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Abstract

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ANTIBACTERIAL INFLUENCE OF SILVER NANOPARTICLES ON MULTI-RESISTANT STRAINS OF *K. PNEUMONIAE* ISOLATED AT HOSPITALS

Introduction. Overuse and misuse of antibiotics in humans, animals, and agriculture has led to the widespread rise of antibiotic resistance and strengthened nosocomial pathogenes' impact. *Klebsiella pneumoniae* became an increasing threat to public health. Nanomaterials are promising alternatives to conventional antibiotics in the fight against multi-resistant germs. Silver nanoparticles are well-known metallic nanoparticles with antimicrobial activity.

Our research aimed to evaluate the spreading of *K. pneumonia* resistant to antibiotics at hospital and assess the effectiveness of Ag NPs against multi-resistant clinical strains of *K. pneumoniae*.

Material and methods. *K. pneumoniae* strains were isolated and identified with the use of conventional bacteriological techniques. Susceptibility of the microorganisms was assessed to inhibitors of β-lactamases, carbapenems, macrolides, oxazolidinones, and other groups of antibiotics with use Kirby-Bauer disk diffusion method. The capability of AgNPs to inhibit attachment and multiplication of the *K. pneumoniae* multi-resistant strains was tested with the use of serial microdilution method, resazurin assay, and SEM.

Results. *K. pneumoniae* was isolated from 13.7% of samples predominantly at the microbial association (97.5%). The microorganisms were resistant to five or more antibiotics in 73.2% of cases. AgNPs possess antimicrobial activity against tested strains at concentrations varied from 1.25 μg/ml to 2.5 μg/ml and kill all germs in 3 hours of incubation. AgNPs inhibited biofilm formation at initial stages and destroyed the mature (2 days) biofilm with Ag NPs treatment at concentrations 20-40 μg/ml. The effectiveness of mature *K. pneumoniae* biofilm treatment with AgNPs depended on biofilm age. The SEM images of the two-days biofilm reveal lysis of the bacterial cells after the cocultivation with Ag NPs but SEM analysis detected the maintaining of the three-dimensional structure in the case of a five-day biofilm after cocultivation with AgNPs.

Conclusions. The distribution of *K. pneumonia* among patients with laryngeal pathology and its sensitivity to eleven antibiotics were examined. There was revealed the high rate of *K. pneumonia* multi-resistant strains. Ag NPs have strong antibacterial and antibiofilm potential against multi-resistant *K. pneumoniae*. Therefore, our results highlight that the Ag NPs have promising antimicrobial and anti-biofilm abilities against multi-resistant clinical strains of *K. pneumoniae*.

Keywords: antimicrobial activity, silver nanoparticles, antibiotic, sensitivity, *K. pneumoniae*.

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Резюме

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АНТИБАКТЕРІАЛЬНИЙ ВПЛИВ НАНОЧАСТИНОК СРІБЛА НА ПОЛІРЕЗИСТЕНТНІ КЛІНІЧНІ ШТАМИ *K. PNEUMONIAE*

Вступ. Надмірне та неправильне використання антибіотиків для лікування людей, тварин та в сільському господарстві призвело до широкого зростання антибіотикорезистентності та посилення ролі внутрішньолікарняних патогенів. *Klebsiella pneumoniae* стає все більшою загрозою для здоров'я населення. Наноматеріали є перспективною альтернативою звичайним антибіотикам у боротьбі з мультирезистентними мікробами. Наночастинки срібла є добре відомими металеві наночастинки з антимікробною активністю.

Метою нашого дослідження було оцінити поширення стійких до антибіотиків штамів *K. pneumonia* серед пацієнтів та оцінити антимікробну ефективність НЧ Ag щодо мультирезистентних клінічних штамів *K. pneumonia*.

Матеріали та методи. Штами *K. pneumonia* були виділені та ідентифіковані з використанням класичного бактеріологічного методу. Чутливість мікроорганізмів до інгібіторів β-лактамаз, карбопенемів, макролідів, оксазолідонів та інших груп антибіотиків оцінювали диско-дифузійним методом Кірбі-Бауера. Здатність НЧ Ag пригнічувати прикріплення та розмноження мультирезистентних штамів *K. pneumoniae* перевіряли із використанням методу серійних розведень, тесту на редукцію резазурину та СЕМ.

