Antonie van Leeuwenhoek (2015) 107:1387–1399
DOI 10.1007/s10482-015-0433-3

ORIGINAL PAPER

Biofilm formation and fibrinogen and fibronectin binding activities by *Corynebacterium pseudodiphtheriticum* invasive strains

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Received: 8 October 2014 / Accepted: 18 March 2015 / Published online: 1 April 2015

Abstract Biofilm-related infections are considered a major cause of morbidity and mortality in hospital environments. Biofilms allow microorganisms to exchange genetic material and to become persistent colonizers and/or multiresistant to antibiotics. *Corynebacterium pseudodiphtheriticum* (CPS), a commensal bacterium that colonizes skin and mucosal sites has become progressively multiresistant and responsible for severe nosocomial infections. However, virulence factors of this emergent pathogen remain unclear. Herein, we report the adhesive properties and biofilm formation on hydrophilic (glass) and hydrophobic (plastic) abiotic surfaces by CPS strains isolated from patients with localized (ATCC10700/Pharyngitis) and systemic (HHC1507/Bacteremia) infections. Adherence to polystyrene attributed to hydrophobic interactions between bacterial cells and this negatively charged surface indicated the involvement of cell surface hydrophobicity in the initial stage of biofilm formation. Attached microorganisms multiplied and formed microcolonies that accumulated as multilayered cell clusters, a step that involved intercellular adhesion and synthesis of extracellular matrix molecules. Further growth led to the formation of dense bacterial aggregates embedded in the exopolymeric matrix surrounded by voids, typical of mature biofilms. Data also showed CPS recognizing human fibrinogen (Fbg) and fibronectin (Fn) and involvement of these sera components in formation of "conditioning films".

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These findings suggested that biofilm formation may be associated with the expression of different adhesins. CPS may form biofilms in vivo possibly by an adherent biofilm mode of growth in vitro currently demonstrated on hydrophilic and hydrophobic abiotic surfaces. The affinity to Fbg and Fn and the biofilm-forming ability may contribute to the establishment and dissemination of infection caused by CPS.

Keywords *Corynebacterium pseudodiphtheriticum* · Fibrinogen · Fibronectin · Biofilm

Introduction

Corynebacterium pseudodiphtheriticum emerged as an opportunistic pathogen responsible for nosocomial infections associated with high mortality of immunocompromised hosts. On various occasions patients suffering from *C. pseudodiphtheriticum* infections had undergone transplants or underlying medical conditions such as chronic obstructive pulmonary disease, malignancies and AIDS. The pathogen is frequently resistant to several antimicrobial agents and may also cause disease in immunocompetent individuals (Nathan et al. 1982; von Graevenitz et al. 1998; Ahmed et al. 1995; Martins et al. 2009; Olender and Niemcewicz 2010) Infections caused by *C. pseudodiphtheriticum* include exudative pharyngitis (Izurieta et al. 1997), necrotising tracheitis (Colt et al. 1991), tracheobronchitis (Craig et al. 1991), pneumonia (Morinaga et al. 2010), bacteremia (Das et al. 2003) and endocarditis (Morris and Guild 1991). *C. pseudodiphtheriticum* have been also associated with catheter-related intravenous infections, including neonates (Camello et al. 2003, 2009).

Microbial adhesion and biofilm formation may pose a public health problem. More than 60 % of the bacterial infections currently treated by physicians are considered to involve biofilm formation. Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms. Taking into account the aging population and the increasing number of implantable medical devices available, it is expected that the number of infections associated with biofilms (Stewart and Costerton 2001; Sousa et al. 2011) caused by opportunistic pathogens will increase. Biofilm infections develop preferentially on inert surfaces (commonly on medical devices), but they can also form on living tissues, as in the case of invasive infections. Successful treatment in these cases depends on longterm, high-dose antibiotic therapies and the removal of any foreign-body material (Costerton et al. 1999; Sousa et al. 2011).

A better understanding of the biology and recognition of the virulence potential of *C. pseudodiphtheriticum* strains will certainly help to effectively prevent the infections caused by them (Souza et al. 2012). Therefore, the present study was undertaken to demonstrate the adhesive properties and biofilm formation on hydrophilic (glass) and hydrophobic (plastic) abiotic surfaces by *C. pseudodiphtheriticum* isolated from patients with localized and systemic infections. Fibrinogen (Fbg) and fibronectin (Fn) binding properties and the involvement of these human sera components in formation of "conditioning films" by *C. pseudodiphtheriticum* were also investigated.

Materials and methods

Origin of bacterial strains, phenotypic characterization and culture conditions

Two *C. pseudodiphtheriticum* strains, partially studied for their virulence properties and presenting an aggregative-like adherence pattern (AA-like) to human epithelial (HEp-2) cell line (Souza et al. 2012) were used in this investigation: ATCC10700 strain isolated from the upper respiratory tract of a patient with exudative pharyngitis (USA) (Izurieta et al. 1997) and HHC1507 strain isolated from blood of a patient with bacteremia (Brazil) (Camello et al. 2009).

Phenotypic characterization of microorganisms was previously carried out by the semi-automated API-Coryne system 3.0 version (bioMérieux) according to manufacturers instructions. Both strains produced the numerical pattern 3001004, which was identified by the API Coryne database as *C. pseudodiphtheriticum* with 96.7 % probability (T = 0.97) with the API-Coryne web decoding system (Martins et al. 2009; Souza et al. 2012; Almuzara et al. 2006). Stock cultures in 10 % skim milk with 25 % added glycerol were maintained at −70 °C and recovered as required by cultivation in Trypticase Soy Broth (TSB; Difco) (Hirata et al. 2002).

