Abstract

Introduction: *Clostridioides difficile* biofilms are believed to protect the pathogen from antibiotics, in addition to potentially contributing to recurrent infections.

Methodology: Biofilm production of 102 *C. difficile* isolates was determined using the crystal violet staining technique, and detachment assays were performed. The expression levels of *cwp84* and *slpA* genes were evaluated by real-time PCR on selected isolates.

Results: More than 70% of isolates (75/102) were strong biofilm producers, and the highest detachment of biofilm was achieved with the proteinase K treatment (>90%). The overall mean expression of *cwp84* was higher in RT027 than in RT001 ($p = 0.003$); among strong biofilm-producing strains, the *slpA* expression was lower in RT027 than in RT001 ($p < 0.000$).

Conclusions: Proteins seem to have an important role in the biofilm's initial adherence and maturation. *slpA* and *cwp84* are differentially expressed by *C. difficile* ribotype and biofilm production level.

Keywords: *Clostridioides difficile*, biofilm, ribotype 027, ribotype 001, *cwp84*, *slpA*.

Introduction

Clostridioides difficile is a Gram-positive, strictly anaerobic, spore-producing bacterium, and it is the most common infectious cause of nosocomial diarrhoea [1]. While *C. difficile* spores are essential for the transmission and persistence of *C. difficile* infection, other factors such as intestinal colonization and the formation of bacterial communities in the intestine can also contribute to the pathogenesis and persistence of the disease. Nevertheless, these factors have not been extensively investigated [2].

For several pathogens, the ability to form biofilms has been associated with recurrent infections [3]. The biofilm matrix protects bacteria by providing a closed environment and generally consists of an extracellular polymeric substance, which can comprise proteins, DNA, and polysaccharides [4]. The biofilm matrix can act as an initial physical barrier that prevents the penetration of antimicrobial agents; other attributes such as the bacteria's physiological state can also contribute to drug resistance [4].

The surface-layer protein A (SlpA, encoded by *slpA*) is a precursor involved in adhesion and is cleaved by the cell wall cysteine protease (Cwp84, encoded by *cwp84*), to form the high and low molecular weight subunits in the S-layer of *C. difficile* [2]. Exposure to subinhibitory concentrations of ampicillin and clindamycin increases the expression of genes encoding colonization factors, such as Cwp66, P47, Fbp68, and Cwp84 [5].

The aim of the present study was to analyse biofilm production and the expression of genes that encode proteins involved in the biofilm production of circulating *C. difficile* strains from Mexico.

Methods

Isolates and evaluation of biofilm production

In this study, 102 strains with previously characterized ribotypes were included [6]. Strains were obtained from patients with a confirmed *C. difficile* infection diagnosis from two Mexican tertiary-care hospitals; only one isolate per patient was included (Table 1).

The evaluation of biofilm production was determined by crystal violet staining using the conditions described by Dawson *et al.* [7] with the following modifications: 96-well microplates (Corning Inc, NY, USA) were used and the biofilm was incubated for 48 h at 37°C in anaerobiosis. After incubation, absorbance at 595 nm (planktonic cells) was measured, and an additional fixing/drying step was performed for one hour at 60°C. The biofilm was stained with 0.1% Hucker's crystal violet, and the stain was dissolved with 30% acetic acid for 30 min. The experiments were performed per triplicate for each isolate. The biofilm index (BI) was calculated [8], and isolates were classified as nonproducer (BI < 0.9), weak producer (BI, 0.9–1.2), or strong producer (BI > 1.2).

Detachment assays

Detachment assays were performed on selected isolates as previously described[8]. Isolates were classified as demonstrating no detachment (<10%), intermediate detachment (10–50%), moderate detachment (51–75%), or strong detachment (>75%).

Evaluation of the expression of genes associated with adherence

The expression levels of genes *slpA* and *cwp84* were evaluated by real-time PCR. Total RNA was extracted using the QIAmp DSP viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Standard curves were constructed for the target genes (*slpA* and *cwp84*) and the endogenous gene (16S rRNA). Oligonucleotides and PCR conditions reported by Deneve *et al.* were used [5]. The results

obtained for each isolate and gene were normalized using the expression levels of the strain *C. difficile* ATCC 9689. Overexpression was defined by relative expression that increased 300% compared with the calibrator strain.

Statistical analysis

The associations of each ribotype with levels of biofilm production as well as the results of the detachment assays were determined by the chi-square test; the Mann-Whitney test was used to determine differences in the results of the expression assays. For both analyses, the SPSS Statistics version 22.0 software (IBM Corporation, Somers, NY) was used, and a P value less than 0.05 was considered to be statistically significant.

Results

Biofilm production and detachment assays

More than 70% (75/102) of the isolates were classified as strong biofilm producers, most of which were RT027 (Table 1). No association was found between the ribotypes and strong biofilm production ($p > 0.05$). Biofilm detachment assays were performed in 72 strong biofilm-producing strains: 12 RT001 isolates and 60 RT027 isolates. Detachment percentages higher than 90% were obtained for both ribotypes with proteinase K treatment. When $NaIO_4$ was used for treatment, approximately 60% of the isolates in both ribotypes had intermediate and moderate detachment. When DNase I was used, approximately 50% of the isolates had a strong detachment in RT027; a similar proportion was found in RT001 but with intermediate detachment (Table 2).

Expression of *cwp84* and *slpA*

Forty-four selected isolates were included for *cwp84* and *slpA* expression assays: 17 RT001 isolates (5 nonproducers and 12 strong producers) and 27 RT027 isolates (13 nonproducers and 14 strong producers).

The overall mean expression of *cwp84* was significantly higher in RT027 than in RT001, regardless of biofilm production classification ($p = 0.003$). Similar results were found in non-biofilm-producing strains ($p = 0.049$). By contrast, among strong-producing strains, the mean *slpA* expression was significantly lower in RT027 than in RT001 ($p < 0.000$) (Table 2).

