Effect of the combination of clarithromycin and amikacin on *Pseudomonas aeruginosa* biofilm in an animal model of ureteral stent infection

Oscar Cirioni1*, Roberto Ghiselli2, Carmela Silvestri1, Daniele Minardi3, Eleonora Gabrielli1, Fiorenza Orlando4, Massimiliano Rimini2, Lucia Brescini1, Giovanni Muzzonigro3, Mario Guerrieri2 and Andrea Giacometti1

1Clinic of Infectious Diseases, Università Politecnica delle Marche—Ospedali Riuniti, Ancona, Italy; 2General Surgery and Surgery Methodology Clinic, Università Politecnica delle Marche—Ospedali Riuniti, Ancona, Italy; 3Institute of Maternal and Children’s Sciences-Urology, Polytechnic University of the Marche Region, A.O. Ospedali Riuniti, Ancona, Italy; 4Experimental Animal Models for Aging Units, Research Department, I.N.R.C.A. I.R.R.C.S., Ancona, Italy

*Corresponding author. Tel: +39-071-5963715; Fax: +39-071-5963468; E-mail: anconacmi@interfree.it or o.cirioni@univpm.it

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**Objectives:** An experimental study was performed to evaluate both *in vitro* and *in vivo* the efficacy of clarithromycin coating combined with systemic amikacin in preventing ureteral stent biofilm infection due to *Pseudomonas aeruginosa*.

**Methods:** The activities of the two antibiotics were studied *in vitro* in the absence or in the presence of biofilm. For the *in vivo* study we evaluated a control group without bacterial challenge to evaluate the sterility of the surgical procedure, a challenged control group that did not receive any antibiotic prophylaxis and three challenged groups that received (i) 15 mg/kg intraperitoneal amikacin immediately after stent implantation, (ii) clarithromycin-coated ureteral stents where 0.2 cm² sterile ureteral stents were incubated in 10 mg/L clarithromycin solution for 30 min immediately before implantation, and (iii) intraperitoneal amikacin plus a clarithromycin-coated ureteral stent at the above concentrations.

**Results:** The *in vitro* studies showed that the biofilm was strongly affected by the presence of clarithromycin and, in its presence, amikacin had MICs and MBCs lower than those obtained in the absence of clarithromycin. For the singly treated groups, intraperitoneal amikacin showed the strongest effect on bacterial numbers. A clarithromycin coating combined with systemic amikacin showed an efficacy that was higher than that of each single compound.

**Conclusions:** The prevention of ureteral stent *Pseudomonas* biofilm infection was enhanced by impregnation of the stent with clarithromycin combined with systemic amikacin.

**Keywords:** antibiotics, medical devices, bacterial biofilm, quorum sensing

**Introduction**

The presence of catheters in the urinary tract provide an ideal condition for biofilm development, inducing complications.1–4 Biofilm is an irreversibly encapsulated structured community of microorganisms within a self-developed polymeric matrix that is able to adhere to various biotic and abiotic surfaces.5–7 Biofilm is formed by a mixed population of rapidly and slow- or non-growing bacteria. These different physiological states may give protection, as slow-growing or stationary-phase cells have more tolerance to antibiotics.8–11 *Pseudomonas aeruginosa* is an opportunistic human pathogen that can colonize these surfaces and represents an important source of nosocomial infections.12,13 It is a species with natural resistance to many antimicrobials, including macrolides. This organism presents two forms: a planktonic form that is motile with a single polar flagellum, and a sessile form through which bacteria attach to abiotic surfaces or organic substances, leading to biofilm formation.14–16 It is also equipped with type IV pili, which confer twitching motility once bound to smooth surfaces. These pili allow single bacterial cells to attach to each other and contribute to the generation of biofilm. Biofilm formation is also controlled by a system of bacterial intercommunication, known as quorum sensing (QS). It is crucial in determining the density of the bacterial population, and it increases locally as more bacteria attach.16,17