Biofilm formation to glass surfaces

Two *C. pseudodiphtheriticum* strains ATCC10700 and HHC1507 were tested for glass surface adherence. The adherence assay employed is based on the assay described by Mattos-Guaraldi and Formiga (1991) for *Corynebacterium diphtheriae*.

Glass tubes (13 × 100 mm) containing 4 ml of TSB medium (Trypticase Soy Broth, Baltimore Biological Laboratory, Inc.) were inoculated with one *C. pseudodiphtheriticum* strain each and incubated stationarily for 48 h at 37 °C. Each culture was gently shaken by hand for 5 s to remove those cells which grew in close contact with the glass surface but did not actually adhere. The culture fluid was then added and the tubes were incubated for 48 h. This procedure was repeated twice. After pouring off the culture fluid for the third time the tubes were scored on a scale of I to IV for adherence: I (strong), confluent coat of cells on sides of tube and localized adherence on glass surface where culture medium is in contact with air; II (intermediate), confluent coat of cells on sides of tube; III (weak), localized adherence on glass surface where culture medium is in contact with air; IV (negative), no visible adherence.

Morphological features of biofilms produced on glass coverslips by *C. pseudodiphtheriticum* strains were investigated by confocal scanning laser microscopy (CSLM) (Chávez de Paz et al. 2011). Microorganisms were classified as adherent when creating confluent layers of bacterial cells on glass surfaces (Mattos-Guaraldi and Formiga 1991). The LIVE/DEAD staining kit (Invitrogen) using fluorescent markers targeting cell membrane integrity was used to assess membrane integrity of microorganisms and confocal imaging was performed using a Zeiss—Meta LSM 510. The LIVE/DEAD mixture was prepared by mixing components A+B in the kit, consisting of SYTO9® and propidium iodide, at a ratio of 1:100 with the inoculum medium (TH). Briefly, 1 ml bacterial suspensions was applied to glass coverslips placed in 24-well polystyrene cell culture plates (Nunc). After incubation at 37 °C for 48 h, the content of each well was aspirated and washing with phosphate-buffered saline (PBS) (0.01 M; pH 7.2). Biofilms were stained with 2 µl SYTO9® and 2 µL propidium iodide in 3 ml PBS. Biofilms were incubated at room temperature for 10 min and washed 2× with PBS. The fluorescence from stained cells was viewed by CSLM. The LIVE/DEAD biofilm viability kit provides a two-color fluorescence assay of bacterial viability growing in biofilm communities, based on membrane integrity. When used alone, the SYTO9® green fluorescent nucleic acid stain generally labels all bacteria in a population—those with intact membranes and those with damaged membranes. In contrast, propidium iodide red-fluorescent nucleic acid stain penetrates only bacteria with damaged membranes, causing a reduction in the SYTO9® stain fluorescence when both dyes are present.

In vitro model of catheter infection

Polyurethane 16-gauge percutaneous nephrostomy catheters (Deseret Pharmaceutical Co) were used in the in vitro model system for evaluation of bacterial adherence and biofilm formation on catheter surfaces. Sterile 4 cm segments of polyurethane catheters were immersed for 24 h in 10^6 CFU ml^{-1} suspended in TSB medium (Franson et al. 1984; Gomes et al. 2009). After 24 h the catheter segments were removed, gently rinsed in PBS (0.01 M; pH 7.2) to eliminate loosely attached planktonic cells.

Quantitative catheter culture and semiquantitative roll plate technique

Catheter infection by *C. pseudodiphtheriticum* strains was evaluated by a quantitative (Dooley et al. 1996) and a semiquantitative roll plate method (Maki et al. 1977) using Columbia agar base (CAB; Oxoid) plates supplemented with 5 % sheep blood at 37 °C for 24 h.

Scanning electron microscopy (SEM)

Sections of polyurethane catheters infected with *C. pseudodiphtheriticum* were fixed in 2.5 % glutaraldehyde, post-fixed in 1 % osmium tetroxide and dehydrated in a graded series of ethanol. Subsequently, catheter segments were subjected to critical point drying with carbon dioxide, covered with gold–palladium to a 10 nm layer and examined with a JEOL JSM 5310 scanning electron microscope. Infected catheter segments with *C. diphtheriae* BR-CAT5003748 (Gomes et al. 2009) and *Staphylococcus epidermidis* strains were used as positive controls (Franson et al. 1984).

Conjugation of fibrinogen and fibronectin to biotin

Experiments were performed as previously described (Sabbadini et al. 2010; Harlow and Lane 1988). Human plasmatic proteins were conjugated to N-hydroxysuccinimidobiotin in dimethyl sulfoxide biotin (Sigma). Proteins were dissolved in 0.1 M NaHCO3 (pH 9.0) to a total protein concentration of 1.0 mg ml^{-1} and added of 20 µL of N-hydroxysuccinimidobiotin in dimethyl sulfoxide. The mixture was incubated at 20 °C for 4 h, and the reaction was mixture by dialyzed against PBS (phosphate buffered saline 0.01 M; pH 7.2) added 0.02 % sodium azide at 4 °C overnight.

