Prolonged Exposure to Antimicrobials Induces Changes in Susceptibility to Antibiotics, Biofilm Formation and Pathogenicity in *Staphylococcus aureus*

Mbarga M. J. Arsene¹, Podoprigora I. Viktorovna¹, Volina E. Grigorievna¹, Anyutoulou K. L. Davares¹, Das M. Sergeeva¹ and Sharova I. Nikolaevna¹

¹Department of Microbiology and Virology, Institute of Medicine, RUDN University, Moscow, Russia.

Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i34B31856

Editor(s): (1) Q. Ping Dou, Wayne State University, USA. (2) P. Veera Muthumari, V.V. Vanniaperumal College for Women, India. (3) Giuseppe Murdaca, University of Genoa, Italy.

Reviewers: (1) Larissa Alves Lopes de Souza, Federal University of Ceará, Brazil. (2) A. Ranganadha Reddy, VFSTR University, India.

Complete Peer review History: http://www.sdiarticle4.com/review-history/70505

Received 25 April 2021
Accepted 01 July 2021
Published 02 July 2021

Original Research Article

**ABSTRACT**

**Introduction:** Frequent exposure to certain biocidal agents such as hypochlorous acid (HOCl), triclosan and benzalkonium chloride (BAC) has been reported to induce significant changes in *Staphylococcus aureus*. However, very few studies of this type have been conducted with conventional antimicrobials.

**Aim:** The current investigation aimed to explore the phenotypic changes (susceptibility to antibiotics, biofilm formation and relative pathogenicity) that occur in *S. aureus* after recurrent exposure to antimicrobials.

**Methods:** We compared the effects of long-term exposure to ampicillin, cefazoline, kanamycin and silver nanoparticles (AgNPs) on their susceptibility to antibiotics, biofilm formation, growth rate and pathogenicity in *Staphylococcus aureus* ATCC 6538. The minimum inhibitory concentrations (MIC) were determined using the microplate microdilution method and the bacteria were exposed to increasing concentrations of each antimicrobial (MIC/2 to MIC) prepared in the BHIB for 8 days.

*Corresponding author: E-mail: josepharsenembarga@yahoo.fr;*
The sensitivity of bacteria to antibiotics was assessed using the Kirby-Bauer disc diffusion method, the biofilm formation with crystal violet bacterial attachment assay and relative pathogenicity was assessed through a Galleria mellonella waxworm model.

**Results:** The data in this investigation indicate that long-term exposure to antimicrobials may induce several changes in *S. aureus*. The exposure to ampicillin induced resistance to ceftazidime, tetracycline and ceftriaxone while the susceptibility to ceftazidime decreased in bacteria exposed to cefazolin and Kanamycin. Meanwhile, exposure to AgNPs induced some changes in susceptibility to trimethoprim and ceftazidime without causing resistance. Similarly, the strains exposed to ampicillin and kanamycin grew more rapidly and produced more biofilms than the control strains whereas the strains exposed to the AgNPs produced less biofilms. On *G. melonella* model, cefazolin seems to have attenuated the pathogenicity while the 3 other strains were more pathogenic than the controls.

**Conclusion:** Long term exposure of *S. aureus* to antibiotics and AgNPs induces several changes in susceptibility to other antibiotics, growth rate, biofilm formation and pathogenicity; and these changes should be taken into account when choosing antibiotics for treatment of diseases caused by *S. aureus*.

**Keywords:** Antibiotic resistance; staphylococcus aureus; exposure; silver nanoparticles; kanamycin; ampicillin; cefazolin; biofilm; adaptation.

1. **INTRODUCTION**

Infectious diseases are among the most prevalent causes of human death worldwide [1]. These infections are more serious due to the growth of antibiotic resistance worldwide. Recent estimates have shown that antibiotic resistance is responsible for 700,000 annual deaths worldwide, 230,000 of which have resulted from multidrug-resistant tuberculosis [2,3]. The World Health Organization estimates that if nothing is done to address this problem, drug-resistant diseases may cause 10 million deaths each year by 2050 and damage to the economy as catastrophic as the 2008-2009 global financial crisis [2]. Furthermore, economically (linked directly or not to agriculture and animal breeding), antimicrobial resistance could force up to 24 million people into extreme poverty by 2030 [2].