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The importance of QS in the pathogenesis of *P. aeruginosa* infections is under study.\(^9,14,18\) The underlying mechanism of QS is the production of diffusible chemical signal molecules by the bacteria (autoinducers) that interact with specific receptors on self and neighbouring cells, which in turn regulate expression of specific target genes. By integrating this with other environmental signals and stimuli, bacteria are capable of exhibiting complex responses and take part in sophisticated interactions.\(^14,19\) The acyl homoserine lactone (AHL)-based cell-to-cell signals (autoinducers) 3-oxo-C\(_2\)-homoserine lactone (HSL) and C\(_2\)-HSL participate in a regulatory network that controls the production of several extracellular virulence factors and are also important for biofilm formation. AHLs have been detected on urethral catheters and in vitro using a *P. aeruginosa* biofilm model, as well as in lung secretions of patients with cystic fibrosis.\(^20\) The impact of *Pseudomonas*-related infection on morbidity and mortality remains high because of the limited therapeutic options available due to intrinsic and acquired resistance to antimicrobial agents. Macrolides are normally used against Gram-positive bacteria, whereas many Gram-negative bacilli are intrinsically resistant. Their mechanism of action is multiple and may include an anti-inflammatory effect, inhibition of a key enzyme in the alginate synthesis pathway, modulation of the production of QS bacterial virulence factors and inhibition of protein synthesis after prolonged exposure.\(^14\) Many antimicrobial agents were evaluated in several in vitro and in vivo biofilm models.\(^13,15,16\) The potential clinical value of antimicrobial agents that control and prevent *P. aeruginosa* infections by interfering with cell-to-cell signalling has recently been underlined. Interruption of QS and inhibition of the transcription of biofilm-controlling genes or genes involved in cell attachment might also prove to be a successful strategy in inhibiting biofilm infections by interfering with various stages of biofilm maturation. The purpose of the current study was to evaluate the efficacy of clarithromycin coating combined with systemic amikacin in an animal model of ureteral stent infection due to a slime-producing clinical isolate of *P. aeruginosa*.

**Materials and methods**

**Organisms**

A clinical isolate of *P. aeruginosa* (AN207), a slime producer, was used in this study. Moreover, another five *P. aeruginosa* clinical strains isolated from patients hospitalized in our department were used for interaction studies.

**Antimicrobial agents**

Preparation of sterile stock solutions of clarithromycin (Abbott Italia, Aprilia, LT, Italy) and amikacin (Sigma-Aldrich, Milan, Italy) was performed according to the manufacturers’ instructions. The antibiotic dosages used in the in vivo experiments corresponded to amikacin and clarithromycin plasma concentrations achievable in humans 4 – 6 h after a 500 mg dose administered every 12 h.\(^24,25\)

**Adherent biofilm formation for susceptibility testing**

To develop biofilms, 50 \(\mu\)L of tryptic soy broth (TSB) (Oxoid S.p.A., Milan, Italy) containing 10\(^6\) cfu/mL of bacteria were added under aseptic conditions to each well of a tissue-culture-treated polystyrene 96-well plate (Becton-Dickinson) containing 150 \(\mu\)L of TSB/2% glucose. After 24 h of incubation at 37°C, the growth medium was discarded and each well was washed three times with PBS under aseptic conditions by agitation at 180 rpm for 1 min to remove non-adherent cells. To evaluate the formation of adherent biofilm, the remaining attached bacteria were fixed with 0.2 mL of 99% methanol per well, and after 15 min plates were emptied and left to dry. Plates were then stained for 5 min with 0.2 mL of 2% Crystal Violet (used for Gram staining) per well. Excess stain was rinsed off by placing the plate under running tap water.\(^26,27\) The plates were air dried and the dye bound to the adherent cells was resolubilized with 0.2 mL of 33% volume-to-volume (v/v) glacial acetic acid per well. The optical density (OD) of each well was determined photometrically at 570 nm by using the MR 700 Microplate Reader (Dyna-tech Laboratories, Guernsey, UK). The 0.00 value (negative control) was determined for every plate by measuring the OD of a well filled with PBS solution. The cut-off OD for the microtitre-plate test was defined as three standard deviations above the mean OD of the negative control. The same experiment was performed three times with and without the addition of 10 \(\mu\)g of clarithromycin in Mueller–Hinton (MH) broth in each well. Biofilms were also observed by light microscopy. Tests were performed in triplicate.

**Susceptibility testing with adherent cells**

The MIC and MBC were determined with modifications for use in the biofilm test. Biofilms were washed with PBS in order to discard unbound bacteria. Subsequently, serial 2-fold dilutions of antibiotic in MH broth were added to wells containing adherent organisms. The polystyrene plates were incubated for 18 h at 37°C in air. The MIC was taken as the lowest amikacin concentration at which observable growth was inhibited. To determine the MBC, the MH broth containing amikacin was removed from each well and replaced with antibiotic-free MH broth; the plates were incubated again for 18 h at 37°C in air. The MBC was taken as the lowest concentration of amikacin that resulted in no bacterial growth following removal of the drug.\(^26,27\) In addition, to investigate the effect of clarithromycin pre-treatment on bacterial susceptibility to amikacin, the MIC and MBC of amikacin were again determined after pre-treatment of cells by incubation for 30 min at room temperature in 10 mg/mL clarithromycin solution immediately before susceptibility testing.