Enzyme-linked immunosorbent assay (ELISA) for determining bacterial interaction with fibrinogen (Fbg) and fibronectin (Fn)

Briefly, microorganisms were previously washed three times with PBS and resuspended in 0.1 M carbonate buffer, pH 9.6 at a concentration of approximately 10^8 CFU ml^{-1} [0.2 optical density (OD_{660}) in a B295II spectrophotometer (Micronal)]. Each well of flat-bottomed microtitre plates (Nunc-Immuno MaxiSorb) were coated with 100 µl bacterial suspensions at 37 °C for 1 h. The wells were treated with PBS with 1 % (w/v) bovine serum albumin (BSA), for 1 h, and 0.05 % (w/v) Tween-20 (PBST–BSA) washed three times with PBST. Aliquots of Fbg and Fn solution in PBST–BSA (30 µg ml^{-1}) were added to each well and the microplates were incubated at 37 °C for 1 h. Any unbound protein was removed by washing the wells with PBST. Bacterial interaction with Fbg and Fn were revealed with streptavidin–alkaline phosphatase. The intensity of Fbg and Fn-binding by *C. pseudodiphtheriticum* strains was expressed in OD_{405} values (Bio-Rad Model 550 spectrophotometer) and compared statistically by the Tukey test using GraphPad Prism. Differences were considered significant at $p < 0.05$.

Biofilm formation on polystyrene surfaces

Biofilm formation on negatively charged polystyrene surface was determined quantitatively in 96-well flat-bottomed microtiter plates according to methods previously described (Stepanovic et al. 2000; Gomes et al. 2009). Aliquots of 200 µl of bacterial suspensions (0.2 OD_{570}) were applied to microplate wells. After incubation at 37 °C for 24 h the supernatants were aspirated and the wells were washed three times with 200 µl of PBS (0.01 M; pH 7.2). The remaining attached bacteria were fixed with 200 µl of 99 % methanol and stained with 2 % crystal violet. Negative controls contained TSB only. The bound dye was then solubilized with 160 µl of 33 % glacial acetic acid and the OD_{570} of the solution was measured by using an enzyme immunosorbent assay reader (BioRad, model 550). The cut-off OD (OD_c) was defined as the mean OD of the negative control. All strains were classified into the following categories: non-adherent (0: $OD \leq OD_c$), weakly (+: $OD_c < OD \leq 2\times OD_c$), moderately (++: $2\times OD_c < OD \leq 4\times OD_c$) or strongly (+++: $4\times OD_c \leq OD$) adherent, based upon the ODs of bacterial films.

Influence of Fbg and Fn on biofilm formation

Biofilm formation was determined in 96-well flat-bottomed polystyrene microtiter plates as described above but with some modifications. In these experiments, the wells of microplates were pre-treated with Fbg and Fn at a concentration of 50 µg ml^{-1} overnight at 4 °C. Fbg and Fn coated wells containing 200 µl of TSB medium without bacteria were used as negative controls (Gomes et al. 2009; Lembke et al. 2006).

Results

Biofilm formation on hydrophilic material

The results of adherence to glass tests for both 10700/Pharyngitis and HHC1507/Bacteremia strains are given in Table 1. The *C. pseudodiphtheriticum* strains were able to adhere to a glass surface independently of site of isolation of bacterial strains. The adherence levels of *C. pseudodiphtheriticum* strains are presented in Table 1. We observed that both ATCC10700/Pharyngitis and HHC1507/Bacteremia strains exhibited strong (I pattern) adhesion to the glass surface, similarly to *C. diphtheriae* strains observed for Mattos-Guaraldi and Formiga (1991).

CSLM assays demonstrated that both ATCC10700/Pharyngitis and HHC1507/Bacteremia strains were able to adhere to and to produce biofilm on hydrophilic glass surfaces and suggested predominance of alive bacterial cells in the mature stage biofilm structure. In

Table 1 Adherence to glass and biofilm formation on polystyrene surfaces in absence and presence of fibrinogen (Fbg) and fibronectin (Fn) by *Corynebacterium pseudodiphtheriticum* strains

C. pseudodiphtheriticum strains	Glass tube	Polystyrene binding (OD ± SD)							
		Control w/o bacteria	Test w/bacteria	Category	Control Fbg w/o bacteria	Test Fbg w/bacteria	Category	Control Fn w/o bacteria	Test Fn w/bacteria	Category
ATCC10700/ Pharyngitis	I	0.095 ± 0.008	0.239 ± 0.084*	++	0.168 ± 0.066	0.686 ± 0.146**	+++	0.146 ± 0.009	0.367 ± 0.036***	++
HHC1507/Bacteremia	I	0.095 ± 0.008	0.730 ± 0.071* $p < 0.05$*	+++	0.168 ± 0.066	0.432 ± 0.116** $p < 0.05$*	++	0.146 ± 0.009	0.333 ± 0.021*** $p < 0.05$*	++

Adherence to glass surface: I (strong), confluent coat of cells on sides of tube and localized adherence on glass surface where culture medium is in contact with air; w, with; w/o, without; OD + SD, Optical Density at $\lambda = 570$ nm ± SD; The cut-off OD (OD_c) was defined as the mean OD of the negative control. Microorganisms were classified into the categories: non-adherent (0: $OD < OD_c$), weakly (+: $OD_c < OD \leq 2\times OD_c$), moderately (++: $2\times OD_c < OD \leq 4\times OD_c$), or strongly (+++: $4\times OD_c \leq OD$) adherent, based upon the ODs of bacterial films (Stepanovic et al. 2000; Gomes et al. 2009). Data were from three independent experiments and compared statistically by the Tukey test using GraphPad Prism 6.0. Differences were considered significant at $p < 0.05$

addition 2-days-old biofilms in a mature stage exhibited voids within microcolonies of viable bacteria with membrane integrity of cells stained fluorescent green and dead bacteria with damaged membranes stained fluorescent red (Fig. 1).

