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Effect of nalidixic acid on the morphology and protein expression of *Pseudomonas aeruginosa*

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ABSTRACT

Objective: To determine the effect of nalidixic acid on the morphology and protein expression of *Pseudomonas aeruginosa* (*P. aeruginosa*). **Methods:** Nalidixic acid solution of 1 600 μg/mL was prepared. The minimum inhibitory concentration (MIC) for *P. aeruginosa* was determined with tube dilution test. The effect of nalidixic acid on the morphology of *P. aeruginosa* was studied using light microscope and scanning electron microscope. Changes in protein profile were studied using SDS-PAGE. **Results:** The MIC of nalidixic acid was 700 μg/mL against *P. aeruginosa*. The exposure of *P. aeruginosa* to different concentrations of nalidixic acid resulted in deformation of most of the growing cells. At the concentration of 600 μg/mL most of the cells turned into elongated and adhere to each other while some of the cells were bulged. The intensity of protein bands were changed when they exposed to nalidixic acid. **Conclusions:** The present findings suggest that the morphology and protein expression of *P. aeruginosa* is greatly affected by nalidixic acid.

1. Introduction

Antibiotics are natural products of fungus or bacteria that suppress or inhibit the growth of other bacteria by interfering with their vital biological processes. These biological processes include DNA replication and translation[1] which alters the bacterial morphology or even cause their death. It is important to note that the majority of antibiotics are originated from natural product sources[2]. However, some are synthetic, such as nalidixic acid that belongs to quinolones group. Quinolones antibiotics were widely used as bacterial inhibitors[3]. After the introduction of nalidixc acid, many thousands of quinolones derivatives have been produced and analyzed for antibacterial activity[4]. The potential of antimicrobial agent rely on the inhibition of bacterial growth that often requires a high concentration of agent being used[5]. Among susceptibility tests, broth dilution susceptibility test was the first to be developed and still serves as the reference method[6]. However, the use of broth dilution tests relies on pointing the minimal inhibitory concentration (MIC) that is simply defined as the lowest concentration of antimicrobial agent at which no growth is detected for a given bacterial strain.

Pseudomonas aeruginosa(*P. aeruginosa*) are mostly free living Gram negative bacteria found in water and soil. However, some are opportunistic pathogens to human and is considered to be an important nosocomial pathogen[7]. Wounds resulted from burns, malignancy, and surgery are often colonized and subsequently infected with this bacterium. This organism has an outer membrane serve as an additional protection layer that plays a keystone in the interaction of the cells with the surrounding environment[8]. The outer membrane enables *P. aeruginosa* to adapt to a wide variety of commonly used antibiotics[9]. Only

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few antimicrobial agents, including nalidixic acid, show potential activity against these species. So the aim of this study was to find out the effect of nalidixic acid on the morphology and protein profile of *P. aeruginosa*.

2. Materials and methods

2.1. Preparation of antimicrobial agent

0.08 g of nalidixic acid was dissolved in 6 mL of methanol and the contents were brought to a final volume of 50 mL with nutrient broth to make a stock solution. The final concentration of the nalidixic acid stock solution was 1 600 μg/mL.

2.2. Identified functional domains

P. aeruginosa (ATCC25668) was used in this study. Bacterial stock subcultures were prepared in 500 μL glycerol eluents and stored at −80 ℃.

2.3. Broth susceptibility test

Ten different concentrations of nalidixic acid (100 μg/mL to 1 000 μg/mL) MIC tubes besides the control tube were prepared and the volume of tubes content was brought to 4 mL with nutrient broth. The sets were then inoculated with a loopful of *P. aeruginosa*. The MIC sets were incubated in a shaker incubator at 37 ℃ for about 18–20 h. The MIC was confirmed by carrying minimal bactericidal concentration (MBC). The MBC was tested using sequential subcultures of nutrient broth on agar plates[6].

2.4. Spectrophotometeric analysis of bacterial growth

Bacterial growth of each tube was determined using spectrophotometer at wavelength of 600 nm. ANOVA test using SPSS software was applied to test the significant difference in the bacterial growth absorbance.

2.5. Effect of nalidixic acid on the morphology of *P. aeruginosa*

Morphological changes of *P. aeruginosa* due to exposure to nalidixic acid were studied by using light microscope and scanning electron microscope.

2.5.1. Light microscope

Gram stained slides were analyzed under light microscope and the morphological changes of bacterial cells as a result of an increase of nalidixic acid concentration were compared with the control. The abnormal cells were recorded.