Результати. *K. pneumonia* було виділено з 13,7 % зразків, переважно в мікробних асоціаціях (97,5 %). У 73,2 % випадків мікроорганізми були стійкі до п'яти та більше антибіотиків. НЧ Ag демонстрували антимікробну активність проти досліджуваних штамів у концентрації від 1,25 мкг/мл до 2,5 мкг/мл та спричинювали загибель усі мікроорганізмів через 3 години інкубації. НЧ Ag інгібували утворення біоплівок на початкових етапах та руйнували зрілу (2-х денну) біоплівку у концентрації 20–40 мкг/мл. Ефективність антибіоплівкової дії срібла залежала від віку плівок , утворених *K. pneumoniae*. На СЕМ зображеннях 2-х денних плівок наявний лізис бактеріальних клітин, у той же час аналіз 5-ти денних біоплівок виявив збереження трьохмірної структури.

Висновки. У цьому дослідженні вивчено поширення *K. pneumoniae* серед пацієнтів з ларингологічною патологією та визначено чутливість мікроорганізмів до одинадцяти антибіотиків. Виявлено високий рівень мультирезистентності серед штамів

K. pneumonia. НЧ Ag мають потужний антибактеріальний та антибіоплівковий потенціал проти мультирезистентних *K. pneumoniae*. Таким чином, наші результати підкреслюють, що НЧ Ag мають багатообіцяючі антимікробні та антибіоплівкові властивості проти мультирезистентних клінічних штамів *K. pneumoniae*.

Ключові слова: антимікробна активність, наночастинки срібла, антибіотик, чутливість, *K. pneumoniae*.

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Introduction/Вступ

Klebsiella pneumonia is one of the increasingly challenging species among *Enterobacteria spp*. The bacteria are often detected in foods, sewage, soil, plants, and the gastrointestinal tracts of animals [1]. It takes the second position after *E. coli* as a pathogen responsible for various community-associated and nosocomial infections [2]. The pathogen is responsible for a comprehensive list of human diseases ranging from wound infections to septicemia, and these infections may impact human health. Inadequate use of antibiotics facilitates the formation of broad-spectrum drug resistance among microorganisms. It leads to a rise in hospital-associated infections numbers, and it increases the mortality and financial burden on public health as well [3]. Several studies indicate *K. pneumoniae* is associated with high rates of antibiotic resistance [4]. In spite of the importance *K. pneumonia* as nosocomial pathogen, the data about its meaning as a causative agent and susceptibility to antimicrobial substances are limited [5]. It is also known as a pathogen with a high capability to biofilm formation due to various virulence factors [6]. Infections caused by biofilm-forming bacteria are difficult to treat; therefore, searching for new antimicrobials with antibiofilm activity is a hot topic for public health.

Applying new approaches and new materials in medical practice is crucial to dealing with antibiotic resistance. The antimicrobial properties of metals have been known for a long time. However, their use as antimicrobials was limited due to their toxicity. Due to the development of nanomedicine, the interest in nanometals, especially silver and its salts, has increased. Silver nanoparticles possess several antimicrobial mechanisms; thus, bacteria can form the resistance slightly [7]. Apart from that, the metals are stable under conditions currently found in the industry. Over the past few decades, there was a huge rise in information on AgNPs antimicrobial activity [8]. However, several unresolved issues still stay. There are several drawbacks such as an easy aggregation of nanoparticles, the uncontrolled release of silver ions, and poor stability of solution that need to be solved.

Thus, the purpose of our research was to evaluate the spreading of *K. pneumoniae* strains resistant to antibiotics at hospitals and assess the antimicrobial effectiveness of the Ag NPs against them.