Biofilm formation on hydrophobic plastic surfaces

In vitro catheter adhesion

Segments of polyurethane catheters were colonized in vitro by the *C. pseudodiphtheriticum* ATCC10 700/Pharyngitis and HHC1507/Bacteremia strains. A large amount of biofilm exhibiting bacterial microcolonies and amorphous material was observed on catheter inner surfaces infected with both *C. pseudodiphtheriticum* ATCC10700/Pharyngitis and HHC1507/Bacteremia strains (Fig. 2). Similarly to *S. epidermidis* strains used as a positive control, in some areas the cocci were coated with slime (Fig. 2f) and for *C. diphtheriae*, bacterial cells coated with slime (condensed glycocalyx) were also observed on polyurethane surfaces (Fig. 2e). Evaluation of adherence and viability of micro-organisms on polyurethane catheter segments by the semiquantitative roll plate method revealed >15 CFU viable cells of the *C. pseudodiphtheriticum* strain extensively adhered to the surfaces of polyurethane catheters as illustrated in Fig. 2a. The quantitative catheter culture assays showed $>5.0 \times 10^4$ CFU viable cells of the *C. pseudodiphtheriticum* strains adhered to the surfaces of polyurethane catheters. Therefore, strains ATCC10700/Pharyngitis and HHC1507/Bacteremia showed properties similar to those of other human pathogens that are biofilm producers. As for *C. diphtheriae* BR-CAT5003748 (Gomes et al. 2009), we have observed the ability of the *C. pseudodiphtheriticum* strains to produce biofilm on both the luminal and external surfaces of polyurethane catheters. Micrographs displayed in Fig. 2 showed lumen (Fig. 2b) and external (Fig. 2c) surfaces of polyurethane catheters colonized in vitro by the HHC1507/Bacteremia strain.

Fbg and Fn binding activities and influence on biofilm formation

Results obtained by the ELISA demonstrated Fbg (30 μg ml^{-1}) and Fn (30 μg ml^{-1}) binding activities

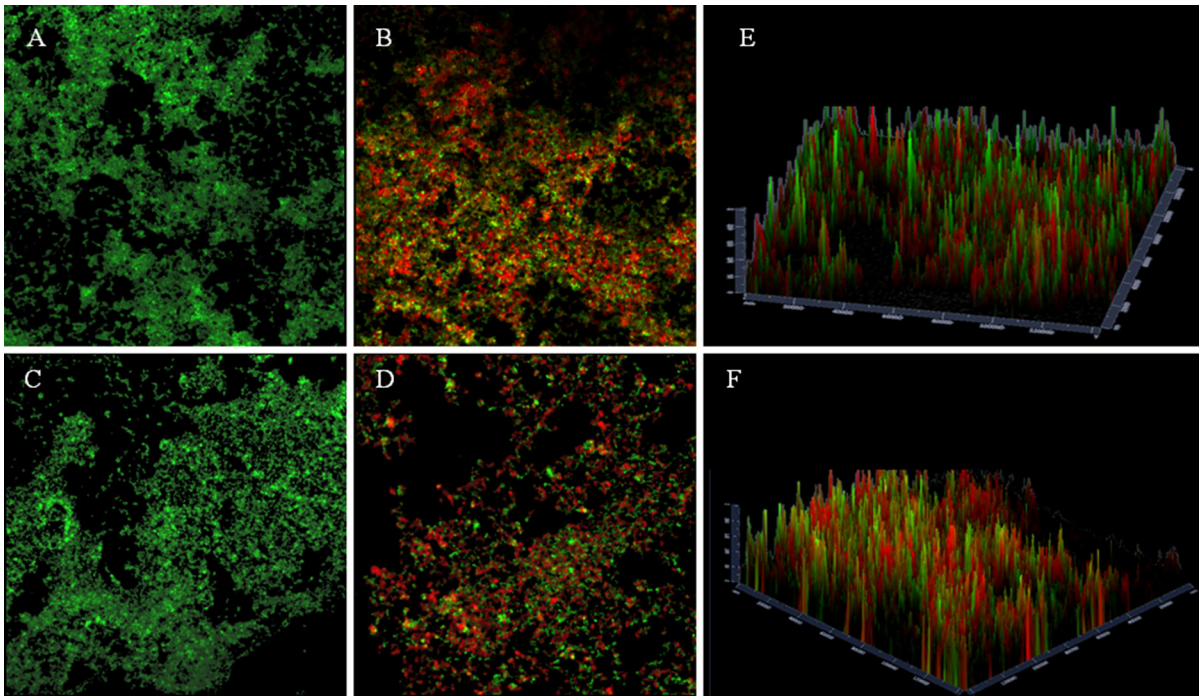

Fig. 1 Confocal laser scanning microscopy (CLSM) assays for examination of bacterial viability and biofilm's structural architecture on glass surfaces of *Corynebacterium pseudodiphtheriticum* (**a**, **b**, **c**) ATCC10700/Pharyngitis strain and (**d**, **e**, **f**) HHC1507/Bacteremia strain. Biofilms in a mature stage (2-day-old) exhibited voids within microcolonies. Microorganisms with intact cell membranes (alive) stained fluorescent green and bacteria with damaged membranes (dead) stained fluorescent red in biofilm communities. Magnification ×40 (**a**, **b**, **c**, **d**) and scale of fluorescence intensity (**c**, **f**)

to both *C. pseudodiphtheriticum* strains (Table 2). Fbg and Fn bound with greater affinity to the ATCC10700/Pharyngitis strain when compared with the HHC1507/Bacteremia strain ($p < 0.0001$).