2.5.2. Scanning electron microscopy (SEM)

100 μL *P. aeruginosa* suspension of different concentrations were fixed with 100 μL 2.5% cacodylate-buffered glutaraldehyde for 4 h. A loop full of the different concentrations were mounted on aluminum stubs and allowed to dry for 40 min. Stubs were then sputter coated with pure gold and examined using SEM (JEOL JSM 5600 LV ×300 000). Each stub was placed on the stage of SEM and about 10 random SEM fields, at high magnification were examined and images were captured.

2.6. Protein extraction

Initially bacterial cells were washed using phosphate buffer saline. Then sonication was performed at a constant time intervals (3×10 min). 10 mM Tris (pH 7.6) buffer was used to disrupt the cells by means of mechanical shearing. Then the contents were centrifuged at a speed of 8 000 *g* for 5 min to remove the unbroken cells and the pellet was discarded. To the supernatant 100 μL of 2% SDS was added to solubilize proteins and centrifuged at 13 000 *g* for 10 min to pellet the phospholipids. After discarding the pellet, 100 μL methanol was added to the supernatant in order to precipitate proteins and the contents were centrifuged for 20 min at 16 000 *g*. Finally, the supernatant was discarded and 100 μL of 20% SDS was added to the pellet and the extracted proteins were used for SDS-PAGE.

2.7. Analysis of bacterial proteins by SDS-PAGE

Gels were prepared by using 5% stacking gel in 1 M Tris (pH 6.8) buffer and the 12% separating gel in 1 M Tris buffer (pH 8.8). Gels were stained using coomassie blue solution (contain 40% methanol, 10% acetic acid and 0.25% coomassie stain) for 4 h. The gels were then washed with deionized water four times and then destained using destaining solution (5% methanol, 10% acetic acid and Milli-Q-water).

3. Results

3.1. MIC

10 different concentration of nalidixic acid, each concentration with three replicates, were analyzed for MIC. The MIC was visually determined to be about 700 μg/mL. No further growth was detected after this point. MBC technique was used to confirm that growth of *P. aeruginosa* did not occur beyond the MIC point (Figure 1).

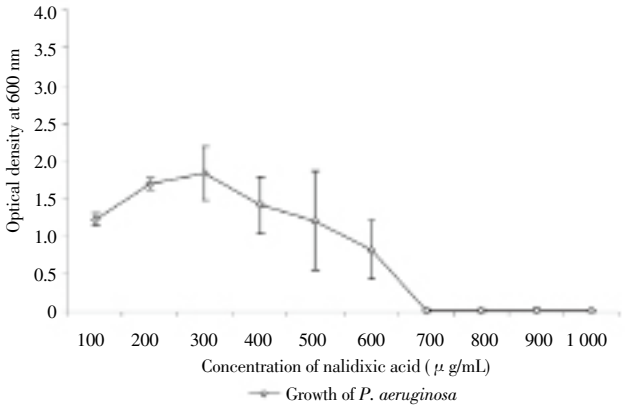

Figure 1. Growth of *P. aeruginosa* at different concentrations of nalidixic acid.

The response of *P. aeruginosa* to different concentrations of nalidixic acid varies considerably. The maximum mean absorbance detected was 1.82±0.09. However there was a highly significant (P< 0.05) decline in *P. aeruginosa* growth beyond 600 μg/mL.

3.2. Determination of morphological changes

Morphological changes of *P. aeruginosa* due to different nalidixic acid concentrations were determined microscopically.

3.2.1. Qualitative assessment

Qualitaive assessment was undertaken to assess the morphological changes. Before the exposure to nalidixic acid (control), the cells of *P. aeruginosa* appeared as normal rod shape cells (Figure 2). The exposure of *P. aeruginosa* to different concentrations of nalidixic acid concentrations (100 to 500 μg/mL) resulted in deformation of most of the growing cells (Figure 3). As nalidixic acid concentration was increased to 600 μg/mL most of the cells became elongated and had tendency to adhere to each other where some of the cells remained bulged (Figure 4 A–C). At higher concentrations of nalidixic acid bacterial cells were not seen in SEM micrograph (Figure 5A), only few cellular debris were observed under light microscopy (Figure 5B).