Material and methods. During 2019–2021, we examined 300 samples from patients with laryngeal pathology. Patients were comprehensively examined following standards of care. To determine the microbial profile of the examined biotopes microbiological study of nasopharyngeal smears was performed in the bacteriological lab of Sumy State University. The sensitivity of the microbes to antibiotics was assessed with the use Kirby-Bauer method on Mulller-Hinton agar. Antimicrobial susceptibility was assessed to inhibitors of β-lactamases, carbapenems, macrolides, oxazolidinones, and other groups with the use of the recommendation of the National Committee for Clinical Laboratory Standards. To evaluate the influence of silver nanoparticles on the *K. pneumoniae* vitability and biofilm formation, we selected multi-resistant microorganisms.

The AgNPs were prepared at NanoWave (Warsaw, Gdansk, Poland) and previosly described [9] at 3 g/L silver concentration. Nanoparticles were cube-shaped with size from 80 nm to 800 nm and smaller silver nanoparticles of spherical shape adhered on it. EDX confirmed the chemical purity of obtained AgNPs with a residual quantity of Cl and Na.

The silver nanoparticles' antimicrobial activity against *K. pneumoniae* strains was examined with determination of the minimal inhibitory concentration (MIC), the kill kinetic, inhibition of biofilm formation, and influence on biofilm viability.

MIC was assessed with the use of the broth microdilution method. At first, the serial dilutions of the AgNPs were prepared, and 20 µl of each concentration was put into a polystyrene 96-well plate. Inoculum of the fresh cultures of *K. pneumoniae* was prepared in Mueller-Hinton broth at concentration 5×10^5 CFU/ml. Then 180 µl of bacterial suspension was added to the silver solution to reach the desired concentrations (0.31–40 µg/ml). Plates were incubated at 37 ºC for 24 h. The assays were performed in triplicate. The MIC was the lowest concentration of the AgNPs completely inhibited visual growth of germs.

The time-dependent dynamic of bacteria-killing was conducted at bacteria concentration 5×10^5 CFU/ml and AgNPs concentration equal MIC. Bacteria incubation in Muller-Hinton broth at 37 ºC has followed the aliquots (10 µl) inoculation from each well onto Mueller-Hinton agar in 1, 3, 6, 12, 24 hours. Then formed colonies were counted.

The influence of AgNPs on biofilm formation was detected by assessing the formed biofilm biomass with gentian violet staining. The bacteria were incubated in 96-well microtiter plates containing AgNPs solutions at the half and 1 MIC concentrations for 24 hours. Then non-adherent cells were removed from the plate, and the biofilm mass attached to the wells was stained with 0.1% crystal violet (30 minutes). After that, the dye was dissolved with ethanol. The optical density of solubilized crystal violet was measured by the Thermo Scientific Multiscan FC microplate photometer ESW 1.01.16 (wavelength 595 nm). All tests were done in triplicate. The coefficient of the microbial biomass reduction was calculated as a proportion of the optical density of the tested sample to the optical density of control in the percentage equivalent.

The microorganisms were incubated initially for different time intervals (48 and 120 hours) to examine the silver nanoparticles effect on preformed biofilms. Then AgNPs at concentrations 10, 20, 30, and 40 µg/ml were added to the wells and incubated for 24 h. It was followed the resazurin assay and crystal violet staining. The percentage of cell viability was calculated according with protocol provided by manufacturer.

AgNPs action on the biofilms structures was assessed with SEM. The glass slides were submerged into inoculums of *K. pneumoniae* in Mueller-Hinton broth and were incubated for 0, 48, 120 h. After that, glass slides were incubated with AgNPs at concentrations 10, 20, 30, and 40 µg/ml for 24 h. Then they were fixed in 2.5% glutaraldehyde, washed in buffer, and dehydrated with a series of 50, 70, 90, and 100% ethanol. The glass slides were coated with silver and examined under scanning electron microscopy (SEM, Hitachi S-3000N).

One-way ANOVA multiple comparisons with the Tukey's post-hoc analysis was used to assess the difference between groups using GraphPad Prism 8.0 software. p value of < 0.05 was considered statistically significant.