C. pseudodiphtheriticum produced biofilm not only on uncoated polystyrene surfaces but also on polystyrene surfaces coated with Fbg and Fn at lower concentrations (50 μg ml^{-1}) than that found in the human plasma (2.5 mg ml^{-1}) (Table 1). Both strains produced biofilm on Fbg or Fn-coated surfaces at similar intensity levels. Analysis of the results performed by the methods described by Stepanovic et al. (2000) showed the ATCC10700/Pharyngitis strain classified into the category of strong biofilm producer on Fbg-coated surfaces and into the category of moderate biofilm producer on uncoated and Fn-coated polystyrene surfaces. The HHC1507/Bacteremia strain classified into the category of strong biofilm producer on uncoated polystyrene surfaces was classified into the category of moderate biofilm producer on both Fbg and Fn-coated polystyrene surfaces.

Biofilm formation on polystyrene surfaces

Both ATCC10700/Pharyngitis and HHC1507/Bacteremia strains were able to adhere to and to produce biofilm on hydrophobic plastic surfaces at different intensity levels as presented in Table 1.

C. pseudodiphtheriticum ATCC10700/Pharyngitis strain strongly adherent to the glass (I pattern) was classified as moderately producing biofilm on polystyrene. Unlike of *C. pseudodiphtheriticum* ATCC10 700/Pharyngitis strain, the HHC1507/Bacteremia strongly adherent to the glass (I pattern) was classified as strongly producing biofilm on polystyrene. When the polystyrene surfaces were coated with Fbg a significant increase of OD was observed (from 0.239/moderately adherent to 0.686/strongly adherent) post-incubation with ATCC10700/Pharyngitis, suggesting the influence of Fbg in biofilm formation by *C. pseudodiphtheriticum*. The *C. pseudodiphtheriticum* HHC1507/Bacteremia strain strongly producing biofilm on polystyrene, differently to the

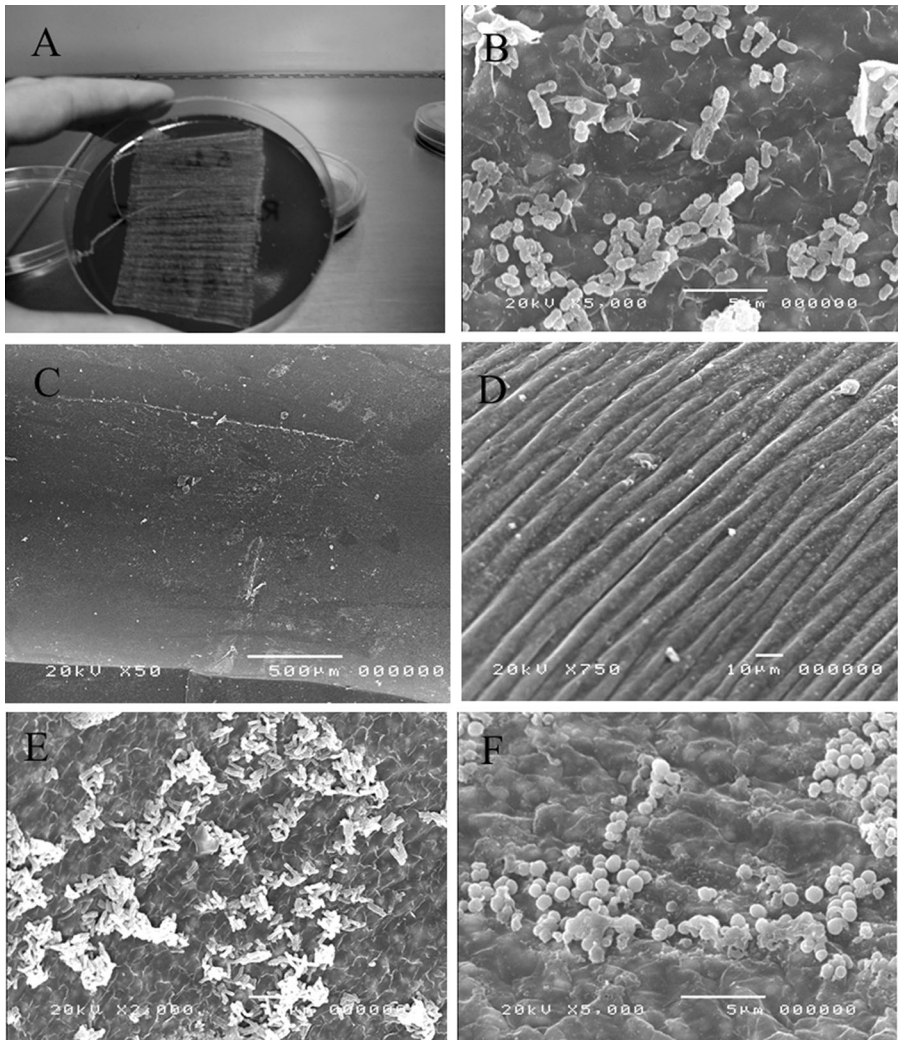

Fig. 2 Biofilm production in vitro on polyurethane catheter by *Corynebacterium pseudodiphtheriticum*. **a** The Maki's roll plate method indicated bacterial confluent growth on the surfaces of polyurethane catheter. Scanning electron micrographs showed: **b** bacterial microcolonies and amorphous material on the catheter inner surface; **c** biofilm formation on the external surface of the catheter; **d** surface of an unused sterile polyurethane catheter characterized by multiple gentle linear irregularities; **e** biofilm formation by *Corynebacterium diphtheriae* BR-CAT5003748 strain and **f** *Staphylococcus epidermidis* strains (positive controls)

ATCC10700/Pharyngitis strain, post-incubation with polystyrene coated with Fbg and Fn was the significant reduction of OD (from 0.730/strongly adherent to 0.432/moderately adherent and 0.730/strongly adherent to 0.333/moderately adherent, respectively). In contrast, differences in capacity for biofilm formation were not observed on polystyrene by *C. pseudodiphtheriticum* ATCC10700/Pharyngitis strain after polystyrene coated with Fn. The formation biofilm processes of the ATCC10700 and HHC1507 strains involved Fbg and Fn at different levels ($p < 0.05$). The ATCC10700 showed higher ability to adhere to polystyrene with Fbg than did the HHC1507. The data indicate that the *C. pseudodiphtheriticum* strains studied exhibited differences in their ability to adhere to polystyrene, mechanism that can be attributed to hydrophobic interaction between the bacteria and the negative charge of the plastic. Furthermore, the clinical strains in formed biofilms with the reverse intensities when the surfaces were coated with Fn and Fbg.

