

Differences in biofilm formation and aggregative adherence between β -lactam susceptible and β -lactamases producing *P. mirabilis* clinical isolates

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SUMMARY

Biofilm formation of multidrug resistant (MDR) extended-spectrum β -lactamase (ES β L) producing *Proteus mirabilis* isolates from a long-term care and rehabilitation facility (LTCRF) in Northern Italy was evaluated.

A total of 10 strains, 4/10 producing the acquired AmpC β -lactamase CMY-16, 3/10 producing the ES β L TEM-92 and the remaining negative for the presence of β -lactamase genes, were studied using standard adherence assays on titer plates. Tests were performed in three different media, including Luria Bertani (LB), LB diluted and urine. Three representative strains were also tested for biofilm production in microtiter in presence of β -lactam sub-MIC concentrations. The same isolates were screened for aggregative adherence onto monkey kidney cells (LLC-MK₂). All strains studied were capable of biofilm formation, though at different levels. The β -lactamase positive strains were statistically better significant in biofilm formation than negative ones regardless of growth medium. Cellular adherence assays showed a preferential ability of all isolates, regardless of β -lactamase production, to adhere to inert surfaces rather than to cells. Although the results did not fully support a direct correlation between β -lactamase production and biofilm formation, both mechanisms can greatly contribute to bacterial persistence in the urinary tract.

KEY WORDS: *Proteus mirabilis*, β -lactamases, Biofilm

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INTRODUCTION

Proteus mirabilis is a common causative organism of urinary tract infections (UTIs) in patients with functional or anatomic urinary tract abnormalities or with chronic instrumentation (Tolkoff-Rubin and Rubin, 1986). In these patients, UTIs are particularly difficult to treat and the bacterium can persist causing not only cystitis and acute pyelonephritis, but also the formation of bladder and kidney stones that can lead to the obstruction of urinary tract and catheters (Griffith *et al.*,

1976). *P. mirabilis* has evolved numerous virulence factors that are important for causing UTIs, including bacterial adhesion to the uroepithelium mediated by fimbriae (Coker *et al.*, 2000).

Many studies have suggested that mannose-resistant like fimbriae (MR/P) play a role in the *P. mirabilis* virulence observed during UTIs (Bahrani *et al.*, 1991). The fimbriae-mediated adhesion to tissue culture cells revealed their predisposition to adhere specifically to human renal tubular epithelial cells and to exfoliated cells of urinary sediment (Sareneva *et al.*, 1990; Cook *et al.*, 1995; Rocha *et al.*, 2007).

MR/P fimbriae dictate the localization of bacteria in the bladder and contribute to biofilm formation (Jansen *et al.*, 2003), a process essential for the establishment of catheter-associated urinary tract infections (CAUTIs).

P. mirabilis forms calcium and magnesium phosphate crystal aggregates, so bacteria are incor-

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porated into a crystalline biofilm (Morris *et al.*, 1999; Stickler *et al.*, 2006). This allows the persistence of *P. mirabilis* in the urinary tract by protecting the microorganism from antibiotics and the host's immune response, and contributes to surfaces adhesion (Hoyle *et al.*, 1992).

Bacteria growing as biofilm develop significant phenotypical, biochemical and morphological differences from their planktonic counterparts, including their extreme resistance to treatment with biocides and detergents and their high tolerance to prolonged antibiotic therapy in human infections (Stewart *et al.*, 2001).

The eradication of the microorganism appears particularly critical if *P. mirabilis* expresses additional resistance mechanisms, such as the production of hydrolytic enzymes. *P. mirabilis* is naturally susceptible to β -lactams, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (O'Hara *et al.*, 2000). However, drug resistance has been increasingly reported for this species, and the production of TEM-, CTX-M-, PER- types extended-spectrum β -lactamases (ES β Ls) and CMY/LAT class C β -lactamases (CBLs), is becoming of great concern also in Italian settings (Stürenburg *et al.*, 2003; Luzzaro *et al.*, 2009; Migliavacca *et al.*, 2007a; Migliavacca *et al.*, 2007b). Moreover, co-resistance to aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole has frequently been reported among ES β Ls-positive *P. mirabilis* strains (De Champs *et al.*, 2002; Winokur *et al.*, 2001; Luzzaro *et al.*, 2002).