*Staphylococcus aureus* is one of the most common Gram-positive human pathogens that causes an array of infections ranging from minor skin infections and food poisoning to more serious infections including toxic shock syndrome, osteomyelitis, endocarditis, necrotizing pneumonia, and sepsis [4,5]. *S. aureus* owes its pathogenicity to various virulence factors that allow it to escape the host's immune system [5]. Among these virulence factors, the best known are leucocidin ED (LukED), exfoliative toxins, *Staphylococcal* protein A, enterotoxins, immune-modulatory factors and *Staphylococcal* accessory regulator gene sarA which controls several virulence determinants, including biofilm formation, hemolysins, and DNase [5-7]. In addition, *S. aureus* is notorious for its ability to acquire and/or develop resistance to antibiotics [5]. The acquisition mechanism of resistance in *S. aureus* is identical to that of other bacteria and this is mainly due to the enzymatic degradation of antibiotics, the modification of the target of the antibiotic, the change in membrane permeability and use of efflux pump, alternative metabolic pathways and interbacterial transmission of resistance genes through the horizontal transfer [8-14]. This attribute coupled with the high burden of *S. aureus* infections is a serious problem for treatment of staphylococcal infections [9].

However, it has been reported that exposure of *S. aureus* to sub-minimal inhibitory concentrations (sub-MICs) of antibiotics can induce considerable changes in the expression of virulence genes [5,15-20]. During treatment, bacteria exposed to sub-MICs of antibiotics can become resistant, more pathogenic and can interfere with treatment or possibly make it difficult for potential future infections [5]. Therefore, the choice of antibiotics should consider the possibility of the above-mentioned changes in order to prevent them.

The present investigation aims to assess effects of long-term exposure to ampicillin, cefazoline, kanamycin and silver nanoparticles (AgNPs) on susceptibility to antibiotics, biofilm formation, growth rate and pathogenicity in *Staphylococcus aureus*. 
2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

In this investigation, we used the standard strains *Staphylococcus aureus* ATCC 6538 provided by the laboratory of microbiology and virology of the Peoples’ Friendship University of Russia. All the cultures were made on BHIB (Brain Heart Infusion Broth) (HiMedia™ Laboratories Pvt. Ltd., India) and Muller Hinton Agar (MHA HiMedia™ Laboratories Pvt. Ltd., India) and incubated aerobically at 37°C for 18-24 h.

2.2 Stock Solutions of Antibiotics and AgNPs

Stock solutions of antimicrobial were each prepared at a concentration of 1024 µg/ml and dilutions were made as required. Ampicillin, cefazolin, and kanamycin were prepared in physiological water (NaCl 0.9%) and 2 nm silver nanoparticles (Nanoserebro Argitos, OOO NPP Sintek Nano, Russia) were prepared in distilled water. All the solutions were sterilized by microfiltration (0.45 µm) prior to use.

2.3 Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC and MBC were determined as described previously [21]. Briefly, 5-ml overnight cultures of test bacteria were prepared in BHIB prior to overnight incubation (18 to 24 h) at 37°C. Cultures were centrifugated and diluted to a visual turbidity equivalent to a 0.5 McFarland, then the stock solutions of antimicrobials (100 µL) above mentioned were submitted to serial twofold dilutions in sterile BHIB (100 µL) on U-bottom 96-well microplates before the addition of the bacterial inoculum (10 µL). The plates were incubated overnight (18 to 24 h) at 37°C. The MIC was defined as the lowest concentration of antimicrobial at which growth was completely inhibited. After incubation, MIC was considered the lowest concentration of the tested material that inhibited the visible growth of bacteria. Furthermore, MBCs were determined by subculturing the wells without visible growth (with concentrations ≥ MIC) on MHA plates. Inoculated agar plates were incubated at 37°C for 24 h. MBC was considered the lowest concentration that did not give any bacterial growth on agar.