Table 2 Fibrinogen (Fbg) and fibronectin (Fn) binding to *Corynebacterium pseudodiphtheriticum* strains evaluated by ELISA

C. pseudodiphtheriticum strains	ELISA (OD ± SD)	
	Fbg (30 μg ml^{-1})	Fn (30 μg ml^{-1})
ATCC10700/Pharyngitis	0.345 ± 0.2040	0.414 ± 0.3838
HHC1507/Bacteremia	0.302 ± 0.0553	0.248 ± 0.0297
	$p < 0.0001$	$p < 0.0001$

OD ± SD, Optical Density ± Standard Deviation; The intensity of human Fbg (30 μg ml^{-1}) and human Fn (30 μg ml^{-1}) binding by *C. pseudodiphtheriticum* strains was expressed in OD values at $\lambda = 450$ nm (Bio-Rad Model 550 spectrophotometer). Data were from three independent experiments and compared statistically by the Tukey test using GraphPad Prism 6.0. Differences were considered significant at $p < 0.05$

Discussion

Pathogenic microorganisms may have an array of mechanisms that facilitate their attachment to abiotic and biotic surfaces and ability to gain access to adjacent tissues and circulatory system (Franson et al. 1984; Costerton et al. 1999; Smani et al. 2012). Of particular concern is the threat of bacterial biofilm development, since biofilm-mediated infections are difficult to diagnose and effective treatments are lacking. In addition to conventional resistance mechanisms, biofilm sessile bacteria also utilize biofilm-specific mechanisms of tolerance (Hoiby et al. 2010). The matrix and layers of cells within the biofilm create a physical barrier to slow the diffusional penetration of antibiotics. Metabolic activity in the outer biofilm can create acidic or anoxic areas that might contribute to antibiotic degradation (Huang et al. 1995). Biofilm structure also inhibits the adaptive and innate immune responses of the host there is a correlation with elevated inflammatory parameters, and the local tissue damage caused by inflammation might release nutrients that then become available to the biofilm bacteria, exacerbating the disease (Jensen et al. 2010).

Some corynebacteria species were found to adhere to available biotic and abiotic surfaces and/or to form biofilms, including *Corynebacterium jeikeium, Corynebacterium macginleyi* and *C. diphtheriae* (Wang et al. 2001; Kwaszewska et al. 2006; Suzuki et al. 2007). Previous investigations also demonstrated the ability of *C. pseudodiphtheriticum* to adhere to, survive and/or multiply within epithelial cells and also suggested differences in the virulence mechanisms of the clinical isolates, including ATCC10700/Pharyngitis and HHC1507/Bacteremia strains (Souza et al. 2012). Microorganisms were also capable to survive and multiply in the extracellular environment at least for 24 h post-infection. Different from the ATCC10700/Pharyngitis strain, the HHC1507/Bacteremia strain did not persist in the intracellular compartment and viable bacteria were observed in the supernatants of infected monolayers. Interestingly, the ATCC10700/Pharyngitis and HHC1507/Bacteremia strains exhibited an aggregative adherence (AA) pattern that was previously associated with invasiveness and intracellular survival of some human pathogens, including *C. diphtheriae* (Hirata et al. 2008) and enteroaggregative *Escherichia coli* (EAEC) (Pereira et al. 2008).

It is generally recognized that the pathogenesis of EAEC can be divided into different stages including adherence, colonization and biofilm formation. EAEC adherence is shown to rely on multiple different factors that altogether contribute in various ways to display the AA phenotype. It has been suggested that there is a correlation between the ability to express AA fimbriae (AAF) and the capability to form biofilm (Harrington et al. 2006; Huang et al. 2006; Fujiyama et al. 2008). Yanagawa and Honda (1976) showed that the ATCC10700 strain possessed a large number of pili, ranging from dozens to more than a hundred, in 91–100 % of the bacterial cells.

To the best of our knowledge, this is one of the first studies concerning adhesion to abiotic surfaces and biofilm formation by *C. pseudodiphtheriticum.* Currently, both AA-like ATCC10700/Pharyngitis and HC 1507/Bacteremia *C. pseudodiphtheriticum* strains showed the ability to adhere to available abiotic and/or biotic surfaces and to form biofilms. Data indicated a correlation between the AA phenotype and biofilm formation by *C. pseudodiphtheriticum* strains. Additional

studies remain necessary for determining if pili, among other bacterial surfaces components, are involved in AA adherence to epithelial cell surfaces and/or adherence to abiotic surfaces of *C. pseudodiphtheriticum* strains.