Results. We have examined 300 samples from patients with laryngeal pathology and isolated 41 strains of *K. pneumoniae* (13.7%). The microorganisms were isolated predominantly in a microbial association in 97.5%. The *K. pneumoniae* was associated with gram-positive (*Staphylococcus spp., S. pyogenes*), gram-negative (*Enterobacrerium spp., P. aeruginosa*) bacteria, and *C.albicans*. According to antibiotic resistance profiles (Fig. 1), all tested strains of *K. pneumoniae* were susceptible to Levofloxacin and Imipenem. The biggest amount of *K. pneumoniae* strains were resistant to Azitromycin (9.1%). Analyses of antibiotic-resistant profile revealed that 73.2% of isolates were resistant against five or more antibiotics, and almost a quarter of strains was resistant to carbapenem (Imipenem).

The evaluation of the AgNPs antimicrobial activity against the tested strains revealed the MIC varied from 1.25 µg/ml to 2.5 µg/ml. The increase of AgNPs concentration in 2 times caused the total killing of microbes. The time-kill kinetics profile of AgNPs against *K. pneumoniae* at MICs demonstrates a gradual drop of the viable cells numbers over the experimental time. The numbers of bacteria cells reach 0 log10 CFU/ml to 3 h incubation (Fig. 2)

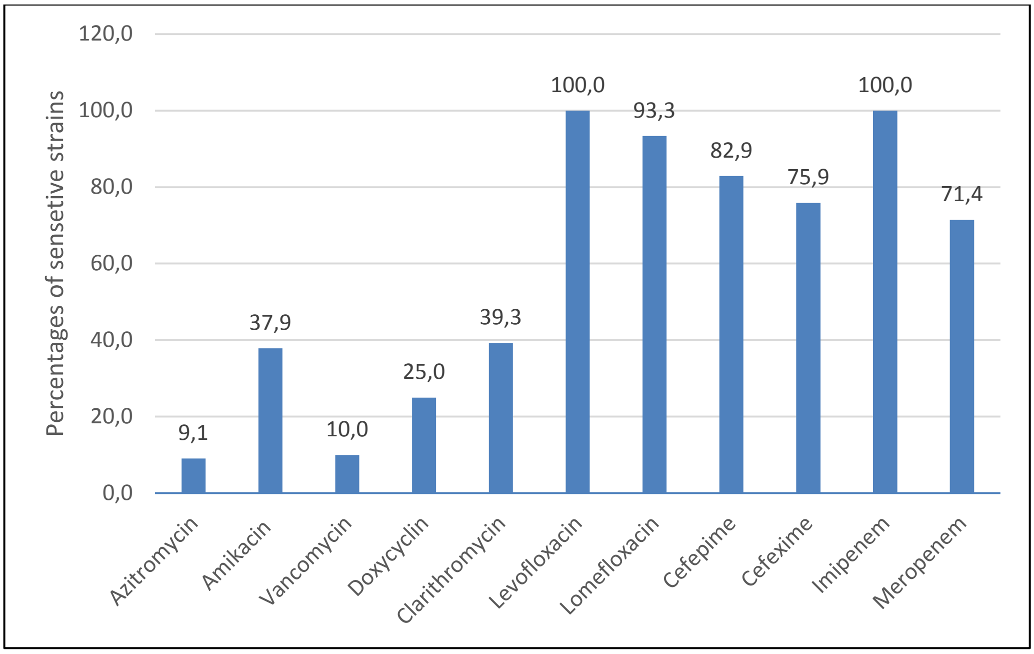

Figure 1 – K. pneumoniae antibiotics susceptibility profile

Examination of the antibiofilm effectiveness for AgNPs at 0.5 and 1 MIC was performed on the initial attachment and maturation stages. Evaluation of the AgNPs' ability to inhibit adhesion and a biofilm formation with *K. pneumoniae* at concentrations 0.5 and 1.0 MIC on the initial stage showed a slight decrease in the biofilm mass and quantity of living cells compared with non-treated bacteria (Fig. 3).