2.4 Long-Term Exposure of Bacteria to Antibiotics and Silver Nanoparticles

Bacteria were exposed to increasing concentrations of ampicillin, cefazolin, kanamycin and AgNPs using U-bottom 96-well microplates. 8 concentrations of antimicrobial were each prepared in sterile BHIB then the mixture was sterilized by microfiltration prior to use. The concentrations varied from MIC/2 to MIC with an increment of MIC/16. For each antimicrobial, 200 µL of the preparation of MIC/2 concentration was introduced into line 1 of the microplate, then MIC/2 + 2MIC/16 in line 2, MIC/2 + 3MIC/16 in line 3... and MIC in line 8. 15 µL of overnight culture of *S. aureus* ATCC 6538 prepared at a concentration equivalent to 0.5 of McFarland was inoculated in the first line and after 24 h of incubation at 37°C, the wells of line 1 were homogenized and 15 µL was transferred to line 2 of the corresponding columns and the same operation was repeated for the following lines until the 8th day [21]. Bacteria that underwent passaging 8 times on antimicrobial-free medium were also included and considered as the controls. During incubation, the microplates were placed in a container containing distilled water to limit water loss by evaporation. After successive passages, the bacteria were kept at ~80°C in cryovials (Cryoinstant; Deltalab, Spain) for subsequent testing.

2.5 Sensitivity of Bacteria to Antibiotics

The modified Kirby-Bauer’s disc method described in our previous study [22] was used to assess the sensitivity to antibiotics of the original *S. aureus* ATCC 6538 and the mutant’s strains obtained. Briefly, after bringing the bacteria to room temperature (25°C), they were cultured at 37°C for 24 hours in sterile BHIB. 1.5ml of each overnight culture was centrifuged (Eppendorf Centrifuge 5415 R) for 10 minutes at 3000 RCF and the centrifugate was collected, washed 3 times with Phosphate buffer saline (PBS) and resuspended in 5ml of physiological water to obtain a concentration equivalent to 0.5 McFarland. 100µL of the culture was plated on Muller Hinton Agar (MHA) (HIMEDIA®, Ref 173-500G) and the antibiotic discs were placed aseptically using a dispenser. After 18-24 hours of incubation, the inhibition diameters were measured and interpreted referred to the Clinical & Laboratory Standards Institute [23]. The petri dishes were again incubated for 48 hours at 37°C and the bacteria of the second growth in the inhibition zones were isolated and subjected.
to a second antibiogram as described above. The 8 antibiotics used were: tetracyclin (TE), 30 µg/disc, cefazolin/ clavulanic acid (CAC), 30/10 per disc; ceftazidime (CAZ), 30 µg/disc; ceftriaxone (CTR), 30 µg/disc; ciprofloxacin (CIP), 30 µg/disc; imipenem (IMP), 10 µg/disc; nitrofurantoin (NIT), 200 µg/disc and trimethoprim (TR), 30 µg/disc [21].

2.6 Evaluation of Biofilm Formation by Crystal Violet Bacterial Attachment Assay

The biofilm formation of original strain and exposed strains was assessed in sterile 96-well microtiter plate. 200 µL of sterile BHI was introduced in each well and was inoculated by the corresponding overnight culture (18 to 24 h at 37°C and 100 rpm) centrifugated and resuspended in physiological water to obtain a visual turbidity equivalent to 0.5 of McFarland as described above. Sterile controls were also included. The plates were incubated statically for 48 h at 37°C. 100 µL of the medium was transferred in the corresponding well in another microtiter plate for planktonic measurement. The remaining medium was removed from the wells and replaced with 200µL of 1% (w/v) crystal violet solution during 90s. The wells were rinsed three times with distilled water prior to drying at 37°C. The biofilm-bound crystal violet was solubilized in 200 µL of 100% ethanol and the A450 was determined using microplate reader (Uniplan, ZAO Pikon, Moscow, Russia) and compared with the negative controls. The negative control was the well with the BHI free of microorganisms. Each test was repeated 6 times and each repeat was read 3 times.

2.7 Planktonic Measurement

Free bacteria were assessed simultaneously with the biofilm assay. The 100 µL of medium transferred to another microtiter plate during the biofilm formation test were diluted with 100 µL of physiological water. Free bacteria were evaluated by determining A450 with microplate reader (Uniplan, ZAO Pikon, Moscow, Russia) and compared with the negative controls (with the BHI free of microorganisms).

