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INFLUENCE OF GENTAMICIN ON ENTEROCOCCI BIOFILM FORMATION

**Myronenko L.G., Peretyatko O.G.,
Iagniuik J.A., Martynov A.V.**

Mechnikov Institute of Microbiology and Immunology

Introduction

Today, it is well established that almost 80% of all infectious diseases are caused by microorganisms that exist in the form of biofilms. Microorganisms in the biofilms acquire signs of increased resistance to antibiotics, disinfectants and other aggressive environmental factors, complicate the course of infectious diseases and play an important role in their chronicity. Scientists receive more and more evidence that features of the infectious process in pneumonia, angiogenic sepsis, uroinfections, infectious endocarditis, cystic fibrosis, chronic bacterial prostatitis, periodontitis, acute otitis media are related to biofilm formation [1-3].

Formation of biofilms by hospital strains of bacteria poses a serious threat to the practical medicine. Enterococci, foremost *Enterococcus faecium* and *Enterococcus faecalis*, are the third most common cause of hospital infections, most of which involve the use of permanent medical equipment. Internal hospital infections gain particular importance in intensive care units and in surgical hospitals, since the formation of biofilms is the cause of severe catheter and fan associated infections, sepsis, pneumonia and endocarditis. It should be noted that ineffective antibiotic therapy of infections, accompanied by the formation of biofilms, also leads to significant economic losses [4, 5].

In this regard, the study of the influence of antimicrobial agents on the processes of formation of enterococci biofilms and the search for compounds capable of enhancing the inhibitory effect of antibiotics on the enterococci biofilm formation is an extremely important task of medical science.

The aim of the research was to study the effects of gentamicin and gentamicin in combination with a penetrator on processes of enterococci biofilm formation.

Materials and methods

The objects of the study included 3 strains of bacteria genus *Enterococcus*, obtained from the bacteria museum of the Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine: *E. faecalis* ATCC 29212, *E. faecalis* IMI (X) 49 p, *E. faecium* IMI (X) 80. Strain *E. faecalis* ATCC 29212 is a pharmacopeidic, clinical strain *E. faecalis* IMI (X) 49 p was isolated from the surface of the trophic ulcer of a patient with diabetes and with "diabetic foot" syndrome, clinical strain *E. faecium* IMI (X) 80 was isolated from the

cerebrospinal fluid of a patient with a postoperative meningitis. Our previous studies have shown that these strains are characterized by high biofilm formation capability. While conducting the research, the following materials were used: gentamicin 4% solution for injections produced by Darnitsa (Ukraine), penetrator produced by Himstatus (Ukraine), *Enterococcus* Agar produced by Farmactiv (Ukraine), trypticase soy broth produced by HiMedia (India).

To study the influence of compounds on biofilm formation, a photometric method in our modification was used [6]. Preparation of microorganisms suspensions with a specified concentration of microbial cells was performed on the McFarland scale using the Densi-La-Meter electronic device (PLIVA-Lachema Diagnostika, Czech Republic). The optical density (OD) of eluates from enterococci biofilms, stained with crystal violet, was measured with the SF-56L spectrophotometer at a wavelength of 590 nm.

Statistical processing of the obtained data was carried out by means of nonparametric statistical methods using Microsoft Excel 2007 and STATISTICA 6.0 programs. The validity of the differences between the two related samples was assessed by the Wilcoxon test and the Sign test.

The effect of the compound was evaluated using biofilm inhibition index (BII), which was calculated according to the formula: $[(OD \text{ positive control} - OD \text{ tested}) / OD \text{ positive control}] \times 100\%$. Reduction of the OD value by more than 25% in the experiment relative to OD positive control was considered as a positive effect (oppression of biofilm formation under the influence of the compound) [7].

Results and discussion

The impact on enterococci biofilm formation was investigated for the gentamicin at 64 mcg/ml, 32 mcg/ml, 16 mcg/ml, 4 mcg/ml concentrations and gentamicin at similar concentrations in combination with a penetrator with a volume fraction of 1,0%.

