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RESEARCH ARTICLE

Effect of different antibiotics on biofilm produced by uropathogenic *Escherichia coli* isolated from children with urinary tract infection

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ABSTRACT

Recurrent urinary tract infections (UTIs) occur frequently in children and women. Intracellular bacterial communities (IBCs) and biofilm formation by *Escherichia coli* are risk factors for recurrence. The aim of this study was to evaluate the effect of different antibiotics on biofilms by *E. coli* strains isolated from children with UTI and to correlate virulence factors and IBCs with biofilm formation. A total of 116 *E. coli* strains were tested for biofilm formation using the crystal violet microplate technique. 58.6% of the strains did not produce biofilm, while 16.4%, 18.1% and 6.8% formed weak, moderate and strong biofilms, respectively. No correlation was found between the ability to form biofilms and the presence of IBCs. Biofilm formation was significantly associated with pili P codifying genes, whereas other virulence factors were not statistically associated. Antibiotics, including ampicillin, cephalothin, ceftriaxone, ceftazidime, amikacin and ciprofloxacin, were evaluated at different concentrations after 48 h of biofilm formation. Except ampicillin, the other antibiotics tested induced a significant reduction of biofilm biomass. In the case of recurrent UTIs potentially associated with the presence of biofilm, the use of third-generation cephalosporin, fluoroquinolones and aminoglycosides could be recommended. These antibiotics demonstrated to reduce biofilm biomass produced even by resistant strains.

Keywords: biofilm; antibiotics; urinary infection; uropathogenic *Escherichia coli*; treatment; children

INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, being uropathogenic *Escherichia coli* (UPEC) the most frequent etiological agent (80%–90%) (Habib 2012; Robino *et al.* 2014a). Forty percent of women and 12% of men will have an UTI episode at least once in their life. In children, up to 8.4% of girls and 1.7% of boys will have an UTI episode in the first six years. A total of 20% to 40% of women and children would suffer a recurrent episode of UTI in the following 6–12 months after the first episode (Garin *et al.* 2006; Elder 2009; Habib 2012).

Different risk factors related to UTI recurrency in children that either depends on the host or on the microorganism have

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been described. Several factors of the host such as age, gender, circumcision, vesicoureteral reflux and bladder-bowel dysfunctions are believed to increase the risk for recurrent UTI (Downs 1999; Garin *et al.* 2006; Keren *et al.* 2015). Considering the microorganism, recurrent UTI may be due by the same strain that involved the original infection in 25%–82% of cases, defined as relapse, or by a different strain other than that involved in the original infection, defined as re-infection. In the cases of relapse, the microorganism may come from the intestinal microbiota or from intracellular bladder reservoirs (intracellular bacterial communities [IBCs] or quiescent Intracellular reservoirs) (Karkkainen *et al.* 2000; Ejrnaes *et al.* 2006; Mysorekar and Hultgren 2006; Czaja *et al.* 2009; Hooton 2012). In a previous study, our group reported the presence of IBC in 36.8% of children with UTI, being its presence associated to recurrence (Robino *et al.* 2014b).

Relapses commonly occur by *Escherichia coli* biofilm producer strains, being responsible for the persistence of *E. coli* in the vaginal reservoir and the bladder epithelium (Sanchez *et al.* 2013). Biofilm is defined as a microbial-derived sessile community characterized by cells that are irreversibly attached to a substratum or interface on each other and embedded in a matrix of extracellular polymeric substances that they have produced (Flemming and Wingender 2010). Biofilm is produced in response to environmental changes, altering bacterial gene expression of surface molecules, virulence factors and metabolic status (Donlan 2002; Donlan and Costerton 2002; Flemming and Wingender 2010).

Five stages are described for biofilm production, although some variations between different bacterial species could be observed. The first stage comprises the reversible attachment of planktonic bacteria to surfaces. In this period, the bacterial flagella may play an important role (Donlan 2002; Toutain *et al.* 2007). After this, an important change in gene expression happens and bacteria start to produce adhesins as type 1 pili, curli fibers and antigen 43 which contribute to the irreversible attachment to surfaces (Lemon, Higgins and Kolter 2007). The third stage comprises the formation and production of the extracellular polysaccharide matrix. In the case of *E. coli*, the matrix is composed of cellulose, polyglucosamine and colonic acid (reviewed by Soto 2014). The biofilm starts to grow and it acquires its three-dimensional structure known as macrocolony (reviewed by Soto 2014). Finally, the last stage happens when bacteria change to a planktonic state and detach from the biofilm. This could lead to a new biofilm formation on other surface and several factors could trigger bacteria detachment (reviewed by Soto 2014).

Bacteria in biofilms are protected from host immune response, being resistant to phagocytosis, and more resistant to conventional antibiotic treatments than their planktonic counterparts. Antibiotic resistance in biofilms has been widely studied in the last years, proposing that it is an adaptive and reversible situation, demonstrated by the recovery of the original susceptibility when bacteria return to a planktonic state (Keren *et al.* 2004). It is proposed that antibiotic resistance in biofilm occur because of different mechanisms, like a limitation of antibiotic diffusion through the matrix, horizontal transmission of resistance genes, inactivation of the antibiotic by changes in metal ion concentrations and pH values and the metabolic inactive bacterial status. The level of resistance depends on the biofilm formation stage, being more susceptible bacteria in the initial reversible step (reviewed by Soto 2014).

The objective of the present work was to evaluate the effect of different antibiotics, commonly used for the treatment of UTI, on biofilm, and to determine if the presence of virulence factors genes or IBCs were associated with biofilm production.