2.8 Filtered Cells Ono 0,45 µm Pore Size Filters

It has been reported that S. aureus are able to decrease their size to cope with stress conditions [24]. This change in size was evaluated by filtration as previously reported [24]. Briefly, the original S. aureus ATCC 6538 strain, the strain passed in the BHI free of antimicrobials and the 4 mutant bacteria resulting from the exposure to the antimicrobials were cultured in the BHI as described above then centrifuged and washed in PBS and finally resuspended in PBS. The suspension was then filtered through a 0,45-lm pore size filters (Millipore Corp., Bedford, MA, USA) and culturable bacteria in the filtrate were assayed by plating on BHI plates after serial dilutions.

2.9 Galleria mellonella Pathogenesis Assay

Relative pathogenicity was assessed as described by Henly et al. [25] and Abid et al. [24] with slight modifications. Briefly, final larval-stage G. mellonella (ECO BAITS, Moscow, Russia) was stored in the dark at 4°C for less than 7 days, before randomly assigning 20 to each treatment group and incubating at 37°C for 30 min. After this acclimatation phase, aliquots (10µL) of each suspension (eq 0.5 of McFarland) of overnight S. aureus strains were injected into the hemocoel of each larva via the last left proleg. Larvae were incubated at 37°C in sterile petri dishes and the number of surviving individuals was recorded after 12, 24, 48 72 and 96 hours. The group injected with sterile PBS were used as negative controls. The larvae were considered dead when they became unresponsive to touch and appeared black.

2.10 Statistical Analysis

All experiments were carried out at least in triplicate. The statistical significance was set at p<0.05. T-test, principal component analysis (PCA) and Ascending Hierarchical Classification (AHC) were carried out using the statistical software XLSTAT 2020 (Addinsof Inc., New York, USA). All the other graphs were plotted by Excel software or SigmaPlot 12.5 (Systat Software, San Jose, CA, USA).

3. RESULTS AND DISCUSSION

3.1 Changes in MIC and MBC after Long Exposure to AMPICILLIN, CEFAZOLIN, KANAMYCIN and Silver Nanoparticles

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)
were determined for the strain used (S. aureus ATCC 6538) and their analogues obtained after 8 repeated passages either in the absence of specific antimicrobial or in the presence of kanamycin, cefazolin, Ampicillin or Silver nanoparticles (Tables 1). The changes in sensitivity were calculated and interpreted as the fold change relative to the control strain. No variation (0-fold) was observed between the initial bacterium and the bacterium which underwent 8 repeated passages in the BHIB antimicrobials-free. Except for Kanamycin, bacteria exposed to antimicrobials had developed cross-adaptation to other antimicrobials. Indeed, with regard to MIC there was ≥2-fold increase in 3/4 strains for Ampicillin, Cefazoline and AgNPs while no change was observed on Kanamycin. As for MBC there was a ≥2-fold increase in 4/4 isolates for Ampicillin and 1/4 for Cefazoline and Kanamycin, and 2/4 for AgNPs. All bacteria had either kept the same sensitivity to antimicrobials or developed resistance which resulted in increased MIC or MBC. Contrary to the observations of Henly et al. [25] on similar work carried out with biocides such as triclosan, triclosan, polyhexamethylene biguanide (PHMB), benzalkonium chloride (BAC) and silver nitrate, in our investigation no increase in sensitivity was observed following the exposure to antimicrobials. In addition to the obvious acquisition of cross-resistance, we noticed that bacteria exposed to a specific antimicrobial were less sensitive to that antimicrobial compared to others. For example, the strain exposed to AgNPs had an 8-fold increase for MIC of AgNPs while the strains exposed to Kanamycin and Ampicillin only had a 2-fold increase. Similarly, although no variation was observed in any strain from the MIC of Kanamycin, the bacteria exposed to this antibiotic were the only bacteria to have a 2-fold increase in MBC to Kanamycin.