The aim of this research was to investigate biofilm formation in 10 previously characterized *P. mirabilis* strains producing the CMY-16 CBL and the TEM-92 ES β L. All the strains studied were obtained from urine of patients hospitalized in a long term care rehabilitation facility (LTCRF) in Northern Italy. Correlations between biofilm production, β -lactamase presence and β -lactam susceptibility were also investigated using both inert surfaces and cell cultures.

MATERIALS AND METHODS

Bacterial strains

A total of 10 *P. mirabilis* strains, including 4 CMY-16 producers, 3 TEM-92 producers and 3 β -lactamase negative (BL negative) susceptible to β -

lactams and representative of clinical isolates responsible of UTIs was investigated.

Bacterial strains were collected from November 2004 through June 2005 from urinary samples of patients hospitalized at the "Santa Margherita" LTCRF of Pavia (Northern Italy).

The CMY-16 producing strains studied were named Pm 48 SMT, Pm 50 SMT, Pm 77 SMT and Pm 78 SMT, all clonally related by pulsed field gel electrophoresis (PFGE). This CMY-16 *P. mirabilis* clone was diffused also at Maugeri, Redaelli and Melegnano LTCRFs, as previously described (Migliavacca *et al.*, 2007b).

The TEM-92 producing strains studied were the following Pm 1 SMT, Pm 2 SMT and Pm 33 SMT, all belonging to the most prevalent clone A and detected in S. Margherita LTCRF together with at least three other different *P. mirabilis* TEM-92 clonal lineages, named B, C, D (Migliavacca *et al.*, 2007a). With the aim to evaluate possible differences in biofilm formation in comparison with CMY-16 and TEM-92 producers, Pm 5 SMT, Pm 20 SMT and Pm 23 SMT clinical isolates, previously screened as negative for the presence of β -lactamases were also used (Migliavacca *et al.*, 2007a, Migliavacca *et al.*, 2007b).

Biofilm formation assays

Biofilm formation in microtiter plates was performed as described by O' Toole (O'Toole and Kolter, 1998), with minimal modifications.

Briefly, bacterial cells were grown overnight in sterile 96-well polystyrene microtiter plates (NUNC, Naperville, IL) at 37°C in three different media: Luria Bertani medium (LB), LB diluted 1:4 (LB1/4) and urine. Bacterial growth in the liquid culture was determined by optical density at 600 nm (OD_{600nm}) and the liquid culture was removed. Microtiter plates were washed with 0.1M phosphate buffer (pH 7.0), and the biofilm cells attached to the microtiter plate wells were stained for 20 min with 1% crystal violet (CV) in ethanol, washed and dried. Crystal violet staining was visually assessed and the microtiter plates were scanned. For semi-quantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 70% ethanol. The absorbance at 600 nm (Abs 600 nm) of the resuspended CV was determined and normalized to the OD 600 nm of the corresponding grown cell density: this value was defined as "adhesion units".

Biofilm formation in presence of β -lactams

The three strains Pm 78 SMT, Pm 1 SMT and Pm 20 SMT chosen among a CMY-16-, a TEM-92-producer and an β -lactamases non producer were tested for biofilm formation in the presence of imipenem (IMI) and the piperacillin-tazobactam (TZP) association respectively.

For each isolate, the biofilm quantity in 2-fold serial dilutions of IMI and TZP was determined in microtiter plates using an initial bacteria inoculum of 10^5 CFU ml⁻¹.

All experiments were performed in triplicate at 37°C in urine and LB.

Statistical analysis

Two general linear models were fitted to estimate the amount of biofilm produced by *P. mirabilis*, to assess the statistical significance of the differences between different strains (CMY-16 and TEM-92 positive and β -lactamase negative strains) and to obtain the relevant confidence intervals. The response analysed was the natural logarithm of the biofilm level. The explanatory variables included growth medium in both models and respective strains or enzymatic variants. The confidence intervals have a coverage probability of 0.95. All computations were carried out using the statistical package R (R Development Core Team, 2008).

Congo Red assay

Congo Red dye, which binds to extracellular polysaccharides, was prepared (Römling *et al.*, 2000; Brombacher *et al.*, 2006). The constituents of the media were casaminoacids (1%), yeast extract (0,15%), bacto agar (2%), (Difco laboratories, Michigan, USA), Congo Red dye (0,004%) and Comassie brilliant blue (0,002%) (Sigma-Aldrich

Chemie, Steinheim, Germany). Congo Red dye was prepared as a concentrated watery solution and added to the media when the agar had cooled to 55°C. Plates of the medium were inoculated and incubated overnight at 37°C.

