Antimicrobial interference of a subinhibitory concentration of azithromycin on fimbrial production of *Porphyromonas gingivalis*

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The first pathogenic mechanism of *Porphyromonas gingivalis*, which is mainly responsible for adult periodontal diseases, is the attachment of fimbriae appendages to oral epithelial cells. The ability of a subinhibitory concentration of azithromycin to inhibit the expression of fimbriae in various strains of *P. gingivalis* isolated was investigated. A one-eighth subinhibitory concentration of azithromycin was evaluated *in vitro*. The antibiotic was active in 75% of *P. gingivalis* strains isolated, judged by both electrophoresis and transmission electron microscopy. The results indicate that the subinhibitory concentration of azithromycin is capable of blocking the pathogenic mechanism of *P. gingivalis in vitro*, and, therefore, can be used *in vivo* as a treatment for recurrent periodontitis.

**Introduction**

*Porphyromonas gingivalis* is a Gram-negative oral anaerobe implicated in the aetiology of human periodontal diseases, particularly adult periodontitis. Pathogenesis is based on the production of: capsule, liposaccharide, proteolytic enzymes such as collagenase, trypsin-like activity, and emolisin. The first step involves microbial colonization and attachment of bacteria to the teeth or to oral surfaces. *P. gingivalis* adheres to the epithelial cells and saliva proteins using filamentous surface appendages or fimbriae.

Azithromycin, the first antibiotic belonging to the subclass of the azalides, has been shown to be more effective than other macrolide antibiotics against many common pathogens. In a previous study, azithromycin was found to have a good activity against almost all periodontopathic bacteria, and it rapidly achieves high and sustained tissue concentrations with oral dosing. Concentrations of antibiotics lower than those necessary to inhibit growth can alter the ability of bacteria to adhere to epithelial cells, lower enzyme and toxin production, and increase microbial susceptibility to the host’s immune defence.

Other researchers have described the ability of subinhibitory concentrations (sub-MICs) of azithromycin to inhibit, in vitro, motility, flagellar synthesis, and virulence factors in *P. mirabilis* and *P. aeruginosa*. Moreover, Gorbay & McGregor demonstrated the activity of sub-MICs of azithromycin in decreasing pilin subunit synthesis and gonococcal adherence to human mucosal cells.

The purpose of the present study was to assess whether the sub-MICs of azithromycin could affect, in vitro, the fimbrial production of *P. gingivalis*.

**Materials and methods**

**Bacterial strains**

Fifteen *P. gingivalis* strains were freshly isolated in the microbiology laboratory of the Microbiology Institute of Catania University. The source of isolates was subgingival plaque samples of patients admitted to the Institute of Dentistry, Section of Periodontology of the University of Catania in the acute phase of recurrent periodontal disease. *P. gingivalis* 381 was used as a control strain.

**Antimicrobial agent**

Azithromycin was kindly supplied by Pfizer Italiana, Rome. The agent was stored at 4°C and solutions were prepared on the day of use according to the manufacturer’s instructions.

**Subinhibitory concentration of azithromycin**

MICs were determined by a broth dilution method using Brucella broth (BBL Microbiological Systems, Cockeys...
ville, MD, USA) supplemented with 5 mg/L haemin (Sigma Chemical Co., St Louis, MO, USA) and 0.4 mL/L vitamin K$_1$ (growth medium). The antibiotic was diluted on to a series of microplates containing two-fold dilutions of azithromycin, range 25 to 0.001 mg/L. Each isolate and the P. gingivalis strain 381 were inoculated at a final inoculum of 10$^5$ cfu/mL in separate wells. The plates were incubated for 48 h at 37°C in anaerobic atmosphere produced by GasPak (Oxoid, Unipath Spa., Milan, Italy). The MIC was recorded as the lowest concentration inhibiting macroscopic growth of each strain under investigation. P. gingivalis strains were considered sensitive to azithromycin if their MIC value was ≤2 mg/L.