**Susceptibility testing with planktonic bacteria**

MICs and MBCs were determined according to the procedures outlined by the CLSI.\(^18\) Experiments were performed in triplicate.

**Synergy studies**

In interaction studies, six clinical isolate strains were used to test the antibiotic combinations by a chequerboard titration method by using 96-well polypropylene microtitre plates. The fractional inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the equation FIC index = FIC\(_A\) + FIC\(_B\) = (MIC\(_A\)/MIC\(_A\)\(_\text{c}\)) + (MIC\(_B\)/MIC\(_B\)\(_\text{c}\)) where A and B are the MICs of drug A and drug B in the combination, MIC\(_A\) and MIC\(_B\) are the MICs of drug A and drug B alone, and FIC\(_A\) and FIC\(_B\) are the FICs of drug A and drug B. The FIC indexes were interpreted as follows: \(>0.5\), synergy; 0.5 - 4.0, indifferent; and \(>4.0\), antagonism.\(^29\)

**Animal model**

Adult female Wistar rats (weight range 190 – 240 g) \((n=5)\) were used. The study included a control group \((C_0)\) without bacterial challenge to evaluate the sterility of the surgical procedure, a challenged control group \((C_1)\) that did not receive any antibiotic prophylaxis, and three challenged groups that received amikacin 15 mg/kg intra-peritonally, immediately after stent implantation; clarithromycin-coated ureteral
stents, where 0.2 cm² sterile ureteral stents (Biosoft®Duo, Porges-Mentor, France) were incubated in 10 mg/L clarithromycin solution for 30 min immediately before implantation; and intraperitoneal amikacin plus a clarithromycin-coated ureteral stent at the above concentrations. Experiments were performed in duplicate (10 groups, each comprising five animals). For statistical analysis, the data were pooled and referred to all 10 animals from each pair of groups.

The rats were anaesthetized by an intramuscular injection of ketamine and xylazine (30 mg/kg and 8 mg/kg, respectively), the hair was shaved and the skin cleansed with a 10% povidone-iodine solution. The bladder was sutured with 000 surgical silk. After the surgical intervention, a saline solution (1 mL) containing 2 × 10⁷ cfu/mL was prepared. The clinical isolate was inoculated into the bladder using a tuberculin syringe. Some of the animals received intraperitoneal amikacin immediately after stent implantation. The animals were returned to individual cages and thoroughly examined daily. Twenty-four hours after ureteral stent placement, urine cultures were performed through a transvesical sample taken by an insulin syringe to verify sterility or infection. Ureteral stents were explanted at day 5 following implantation and biofilm bacteria enumerated. The limit of detection for this method was approximately 10 cfu/mL. Toxicity was evaluated on the basis of the presence of any drug-related adverse effects (e.g. local signs of local inflammation, anorexia, weight loss, vomiting, diarrhoea, fever and behavioural alterations). The study was approved by the Animal Research Ethics Committee of the I.N.R.C.A.-I.R.R.C.S., Polytechnic University of Marche, Ancona, Italy.

**Statistical analysis**

MIC values are presented as the geometric mean of three separate experiments. Quantitative culture results regarding the in vivo experiments were presented as mean ± SD of the mean and the statistical comparisons between groups were made using analysis of variance (ANOVA) on the log-transformed data with the Tukey–Kramer Honestly Significant Difference Test. Significance was accepted when the P value was ≤ 0.05.

**Results**

**In vitro studies**

Biofilm was photometrically confirmed when the strain showed a mean OD₅₇₀nm of 0.823 ± 0.077. Without clarithromycin pre-treatment, amikacin against the adherent bacteria showed MIC and MBC values of 4.0 and 16.0 mg/L, respectively. Interestingly, after clarithromycin pre-treatment, amikacin exerted higher activity (MIC 1.0 mg/L, MBC 2.0 mg/L). When susceptibility tests were performed according to the procedures outlined by the CLSI, they produced values 2 × to 8× lower (Table 1).

In the combination studies, synergy was observed in five (including AN207) of the six strains evaluated (range 0.187–0.750) (Table 2).

**In vivo studies**

The uncontaminated control group had no microbiological evidence of stent infection. All rats included in the challenged but untreated control group demonstrated evidence of infection, with quantitative culture results showing 6.73 ± 6.04 log₁₀ cfu/mL. In the contaminated groups, rats that received intraperitoneal amikacin showed bacterial counts of 3.46 ± 2.69 log₁₀ cfu/mL.