E. coli PHL 628 strain, a spontaneous curli-producing mutant, was used as a reference standard (Brombacher *et al.*, 2006). The experiments were performed in triplicate.

Assay for the evaluation of biofilm vitality after exposure to β -lactams

Biofilm formation in microtiter plates was determined in triplicate as previously described (O'Toole and Kolter, 1998) in LB and in urine with $\frac{1}{2}$ MIC concentration, with $\frac{1}{4}$ MIC concentration (on the bases of the MIC values previously determined in each medium and reported in Table 1) and without antibiotics. After overnight incubation each well was washed three times with phosphate buffered saline (PBS) and 100 μ l of supernatant was drawn. Bacterial serial dilutions (1:10) in water were plated on MacConkey agar and incubated overnight at 37°C. The colonies were then counted to calculate the CFU mL⁻¹.

Well surfaces were vigorously scraped with a sterile tip, and resuspended in a 100 μ l of sterile water. As performed for planktonic cells, serial dilutions were plated and incubated overnight and the colonies were counted to calculate the CFU mL⁻¹.

Adherence assays to LLC-MK₂ cells

P. mirabilis isolates were tested for adherence to monkey kidney cell cultures (LLC-MK₂) as described (Cravioto *et al.*, 1979). Semi-confluent cell monolayers in 24-well tissue culture plates (Corning Inc, Corning, NY, USA) containing a

TABLE 1 - TZP and IMI MICs in urine and LB of representative clinical isolates tested for biofilm formation.

Strain	MIC TZP urine	MIC TZP LB	MIC IMI urine	MIC IMI LB
PM 78 SMT CMY-16 producer	4 mg/L	2 mg/L	4 mg/L	4 mg/L
PM 1 SMT TEM-92 producer	0,5 mg/L	0,5 mg/L	4 mg/L	4 mg/L
PM 20 SMT BL negative	0,5 mg/L	0,5 mg/L	2 mg/L	2 mg/L

coverslip were incubated at 37°C with 40 μL of bacterial cultures in LB and 960 μL of Dulbecco's modified Eagle's medium (Euroclone, Italy). After 1 h and 4 h, cell monolayers were washed with PBS six times and stained with May-Grunwald-Giemsa (Sigma-Aldrich Chemie, Steinheim, Germany). The cover slips were air-dried, mounted on glass slides and examined in a light microscope with a 100 oil immersion lens.

To quantify the number of bacteria adherent to the epithelial cells and coverslips, no adherent bacteria were removed from the monolayers by washing with PBS and the cell monolayers were lysed with 400 μL (1%,v/v) of Triton X-100 solution (Sigma-Aldrich). After 10 min of incubation at room temperature, 1.6 mL of PBS were added to the wells and homogenized by pipetting several times. Serial dilutions (1: 10) in PBS were plated on MacConkey agar and incubated overnight; the colonies were then counted to calculate the CFU mL^{-1} (Rocha *et al.*, 2007). The same strains were also tested for biofilm forma-

tion in the presence of IMI and TZP. The assays were performed in triplicate.

Detection of MR/P fimbriae by PCR

The *P. mirabilis* isolates were screened for the presence of the genetic sequences of *mrpA*, major subunit of MR/P fimbriae, as described by Rocha *et al.* (2007).

RESULTS

The ability of *P. mirabilis* strains to form biofilm, measured as surface adhesion to polystyrene microtiter plates, was tested. The results suggest that biofilm formation is strongly affected by growth media: all strains, regardless of *bla* genes presence, resulted more proficient to produce biofilm in urine and in LB 1/4 than in LB medium (Figure 1). However, a variability in biofilm amount produced among CMY-16, TEM-92 and BL negative strains in each medium was detected.