The effect of one-eighth MIC azithromycin on the pilation of P. gingivalis strains was then evaluated. In particular, P. gingivalis strains were incubated with one-eighth MIC concentration of azithromycin diluted in growth medium in anaerobic atmosphere for 12 h at 37°C. The colonies were further incubated in the same medium in anaerobic atmosphere for 5–7 days. Bacterial cultures of the same strains were prepared without antibiotic treatment.

**Fimbrial extraction**

Using a modification of the method of Yoshimura et al. and Lee et al., fimbrial protein was extracted from each strain, with and without subinhibitory azithromycin, to evaluate the activity of the antimicrobial agent on piliation. Cells of P. gingivalis were added to growth medium, and then incubated for 5 days at 37°C in anaerobic atmosphere. Cells were harvested by centrifugation at 8000g for 20 min at room temperature, then washed in 20 mM Tris buffer, pH 7.4, with the addition of 0.15 M NaCl and 10 mM MgCl$_2$ by repeated pipetting, and stirred for 4 h at room temperature. The supernatant obtained by centrifugation at 10,000g for 30 min at 4°C was brought to 40% saturation of ammonium sulphate and incubated overnight at 4°C. The protein was collected by centrifugation at 10,000g for 25 min at 4°C and suspended in a small volume of 20 mM Tris-HCl (pH 7.0). The ammonium sulphate was eliminated by overnight dialysis in Tris buffer.

Protein determination of each strain of P. gingivalis was evaluated using the Lowry method.

**SDS–PAGE**

The ability of subinhibitory azithromycin to inhibit the expression of fimbrial production was investigated by running fimbrial extracts through a 10% SDS–polyacrylamide gel following Laemmli’s method. Briefly, fimbrial extract from each P. gingivalis strain was treated with 2% SDS in 0.1 M Tris–HCl (pH 6.8) by 20 passes in a syringe, pipetted into each slot, and separated in a Protein II Electrophoresis Cell (Bio-Rad, Milan, Italy) by application of 18 mA overnight at 4°C. After PAGE, the SDS–polyacrylamide gel was stained with Coomassie brilliant blue to visualize protein bands. The high and low range of the 

**Table I.** Fimbrial production after subinhibitory concentrations (one-eighth MIC) of azithromycin treatment against P. gingivalis strains isolated

<table>
<thead>
<tr>
<th>Strain$^a$</th>
<th>MIC (mg/L)</th>
<th>Subinhibitory concentration (mg/L)</th>
<th>SD$^b$–PAGE 43 kDa a protein</th>
<th>TEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5</td>
<td>1.56</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>0.78</td>
<td>0.09</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>0.78</td>
<td>0.09</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>0.39</td>
<td>0.04</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>0.04</td>
<td>0.006</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>0.02</td>
<td>0.002</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>0.19</td>
<td>0.02</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>0.19</td>
<td>0.02</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>0.39</td>
<td>0.04</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>0.78</td>
<td>0.09</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>0.04</td>
<td>0.006</td>
<td>–</td>
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<tr>
<td>12</td>
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<tr>
<td>13</td>
<td>0.78</td>
<td>0.09</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>0.19</td>
<td>0.02</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>0.04</td>
<td>0.006</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>1.56</td>
<td>0.19</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$1–15, P. gingivalis strains isolated from periodontal patients; 16, P. gingivalis strain 381. TEM, transmission electron microscopy.
molecular calibration proteins (Bio-Rad) and *P. gingivalis* 381 fimbrial extract, with and without the contact with subinhibitory azithromycin, were included in each test. The piliation of the bacteria was considered positive if the 43 kDa fimbrial protein band was present.

Electron microscopy

To assess subinhibitory azithromycin effects on the pilin subunit content of *P. gingivalis*, all strains, with and without the contact with the antibiotic, were observed by transmission electron microscopy. Each *P. gingivalis* strain was suspended in an equal volume of phosphate saline buffer (PBS) containing 0.25% formalin. After 1 h fixation, a drop of the cells was placed on a Formvar-coated copper grid (SIC, Rome, Italy) and allowed to stand for 15 min. Excess suspension was drained off and the adherent bacteria were negatively stained with 1% phosphotungstic acid (Merck, Milan, Italy), pH 7.0, for 15 s, drained, and observed by electron microscopy. The observation of the presence of fimbriae emanating from the surface of the bacteria was considered a positive result.