Discussion

For *C. difficile,* biofilm represents a closed environment that protects the bacterial population from the pressure of antibiotics, but it can also promote the recurrence of the disease [2]. In this study, we analysed the biofilm production and expression of genes related to the adhesion of circulating *C. difficile* strains from Mexico.

Most of the analysed isolates were strong biofilm producers, regardless of the ribotype. RT027 isolates were more likely to be strong biofilm producers (63/82, 76.8%), with no statistical significance detected. Similarly, the analysis of biofilm production in 37 strains showed no correlation between the ability to form a biofilm and the ribotype [9].

In the present study, the participation of proteins, carbohydrates, and extracellular DNA as components of the matrix was evaluated. Previous studies have shown that proteins are incorporated into the matrix during the maturation of biofilm and are required for the assembly of the biofilm [10]. In our study, all strains showed detachment higher than 90% when the 48-h biofilm is treated with proteinase K, reinforcing the important role of proteins in the initial adherence and/or the biofilm maturation process.

Subinhibitory concentrations of metronidazole have been reported to increase the biofilm production of *C. difficile* strains[11]. A thick biofilm characterized by layered aggregates has been demonstrated by confocal laser scanning microscopy (CLSM) imaging. The CLSM provided evidence for the binding of concanavalin A to exopolysaccharide (EPS) residues of matrix [11]. In our study, carbohydrate contribution to the extracellular matrix was variable, suggesting differences in the type of carbohydrate and their proportions.

Extracellular DNA (eDNA) has also been described as an essential component of *C. difficile* biofilm, but in lower proportions than proteins [12]. It has been suggested that eDNA in the biofilm matrix results from phage-mediated bacterial cell lysis and other unknown eDNA release mechanisms [13]. In previous studies, the staining pattern of the DOC-induced biofilm with BOBO-3 and SYPRO Ruby Red showed a net-like structure that implicates eDNA and proteins in the biofilm matrix. The presence of eDNA in the DOC-induced biofilm matrix was demonstrated on an agarose gel, and treatment with DNase dispersed previously formed biofilm [10]. Our results suggest that DNA plays an essential role in the biofilm of RT027, considering the detachment rates in this ribotype.

In the present work, we evaluated the expression of *slpA* and *cwp84* genes in biofilm; however, we found low expression of these genes. The low expression observed may be explained by the model used because the expression was not induced by stressing the cells (either by antibiotic or other conditions), which may have promoted the adherence and formation of biofilm [5]. Similarly, the ligands for these adhesins are present on host tissue but not in polystyrene plates; thus, increased expression of these adhesins may depend on the presence of the ligands [14]. Also, these genes may not be expressed at high levels once a biofilm is established [14].

A limitation of our study was the use of one species biofilm because *in vivo* biofilms are multispecies and complex. It has been demonstrated that *C. difficile* aggregates with *Fusobacterium nucleatum*; together, these species produce mature biofilms by means of the *Fusobacterium* protein RadD [15]. Therefore, attachment and stability mechanisms may differ from *in vitro* studies, and key species may be needed to establish biofilms and facilitate attachment and survival. Another limitation is the proportion of isolates per included ribotype. The strains were recovered from patients of two tertiary hospitals from 2011 to 2016. However, in 2014, there was an outbreak due to the RT027 strain in one of the hospitals, which may have generated ribotype proportion bias.

In conclusion, most *C. difficile* clinical isolates were strong biofilm producers. Proteins may have an essential role in the initial adherence and maturation of the biofilm matrix and the contribution of DNA and carbohydrates was variable, suggesting a complex mechanism of biofilm production. The low levels of expression of genes associated with adherence, highlights the need to find suitable models for the study of the *C. difficile biofilm* to understand its role in the pathogenesis.

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Table 1. Ribotypes of *C. difficile* strains classified by biofilm production

Classification	n (%)	Ribotype (n)
Nonproducer	22 (21.6)	027 (15), 001 (7)
Weak producer	5 (4.9)	027 (4), 001 (1)
Strong producer	75 (73.5)	027 (63), 001 (12)

Table 2. Detachment assay results by *C. difficile* ribotype and relative expression of *cwp84* and *slpA.*

Ribotype	**001 (n=12)**		**027 (n=60)**		***p***
Proteinase K					
Strong detachment, n (%)	12 (100)		60 (100)		n/a
$NaIO_4$					
No detachment, n (%)	2 (16.7)		5 (8.3)		
Intermediate, n (%)	4 (33.3)		21 (35)		
Moderate, n (%)	4 (33.3)		18 (30)		
Strong, n (%)	2 (16.7)		16 (26.7)		0.722
Dnase I					
No detachment, n (%)	0 (0)		0 (0)		
Intermediate, n (%)	7 (58.3)		10 (16.7)		
Moderate, n (%)	2 (16.7)		16 (26.7)		
Strong detachment, n (%)	3 (25)		34 (56.7)		0.089
Expression assays (Mean expression ± SD)					
cwp84	**n**	**001**	**n**	**027**	***p***
All isolates	17	0.47 ± 0.45	27	1.28 ± 0.88	0.003
Nonproducers	5	0.56 ± 0.48	13	1.6 ± 0.97	0.049
Strong producers	12	0.43 ± 0.46	14	0.99 ± 0.68	0.105
slpA					
All isolates	17	2.45 ± 1.42	27	2.16 ± 4.45	0.795
Nonproducers	5	2.73 ± 1.3	13	3.7 ± 0.51	0.237
Strong producers	12	2.34 ± 1.51	14	0.77 ± 1.65	0.000

SD, standard deviation; n/a, not applicable.