Analysis of the results showed that when applying gentamicin at concentration of 8 mcg/ml, the average value of the optical density of eluates significantly decreased compared to the control value and equaled $(0,398 \pm 0,02) OD_{590}$ against $(0,790 \pm 0,07) OD_{590}$ respectively ($p=0,008$). With a further increase in the concentration of gentamicin to 16 mcg/ml, 32 mcg/ml and 64 mcg/ml (Fig. 1), the average values of the optical density amounted to $(0,396 \pm 0,03) OD_{590}$, $(0,343 \pm 0,01) OD_{590}$ and $(0,365 \pm 0,02) OD_{590}$ respectively, and were also significantly different from the control value ($p < 0,05$).

It should be noted that the statistical analysis of the results did not show a significant difference between the parameters of the enterococci biofilm formation under influence of different concentrations of gentamicin ($p > 0,05$).

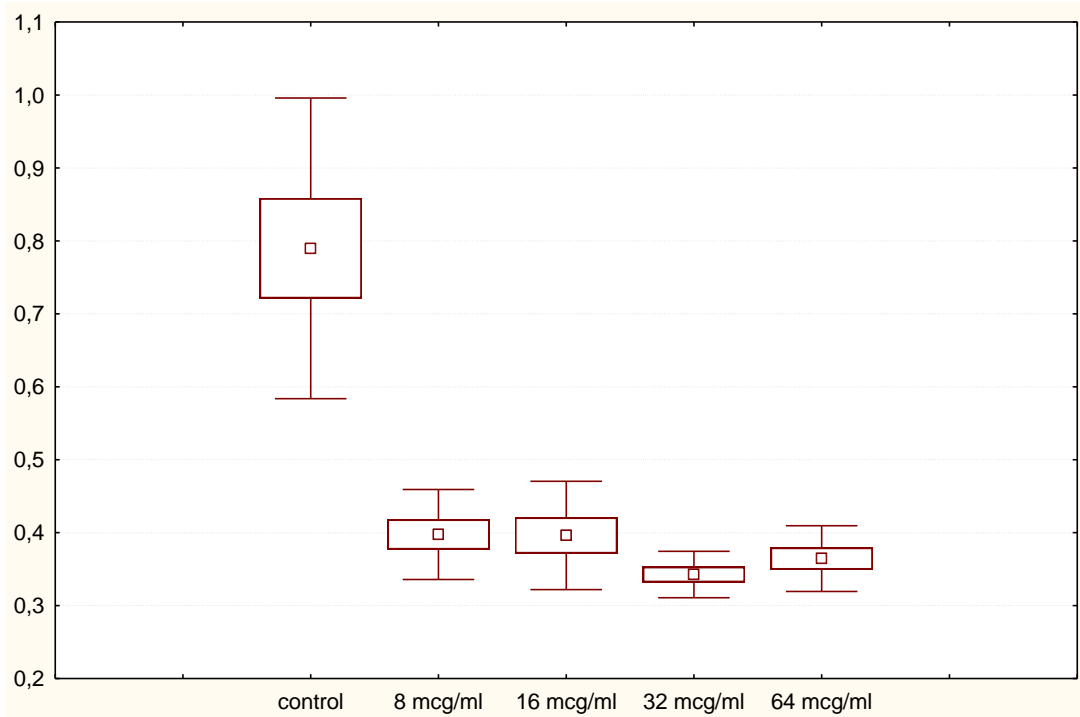


Fig. 1. Influence of gentamicin in various concentrations on enterococci biofilm formation

The inhibitory effect of gentamicin on biofilm formation was also confirmed by the inhibition index calculation. Thus, when applying gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml, the index of inhibition equaled 53,8%, 56,6%, 49,9% and 49,6% respectively. Statistical analysis did not show any significant difference between the inhibition indexes when using different concentrations of gentamicin ($p > 0,05$).

It should be noted that a higher inhibitory effect of gentamicin was identified for the formation of *E. faecium* biofilms than for *E. faecalis* ones. Thus, when applying gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml for *E. faecalis* biofilm formation, the inhibition index was equal to 45,5 %, 46,7 %, 49,6 % and 48,5 %, for *E. faecium* – 54,8 %, 53,5 %, 65,3 % and 60,2 % respectively (Fig. 2).