MATERIALS AND METHODS

Bacterial strains

A total of 116 uropathogenic *Escherichia coli* (UPEC) strains isolated from urine samples from children with UTI were studied. All the strains were characterized in a previous study and classified according to the presence or absence of IBCs, or isolated intracellular bacteria (IB), phylogenetic groups and virulence factors (Robino *et al.* 2014b). The distribution into these groups was as follows: 25 isolates produced IBC (19.8%), 17 produced IB (16.3%) and 74 isolates did not produce IBC or IB (61.2%). UPEC isolates were also classified into phylogenetic groups, 52 belonging to group D, 38 to B2, 19 to group A and 7 to group B1. In this work, FimH expression was evaluated by hemagglutination. This assay was performed as described by Hultgren *et al.* (1986) with modifications.

Antibiotic susceptibility was assessed, by minimum inhibitory concentration (MIC), using the Vitek ® 2 compact systems (bioMérieux, Marcy-l'Étoile, France). The interpretation of MIC results was done following Clinical Laboratory Standard recommendations (CLSI 2015). These strains were stored in glycerol at –80°C. Bacteria were grown in either Luria-Bertani (LB) broth or Luria-Bertani agar (LA) at 37°C.

Those isolates with a positive extended spectrum beta-lactamase (ESBL) screening test underwent molecular confirmation by PCR using specific primers for ESBL genes frequently detected in Uruguay (blaCTX-M, blaTEM, blaPER-2 and blaSHV) (Bado *et al.* 2010).

Antibiotics

The antibiotics used in the present work are commonly used for the treatment of UTI and three classes of antimicrobial agents were represented: quinolones, aminoglycosides and beta-lactams. Antibiotics were used at the following concentrations: ampicillin (AMP: 300, 500 and 1000 μg/ml), cephalothin (CEFA: 1200, 1600 and 2000 μg/ml), ceftriaxone (CRO: 500, 1000 and 1500 μg/ml), ceftazidime (CAZ: 500, 1000 and 1500 μg/ml), amikacin (AK: 500, 1000 and 2000 μg/ml) and ciprofloxacin (CIP: 2, 3 and 5 μg/ml) (Table 1). The selection of these concentrations was defined according to antibiotics bioavailability in human urine (Lorian 2005).

Biofilms and antibiotics assays

Biofilm formation assays were performed as previously described with few modifications (Pratt and Kolter 1998). The strains were grown overnight in LB at 37°C under static conditions. Aliquots of 20 μl from overnight cultures were inoculated into 180 μl of LB in 96 flat-bottomed well, polystyrene microtitre plates (Deltalab), and incubated for 48 h at 37°C without shaking. The optical densities (ODs) were read at $\lambda = 600$ nm for bacteria growth. Planktonic bacteria were removed and fresh LB was added to each well with or without antibiotics as indicated (Table 1). After additional 24 h of incubation, the wells were washed with PBS three times and stained with 200 μl of 1% crystal violet for 15 min at room temperature. Then, the plates were washed to remove the dye excess and CV was solubilized with 200 μl of 95% ethanol. The ODs were read at $\lambda = 590$ nm for stained biofilms using a Microplate Reader (Varioskan, Thermo Scientific).

All the measures were performed in triplicate for all strains, and the means and standard error were calculated for all

Table 1. Antibiotics, mechanisms of action and concentrations.

Antibiotics	Family	Mechanism of action	Urine concentration (μg/ml)	Concentrations tested (μg/ml) 1	2	3
Ampicillin	Beta-lactams	Inhibition of cell wall synthesis	>1000	300	500	1000
Cephalothin				1200	1600	2000
Ceftriaxone				500	1000	1500
Ceftazidime				500	1000	1500
Amikacin	Aminoglycosides	Inhibition of protein synthesis	170–1720	500	1000	2000
Ciprofloxacin	Quinolones	Inhibition of DNA replication	2	2	3	5

experiments. Strains were classified as follows: OD ≤ ODc = no biofilm producer; ODc< OD ≤ (2 × ODc) = weak biofilm producer; (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producer; and (4 × ODc) < OD = strong biofilm producer where ODc were control wells with media without bacteria inoculation (Villegas *et al.* 2013).

Real-time PCR

Real-time PCR was performed in order to assess the expression of two different ESBL genes, blaCTX-M-9 and blaCTX-M-2, within biofilm and planktonic cells.

Escherichia coli culture was incubated 48 h at 37°C in 6-well flat-bottom plates (Greiner CELLSTAR®) with 5 ml LB and LB supplemented with 500 and 1500 mg/ml of ceftriaxone for others 24 h. Planktonic cells were removed and retained into 1 ml RNA later (Ambion), and the biofilms were washed with PBS and then 1 ml of RNA later were added. RNA extraction was performed immediately using PureLink RNA mini kit (Ambion) according to the manufacturer's recommendation. The total RNA was treated with DNAse I (Invitrogen) to remove any contamination with genomic DNA. The RNA was quantified using a NanoDrop spectrophotometer (Thermo) and cDNA was synthesized from 8 ng of purified RNA using random primers and M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendation. The real-time PCR primers sequences were designed to amplify CTX-M-9 gene (forward: GCGACAATACCGCCATGAAC; reverse: TAAGCTGACGCAACGTCTGT) and CTX-M-2 gene (forward: AAAACCGGCAGCGGAGATTA; reverse: CTGCTCCGGTTGGGTAAAGT), and the *rpoA* gene was used as the housekeeping control (Steyert *et al.* 2012). Quantitative Real-Time PCR Detection System (BioRad) was used to analyze PCR products with Syber Green (Invitrogen) fluorescence after the following program: one cycle of 2 min at 50°C, 15 min at 95°C and 40 cycle of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C. The melting curve 65°C to 95°C, with increments of 1°C/s. The threshold cycle (Ct) of each gene was normalized against the Ct of the *rpoA* gene amplified from the corresponding sample and with Ct of biofilm without antibiotic condition. Fold change was calculated according to the $2^{-\Delta\Delta Ct}$ method (Litvak and Schmittgen 2001).