**Table 1.** Antimicrobial activity of amikacin and clarithromycin against planktonic and adherent cells of a clinical isolate of *P. aeruginosa*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Planktonic cells</th>
<th>Adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>MBC (mg/L)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Clarithromycin plus amikacin</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Wells pre-treated with clarithromycin (10 mg/L clarithromycin in MH broth for 30 min).*

**Table 2.** Results of interaction studies between amikacin and clarithromycin

<table>
<thead>
<tr>
<th><em>P. aeruginosa</em> strain</th>
<th>FIC index</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN207</td>
<td>0.312</td>
</tr>
<tr>
<td>AN345</td>
<td>0.187</td>
</tr>
<tr>
<td>AN527</td>
<td>0.312</td>
</tr>
<tr>
<td>AN609</td>
<td>0.750</td>
</tr>
<tr>
<td>AN117</td>
<td>0.250</td>
</tr>
<tr>
<td>AN448</td>
<td>0.312</td>
</tr>
</tbody>
</table>

The range of drug dilutions used was 0.25–256 mg/L. The FIC indexes were interpreted as ≤ 0.5, synergy; 0.5–4.0, indifferent; and ≥ 4.0, antagonism.

Animals that had clarithromycin-coated stents showed bacterial counts of 4.79 ± 4.23 log₁₀. Interestingly, as shown in Table 3, a clarithromycin coating combined with systemic amikacin was the most efficacious treatment, with an absence of bacterial counts (P < 0.001). Urine cultures confirmed these microbiological data and were negative for both the uncontaminated group and the combined treatment group. For the singly treated groups they were positive, with a bacterial load of 10⁵ for the clarithromycin-treated group and 10⁴ for the amikacin-treated group (Table 3). None of the animals included in any group died or had any clinical evidence of drug-related adverse effects.

**Discussion**

Biofilm on medical devices is up to 1000-fold more tolerant to antibiotics. Its resistance appears to depend on multicellular strategies and includes restricted antimicrobial compounds penetration, heterogeneous metabolic activity of the biofilm cells, differential gene expression of biofilm versus planktonic cells, and QS. One of the methods by which bacterial colonization can be reduced is the use of medical devices pre-coated with antimicrobials and antiseptics. Ureteral stent and urinary catheter materials have been used, such as silicone, polyethylene, polyurethane, biodegradable materials and drug delivery materials, as well as coatings such as silver, heparin,
polytetrafluoroethylene and phosphorylcholine biocides or antibiotics.\textsuperscript{1,32,33} However, the management of those materials that become associated with biofilm-based infection remains problematic. Systemic antibiotic therapy is effective in eliminating circulating bacteria, but it usually fails to protect the surfaces of the materials from colonization, leaving the patient at continued risk of complications or recurrence.\textsuperscript{1,33,36} For these reasons, coatings of these materials are considered useful to decrease bacterial attachment, to stop bacterial growth and to dissolve the biofilm matrix. An alternative approach is to disrupt bacterial cell-to-cell communication that would interfere with the pathogenic potential of the bacteria.\textsuperscript{1,33,36} Bacteria communicate their cell density by the production of chemical signals in a process known as QS. In the case of \textit{P. aeruginosa}, this system regulates biofilm architecture and detachment.\textsuperscript{12,14} The biofilm produced by \textit{P. aeruginosa} and the effect of macrolides on that biofilm have been investigated. The macrolides have been shown to inhibit the activity of many known and potential virulence factors of this organism, including exotoxin A, total protease, elastase, lecinthinase, gelatinase, lipase, pyocyanin and motility.\textsuperscript{15,36}

The aim of the present study was to assess the efficacy of clarithromycin and one of the more commonly used anti-\textit{Pseudomonas} agents, amikacin, in the prevention of ureteral stent-associated \textit{Pseudomonas} infection. We hypothesize that the macrolide might inhibit the virulence factors of the bacteria and thereby enhance the activity of amikacin. Data presented here show that the use of a clarithromycin-impregnated stent with or without conventional antibiotics produced significantly lower bacterial loads. Similar to amikacin, the macrolide caused a significant reduction in bacterial load on the ureteral stent tissue when compared with untreated control animals. Interestingly, when local clarithromycin was combined with systemic amikacin, no evidence of bacteria was detected on the stent or in the urine.