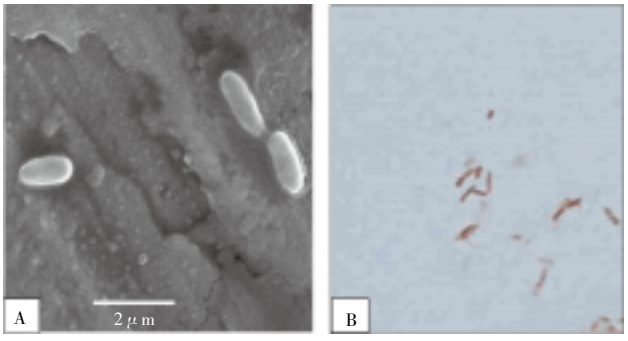

Figure 2. Normal rod shape of *P. aeruginosa* in control without nalidixic acid treatment.
A –SEM, B – light microscope.

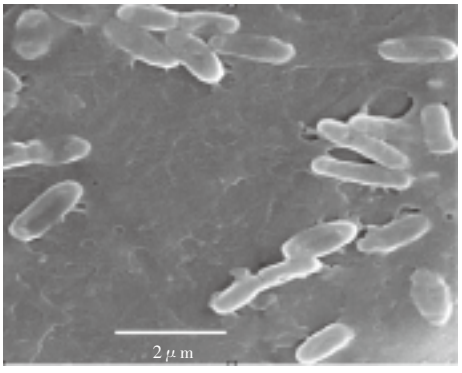

Figure 3. Deformed cells due to low concentration of nalidixic acid under SEM.

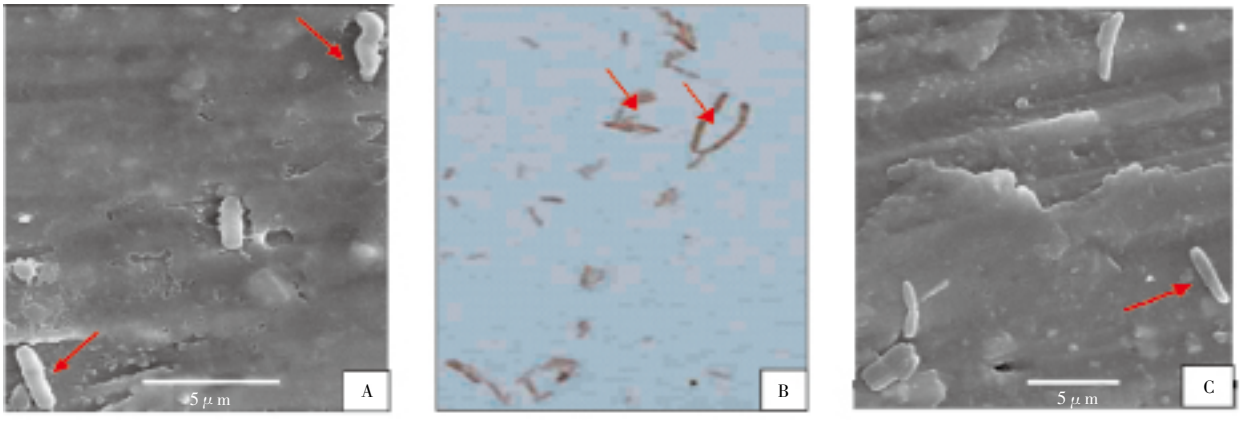

Figure 4. Defromation of *P. aeruginosa* cells.
A–bulging of cells under SEM, Cell elongation and cells tend to adhere under B–light microscope, C–SEM.

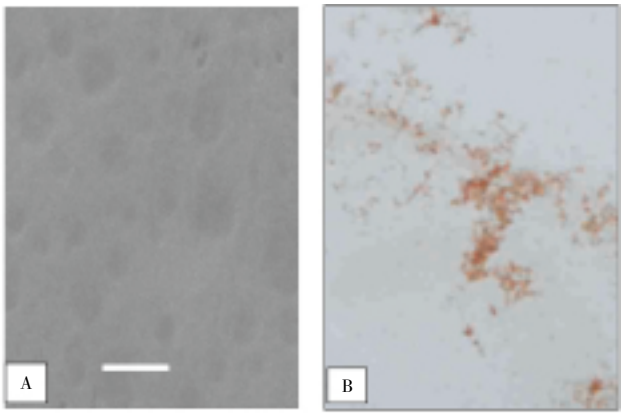

Figure 5. Death of bacterial cells.
A-under SEM and B-light microscope.

3.2.2. Quantitative assessment

Preliminary quantitative assessment of the deformed and abnormal *P. aeruginosa* cells in comparison to normal cells was carried out. Deformed cells increased gradually from 48% to 89% as the concentration of nalidixic acid increased from 100 to 600 μg/mL. Even though the elongation has increased proportionally to the concentration, the bulging of cells decreases with increasing concentration of nalidixic acid. At 400 μg/mL concentration of nalidixic acid 29 elongated cells and 24 bulged cells were observed out of 100 cells. Interestingly the deformation reaches around 50% where number of deformed cells was almost equal to the number of elongated cells.