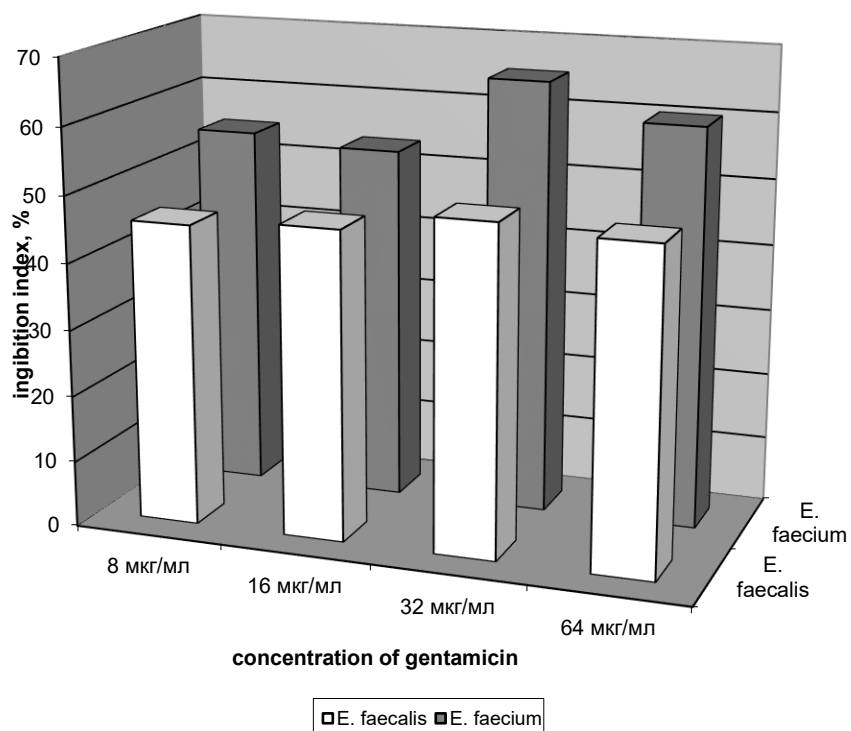


Fig. 2. Gentamicin inhibition index for *E. faecium* and *E. faecalis* biofilm formation

Analysis of the results allowed to conclude that gentamicin at concentration of 8 mcg/ml is capable of preventing the formation of biofilms taken in experiments with enterococci. Further increase in the concentration of gentamicin to 64 mcg/ml did not lead to an increase in its activity relative to biofilm formation. Some scientists believe that with an increase of antibiotic concentration, its activity is reduced by the induction of resistance mechanisms in bacteria. Recent studies have shown that the possible reason for this effect may be the release of enzymes in the lysis of bacterial cells, which leads to inhibition of bactericidal activity of the antibiotic [8, 9].

It is known that most microbial cells in biofilms suffer from the lack of oxygen and nutrients and are in a state of metabolic sleep [10]. Unlike some antibiotics, such as beta-lactams, the bactericidal effect of aminoglycosides (including gentamicin) does not depend on the growth phases of microorganisms, antibiotics in this group show high activity even with respect to microorganisms with low levels of metabolism. However, a negative fact is that aerobic conditions are needed for aminoglycosides activity, while the thickness of biofilm has practically anaerobic conditions, therefore, perhaps, gentamicin can only affect the microbial cells located in the upper layers of the biofilm.

One way of facilitating the transportation of biocides through the extracellular matrix of biofilms to the target of action may be the use of so-called penetrators. Polyethylene glycol – a compound widely used in pharmacy for the development of local medicines (ointments, eye drops, aerosols) as an osmotic agent and formoter (including for injection and tablet dosage forms) was used as a penetrator (PNT) in our studies. When applied at concentrations from 0,5 to 15,0%, polyethylene glycol can significantly increase the penetration of biologically active substances through biological membranes. Polyethylene glycol was not used directly as a means for microbial biofilms penetration, but there are some references to this effect in the literature [11].