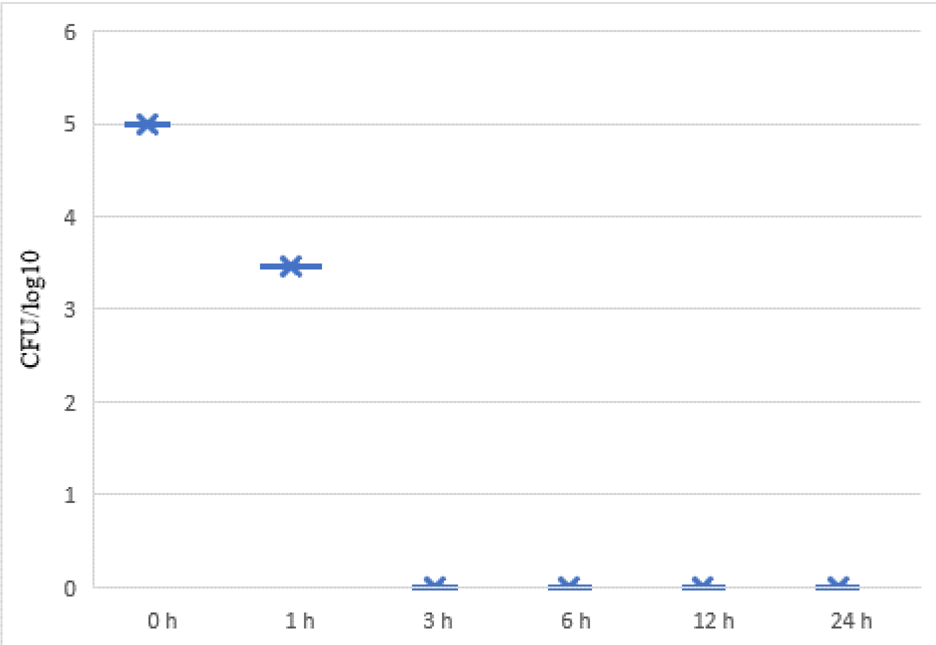

Figure 2 – Time-dependent bactericidal activity of the tested silver nanoparticles against K. pneumoniae

The effectiveness of mature *K. pneumoniae* biofilm treatment with AgNPs depended on biofilm age (Fig.4). It was found the decrease of the biofilm mass by an average of 60% at concentration 20–40 µg/ml. However, there were no differences in *K. pneumoniae* biofilm mass and cell viability on five days biofilm.

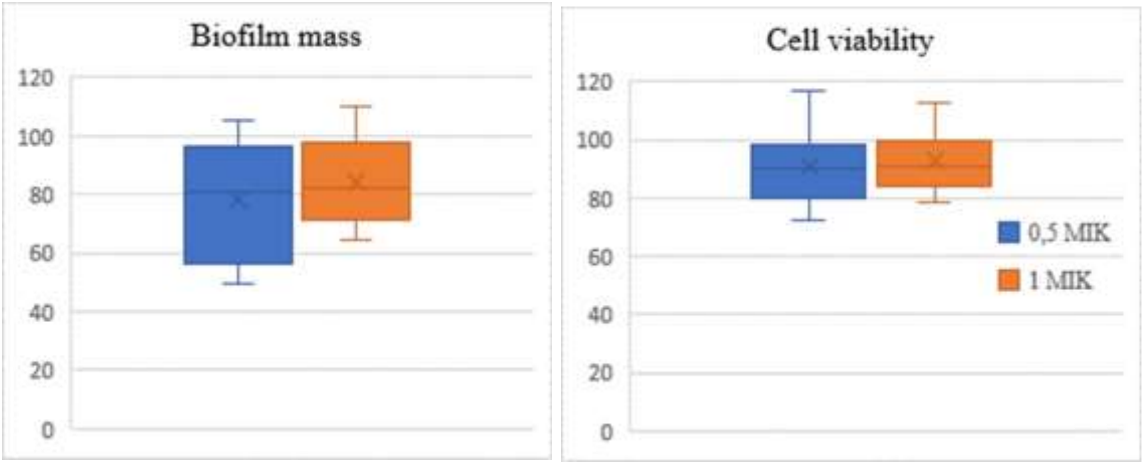

Figure 3 – The percentage of K.pneumoniae biofilm mass and cell viability after treatment with AgNPs at concentration 0.5 and 1.0 MIC on initial stage

The SEM micrographs (Fig. 5) of initial stage biofilm formation at the control group show bacteria adhering tightly to each other. Observation of the bacterial clusters at second- and fifth-day biofilm showed bacteria tethered to each other by a fibrillar network composed of copious amounts of long, flexible pili that extended several microns away from the bacteria.