Statistical analyses

The differences between the treatments groups were first assessed using the Kruskal–Wallis test, and the differences between pairs of groups were further assessed using the Mann–Whitney U test. The relationship between biofilm formation with CBI, phylogenetic groups and virulence factors were determined using χ^2 test ($P < 0.05$).

Table 2. Ability of *E. coli* strains to produce biofilms and IBC or IB.

Presence or absence of IBC/IB	Total (n)	Strong	Moderate	Weak	No producer
IBC	25	1	5	5	14
IB	17	1	3	2	11
Negative	74	6	13	12	43
Total biofilms	116	8	21	19	68

IBC = Intracellular bacterial communities; IB = Intracellular bacteria.

RESULTS

Determination of biofilm production on an abiotic surface, and its relation with CBI /IB, phylogenetic groups and virulence factors

The biofilm formation was evaluated *in vitro* using the standard microtiter plate-based assays. The result for the ODc was 0.21 so the isolates were classified according to OD cutoffs as follows: OD ≤ 0.21 = no biofilm producer; 0.21 < OD ≤ 0.42 = weak biofilm producer; 0.42 < OD ≤ 0.84 = moderate biofilm producer; and 0.84 < OD = strong biofilm producer. The biofilm production was negative in 68 strains (58.6%). The remaining strains were weak (19 strains, 16.4%), moderate (21 strains, 18.1%) and strong biofilm producers (8 strains, 6.8%).

Within the group of biofilm producer strains, 11 formed IBC (22.9%), 6 as IB (12.5%) and 31 did not produce IBC or IB (64.6%). In the group of non-biofilm producers, we observed a similar distribution, since 14 strains formed IBC (20.6%), 11 were observed as IB (16.2%) and 43 did not produce IBC or IB (63.2%). No significant correlation was found between the ability to produce biofilm and the formation of IBC or IB ($p > 0.05$, Table 2) (Robino *et al.* 2014b).

In the biofilm producer group, 27 belonged to phylogenetic group D, 11 to B2, 6 to group A and 4 to B1. No significant association was found between biofilm production and phylogenetic groups.

The presence of the following virulence factors was assessed by multiplex PCR: *afa* (afimbrial adhesin), *papA*, *papC*, *papEF*, *papGII* (genes related to P pili), *fimH* (type 1 pili), *sfa/FocDE* (fimbria), *kpsMII* (type II capsule), *iutA* (aerobactin siderophore). The distribution of virulence factors related to the ability to form biofilms is shown in Table 3. Interestingly, the production of biofilm was significantly associated with the presence of *papA*, *papEF* and *papGII* genes ($P = 0.024$, 0.017 and 0.024, respectively). In 84 out of 116 (72.4%) strains *fimH* genes were detected, 36 in the group of biofilm producer strains and 48 in the group of no biofilm producers ($P \geq 0.05$). Sixty nine out of 84 strains harboring the *fimH* gene expressed FimH protein, as seen by

Table 3. Biofilm production and virulence factors.

Virulence factors	Biofilm producers (N: 48)	No biofilm producers (N:68)	P-value (CI%95, OR)
afa	18	30	NS
sfa/focDE	9	16	NS
kpsMII	36	51	NS
papA	31	29	0.02 (1.2–5.2, OR 2.42)
papC	32	35	NS
papEF	31	27	0.01 (1.3–7.7, OR 3)
papGII	31	29	0.02 (1.2–5.2, OR 2.42)
fimH	36	48	NS
iutA	40	54	NS

The $\chi 2$ test was applied to nominal variables. $P < .05$ was considered significant. Virulence factors genes: *afa* = afirmbrial adhesin; *sfa/focDE* = S and Dra fimbriae; *kpsMII* = type II capsule; *papA, papC, papEF y papGII* = P pili; *fimH* = type 1pili; *iutA* = siderophore aerobactin; NS = no significant.

Table 4. Effect of different antibiotic concentrations on biofilm biomass.

	Concentration (μg/ml)	Mean OD	P-value
Ampicillin	0	0.752 (±0.083)	
	300	0.762 (±0.088)	0.86
	500	0.776 (±0.093)	0.96
	1000	0.795 (±0.081)	0.61
Cephalothin	0	0.909 (±0.112)	
	1200	0.735 (±0.129)	0.021
	1600	0.682 (±0.104)	0.048
	2000	0.581 (±0.085)	0.013
Ceftriaxone	0	0.932 (±0.124)	
	500	0.139 (±0.029)	1.7×10^{-10}
	1000	0.125 (±0.031)	1.0×10^{-10}
	1500	0.136 (±0.030)	2.3×10^{-10}
Ceftazidime	0	0.942 (±0.126)	
	500	0.105 (±0.022)	1.6×10^{-10}
	1000	0.073 (±0.020)	2.7×10^{-10}
	1500	0.076 (±0.021)	2.6×11^{-11}
Amikacin	0	0.646 (±0.082)	
	500	0.132 (±0.047)	2.6×10^{-10}
	1000	0.149 (±0.044)	1.7×10^{-9}
	2000	0.163 (±0.048)	3.9×10^{-9}
Ciprofloxacin	0	0.866 (±0.095)	
	2	0.267 (±0.060)	5.6×10^{-9}
	3	0.350 (±0.076)	4.7×10^{-7}
	5	0.338 (±0.083)	1.2×10^{-7}

Mean OD values ± standard error of each group of antibiotics. P value ≤ 0.05 was considered significant.