Therefore, we studied the effect of combination of gentamicin and polyethylene glycol penetrator on enterococci biofilm formation. According to the research results, it was found that when using gentamicin at a concentration of 8 mcg/ml with 1,0% PNT, the mean value of the optical density of eluates significantly decreased compared to the control value and equaled $(0,172\pm 0,02)$ OD_{590} against $(0,790\pm 0,21)$ OD_{590} respectively ($p=0,008$) (Fig. 3.).

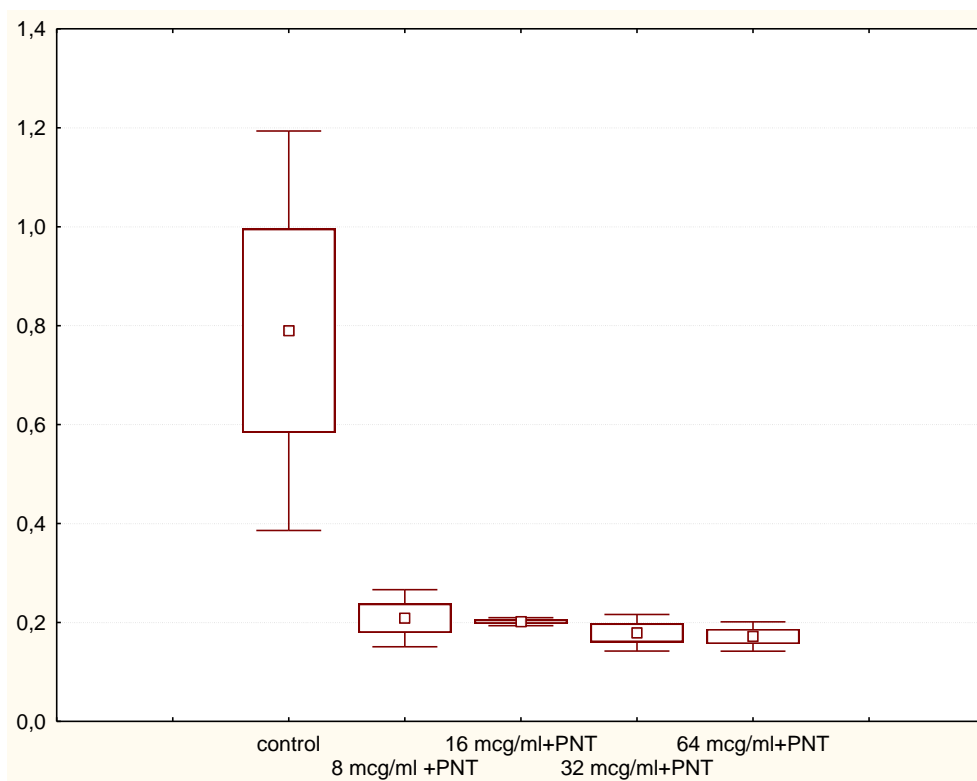


Fig. 3. Influence of gentamicin with 1,0 % PNT on enterococci biofilm formation

It should be noted that the average value of the optical density was also significantly lower compared to the same indicator when using gentamicin at a concentration of 8 mcg/ml without PNT ($p=0,008$). Similar patterns were identified when using gentamicin at concentrations of 16 mcg/ml, 32 mcg/ml and 64 mcg/ml with and without PNT (Fig. 4). Thus, the average value of

the optical density when using gentamicin at concentrations specified before with PNT equaled $(0,202\pm 0,01)$ OD_{590} , $(0,180\pm 0,02)$ OD_{590} and $(0,172\pm 0,02)$ OD_{590} respectively, without PNT – $(0,396\pm 0,03)$ OD_{590} , $(0,343\pm 0,01)$ OD_{590} and $(0,365\pm 0,02)$ OD_{590} ($p=0,008$).