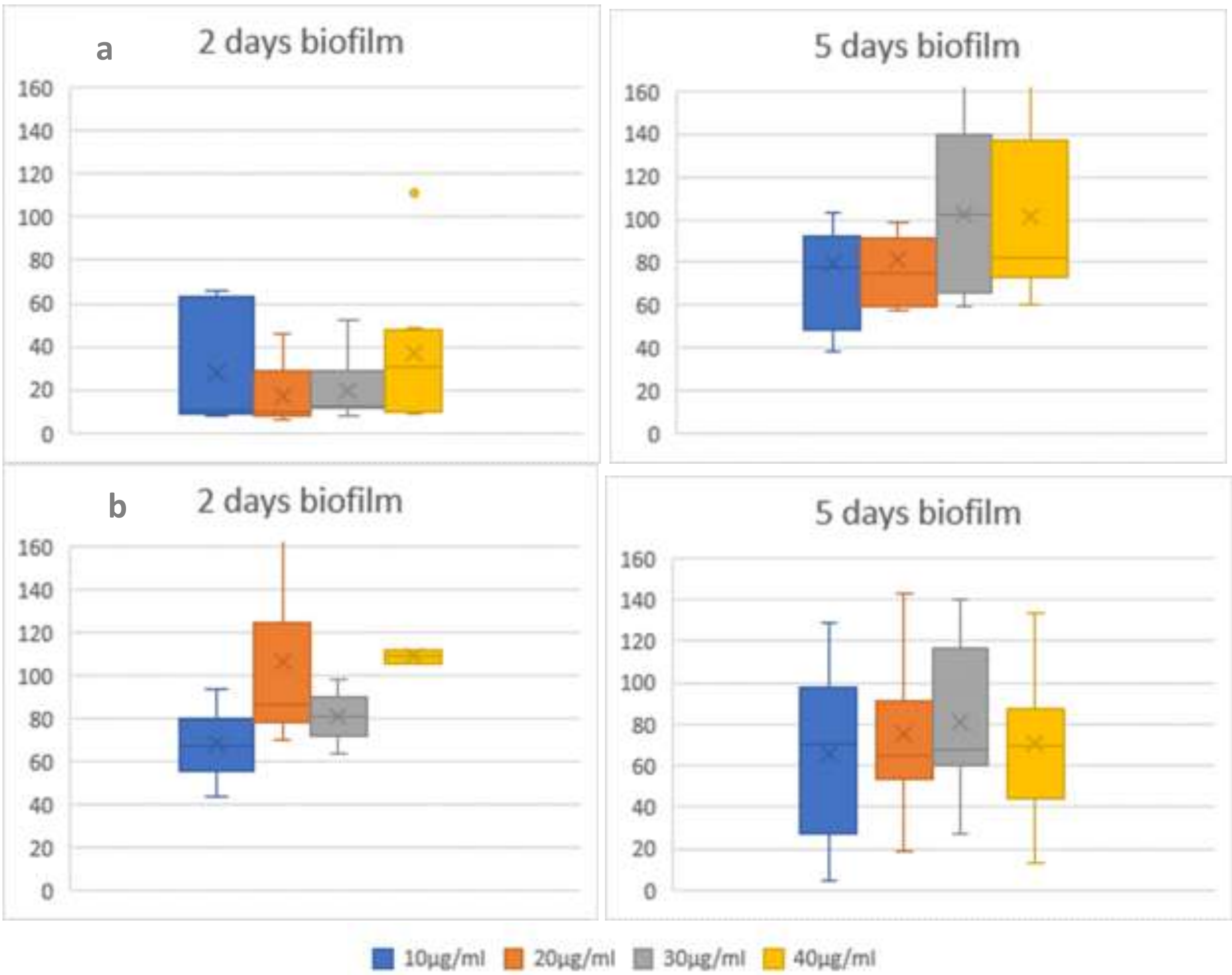

Figure 4 – The percentage of K.pneumoniae biofilm mass (a) and cell viability (b) after mature biofilm treatment with AgNPs