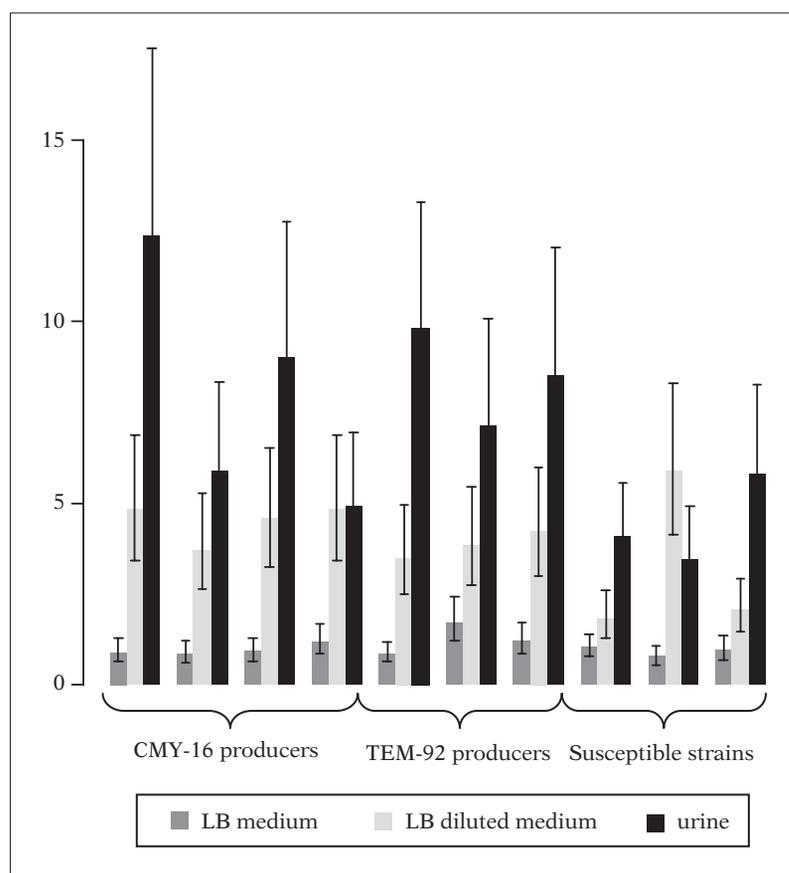


FIGURE 1 - Biofilm formation in the three different media. The confidence intervals, with a coverage probability of 0.95, are shown.

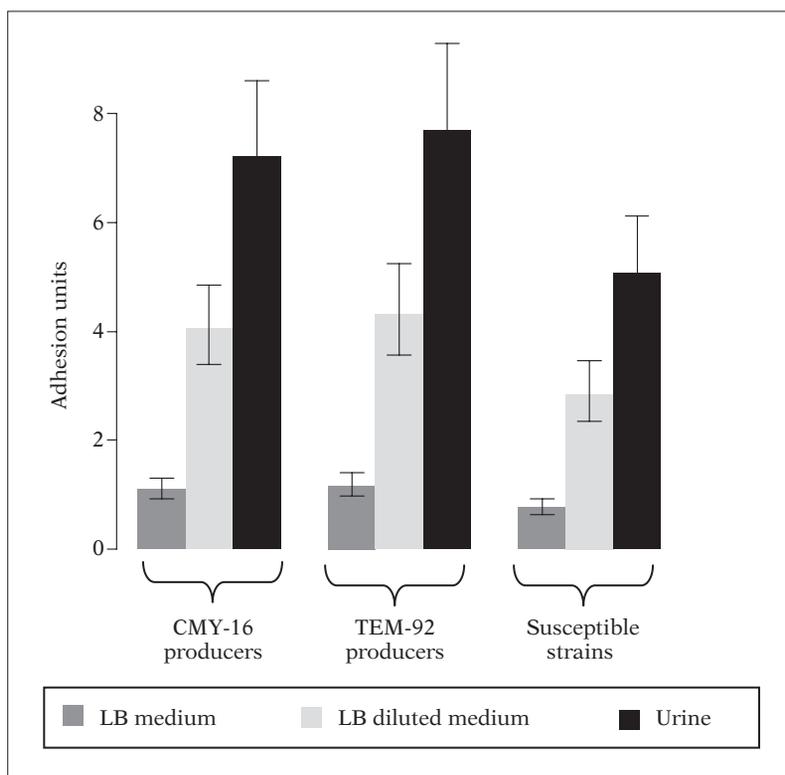


FIGURE 2 - Variability in biofilm amount produced by CMY-16, TEM-92, and susceptible strains. TEM-92 and CMY-16 strains produced 51% (p -value = 0.000157) and 42% (p -value = 0.000715) more biofilm than the susceptible ones respectively. The confidence intervals with a coverage probability of 0.95 are shown.

CMY-16 and TEM-92 ES β L producers were better at biofilm production than BL negative strains. TEM-92 and CMY-16 positive strains produced 51% (p -value = 0.000157) and 42% (p -value = 0.000715) more biofilm than the susceptible ones respectively (Figure 2).