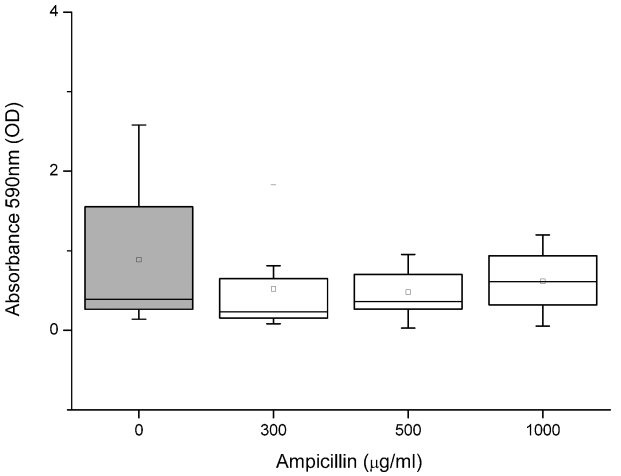

Figure 1. Biofilm quantification with different ampicillin concentrations. Boxplot comparing the effect of ampicillin at 0, 300, 500 and 1000 μg/ml concentrations on susceptible strains.

hemagglutination results. When *fimH* was present, and hemagglutination-positive strains were evaluated with the ability to form biofilm, 35 strains were biofilm producers and 34 not ($P = 0.21$). The presence of other virulence factors was not significantly associated with the ability to form strong, moderate or weak biofilm ($P \geq 0.05$).

In vitro effect of antibiotics on biofilm formation

Distinct antibiotics were evaluated at different concentrations to assess the possible effect on biofilms. The 48 strains evaluated were the ones belonging to the group of biofilm producers and the results are shown in Table 4.

Thirty eight of 48 isolates studied (79.2%) were resistant to ampicillin (all of them with MICs ≥ 32 μg/ml). In order to analyze the effect of this antibiotic on biofilm, we assayed different ampicillin concentrations (300, 500 and 1000 μg/ml). The results showed that there is no significant effect on biofilms in all the concentrations evaluated (Table 4). Even more, in the case of the strains that were susceptible to ampicillin no significant reduction of the biofilm was obtained (Fig. 1). On the other hand, in resistant strains, we observed a slightly increase in the mean OD values when different ampicillin concentrations were compared with no antibiotic addition, although this increase was not significant (Table 5).

In the case of cephalothin, 21 isolates (43.8%) showed resistance to this antibiotic, with MICs ranging from 32 to ≥64 μg/ml. Three different cephalothin concentrations were assayed over biofilm (1200, 1600 and 2000 μg/ml) and the three of them produced a significant reduction of the biofilm biomass ($P \leq 0.05$, Table 4). When we analyzed the resistant isolates, we did not observe any significant effect of cephalothin on biofilm. Otherwise, in susceptible strains, we observed a significant reduction in biofilm biomass for all concentrations showing a dose-dependent behavior (Table 5, Fig. 2).

Ceftriaxone and ceftazidime were evaluated at 500, 1000 and 1500 μg/ml, and the results showed a strong significant reduction in biofilm biomass compared to the biofilms formed without antibiotics (Table 4, Figs 3 and 4). Only two isolates (4.2%) were resistant to ceftriaxone (MIC ≥ 64 μg/ml) and ceftazidime (MIC 16 μg/ml) in the group of strains evaluated. This two strains harbor

Table 5. Effect of different antibiotic concentrations on biofilm biomass in the resistant and susceptible groups.

	Concentration (μg/ml)	OD resistant strains	P-value	OD susceptible strains	P-value
Ampicillin	0	0.716 (±0.083)		0.887 (±0.254)	
	300	0.827 (±0.102)	0.63	0.519 (±0.165)	0.14
	500	0.853 (±0.112)	0.62	0.479 (±0.096)	0.31
	1000	0.842 (±0.095)	0.44	0.617 (±0.124)	0.72
Cephalothin	0	0.863 (±0.129)		0.945 (±0.174)	
	1200	0.857 (±0.193)	0.45	0.641 (±0.174)	0.0049
	1600	0.761 (±0.159)	0.43	0.621 (±0.139)	0.021
	2000	0.702 (±0.127)	0.41	0.486 (±0.114)	0.0023
Ceftriaxone	0	0.449 (±0.152)		0.958 (±0.130)	
	500	0.352 (±0.341)	0.27	0.127 (±0.026)	1.2×10^{-10}
	1000	0.327 (±0.059)	0.19	0.114 (±0.032)	8.2×10^{-11}
	1500	0.262 (±0.088)	0.12	0.129 (±0.031)	2.6×10^{-10}
Ceftazidime	0	0.461 (±0.211)		0.967 (±0.131)	
	500	0.257 (±0.165)	0.17	0.096 (±0.022)	1.6×10^{-10}
	1000	0.128 (±0.012)	0.05	0.07 (±0.021)	4.5×10^{-11}
	1500	0.102 (±0.013)	0.03	0.074 (±0.023)	5.4×10^{-11}
Amikacin	0	0.767 (±0.147)		0.599 (±0.099)	
	500	0.126 (±0.035)	6.0×10^{-5}	0.135 (±0.064)	4.7×10^{-9}
	1000	0.111 (±0.032)	2.3×10^{-5}	0.164 (±0.061)	8.4×10^{-8}
	2000	0.171 (±0.067)	2.3×10^{-4}	0.161 (±0.062)	3.9×10^{-8}
Ciprofloxacin	0	0.866 (±0.182)		0.866 (±0.099)	
	2	0.517 (±0.269)	0.39	0.266 (±0.063)	7.4×10^{-9}
	3	0.451 (±0.295)	0.29	0.358 (±0.078)	8.3×10^{-7}
	5	0.553 (±0.303)	0.44	0.345 (±0.087)	1.9×10^{-7}

Mean OD values ± standard error of every group of antibiotics for susceptible and resistance strains. P value ≤ 0.05 was considered significant.

