**In vitro** activity of ciprofloxacin, moxifloxacin, vancomycin and erythromycin against planktonic and biofilm forms of *Corynebacterium urealyticum*

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Received 14 August 2008; returned 31 October 2008; revised 5 November 2008; accepted 5 November 2008

**Objectives:** To study the ability of *Corynebacterium urealyticum* to produce biofilms and to compare the **in vitro** activity of antimicrobials against planktonic and biofilm-associated organisms.

**Methods:** Biofilm formation on polystyrene plates by three *C. urealyticum* strains was studied in artificial urine under static conditions. The bactericidal activities of ciprofloxacin, moxifloxacin, vancomycin and erythromycin were studied against biofilm-associated organisms, and the results were compared with those obtained against planktonic organisms. Persister biofilm-associated organisms of each strain exposed to antibiotics were retested to determine the MIC of the same antibiotic.

**Results:** The three strains tested consistently produced biofilms. Planktonic organisms was susceptible to ciprofloxacin, moxifloxacin and vancomycin, and their MBC values were two to eight times higher than their corresponding MICs. Bactericidal effect on biofilm-associated organisms required very high antibiotic concentrations; the minimum biofilm bactericidal concentrations for ciprofloxacin, moxifloxacin and vancomycin ranged from 128 to >1024 times their respective MBCs for planktonic organisms. Erythromycin was not bactericidal against either planktonic or biofilm-associated organisms for the single susceptible strain tested. Persister biofilm-associated organisms exposed to erythromycin increased their MIC by a factor >8000, but no changes in susceptibility were observed with the other compounds.

**Conclusions:** This work demonstrates that *C. urealyticum* produces biofilms on polystyrene plates and biofilm-associated organisms are much less susceptible to the bactericidal effect of the antibiotics; and the exposure of *C. urealyticum* to erythromycin may favour resistance selection. Overall, these results may explain the difficulties for bacterial eradication in chronic infections caused by *C. urealyticum*.

Keywords: bactericidal, eradication, resistance, device-related infections, polystyrene

**Introduction**

*Corynebacterium urealyticum* is a slow-growing lipophilic, asaccharolytic and urease-positive organism mainly involved in urinary tract infections (UTIs).1,2 Most infected patients suffer from urologic diseases, have undergone urological manipulations, have a history of previous UTI, have been hospitalized for long periods or have received broad-spectrum antibiotics.2 Prolonged vesical or renal catheterization seems to be the most important risk factor for the development of chronic and more complicated infections.2 It has been previously shown that *C. urealyticum* adheres to abiotic material, such as urinary catheters,3 and some reports suggest that this organism may produce biofilms both **in vitro** and **in vivo**.4–6 It is now accepted that most bacteria grow in biofilms on a wide variety of surfaces rather than free floating or planktonically.7 Organisms in biofilms grow as colonies encased in extracellular matrices of carbohydrates or exopolysaccharides8–10 and *C. urealyticum* is even viable within struvite stones.4,6 Organisms that grow forming aggregates alter their physiology and take advantage of deficiencies in the host clearance mechanisms. Numerous studies have demonstrated that biofilm-grown organisms have an
having a pore size of 0.45 μm. Cial urine was sterilized with a Millipore membrane sterilization unit.

Microorganisms and antimicrobials

Three clinical isolates of *C. urealyticum* isolated in our laboratory and deposited at the ATCC, numbers 43042\(^{2}\), 43043 and 43044, were used.

Ciprofloxacin and moxifloxacin were gifts from Bayer AG (Leverkusen, Germany) and vancomycin and erythromycin were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Planktonic antimicrobial susceptibility testing

MICs were determined by the broth microdilution method following the CLSI recommendations.\(^{19}\) The MBC was read as the lowest antibiotic concentration to kill 99.9% of the initial inoculum.\(^{20}\) Inocula for determining the MIC and MBC values were prepared by direct colony suspension. All determinations were made in triplicate.

Biofilm formation assay

Biofilm formation assays were performed in artificial urine following a previously described method\(^{21}\) with few modifications. The artificial urine was prepared according to a formula previously described\(^{22}\) with minor modifications and contained the following compounds (in g/L): CaCl\(_2\), 0.49; MgCl\(_2\), 0.65; NaCl, 4.6; Na\(_2\)SO\(_4\), 2.3; sodium citrate, 0.65; sodium oxalate, 0.02; KH\(_2\)PO\(_4\), 2.8; KCl, 1.6; NH\(_4\)Cl, 1.0; urea, 25; and trypcase soy broth (bioMérieux, Marcy-l’Étoile, France), 1.0. pH was adjusted to 6.2, and the artificial urine was sterilized with a Millipore membrane sterilization unit having a pore size of 0.45 μm.