Biofilm morphological differences in urine compared to the LB medium were observed.

In LB medium biofilm appeared as a uniform layer without any kind of aggregates. In urine the formation was less homogenous, due to the urease activity of *P. mirabilis* and it was characterized by aggregates of crystals and bacteria.

To verify if the ability to form biofilm was stimulated by sub-inhibitory antibiotic concentrations, an adhesion assay using both IMI and TZP was performed. MICs were determined both in urine and LB for each representative strain studied (Table 1).

Results show an enhanced biofilm formation in presence of increasing antibiotic concentrations, especially for sub-MIC concentrations of both IMI and TZP. All isolates tested, regardless of β -lactamase production, showed the same trend, with the largest increase at $\frac{1}{2}$ of the MIC of IMI and

TZP. Only in the presence of TZP and in urine, the TEM-92 producer showed the largest increase in biofilm formation at $\frac{1}{8}$ of MIC.

Finally, to verify the vitality of sessile cells after exposure to IMI and TZP, the same representative strains were tested.

In this experiment vital cells were counted after overnight incubation in urine at 37°C in the presence of sub-inhibitory concentrations of IMI and TZP. The same trend of vitality was observed for all the *P. mirabilis* strains tested: the sessile cells, exposed to subinibitory antibiotic concentrations, survived more than planktonic cells. In the presence of both drugs, planktonic cells roughly decreased numerically from $\frac{1}{4}$ MIC, regardless of the antibiotic employed, while the number of sessile cells remained constant or tended to increase with the increase in antibiotic concentrations.

P. mirabilis urinary isolates on plates supplemented with the Congo Red dye (CR plates) were analysed.

Only a TEM-92 producing isolate was dark red and similar to the *E. coli* curli-producing reference strain PHL 628. All other isolates displayed a white phenotype. The ability of uropathogenic

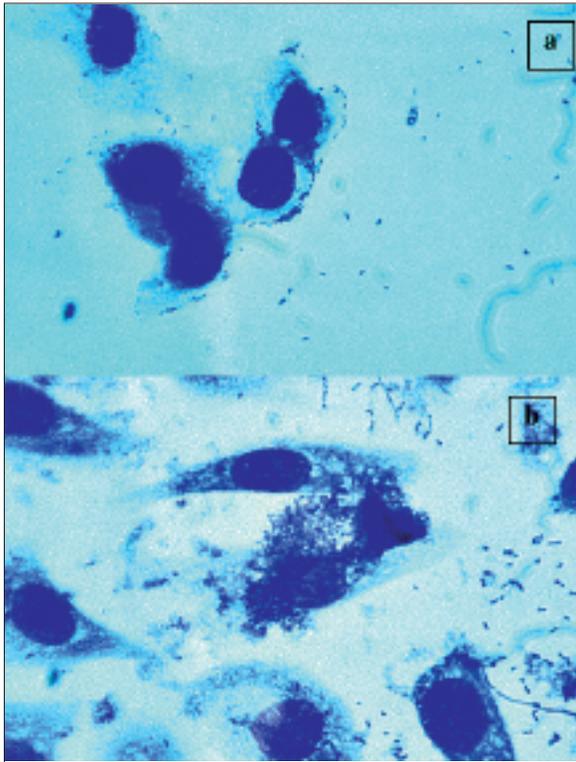


FIGURE 3 - *P. mirabilis* adherence to LLC-MK₂ cells after 1h (a) and 4h (b) of incubation.

P. mirabilis to adhere *in vitro* to epithelial cells was evaluated in LLC-MK₂ cells (Figure 3). After 1 hour of incubation (Figure 3a) a few bacteria forming aggregates in intercellular spaces and most bacteria adhered to the coverslips were observed. Instead, after 4 hours (Figure 3b) a great adherence in intercellular spaces was observed with only few bacteria on the cells, which were particularly arranged in chains along the border of the cytoplasm.

All clinical urinary isolates, including susceptible and β -lactamase producing strains, harboured *mmpA* gene coding for the major fimbrial unit of MR/P fimbriae, involved in the adhesion of *P. mirabilis*.

DISCUSSION

This study investigated the ability of β -lactamase producing *P. mirabilis* to form biofilm.

Ten urinary isolates, representative of strains responsible for UTIs and collected during 2004-

2005 from catheterized patients admitted to the "Santa Margherita" LTCRF in Pavia were analyzed.