Bacteria often switch from a free-living lifestyle to a surface adapted, multicellular lifestyle known as a biofilm. Bacteria in biofilms become highly differentiated from free-living bacteria and often exhibit a developmental sequence characterized into different stages such as: initial attachment, irreversible attachment, maturation and dispersion. Primary adhesion of planktonic bacterial cells is a crucial step in biofilm formation. This initial, reversible microbial adherence is mostly dependent on bacterial cell surface characteristics and on environmental parameters such as the nature of the material surface, availability of substrates and nutrients (Stanley and Lazazzera 2004). It is mainly due to the physicochemical interactions that bacteria firmly adhere to the biomaterial surface during the adhesion process. These comprise van der Waals forces, Lewis acid–base, and electrostatic forces (Rosenberg 1981). The presence of bacterial surface-associated proteins may be associated with cell surface hydrophobicity and initial adhesion (Costerton et al. 1999; Sousa et al. 2011; von Eiff et al. 2002; Oliveira et al. 2003).

In general, biofilm-producing strains demonstrate an increased adherence to plastic surfaces as compared with non-biofilm-producing strains. *C. diphtheriae* and lipophilic skin corynebacteria hydrophobicity was connected with biofilm formation on solid surfaces (Kwaszewska et al. 2006; Mattos-Guaraldi et al. 1999). Similar to *C. diphtheriae*, *Corynebacterium urealyticum* and a number of other bacterial species (Kwaszewska et al. 2006; Mattos-Guaraldi and Formiga 1991; Soriano et al. 2008), the adherence to polystyrene attributed to hydrophobic interactions between cells and this negatively charged plastic surface was also observed for *C. pseudodiphtheriticum*. However, *C. pseudodiphtheriticum* clinical isolates seemed to exhibit differences in their ability of biofilm production on polystyrene surfaces. This activity was significantly higher for the strain associated with invasive disease (HHC1507/Bacteremia strain).

After the initial adhesion to a foreign body surface, bacteria multiply forming microcolonies and accumulate as multilayered cell clusters, a step that involves intercellular adhesion and the synthesis of extracellular matrix molecules, such as proteins and polysaccharides. Microcolony formation is a coordinated, adaptive response that facilitates continued biofilm development and dispersal. Further growth of the attached microorganisms occurs, leading to the formation of dense bacterial aggregates embedded in the exopolymeric matrix surrounded by a network of water channels, typical of mature biofilms (O'Toole et al. 2000; Webb et al. 2003). Mature biofilms can then undergo a detachment process releasing planktonic bacteria that can then colonize another region of the substratum to form new microcolonies (Karatan and Watnick 2009).

In the present investigation, *C. pseudodiphtheriticum* strains were found as effective biofilm producers on glass surfaces. Similarly to the catheter-related *C. diphtheriae* BR-CAT5003748 strain (Gomes et al. 2009), microcolony formation was verified on both hydrophilic (glass) and hydrophobic (polyurethane) surfaces by *C. pseudodiphtheriticum* ATCC10700/Pharyngitis and HHC1507/Bacteremia strains. Extracellular polymeric substances (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix (Vu et al. 2009). *C. diphtheriae* adherence to hydrophilic glass surfaces was attributed to the presence of extracellular matrix molecules, especially polysaccharides with high levels of sialic acid residues (Mattos-Guaraldi and Formiga 1991; Gomes et al. 2009; Moreira et al. 2003).

Cell death inside microcolonies is an important physiological event that plays a role in subsequent differentiation and dispersal of a subpopulation of surviving biofilm cells (Webb et al. 2003). Presently, CLSM images showed microcolonies of viable bacteria with intact cell membranes stained fluorescent green and dead bacteria with damaged membranes stained fluorescent red in *C. pseudodiphtheriticum* biofilm accretions. The free diffusion of low-molecular-weight compounds fluorescein showed the spatial arrangement of cells and indicated the presence of porosity and channels throughout the *C. pseudodiphtheriticum* biofilm. Data suggested that cell death inside microcolonies may play a role in subsequent differentiation and dispersal of a subpopulation of surviving biofilm cells of *C. pseudodiphtheriticum*.

However, the mechanism(s) by which voids were created within microcolonies and by which cells inside disperse in mature biofilms need(s) further investigation.

C. pseudodiphtheriticum produced biofilm on abiotic surfaces, including polyurethane medical devices. Although medical devices may differ widely in design and use characteristics, specific factors determine susceptibility of a device to microbial contamination and biofilm formation such as number and type of organisms to which the device is exposed, composition of the medium in or on the device, device material construction and conditioning films on the device all may influence biofilm formation. Polyurethanes are commonly used in a number of medical applications including catheter and general purpose tubing, hospital bedding, surgical drapes, wound dressings, as well as in a variety of injection molded devices (Donlan and Costerton 2002; Donlan 2008).

The general findings from the literature indicate that the increased synthesis of exopolysaccharide is often related to biofilm-producing bacteria associated with symptomatic catheter infections (Olson et al. 2002). A large amount of biofilm material exhibiting bacterial microcolonies and amorphous material was observed on polyurethane catheter inner surface infected in vitro with *C. pseudodiphtheriticum* and the positive controls (*C. diphtheriae* and *S. epidermidis* strains). Like other Gram-positive pathogens, the nature of the surface irregularities probably did not fully account for trapping *C. pseudodiphtheriticum* on polyurethane catheter surfaces. In accordance with previous observations with *C. diphtheriae* (Mattos-Guaraldi and Formiga 1991; Gomes et al. 2009) the adherence of *C. pseudodiphtheriticum* to polyurethane may be partially explained by the positive electric charges associated with this polymer. The amorphous deposited substances or glycocalyx noted surrounding *C. pseudodiphtheriticum* microcolonies on the surfaces of polyurethane catheters reinforce the fact that the bacteria may produce or attract exopolymeric components that strengthen their attachment to inert surfaces in vitro.