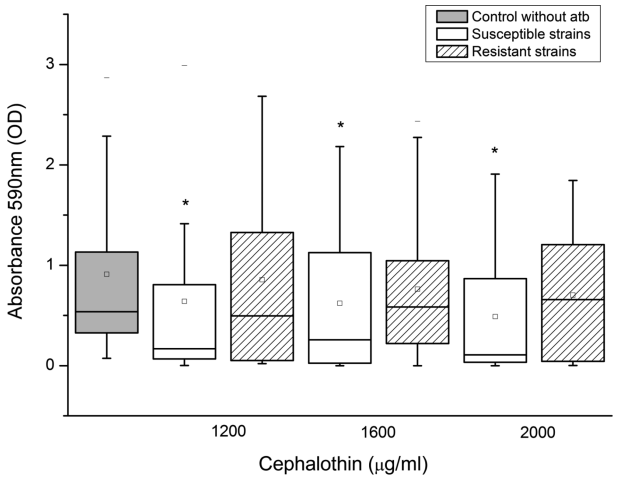

Figure 2. Biofilm quantification with different cephalothin concentrations. Boxplot comparing the effect of cephalothin at 0, 1200, 1600 and 2000 μg/ml concentrations on susceptible strains and resistant strains groups. Asterisk indicates a significant reduction of the biofilm biomass versus group without antibiotics.

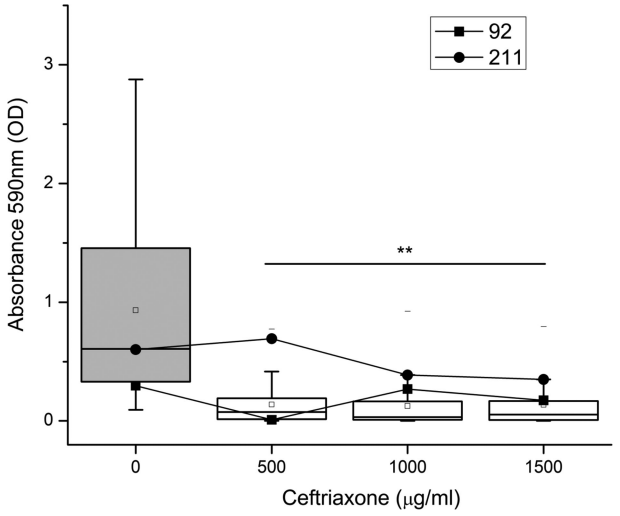

Figure 3. Biofilm quantification with different ceftriaxone concentrations. Boxplot comparing the effect of ceftriaxone at 0, 500, 1000 and 1500 μg/ml concentrations on all strains and with dots and lines the resistant strains (92 and 211). Double asterisks indicate a significant reduction of the biofilm biomass versus group without antibiotics.

bla-CTX-M-2 and bla-CTX-M-9 evaluated by PCR. When biofilm biomass was quantified in these two resistant strains, reduction on biofilm was observed at all concentrations of ceftazidime, being significant only at higher concentrations (1000 and 1500 μg/ml). In the case of ceftriaxone, a reduction was observed in biofilm formation values although they were not significant (Table 5, Figs 3 and 4).

Amikacin exerted a significant reduction of biofilm biomass in susceptible strains when tested at 500, 1000 and 2000 μg/ml (Table 5). Interestingly, in 13 isolates (27%) that were resistant to amikacin, all strains had an important reduction on biofilm, with statistical significance compared with growth without antibiotic (Table 5, Fig. 5).

Ciprofloxacin produced a significant reduction in the biofilm biomass when tested at 2, 3 and 5 μg/ml, with statistical significance compared with the values without antibiotic ($P < 0.05$, Table 4). Three isolates (6.3%) were resistant to ciprofloxacin (MICs ≥ 4 μg/ml). Two of them showed biofilm reduction at all concentrations of antibiotics, but this biofilm reduction was not significant compared to the biofilm formed without antibiotic

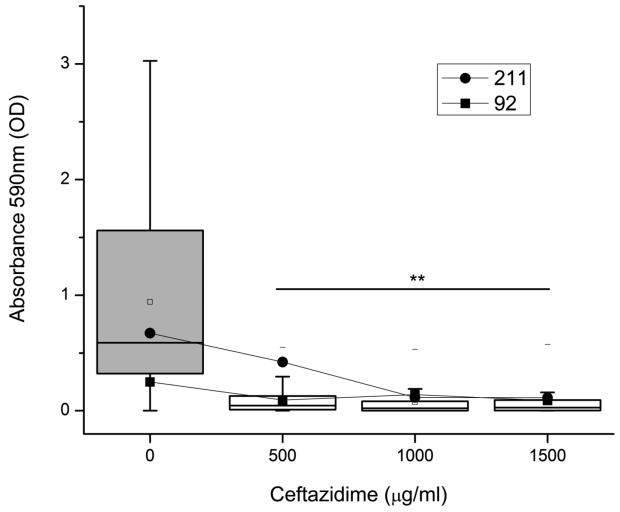

Figure 4. Biofilm quantification with different ceftazidime concentrations. Boxplot comparing the effect of ceftazidime at 0, 500, 1000 and 1500 μg/ml concentrations on all strains and with dots and lines the resistant strains (211 and 92). Double asterisks indicate a significant reduction of the biofilm biomass versus group without antibiotics.

