterial strains, heterogeneity among protein profiles of *C. diphtheriae* has been used for the analysis of epidemic strains (28,29). In the present study, SDS-PAGE demonstrated *C. diphtheriae* endocarditis isolates characterized by a particular protein profile, as exemplified by the HC01 strain in Figure 2. However, the endocarditis isolates showed a protein profile distinct from those presented by *C. diphtheriae* 241 and CDC-E8392 strains isolated from patients with diphtheria. Thus, in addition to the aggregative pattern to HEp-2 cells,

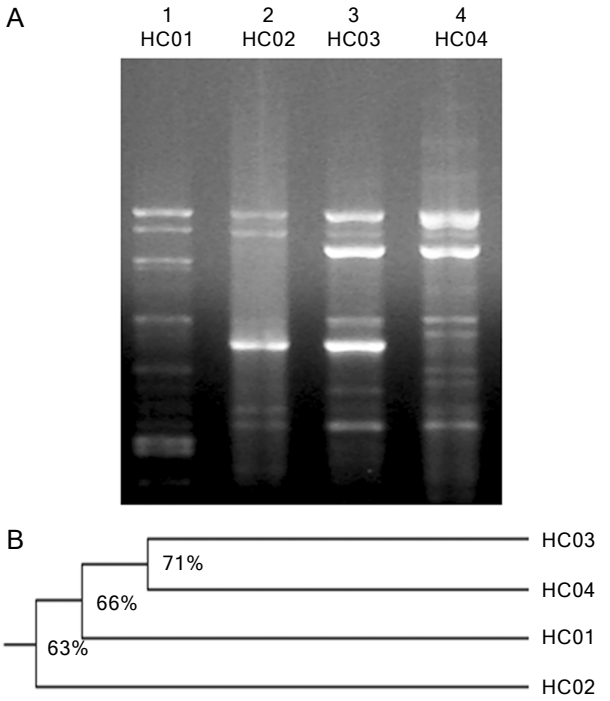

Figure 3. RAPD-PCR patterns of *Corynebacterium diphtheriae* strains isolated from blood samples of patients with endocarditis using the Primer 4 of Kit Ready-to-go. *A*, *Lanes 1-3*, strains of the *mitis* biotype (HC01, HC02, HC03); *lane 4*, strain of the *gravis* biotype (HC04). *B*, Dendrogram portraying the genetic diversity of the Brazilian *C. diphtheriae* invasive strains.

the relationship among *C. diphtheriae* endocarditis isolates was verified by the expression of identical SDS-PAGE protein profiles.

RAPD-PCR can be reliably and reproducibly used for rapidly screening *C. diphtheriae* strains of the predominant epidemic clonal group (30). *C. diphtheriae* endocarditis isolates showed different amplification patterns by RAPD-PCR for all 6 sets of primers used, indicating diversity among *C. diphtheriae* invasive clones. The use of RAPD-PCR was successful for detecting minor differences in closely related toxigenic *C. diphtheriae* endocarditis strains. The primer 4 of RAPD-PCR kit was more discriminating for the *C. diphtheriae* strains evaluated in this study (Figure 3).

Our data demonstrate the destructive feature of infective endocarditis due to *C. diphtheriae* and demonstrate the circulation of different *C. diphtheriae* clones exhibiting invasive properties. The data also suggest the pathogenic role of aggregative-adhering *C. diphtheriae* in invasive disease.

Additional studies are necessary to characterize the full range of virulence factors of the aggregative-adhering *C. diphtheriae* and the molecular pathophysiology of the invasive illness caused by these strains. It has been demonstrated that *E. coli* strains, which present aggregative adherence, are able to form biofilms (31). This property enables aggregative-adhering *E. coli* strains to produce more prolonged diseases. The possibility that *C. diphtheriae* can also form biofilms (15) and the potential of this property to aggravate the clinical outcome of invasive disease merits investigation.

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