The obtained micrograph indicates a considerable reduction in biofilm in the initial attachment stage. In the case of primary cocultivation of the AgNPs and *K. pneumoniae* we detected only the single bacterial cells attached to the surface. These data show that AgNPs decreased the biofilm-forming ability. It could be caused by inhibition of bacteria multiplication or bacterial adhesion.

The images of the two-day biofilm reveal lysis of the bacterial cells after the cocultivation with Ag NPs. The membrane integrity of *K. pneumoniae* was violated in the presence of AgNPs. SEM analysis detected the maintaining of the three-dimensional structure in the case of a five-day biofilm after co-cultivation with AgNPs.

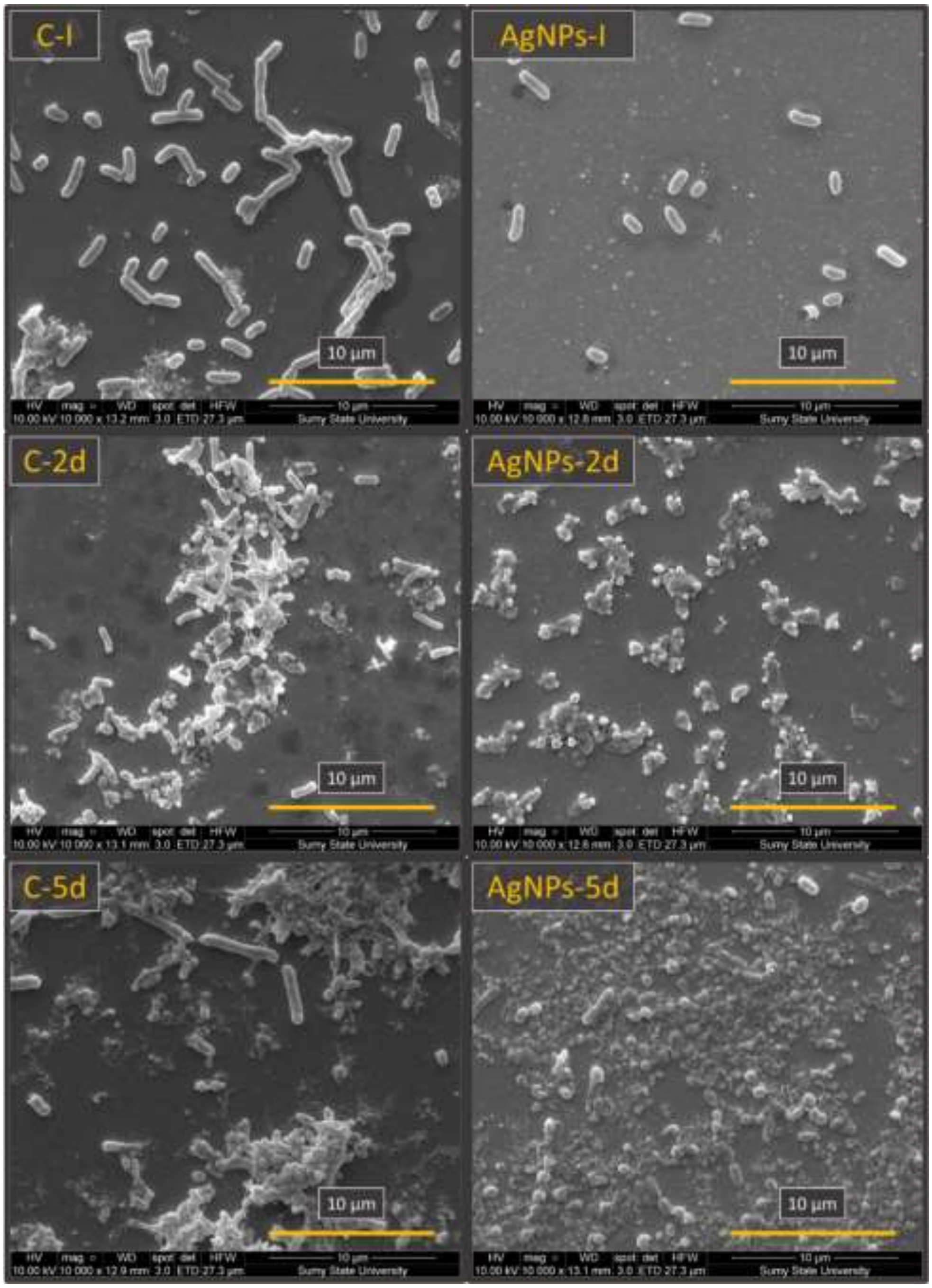

Figure 5 – Scanning electron micrographs of K. pneumonia biofilms formed on the glass (37 °C) at different time intervals after 24 h (0d), 48 h (2d) and 120 h (5d)
K – Control, AgNPs - treated with 20 µg/ml of silver nanoparticles

Disscussion. Many studies indicated the threat of antimicrobial resistance as a complex affected patients, healthcare, and economy in all countries [10]. The ESKAPE pathogens cause concern due to their capacity to escape from antimicrobial therapy, increased duration of treatment, and financial burden on public health [11]. Currently, *K. pneumoniae* is one of the most important species among gram-negative microbes of this group. In this work, we have examined the distribution of *K. pneumoniae* strains among patients with laryngeal pathology. We isolated the bacteria mostly in association from 13.7% of samples. Three-quarters of isolates were multi-resistant. Hera Nirvati et al. [12] isolated a bit higher numbers of *K. pneumoniae* (17.36%), but there was a lower level of antibiotic resistance (54.49%).

According to Hemeg [13], Ag NPs possess a greater surface area, leading to a more controlled release of Ag^{+} and could be a promising antibacterial compound. We have examined the cubic shape AgNPs on the antimicrobial activity against the multi-resistant clinical strains of *K. pneumoniae*. It was revealed the antibacterial effectiveness of AgNPs at concentration 1.25–2.5 µg/ml in 3 hours of cocultivation with *K. pneumoniae*. MIC of tested AgNPs was lower than it was reported by Pareek et al. [14].