All strains were grown in 5 mL tubes with trypcase soy broth (bioMérieux) enriched with 0.1% Tween 80 (Sigma Chemical Co.) at 35.5°C in air for 48 h. Aliquots of 1.3 μL from this culture were inoculated in 130 μL of artificial urine (~10\(^8\) cfu/mL) in 96-well flat-bottomed non-cell-treated polystyrene microtitre plates (Greiner Bio-one, Stuttgart, Germany). Control wells containing only 130 μL of artificial urine without bacteria were included to measure, for instance, potential changes of optical density (OD) associated with precipitation of salts.

These plates were incubated without shaking at 35.5°C for 72 h, and next the medium was removed and the wells were rinsed once with 150 μL of sterile saline to remove planktonic bacteria. After the wells were dried at 35.5°C for 25 min, 130 μL per well of a 1% Crystal Violet (CV) solution (Panreac, Barcelona, Spain) was added for 10 min. Following the staining step, the colorant was discarded and the wells were rinsed four times with 180 μL of distilled water to remove excess stain. Then, the plates were dried at 35.5°C for 1 h. The dye incorporated by the biofilms or present in the control wells was solubilized in 130 μL of absolute ethanol. The ODs of the stained biofilm and control wells were read at λ = 570 nm, as previously described.\(^{21}\) The assays were performed in triplicate, that is, a total of 72 wells per each organism and 24 control wells were tested.

Biofilm antimicrobial susceptibility testing

For the measurement of *in vitro* antimicrobial susceptibility of biofilms, the biofilm was prepared for each strain as described earlier. After the medium was removed, the biofilm was exposed to ciprofloxacin, moxifloxacin, vancomycin and erythromycin at concentrations from 0.03 up to 1024 mg/L in cation-adjusted Mueller–Hinton broth with 4% lysed horse blood. Colony counting was performed in three control wells to determine the initial bacterial inoculum. After 24 h of incubation at 35.5°C, plates were sonicated at 35 kHz (Bandelin Sonorex TK2, Schaltec GMBH, Morfelden-Walldorf, Germany) for 5 min. Material removed was diluted and plated on blood agar for colony counting. The minimum drug concentration that reduced the initial inoculum over 99.9% was defined as the MBBC.\(^{18}\)

In addition, persister biofilm-associated cells of each strain were retested after exposure to antibiotics, in parallel with the non-exposed ones, to determine the MIC of the same antibiotic using the Etest method (AB Biodisk, Solna, Sweden) following the manufacturer’s recommendations.

Statistical analysis

The significance of differences between means was analysed by the double-sided Student’s *t*-test and the Mann–Whitney test (*P* < 0.05).

Results

Biofilm formation

Figure 1 shows the quantification (mean ± SD OD\(_{570}\)) of CV staining of biofilms formed by the three *C. urealyticum* strains...
**Corynebacterium urealyticum** biofilm and antibiotic activity

Table 1. Antimicrobial activity of four antibiotics against three *C. urealyticum* strains as a planktonic population (MIC and MBC, in mg/L) and as a biofilm population (MBBC, in mg/L)

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Strain</th>
<th>MIC</th>
<th>MBC</th>
<th>MBBC</th>
<th>MBBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>ATCC 43042&lt;sup&gt;T&lt;/sup&gt;</td>
<td>0.25</td>
<td>0.5</td>
<td>1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td></td>
<td>ATCC 43043</td>
<td>0.12</td>
<td>0.5</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>ATCC 43044</td>
<td>1</td>
<td>8</td>
<td>&gt;1024</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>ATCC 43042&lt;sup&gt;T&lt;/sup&gt;</td>
<td>0.12</td>
<td>0.5</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>ATCC 43043</td>
<td>0.06</td>
<td>0.25</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>ATCC 43044</td>
<td>0.5</td>
<td>4</td>
<td>512</td>
<td>128</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>ATCC 43042&lt;sup&gt;T&lt;/sup&gt;</td>
<td>0.5</td>
<td>1</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>ATCC 43043</td>
<td>0.5</td>
<td>1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td></td>
<td>ATCC 43044</td>
<td>0.5</td>
<td>1</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>ATCC 43042&lt;sup&gt;T&lt;/sup&gt;</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>NT</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>ATCC 43043</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>NT</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>ATCC 43044</td>
<td>≤0.03</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>ND</td>
</tr>
</tbody>
</table>

NT, not tested as the strain was erythromycin-resistant; ND, not determined.

tested compared with that of control wells (top panel), and the burden of bacterial concentration in the biofilm (bottom panel). The rate defined by OD<sub>570</sub> of the biofilm versus that of control wells (OD<sub>570</sub> biofilm/OD<sub>570</sub> control) was >4.0 for all strains, and such rates indicate biofilm formation by the three *C. urealyticum* strains. No significant differences in the extent of biofilm or bacterial burden were observed among the three strains studied.