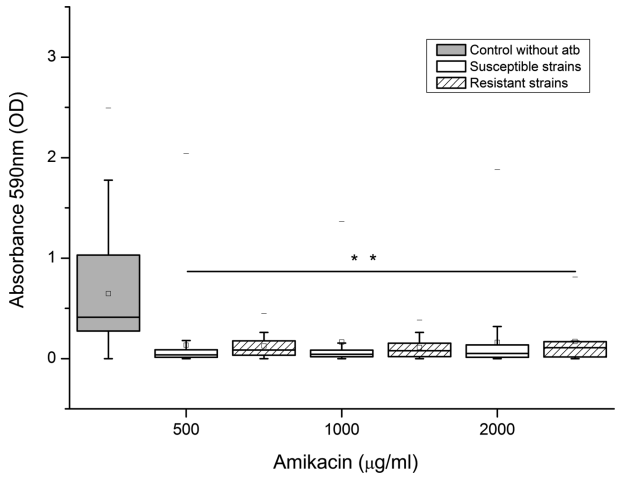

Figure 5. Biofilm quantification with different amikacin concentrations. Boxplot comparing the effect of amikacin at 0, 500, 1000 and 2000 μg/ml concentrations on susceptible strains group and resistant strains group. Double asterisks indicate a significant reduction of the biofilm biomass versus group without antibiotics.

addition. Biofilm biomass of the other strain did not vary when incubated in the presence or absence of ciprofloxacin (Fig. 6).

In summary, among the beta-lactams, ampicillin did not show any effect while cephalothin, ceftriaxone and ceftazidime exerted a significant reduction in the biofilm biomass. Moreover, aminoglycosides and quinolones also induced a significant reduction in the biofilm biomass.

Resistance genes expression in the ESBL producer strains

Quantitative RT-PCR was performed in order to determine the expression of CTX-M-9 and CTX-M-2 in *E. coli* biofilms under different conditions. The CTX-M gene expression was compared during biofilms with and without ceftriaxone supplementation, and in planktonic growth. As shown in Fig. 7, the expression of

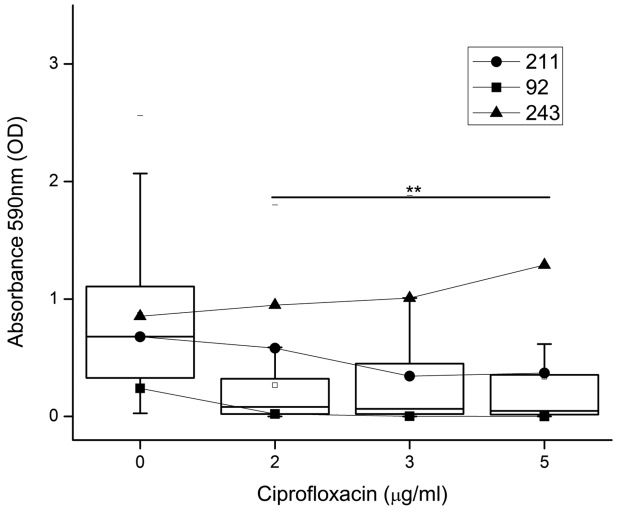

Figure 6. Biofilm quantification with different ciprofloxacin concentrations. Boxplot comparing the effect of ciprofloxacin at 0, 2, 3 and 5 μg/ml concentrations on all strains group and with dots and lines the resistant strains (211, 92 and 243). Asterisk indicates a significant reduction of the biofilm biomass versus group without antibiotics.

CTX-M-9 gene significantly increased in planktonic cells (5.68 ± 1.18) compared with biofilm (1.15 ± 0.42), biofilm with 500 μg/ml (0.28 ± 0.06) and 1500 μg/ml (0.29 ± 0.16) of ceftriaxone. The expression of CTX-M-2 showed a similar tendency that CTX-M-9 but without a statistical significance. The expression of CTX-M-2 was increased in planktonic cells (1.44 ± 0.59) compared with biofilm (1.03 ± 0.14), biofilm with 500 μg/ml (1.13 ± 0.26) and 1500 μg/ml (1.14 ± 0.26) of ceftriaxone.

DISCUSSION

UPEC is the main etiological agent associated with community-acquired UTI (Ronald 2003). One of the most interesting mechanisms described so far used by UPEC to infect the host is the formation of IBCs (Anderson *et al.* 2003) within the superficial bladder cells. These structures have been reported in urine from women with UTI (Garofalo *et al.* 2007; Rosen *et al.* 2007), children (Robino *et al.* 2013, 2014b) and in mouse models of infection (Anderson *et al.* 2004) and there is evidence that these structures count for recurrence (Robino *et al.* 2014b). These IBCs are transient and undergo several distinct morphological changes throughout their development (Justice *et al.* 2004) that mainly consist of three different stages: early, middle and late IBCs (Mulvey, Schilling and Hultgren 2001). The IBC maturation happens between 6 and 8 h after infection in the middle stage. Bacteria shorten and acquire a slow growth rate producing biofilm-like intracellular communities, which appear to be enclosed in an extracellular matrix (Anderson *et al.* 2003). It is in this context that we tested the ability of an entire clinical strain collection in its ability to form biofilms and its relationship with IBC formation. Interestingly, all the strains tested in the present work showed different biofilm abilities varying from strong to non-biofilm producers. Moreover, more than 50% of the strains were non-biofilm producer using the microtiter biofilm assay, and no correlation was found between biofilm formation and the presence or absence of IBC/IB in urine. It is worth to mention that IBC classification was based on the observation of human urine samples (Robino *et al.* 2014b), and this does not discard that a strain that was classified as none IBC/IB could have the capability to do it.