Results

The MIC of azithromycin for each strain and the production of fimbriae in subinhibitory concentrations are shown in Table I. Fifteen out of 16 of the strains were susceptible to azithromycin and only one was resistant to the antibiotic studied. One-eighth of the MIC of azithromycin interfered with fimbrial production. The results obtained by SDS–PAGE showed the presence of the 43 kDa pilin band in all *P. gingivalis* strains without antibiotic treatment. Twelve out of 16 strains (75%) demonstrated a complete absence of the 43 kDa pilin band after contact with subinhibitory azithromycin (Figure 1).

*P. gingivalis* strain 381 showed inhibition of pilin production if a subinhibitory concentration was used, and the production of fimbriae was positive in the absence of antibiotic exposure (Figure 1).

The results obtained by SDS–PAGE were confirmed by the electron microscopy observations. All strains isolated exhibited fimbrial appendages before azithromycin exposure (Figure 2a). With sub-MIC azithromycin treatment, the fimbriae were absent in 12 out of 16 strains of *P. gingivalis* (Figure 2b).

Discussion

The aetiology of periodontal diseases is complex and, in many cases, different bacterial strains are involved in the damage of periodontal tissues. Many workers have described *P. gingivalis* as one of the most prevalent microorganisms in the active pockets of adult periodontal patients. Blocking the pathogenic mechanism of *P. gingivalis* could be a way to eliminate this micro-organism from oral surfaces. Because the early phase of microbial colonization of oral surfaces involves the adherence of

![Figure 1. Gel electrophoresis of fimbrial proteins. s, High and low protein standards; a, *P. gingivalis* strain 381 before antibiotic treatment; b, *P. gingivalis* strain 381 after antibiotic treatment; c, d, e, f, *P. gingivalis* strains 3, 4, 12, 14 after antibiotic treatment.](image-url)
P. gingivalis to the teeth or oral epithelial cells by fimbriae or fimbriae-associated adhesions, this study focused on the use of antibiotics to block the synthesis of fimbrial proteins. Many workers have demonstrated that the use of subinhibitory antibiotics can cause changes in other membrane proteins, and a decrease of piliation.25–27 Concentration of antibiotics in infected periodontal tissues often may be subinhibitory. Subinhibitory azithromycin significantly reduces the percentage of gonococci that express assembled pili on their surfaces, decreasing gonococcal adherence to human mucosal cells.15 A zithromycin, like other macrolide antibiotics, acts on the ribosome to decrease protein synthesis, binding the 50S subunit of the ribosome.28

The present study extends the previous findings described by other workers because the effect of one-eighth MIC azithromycin in vitro blocks fimbrial production on the bacterial surface of the majority of P. gingivalis strains. Electron microscopy confirmed the relationship of this block to the absence of the 43 kDa protein band. All the fimbrial extraction from P. gingivalis strains that showed the absence of the 43 kDa protein band by SDS–PAGE belonged to bacteria which lost fimbrial appendages after azithromycin exposure. The presence of the same protein band detected by electrophoresis was related to the bacteria which remained piliated in the presence of azithromycin.

Subinhibitory azithromycin (one-eighth MIC) failed to inhibit fimbrial production in 25% of the P. gingivalis strains. Further in-vitro studies are in progress to determine whether higher concentrations of the same antibiotic might be used to inhibit the production of fimbriae in those strains.

In conclusion, the present study demonstrates that azithromycin, at subinhibitory concentrations, is capable of inhibiting the expression of the virulence factor of P. gingivalis. The antibiotic exhibits a good activity in the majority of the isolated strains, reflecting its high antibacterial activity in vitro. As azithromycin has shown antibacterial activity at subinhibitory concentrations, further benefits may be expected in vivo, where the half-life is prolonged and tissue distribution high. Azithromycin is likely to be useful in the treatment of recurrent periodontal diseases caused by P. gingivalis.

Acknowledgements

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Figure 2. (a) P. gingivalis 12 strain before antibiotic treatment. (b) P. gingivalis 12 strain after antibiotic treatment.
References


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