**Antimicrobial susceptibility of planktonic and biofilm-associated *C. urealyticum* strains**

The final inoculum used for determining MIC and MBC for planktonic cells was 6.27 log<sub>10</sub> cfu/mL for strains ATCC 43042<sup>T</sup>, ATCC 43043 and ATCC 43044, respectively. The MBBC was determined using a final inoculum of 6.43 ± 0.47, 6.22 ± 0.39 and 6.17 ± 0.27 log<sub>10</sub> cfu/mL for strains ATCC 43042<sup>T</sup>, ATCC 43043 and ATCC 43044, respectively. Table 1 summarizes the antimicrobial susceptibility of planktonic and biofilm-associated cells. All planktonic strains were susceptible to ciprofloxacin, moxifloxacin and vancomycin and MBC values were within two to eight times higher than their corresponding MICs. Only one strain was susceptible to erythromycin but this antibiotic was not bactericidal, even at a concentration >34 000 times its MIC for planktonic cells.

To obtain a bactericidal effect on biofilm-associated cells, ciprofloxacin, moxifloxacin and vancomycin were retested at very high concentrations with MBBC values ranging from 128 to >1024 times their corresponding MBCs for planktonic cells. Erythromycin was not bactericidal against either planktonic or biofilm-associated cells for the only susceptible strain (ATCC 43044).

Persistor biofilm-associated cells of each strain exposed to ciprofloxacin, moxifloxacin and vancomycin were retested against each antibiotic by the Etest method, and the MIC values did not change. However, colonies of strain ATCC 43044 selected at random from biofilm-associated cells that were exposed to 128 mg/L erythromycin changed their MIC from the initial value of ≤0.03 up to >256 mg/L.

**Discussion**

Although the ability of *C. urealyticum* to adhere to some medical devices is known, its capacity to form biofilms on abiotic material has not yet been reported. To our knowledge, this is the first report showing the ability of *C. urealyticum* to form biofilms on abiotic material, a phenomenon that may have medical relevance because many UTIs caused by this organism are catheter-associated infections in both bladder and kidneys.2

As *C. urealyticum* is usually a multidrug-resistant organism,23,24 few antimicrobials are available for treatment. Among the indicated antibiotics, we chose to test two fluoroquinolones, vancomycin and erythromycin; four agents that have been successfully used in some UTIs.2 These antimicrobials, except erythromycin, were active *in vitro* against planktonic cells of the strains tested and bactericidal activity was easily achieved by concentrations slightly above their corresponding MIC values. However, application of MICs and MBCs for choosing therapeutic regimens in chronic or device-related infections involving bacterial biofilms is often ineffective.2 In fact, our results show that the bactericidal activity of the tested antibiotics was severely compromised in biofilm-associated organisms, as MBBC values exceeded the planktonic MBC values by a factor ranging from 128 to >1024. Such concentrations are not easily achieved in urine after therapeutic doses of any of the tested compounds. On the other hand, two out of the three strains tested were erythromycin-resistant and the susceptible strain, both in planktonic and biofilm-associated forms, was not killed by drug concentrations >34 000 times the MIC. The difference in antibiotic susceptibility between planktonic and biofilm-associated...
organisms may result from changes in the microbial physiology and/or differences in the diffusion of antibiotics.\textsuperscript{7,14,25,26}

The lesser activity of antibiotics against biofilm-grown organisms can also be complicated by selection of antibiotic-resistant mutants. We have detected that persister biofilm-associated organisms easily developed resistance to erythromycin, as the MIC increased to more than 8000 times its initial MIC value. This finding may explain the \textit{in vivo} development of erythromycin resistance during the treatment of some UTIs,\textsuperscript{5,17} a phenomenon also observed with other drugs, such as fluoroquinolones,\textsuperscript{7,16,17} doxycycline\textsuperscript{5} and rifampicin.\textsuperscript{5} However, in this study, we did not observe development of fluoroquinolone resistance.

This work demonstrates the ability of \textit{C. urealyticum} to form biofilms on abiotic material and that in order to kill such organisms it is necessary to use very high concentrations of \textit{in vitro} active antibiotics. Of the antibiotics tested, moxifloxacin appears to be one of the most effective drugs to kill biofilm-associated organisms, but after standard therapy the achieved concentrations of this fluoroquinolone are insufficient to eradicate such biofilm-forming bacteria. In addition, many isolates are fluoroquinolone-resistant,\textsuperscript{24} and the risk of selection of antibiotic-resistant mutants is high. All of these factors may explain the difficulties for bacterial eradication in chronic and device-related infections caused by \textit{C. urealyticum}.

\section*{Funding}

This study was supported by a grant from the Fondo de Investigaciones Sanitarias (PI060059), Ministerio de Sanidad y Consumo, Madrid, Spain.

P. N. was aided with a grant from the AlBAn programme for Latin America (European Union, scholarship no. E05D055472BR). V. R.-C. and G. d. P. received post-doctoral and pre-doctoral research contracts from the Comunidad Autónoma de Madrid (COMBACT s-bio-0260/2006 and CPI/0305/2007).

\section*{Transparency declarations}

None to declare.

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