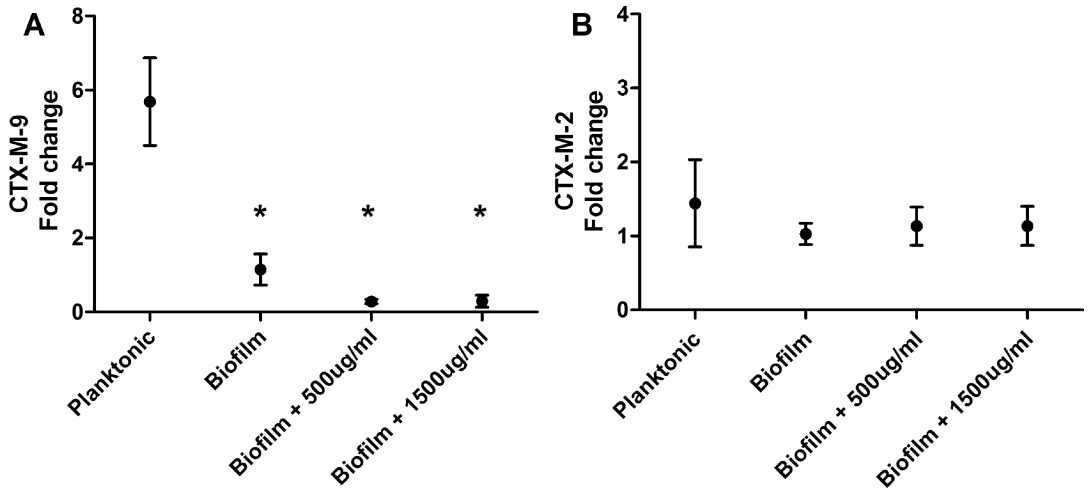

Figure 7. Real-time PCR to evaluate resistance gene expression. Expression of (**a**) CTX-M-9 gene and (**b**) CTX-M-2 gene assayed by real-time RT-PCR on planktonic bacteria, biofilm and biofilm with 500 and 1500 g/ml of ceftriaxone on strain 92 and 211, respectively (these strains are resistant to ceftriaxone). Differences in gene expression were calculated using the $2-\Delta\Delta Ct$ method normalized against expression of the rpoART housekeeping gene. Box plots show minimum, maximum and mean differences in gene expression from three independent replicates. An asterisk (*) indicates a significant difference between biofilm culture ($P < 0.05$ Student's t-test).

Another important mechanism in UTI pathogenesis is biofilm production. The genetic expression in bacteria-forming biofilm varies compared to their planktonic counterparts, and several works have demonstrated the importance of this change (Donlan and Costerton 2002). Biofilm formation is a complex process that comprises different stages, and different factors are involved on each one (Klausen *et al.* 2003). One of the key events in the success of biofilm development is the irreversible attachment to the surface where the synthesis of flagella is repressed. Adhesive organelles such as type 1 fimbriae and curli are important for the irreversible attachment to surfaces in *Escherichia coli* (Sharma *et al.* 2016). However, Hancock *et al.* (2010) demonstrated that genes involved in the biogenesis of type 1, P and F1C fimbriae are downregulated in CFT073 biofilms grown in human urine, indicating that these fimbriae are not critical for biofilm formation, at least during growth in urine.

In the present work, we were able to correlate the presence of *pap* genes and the capability to form in vitro biofilm. Pap genes ('pyelonephritis-associated pili') codify for P pili, which play a major role in pyelonephritis pathogenesis by adhesion to Gal(α1–4)Galβ motifs in the kidney (Lane and Mobley 2007). In another work, Naves *et al.* (2008) found that *papC*, different *papG* alleles, *sfa/foc* DE, *focG*, *hlyA* and *cnf1* were more prevalent in strong biofilm producer strains. Recently, a novel adhesin called UCA-like has also been associated with strong biofilm formation (Wurpel *et al.* 2016). Even though, we observed that the presence of the *fimH* fimbrial gene (type-1 fimbriae) in the UPEC strains evaluated was not able to correlate with biofilm formation under the conditions assayed. This may be explained because of the high prevalence of type-1 fimbriae genes in more than 90% of UPEC strains (Robino *et al.* 2014a). Also the expression of *fimH* is similar in the group of strains that form biofilm and the non-producer strains. The repertoire of adhesins, fimbrial structures and other non-fimbrial adhesins could determine the tropism of the pathogen to host tissues, bacterial–bacterial interactions, formation of IBC and also biofilms (Chahales and Thanassi 2015). The prevention of the first step in adhesion is an interesting strategy that could lead to the reduction of the impact of this type of infection.

Beta-lactam antibiotics act inhibiting the bacterial wall synthesis and include penicillins, aminopenicillins, cephalosporins and carbapenems. In Uruguay, 58% of *E. coli* isolated from children with UTI were resistant to aminopenicillins explained by the presence of broad-spectrum beta-lactamases (like TEM-1, SHV-1) (Robino *et al.* 2014a). In this study, 79% of the isolates were resistant to ampicillin. No reduction in biofilm biomass was observed when ampicillin was tested at different concentrations, even in the susceptible strains. In the susceptible group, the biofilm acts as an antibiotic-resistant mechanism. This may be explained by the scarce penetration of ampicillin in the biofilms as was demonstrated by Anderl, Franklin and Stewart (2000). Other factors that could explain the limited effect of ampicillin on biofilm may be nutrient limitations and the different physiological state of bacteria in the biofilm that becomes tolerant to the antibiotic (Anderl *et al.* 2003).