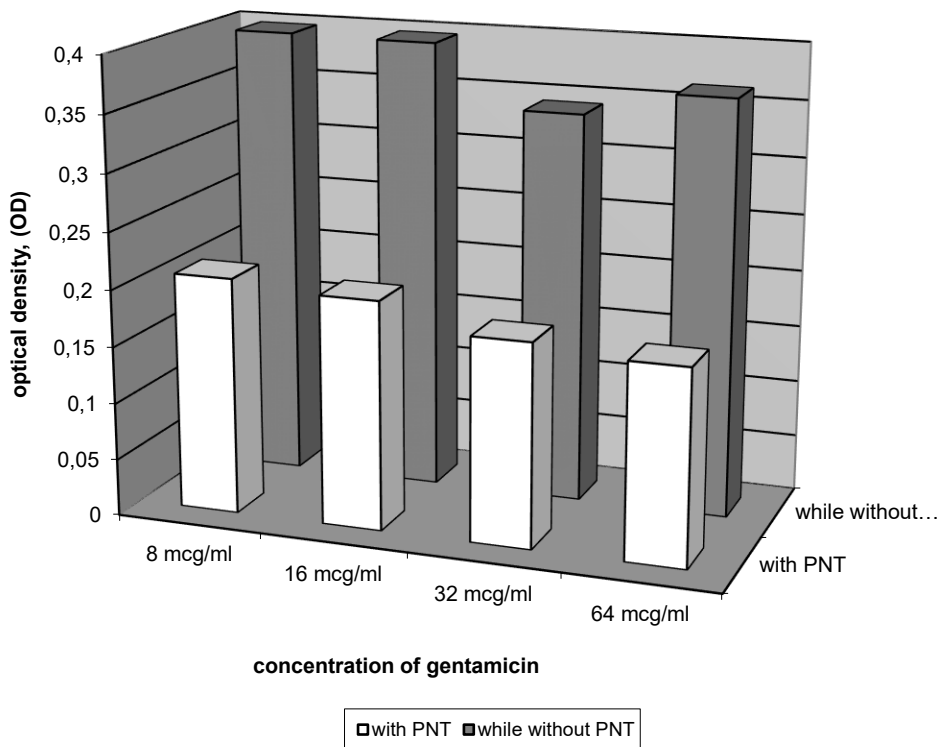


Fig. 4. Influence of gentamicin and gentamicin with 1,0 % PNT on enterococci biofilm formation

Inhibition index analysis showed a statistically significant increase in the suppressing effect of the combination of gentamicin and PNT on the enterococci biofilm formation compared to the effect of gentamicin without PNT ($p < 0,05$).

Thus, the inhibition index of gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml with PNT amounted to 73,5 %, 74,4 %, 77,2 % and 78,2 % respectively, while without PNT – 53,8 %, 56,6 %, 49,9 % and 49,6 % respectively.

Also, a higher suppressing effect of gentamicin with penetrator on enterococci biofilm formation was found for *E. faecium* compared to *E. faecalis*.

Thus, the results obtained suggest that polyethylene glycol can increase the penetration of gentamicin through the biofilm glycocalyx.

Conclusion

1. Gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml shows an inhibitory effect on enterococci biofilm formation (inhibition index varied from 49,6 % to 56,6 %).
2. Inhibitory effect of gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml on enterococci biofilm formation is enhanced under the influence of polyethylene glycol (inhibition index – from 73,5 % to 78,2 %).