All *P. mirabilis* isolates, regardless of β -lactamase production, showed a great capacity to form biofilm, although within a wide range. Therefore in urine it was more apparent compared to other media, in agreement with other authors (Jansen *et al.*, 2003). Morphological differences among biofilm grown in urine and in LB were found in all strains, confirming the crystalline nature of the *P. mirabilis* biofilms, as observed previously (Mobley and Warren, 1987, Morris *et al.*, 1997, Stickler *et al.*, 1998). This property makes *P. mirabilis* capable of forming encrustation and biofilms on patients' catheters (Kunin, 1989; Mobley and Warren, 1987; Ganderton *et al.*, 1992). This ability to adhere to plastic surfaces (Mobley and Belas, 1995) was also detected using LB and LB $\frac{1}{4}$ broth.

All isolates were more capable of biofilm production when grown in defined medium such as LB $\frac{1}{4}$ broth and urine, than in rich medium such as LB. These results are in accordance with previous studies on *E. coli* or other *Enterobacteriaceae* (Castonguay *et al.*, 2006).

Irrespectively of growth medium, β -lactamase producing *P. mirabilis* strains were more adept in forming biofilm than susceptible ones. These results are in apparent contrast with those obtained by Gallant *et al.* (2005) for *E. coli* and *P. aeruginosa* harbouring vectors, and suggest that both bacterial species and clinical isolates can produce biofilm in different ways.

Although adherence assay showed a variable biofilm production variability among CMY-16, TEM-92 and susceptible strains, CR assay did not show any correlation among strains belonging to the same variants (CMY-16 and TEM-92 producing and β -lactams susceptible strains). All strains showed the same light-pink phenotype on CR medium, except a TEM-92 producing strain which showed a dark red phenotype similar to the curli-producing *E. coli* PHL 628 reference strain. In this case, it could produce similar extracellular structures favouring adherence or structures constituted by the same polysaccharides. Interestingly, in these isolates harbouring *mmpA* gene, a heterogeneous phenotype emerged. This could be due both to a different gene expression in this medium or to a different

dye affinity towards the MR/P fimbriae. These results suggest that the susceptibility to antibiotic treatment of BL negative urinary isolates is not only due to the absence of hydrolytic enzymes, but also to their low tendency to form biofilm. The use of antibiotics is currently one of the possibilities for the prevention of biofilm formation. However, even in the presence of antibiotics, bacteria can adhere, colonize and survive on implanted medical devices, as described previously for urinary catheters and urethral stent surfaces. In conventional clinical microbiology the prime concern is for the first rate patient care. This depends on the choice of antibiotics, which are currently tested on bacterial cultures derived from planktonic cells greatly differing from bacteria in biofilm (Tenke *et al.*, 2006).

In this light, three isolates, each producer of a different β -lactamase, was tested for biofilm in the presence of two antibiotics commonly used to treat UTIs.

A biofilm enhancement in relation to antibiotic concentration increase was observed for all strains regardless of the presence of β -lactamase. Non-optimal growth conditions or even cellular stress, such as antibiotic sub-MIC concentration exposure, promoted *P. mirabilis* biofilm formation, as previously described for other species (Bagge *et al.*, 2004; Hoffman *et al.*, 2005). In addition, the biofilm vitality results obtained in this study after exposure to IMI and TZP, confirmed that the sessile cells were resistant to both β -lactams tested as described by Mah *et al.* (2001).

Biofilm increase in the presence of sub-MIC concentrations was similar for all three strains tested and also independent of β -lactamase productions. Likewise, adherence to LLC-MK₂ assay showed no differences among β -lactamase producers and susceptible strains. *P. mirabilis* ability to adhere to inert surfaces rather than cells was evident in this study especially after one hour of incubation at 37°C.

This is in contrast with *P. mirabilis* ability to adhere to epithelial cells previously described (Sareneva *et al.*, 1990; Rocha *et al.*, 2007). Nevertheless these studies used different cellular lines (kidney sections etc.) or different cultural conditions than those used in the present work. Even if a direct correlation between biofilm formation and β -lactamase production was not demonstrated, the results obtained suggest that the

synergy between these two mechanisms can contribute to persistence and chronicization of urinary tract infections.

In conclusion, this study emphasizes the importance of evaluating biofilm impact in catheter-associated urinary tract infection, in addition to the classical resistance determinants.

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