Cefuroxime and ceftriaxone are recommended for the empirical treatment of UTI in adults and children (Gupta *et al.* 2011). The main mechanism of resistance to these antibiotics present in *Enterobacteriaceae* strains is the production of ESBLs (Paterson and Bonomo 2005). In community-acquired UTI in children in Uruguay, *Enterobacteriace*-ESBL producers were detected in around 3% (Robino *et al.* 2014b). The two strains resistant to ceftriaxone and ceftazidime included in this study harbored CTX-M-2 and CTX-M-9 ESBL genes (data not shown).

Third-generation cephalosporins showed an important effect in reducing biofilm biomass, in the case of susceptible and resistant isolates.

We hypothesize that the biofilm reducing effect, even in the case of resistant strains, could be explained by two phenomena: the high concentrations of the antibiotics tested and the absence of expression of ESBL in the biofilm. The antibiotic concentrations tested in this study (determined by the concentration that the antibiotic achieves in urine at the regularly used doses) are considerably above the MIC established to consider an isolate resistant ($\geq$4 ug/ml for ceftriaxone and $\geq$16 ug/ml for ceftazidime) these high concentrations of antibiotic may saturate the ESBL enzymes turning the bacteria susceptible to the antibiotic explaining the reduction of biofilm biomass in the resistant strains (Lorian 2005; CLSI 2015). Another explanation could be the reduction of ESBL expression during biofilm formation. Most of the published studies found a correlation between ESBL genes' presence in *E. coli* and biofilm production, but these studies are based on the presence or on the absence of the genes but did not analyze if ESBL genes were expressed within the biofilm

(Neupane *et al.* 2016). When we evaluated the expression of CTX-M-9 in planktonic cells and in biofilm with and without antibiotic by real-time PCR, we observed that expression of CTX-M-2 and CTX-M-9 was two to four times higher in planktonic cells than in the biofilm. No significant difference was observed between the expression of ESBL under antibiotic stress or not.

In the case of aminoglycosides (amikacin), we detected an antibiofilm effect on the susceptible and on the resistant group of strains. This group of antibiotics inhibits protein synthesis acting on the bacterial ribosome, so this may explain an inhibitory effect on the biofilm synthesis. This inhibitory effect on biofilm was also demonstrated for other groups of antibiotics that act on the inhibition of the bacteria ribosome, such as chloramphenicol and tetracycline (Liaqat, Sumbal and Sabri 2009). Studies on Klebsiella *pneumoniae* biofilm growth mode and antibiotics effect reported that amikacin was able to eradicate the young biofilms but became ineffective when the biofilm age increased because of the enhanced production of exopolysaccharide (Singla, Harjai and Chhibber 2013). However, in our study, biofilm formation was evaluated after 48 h of incubation; considering that the irreversible phase of biofilm occurs after 24–48 h in other uropathogens (Schlapp *et al.* 2011), we can conclude that the biofilm was older or at least after the irreversible stage. In spite of the stage in biofilm maturity, we were able to observe a reduction in biomass when amikacin was tested.

Fluoroquinolones, like ciprofloxacin, inhibit DNA replication and are known to penetrate host cell membranes and accumulate intracellularly. In our study, ciprofloxacin showed a reduction in biofilm biomass in the susceptible and resistant strains. Blango *et al.* studied the effect of different antibiotics on *in vitro* biofilm, and their efficacy to eradicate IBC in a mouse model. They observed that ciprofloxacin and sparfloxacin had an inhibitory effect on biofilm persistence and promoted the reduction of pre-existing biofilm communities. However, none of these antibiotics could completely eradicate IB from bladder tissue in a mouse model (Blango and Mulvey 2010).

The empirical treatment of UTI should consider the local antibiotic sensitivity patterns of the microorganisms isolated with a high frequency. In this context, the present work contributes to the knowledge on antibiotic resistance, biofilm formation and its potential relationship with IBC in a collection of strains isolated from Uruguay.

In the case of recurrent or persistent UTI potentially associated with the presence of biofilm, as in catheterized patients, the use of third-generation cephalosporin, fluoroquinolones and aminoglycosides could be recommended. These antibiotics reduced biofilm biomass even in the case of resistant strains.

Another relevant aspect of UTI recurrence is the potential role of IBC or intracellular biofilms (Robino *et al.* 2014b). Most of the antibiotics used in the treatment of UTI are not able to enter inside the cell except fluoroquinolones so the efficacy of this strategy is under debate. New therapeutic options as chitosan (which induces urothelium exfoliation), forskolin (which increases the AMPc and IB expulsion) or mannosides (which inhibits FimH-mediated adhesion) associated with the antibiotic therapy should be evaluated (Mysorekar and Hultgren 2006; Sivick and Mobley 2010; Cusumano *et al.* 2011; Blango *et al.* 2014).

In conclusion, 41% of UPEC clinical isolates from children with UTI were able to produce biofilm, in which third-generation cephalosporins, fluoroquinolones and aminoglycosides showed to be effective for its reduction *in vitro*. Even though IBCs are described as a structure similar to biofilm, no correlation was found between IB and IBC in uroepithelial cells with the ability of the strains to produce extracellular biofilms.

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