References

1. Jensen P.O., Tolker-Nielsen T. Report from Eurobiofilms 2011 // Future Microbiol. 2011. N. 6. P. 1237-1245.

2. Sidorenko S.V. The role of bacterial biofilms in human pathology // Infections in surgery. 2012. N. 3. P. 16-20.
3. Percival S.L., McCarty S.M., Lipsky B. Biofilms and wounds: an overview of the evidence // Advances in Wound Care. 2015. Vol. 4. N. 7. P. 373-381.
4. Kudo M., Nomura T., Yomoda S., Tanimoto K., Tomita H. Nosocomial infection caused by vancomycin-susceptible multidrug-resistant *Enterococcus faecalis* over a long period in a university hospital in Japan // Microbiol Immunol. 2014. Vol. 58. N. 11. P. 607-614.
5. Furtado I., et al. *Enterococcus faecium* and *Enterococcus faecalis* in blood of newborns with suspected nosocomial infection // Rev. Inst. Med. Trop. Sao Paulo. 2014. Vol. 56. N. 1. P. 77-80.
6. Myronenko L.G., Peretyatko E.G., Iagniuik J.A. The study of Biofilm Formation of Enterococci isolated from Various Ecotopes // Bulletin of Biology and Medicine. 2015. Issue 3. Vol. 2 (123). P. 290-293
7. Bozhkova S.A., Krasnova M.V., Polyakova E.M., Rukina A.N., Shabanov V.V. In vitro biofilm formation of clinical strains of MRSA under the influence of antibiotics in blood sera // Traumatology and Orthopedics of Russia. 2015. N. 3. P. 92-107.
8. Tong Z., Zhang Y., Ling J., Ma J., Huang L., Zhang L. An *In Vitro* Study on the Effects of Nisin on the Antibacterial Activities of 18 Antibiotics against *Enterococcus faecalis*. PLoS One, 2014 <http://dx.doi.org/10.1371/journal.pone.0089209>.
9. Tanouchi Y., Pai A., Buchler N.E., You L. Programming stress-induced altruistic death in engineered bacteria // Mol. Syst. Biol. 2012. Vol. 8. P. 626. Published online 2012 Nov. 20. doi : 10.1038/msb.2012.
10. Gostev V.V., Sidorenko S.V. Bacterial biofilms and infections // Journal of Infectology. 2010. Vol. 2. N. 3. P. 4-15.

11. Du J., Bandara H.M., Du P., et al. Improved Biofilm Antimicrobial Activity of Polyethylene Glycol Conjugated Tobramycin Compared to Tobramycin in *Pseudomonas aeruginosa* Biofilms // *Mol Pharm.* 2015. May 4;12(5):1544-53. doi: 10.1021/mp500846u. Epub 2015 Apr 2.

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preventing the formation of biofilms taken in experiments with enterococci. Further increase in the concentration of gentamicin to 64 mcg/ml did not lead to an increase in its activity relative to biofilm formation. When applying gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml, the index of inhibition equaled 53,8%, 56,6%, 49,9% and 49,6% respectively. A higher inhibitory effect of gentamicin was identified for the formation of *E. faecium* biofilms than for *E. faecalis* ones. Thus, when applying gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml for *E. faecalis* biofilm formation, the inhibition index was equal to 45,5 %, 46,7 %, 49,6 % and 48,5 %, for *E. faecium* – 54,8 %, 53,5 %, 65,3 % and 60,2 % respectively. One way of facilitating the transportation of biocides through the extracellular matrix of biofilms to the target of action may be the use of so-called penetrators. Polyethylene glycol was used as a penetrator (PNT) in our studies. Inhibition index analysis showed a statistically significant increase in the suppressing effect of the combination of gentamicin and PNT on the enterococci biofilm formation compared to the effect of gentamicin without PNT ($p < 0,05$). The inhibition index of gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml with PNT amounted to 73,5 %, 74,4 %, 77,2 % and 78,2 % respectively. Also, a higher suppressing effect of gentamicin with penetrator on enterococci biofilm formation was found for *E. faecium* compared to *E. faecalis*. Thus, the results obtained suggest that polyethylene glycol can increase the penetration of gentamicin through the biofilm glycocalyx. **Conclusion.** Gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml shows an inhibitory effect on enterococci biofilm formation (inhibition index varied from 49,6 % to 56,6 %). Inhibitory effect of gentamicin at concentrations of 8 mcg/ml, 16 mcg/ml, 32 mcg/ml and 64 mcg/ml on enterococci biofilm formation is enhanced under the influence of polyethylene glycol (inhibition index – from 73,5 % to 78,2 %). **Keywords.** Gentamycin, *Enterococcus*, biofilm formation, Polyethylene Glycol - 400