K. pneumoniae possesses the ability to form biofilms that is central to their pathogenicity. A sticky capsule and several adhesive fimbrial or non-fimbrial adhesins are essential to biofilms formation [15]. The investigation of AgNPs anti-biofilm activity showed its slight inhibition effect on biofilm formation at initial stages. We have also detected the destruction of the mature (2 days) biofilm with Ag NPs treatment at concentrations 20–40 µg/ml. The AgNPs used in our experiment demonstrated higher effectiveness than G. Rajivgandhi et al. reported it [16], who describe the *K. pneumoniae* antibiofilm effectiveness at concentration 50–100 µg/ml. It is known that Ag NPs interact with the microbial surface and may lead to the disruption of the cell membrane. Our SEM data confirm this on a two-day biofilm, but the absence of AgNPs effectiveness was detected against a five-day biofilm. Obviously, there is a need to further investigate the mechanisms and substances that protect *K. pneumoniae* mature biofilm.

Conclusions/Висновки

In this study, the distribution of the *K. pneumoniae* among patients with laryngeal pathology and its sensitivity to eleven antibiotics was examined. There was revealed the high rate (73.2%) of *K. pneumonia* multi-resistant strains. Ag NPs has strong antibacterial and antibiofilm potential against multi-resistant *K. pneumoniae*. Therefore, our results highlight that the Ag NPs have promising antimicrobial and anti-biofilm abilities against multi-resistant clinical strains of *K. pneumoniae*.

Prospects for future research/Перспективи подальших досліджень

The further investigation of the AgNPs effect on the mature biofilms formed by *K. pneumoniae* will provide us with new knowledge.

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Conflict of interest/Конфлікт інтересів

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