

ORIGINAL ARTICLE

MICROSCOPIC STUDY OF THE EFFECTS OF SUB-INHIBITORY CONCENTRATIONS OF GENTAMICIN ON CAPSULE PRODUCTION OF *PSEUDOMONAS AERUGINOSA*

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Abstract

Background-The mucoid strains of *Pseudomonas aeruginosa* produce a hyperviscous capsule, which has several roles in pathogenesis. In this study, the *in vitro* microscopic effects of sub-inhibitory concentrations of gentamicin on capsule production by mucoid *Pseudomonas aeruginosa* were investigated.

Methods-The production of a capsule by the mucoid *Pseudomonas aeruginosa* cells cultured on agar media in the presence of sub-inhibitory concentrations of gentamicin was observed by light and electron microscopy.

Results- Capsule production was reduced by 0.5 and 0.25 minimum inhibitory concentrations. The results showed a reduction in capsular size in the presence of sub-inhibitory concentrations of gentamicin. The size of the capsule in the presence of 0.5 MIC gentamicin was less than its size in 0.25 MIC gentamicin.

Conclusion-The results confirm that the production of alginate was reduced and consequently, *P. aeruginosa* infections might be prevented by sub-inhibitory concentrations of gentamicin.

Keywords • Gentamicin • *Pseudomonas aeruginosa* • alginate

Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen and possesses many possible virulence factors.¹ Mucoid strains of *P. aeruginosa* are surrounded by a capsule composed of β 1-4 linked D-mannuronic acid and L-glucuronic acid.²⁻⁵ The capsule provides a protective barrier against antimicrobial agents and the immune system.⁶⁻⁸ Little is known about the effects of sub-inhibitory concentrations of antimicrobial agents on the capsule.⁹ Some influence might be anticipated, since there are rep-

orts showing the effects of sub-inhibitory concentrations of various agents on exoenzymes, which are important virulence factors including exotoxin A, exoenzymes S, elastase, phospholipase C and total protease.¹⁰ At sub-inhibitory concentrations, tobramycin and gentamicin reduce the levels of proteases *in vitro*.¹¹ Erythromycin, clarithromycin and azithromycin inhibit the elaboration of elastase and proteases at concentrations below the minimum inhibitory concentration (MIC) *in vitro*.^{9,12} In a previous study, we showed that 0.5 and 0.25 MIC gentamicin quantitatively reduced the production of alginate in a mucoid strain of *P. aeruginosa*.¹³ In this study we investigated the microscopic effects of 0.5 and 0.25 MIC gentamicin on *P. aeruginosa* capsule production.

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Materials and Methods

Bacteria:

Mucoid *P. aeruginosa* 8821M was donated by Dr. Isabel Sa-Corria, Instituto Superior Tecnico, Lisboa, Portugal.¹⁴ The standard strains of *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used for determination of MIC.¹⁵ The strains were maintained in 10% skimmed milk (Difco Laboratories, Detroit, MI) at -80°C and were subculture on Muller-Hinton agar (Difco Laboratories, Detroit, MI).¹⁶

MIC determination:

Standard solutions of gentamicin ($1.25\text{-}320\ \mu\text{g mL}^{-1}$) (Sigma Chemical Co., Ltd., St. Louis, MO) were prepared by dissolving gentamicin in sterile distilled water and diluting in Muller-Hinton agar. An agar dilution technique using Muller-Hinton agar and an inoculum of 10^4 colony-forming units (CFU) per spot was used for the determination of MIC. The lowest concentration of antimicrobial agent which inhibited visible growth after 16 h incubation at 37°C was defined as the MIC.¹⁵

Microscopic study

After 8h incubation at 37°C in Muller-Hinton broth (Difco Laboratories, Detroit, MI) the mucoid *P. aeruginosa* 8821M was adjusted to 10^3 CFU mL^{-1} with 0.06 M phosphate-buffered saline (PBS) pH 7.2, and 0.1 mL of the resultant suspension was inoculated onto Muller-Hinton agar plates, with or without gentamicin. Plates were incubated for 24 h at 37°C . The formed colonies were collected on a cotton swab and suspended in 10 mL PBS.¹⁶ The suspensions were used for microscopic study.

Light microscopy: The method of Muir was used.¹⁷ A thin film of suspension was prepared and dried in the air. The film was covered with a piece of filter paper the size of the smear and the slide was flooded with Ziehl-Neelsen carbolfuchsin, which was then heated to steaming for 30 sec by a low Bunsen flame. The slide was gently rinsed by 95% ethanol and then with water. Mordant solution was added for 20 sec and was washed with water. Decolorization was performed by ethanol. For a counterstain, 0.3% methylene blue was used for 30 sec and the slide examined under the oil immersion lens. The cells were stained red, and the capsule blue.

Electron microscopy: The method of negative stain was used.¹⁸ A $10\ \mu\text{L}$ droplet of suspension

was applied directly from a pipette to the support film on 400 mesh grid, which was held (by a rubber band) in forceps. After 20 sec, the edge of the droplet was touched to the edge of a filter paper. A $10\ \mu\text{L}$ droplet of 1% phosphotungstic acid (negative stain) was applied to the grid and after a few seconds it was similarly removed, by touching the edge of a filter paper. The layer of suspension and negative stain was allowed to dry at room temperature, after placing it onto a filter paper in a covered petridish. The electron microscope consisted of a model 900 Zeiss transmission electron microscope.

Results

The MIC values of the mucoid strain of *P. aeruginosa* 8821M, *P. aeruginosa* 27853 and *E. coli* 25922 were 8, 2 and $1\ \mu\text{g mL}^{-1}$, respectively. Samples were compared with controls by light and electron microscopy. The results showed a reduction in capsular size in the presence of sub-inhibitory concentrations of gentamicin. The size of the capsule in the presence of 0.5 MIC gentamicin was less than its size in 0.25 MIC gentamicin. The results of a prior study¹³ showed a significant difference between alginate production by control (untreated) cultures and that produced by bacteria grown in the presence of 0.5 and 0.25 MIC gentamicin.

Discussion

The earlier study was a quantitative assay, but this study was qualitative. Comparison of the results confirmed that capsule production was reduced in the presence of 0.5 and 0.25 MIC gentamicin.

Capsule production and consequently biofilm formation, which correlates with the production of alginate, has several roles in the pathogenesis of *P. aeruginosa*.^{1,6-8} Consequently, any treatment which reduces capsule production, might be effective in counteracting the pathogenesis of *P. aeruginosa*, especially in cystic fibrosis and in patients with suppressed immune system.^{1,16} It has been shown that sub-MIC values of gentamicin reduce the level of proteases *in vitro*.¹¹ Other antimicrobial agents reduce the level of exotoxin A.⁹ However, there is no report regarding the effect of gentamicin on capsule production. In this study, we have demonstrated that sub-inhibitory concentrations of

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gentamicin, which can be attained in human lungs or other tissues following a normal dosage reduce capsule production, probably by suppressing biofilm formation.¹⁶ Consequently chronic respiratory tract infections due to mucoid strains of *P. aeruginosa* might be prevented.

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