Biofilm material was also observed on polyurethane catheter external surface infected in vitro with *C. pseudodiphtheriticum*. Data suggested that *C. pseudodiphtheriticum,* like other pathogens from exogenous sources (such as the hands of personnel) or microflora endogenous to patients, may invade via the catheter insertion wound and migrate along the external catheter surface eventually colonizing the catheter segment (Franson et al. 1984).

In natural environments, bacteria mostly adhere to the layer of adsorbed molecules that coats the inert surfaces, the so called "conditioning film", and not directly to the substratum. In vivo, any material surface is rapidly covered by plasma and matrix proteins toward which bacteria may display specific adhesins (Whittaker et al. 1996; Arciola et al. 2005). The stimulation of bacterial biofilm formation by exogenous mammalian proteins has been reported for many human pathogens (Akiyama et al. 1997; Bonifait et al. 2008). Fibronectin (Fn) is a complex glycoprotein found in a soluble form in many body fluids (blood, saliva) and in an insoluble form as a component of cell surfaces, basement membranes, and the extracellular matrices. Soluble plasma Fn interacts with various bacteria and cell surface Fn may serve as a receptor in the adherence of bacteria to host epithelial cells. The ability of various bacteria to interact with different domains on the Fn molecule may play an important role in bacterial adherence and tissue tropism (Hynes 1990). At least ten different proteins from *E. coli* bind to Fn leading to internalization of *E. coli* by human host cells including epithelial cells (Henderson et al. 2011). For *S. aureus*, Fn is targeted by bacteria through Fn-binding proteins (FnBPs), which are anchored to the bacterial cell wall, and these interactions are likely to play a role in the infection process in particular. FnBPs have been shown to mediate not only bacterial adhesion to host cells but also the uptake of bacteria by the cells and biofilm formation (Schwarz-Linek et al. 2003). FnBP-promoted biofilm occurred at the level of intercellular accumulation and not primary attachment to Fn or Fbg (O'Neill et al. 2008).

Many bacterial pathogens exploit mechanisms involved in coagulation systems to colonize exposed tissue matrix proteins or evade immune mechanisms of bacterial clearance. Fibrinogen (Fbg) is a major protein in human plasma and is primarily involved in the coagulation cascade system through its conversion to insoluble fibrin. Fbg synthesis is dramatically upregulated during inflammation or under exposure to stresses such as systemic infections. Both Fbg and fibrin play overlapping roles in blood clotting, fibrinolysis, inflammatory response, cellular and matrix interactions and wound healing (Davis et al. 2001;

Cheung et al. 1991; Mosesson 2005). A comparative proteome analysis of *S. aureus* biofilm and planktonic cells showed that biofilm cells expressed higher levels of protein associated with cell attachment and, in particular, Fbg-binding proteins (Resch et al. 2006). For *Streptococcus suis*, Fbg binding property allowed bacteria to attach to each other through Fbg-mediated cross-bridging (Bonifait et al. 2008) and contributed to the production of biofilm. Fbg binding to *Streptococcus agalactiae* played a significant role in preventing opsonophagocytosis (Tenenbaum et al. 2005).

Like *C. diphtheriae* (Gomes et al. 2009; Souza et al. 2004; Sabbadini et al. 2010), the ability of *C. pseudodiphtheriticum* strains to bind to Fn and Fbg was demonstrated in the present study. Interestingly, Fn and Fbg bound with lower affinity to the blood isolate (HHC1507/Bacteremia strain). Fn and Fbg binding properties, possibly favored the adherence to respiratory epithelial surface by the ATCC10700/Pharyngitis strain. Data suggest that interaction with Fn and Fbg may exhibit a multifactorial nature and be relevant in promoting *C. pseudodiphtheriticum* attachment to host epithelial cells, damaged tissues and/or blood clots, as previously observed for other Gram-positive pathogens (Bonifait et al. 2008; O'Neill et al. 2008).

In addition to the ability of biofilm production directly to hydrophilic and hydrophobic abiotic surfaces, *C. pseudodiphtheriticum* biofilm production was also observed on Fbg and Fn "conditioning films". Nevertheless, the clinical isolates responded inversely in the presence of Fbg and/or Fn bound to the solid surface. When compared to biofilm formation on uncoated polystyrene surfaces, Fbg and/or Fn coated surfaces enhanced biofilm formation by the ATCC10700/Pharyngitis strain and reduced biofilm formation by the HHC1507/Bacteremia strain. Therefore, the expression of Fbg and Fn binding adhesins at different levels may be implicated in biofilm formation on "conditioning films" by *C. pseudodiphtheriticum* strains, as previously reported for *S. suis* (Bonifait et al. 2008). *C. pseudodiphtheriticum* may form biofilm in vivo possibly by an adherent biofilm mode of growth in vitro demonstrated on hydrophilic and hydrophobic abiotic surfaces, including polyurethane catheters. The affinity to human Fbg and Fn and the biofilm-forming ability appears to be a potential virulence trait that may contribute to the establishment and dissemination of infection caused by *C. pseudodiphtheriticum*, including in patients making use of indwelling medical devices. Further studies remain necessary in order to investigate the involvement of EPS in biofilm formation by *C. pseudodiphtheriticum.*

Acknowledgments This work was supported by grant from CAPES, CNPq, FAPERJ, SR-2/UERJ, PNPD (CNPq/CAPES), PAPD (FAPERJ/CAPES) and PRONEX of the Brazilian Ministry of Science and Technology. We are also grateful to Noêmia Rodrigues Gonçalves Alves from Cellular Ultrastructure Laboratory Hertha Meyer-Universidade Federal do Rio de Janeiro, for electron microscopy technical assistance.

Conflict of interest No competing financial interests exist.

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