3.3. Protein profiling by SDS-PAGE

In the present study, the SDS-PAGE analysis showed variation of protein expression as a result of exposure to different concentration of nalidixic acid. Although most of proteins were present at all concentrations of nalidixic acid, expression of some proteins such as P82, P62, P41 and P34 kDa varied with different concentration of nalidixic acid. P34 expression increased at higher concentration of nalidixic acid. On the other hand the expression of P41 decreased and appeared lighter on the gel as the concentration of nalidixic acid increased.

4. Discussions

In the present work, the effect of nalidixic acid on the morphology and protein expression of *P. aeruginosa* was studied. *P. aeruginosa* was found to be 50 times more resistant to nalidixic acid than *Escherichia coli* (*E. coli*) and other enteric bacteria. The outer membrane of Gram-negative bacteria functions as a permeability barrier that protects cells against a large number of antibacterial agents[10]. The stability of an outer membrane is important for its properties as a molecular sieving and exclusion of antimicrobial agents. According to the hypothesis that morphological changes and development is either proceeded by or accompanied simultaneously by chemical changes[11], results of the present work indicates higher concentration of nalidixic acid concentration induces deformation of cells resulting in their elongation and bulging.

P. aeruginosa exposed to nalidixic acid showed that there were significant morphological changes. However, the minimal inhibitory concentration was found to be 700 μg/mL, that strongly suggests that at this concentration nalidixic acid affects the DNA gyrase action, leading to the alteration of protein expression[12] hence the change in bacterial morphology. A significant reduction on the morphology of *P. aeruginosa* in its diagonal length, radius, height, volume and surface area was observed when it was incubated with erythromycin[13].

In this study some cells were as twice longer than normal cells at different concentrations of nalidixic acid. It was also found that deformed bulged cells were very common at lower concentration of nalidixic acid. This suggested that low concentration of nalidixic acid affects the *P. aeruginosa* morphology in a way that disrupts the ribosomal action inhibiting the protein synthesis. However, at higher concentration bulged cells became less common and the elongated cells with a tendency to adhere to each other turned out to be a common feature. This could be due to the inhibition of either DNA gyrase[14] or peptidoglycan transpeptidase[15]. The uncommon phenomenon of bacterial filamentation associated with Gram negative bacilli was observed when exposed to certain cell wall active agents. This is because of inhibition of cross-linking of peptidoglycan cell wall[16]. The two antibiotics caused extreme elongation of cells and cells without septa were produced. According to our search on other studies this particular behavior on variation of bulging and elongation of *P. aeruginosa* at different concentrations of nalidixic acid has not been reported.

The effect of antimicrobial agent on bacterial protein synthesis has been a significant area of research. Organisms respond to the surround stressful environment by increasing the expression of specific proteins to reduce damage occurred to the cell[17]. In *E. coli* lon mutants high pressure treatment induced hyperfilamentation[18]. Filamentation may be a usual response to environmental stresses such as temperatue for at least some strains of *E. coli*[19]. Low water activity induced filamentation of *Salmonella enterica* cells and the surviving filamentous cells maintained their membrane integrity after exposure to low water activity for 21 days[20]. Cell elongation was observed in Listeria monocytogenes Scott A and LO28 when Nacl addition and acidification were applied concomitantly suggests

filamentation as an adaptive mechanism[21-24]. Studies of protein profiling by SDS-PAGE and Western Blotting techniques of *Bacillus subtilis* grown under stress conditions revealed that protein expression varies[25]. *E. coli* also responds to the change in temperature by producing either heat shock or cold shock proteins[5]. The increasing intensity of p34 and decreasing intensity of p41 protein bands corresponding to the increasing concentration of nalidixic acid could be related to the chemical stress given in the form of nalidixic acid.

In conclusion elevation of nalidixic acid concentrations affect cellular morphology of *P. aeruginosa* and expression of its proteins. At lower concentration most of cells were bulged and became elongated at higher concentration of nalidixic acid. Elongated cells had a tendency to adhere to each other. SDS-PAGE revealed variation of P82, P62, P41 and P34. Unlike P82, P62 and P34, P41 expression decreased when nalidixic acid concentration was increased.

Conflict of interest statement

We declare that we have no conflict of interest.

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