In summary, our experiments demonstrate that clarithromycin may enhance the effect of amikacin against some \textit{Pseudomonas} strains. The \textit{in vitro} studies support these data, since the biofilm was strongly affected by the presence of clarithromycin and, in its presence, amikacin showed MICs and MBCs lower than those obtained in its absence. Moreover, the \textit{in vivo} studies showed that synergism was observed in all six strains (including the strain used for the \textit{in vivo} study).

Recent studies have shown that the interaction of clarithromycin with other antibiotics may produce different results: actually the investigations have produced a great variety of data, at times contradictory.\textsuperscript{15,21,37,38} Our results seem to affirm the utility of the studied association. In fact, although \textit{Pseudomonas} organisms are resistant to macrolides, in agreement with several previous studies, our study showed that macrolides may exert some type of activity against this organisms. Various reports have suggested that macrolides act through effects on the immune system, modifying the inflammatory response to infection (as immunomodulatory), or through a direct effect in decreasing the virulence of \textit{Pseudomonas}.\textsuperscript{14,35,39} They can exert their effect on biofilm formation via inhibition of cell-to-cell signalling. In this way, macrolides reduce the production of extracellular virulence factors such as elastase, but also interfere with biofilm formation at different levels. It has also been reported that these antibiotics are able to inhibit \textit{Pseudomonas} glycocalyx polysaccharide production or neopolysaccharide synthesis.\textsuperscript{14,35,39} Clarithromycin was effective in preventing biofilm formation, and when combined with amikacin, was able to improve amikacin activity through a reduction of bacterial adherence in the biofilm.

Biofilm formation represents a protected mode of growth that allows microorganisms to survive in hostile environments and also disperse to colonize new areas.\textsuperscript{3} Our data support the need for active research to develop more efficient cell-to-cell signalling blockers that could, according to the results presented here, reduce \textit{P. aeruginosa} biofilm formation on medical devices such as ureteral stents. Combination antibiotic therapy could be particularly useful in clinical situations in which growth in biofilm might play an important role. In our study, the prevention of ureteral stent \textit{Pseudomonas} biofilm infection was enhanced by impregnation of the stent with clarithromycin combined with systemic amikacin.

### Table 3. Activity of clarithromycin and amikacin against the slime-producing \textit{P. aeruginosa} AN207 clinical isolate in a rat model of ureteral stent infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Stent-bonded drug\textsuperscript{a}</th>
<th>Intraperitoneal drug\textsuperscript{b}</th>
<th>Quantitative stent culture (cfu/mL)\textsuperscript{c}</th>
<th>Urine culture (cfu/mL)\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C\textsubscript{0})</td>
<td>–</td>
<td>–</td>
<td>(&lt;1\times10^5)</td>
<td>(&lt;10)</td>
</tr>
<tr>
<td>Control (C\textsubscript{1})</td>
<td>–</td>
<td>–</td>
<td>(6.73 \pm 6.04)</td>
<td>(6.68 \pm 5.47)</td>
</tr>
<tr>
<td>Group 1</td>
<td>–</td>
<td>amikacin</td>
<td>(3.46 \pm 2.69)\textsuperscript{ef}</td>
<td>(4.78 \pm 4.11)\textsuperscript{ef}</td>
</tr>
<tr>
<td>Group 2</td>
<td>clarithromycin</td>
<td>–</td>
<td>(4.79 \pm 4.23)\textsuperscript{e}</td>
<td>(5.85 \pm 5.25)\textsuperscript{e}</td>
</tr>
<tr>
<td>Group 3</td>
<td>clarithromycin</td>
<td>amikacin</td>
<td>(&lt;1\times10^5)\textsuperscript{g}</td>
<td>(&lt;10)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The ureteral stent segments were impregnated with 10 mg/L clarithromycin.
\textsuperscript{b}Each rat received amikacin 15 mg/kg intraperitoneally.
\textsuperscript{c}Ureteral stents were explanted at day 5 following implantation. Bacterial counts are given in terms of \(\log_{10}\) cfu/mL. The limit of detection for the method was \(<1\) cfu/mL.
\textsuperscript{d}The urine cultures were performed 24 h after ureteral stent placement using a transvesical sample taken by an insulin syringe. Bacterial counts are given in terms of \(\log_{10}\) cfu/mL. The limit of detection for the method was \(<10\) cfu/mL.
\textsuperscript{e}Statistically significant when compared with the untreated control group.
\textsuperscript{f}Statistically significant when compared with the clarithromycin-treated control group.
\textsuperscript{g}Statistically significant when compared with singly treated groups.
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Transparency declarations
None to declare.

References