As reported by others [11-15,26,27], observations made in this study suggest that Staphylococcus aureus may acquire cross resistance and/or direct resistance following exposure to low doses of antimicrobials. Indeed, S. aureus has demonstrated a unique ability to quickly respond to each antibiotic with the development of a resistance mechanism [26]. These resistance mechanisms include spontaneous mutations and positive selection [26], alteration of the target with decreased affinity for the antibiotic [26], trapping of the antibiotic and efflux pumps [11,12,28]. It is important to highlight that under in vivo conditions, other resistance acquisition mechanisms such as complex genetic arrays like staphylococcal chromosomal cassette mec elements or the vanA operon can be acquired by S. aureus through horizontal gene transfer [26,27].

3.2 There is a Change in Susceptibility to Other Antibiotics in some S. aureus having Undergone a Long Exposure to Antimicrobials

Fig. 1 shows the sensitivity of S. aureus to tetracycline (TE), ceftazidime/clavulanic acid (CAC), ceftazidime (CAZ), ceftriaxone (CTR), ciprofloxacin (CIP), imipenem (IMP), nitrofurantoin (NIT), and trimethoprim (TR) before and after exposure to ampicillin, Cefazoline, Ampicillin, Kanamycin and silver nanoparticles (AgNPs). The initial S. aureus ATCC 6538 strain and the strain passed 8 times on BHIB antimicrobial-free were both sensitive to all antibiotics tested with no significant variation in inhibition diameters. However, significant variations were observed in susceptibility to antibiotics in bacteria long exposed to antimicrobials. Indeed, the strain passed in ampicillin became resistant to ceftazidime, ceftazidime/clavulanate (CAC), tetracycline and Ceftriaxone. Likewise, the strain exposed to cefazoline became resistant to ceftazidime, CAC, and Tetracycline while exposure to Kanamycin and AgNPs caused decrease in susceptibility to ceftriaxone and resistance to ceftazidime and CAC. No significant variation was observed in the sensitivity to nitrofurantoin, imipenem, and ciprofloxacin. These results are in accordance with the observations made above on variations in MICs and MBCs. It has been reported that changes in sensitivity such as those observed in this study may be temporary (simple stress response) or permanent (profound physiological and genetic changes) [10,25,28]. Bui et al. [29] reported that S. aureus has an incredible ability to survive, either by adapting to environmental conditions or defending against exogenous stress. The changes in sensitivity observed in this study can be disastrous in the management of infections due to S. aureus given that they can lead to a decrease in the number of treatment options, be the cause of treatment failure, prolong the duration of treatment/hospitalization and even cause death. Therefore, it is imperative to take all these potential changes into account before administering any antibiotics.
3.3 Biofilm Formation, Strain Appearance and Growth Rate

In addition to the changes observed in the susceptibility to antibiotics, the cultures on Muller Hinton Agar of the strains exposed to kanamycin and AgNPs appear different from the original strain (Fig. 2). The strain exposed to kanamycin turned golden while the colonies of the strain resulting from exposure to AgNPs appeared to be very small in size. However, despite this appearance of small size, contrary to the results obtained by Abi et al. [24] all the strains including those exposed to AgNPs were retained by the pores of the 0.45 μm filtration membrane. Meanwhile, the color of the strain exposed to kanamycin can be explained by the production by S. aureus of a golden colored carotenoid pigment staphyloxanthin in response to stress [30]. However, the absence of this change in strains exposed to other antimicrobials raises questions about the specific action mechanism of kanamycin on S. aureus and calls for further research. On the other hand, the golden pigment of Staphylococcus aureus has been reported to impair neutrophil killing and promotes virulence through its antioxidant activity [31,32]. By deduction, the strain exposed to Kanamycin would therefore be potentially more pathogenic than the other analogues.

As shown in Fig. 3 and Fig. 4, strains exposed to Kanamycin and Ampicillin grow faster and produce more biofilm than control strains and their analogues obtained after long exposure to antimicrobials. Bui et al. [29] highlighted that in a multicellular biofilm, the metabolically quiescent bacterial community additionally produces a highly protective extracellular polymeric substance (EPS and there are bacteria within a

Table 1. MICs and MBCs of S. aureus before and after long exposure to antimicrobials

<table>
<thead>
<tr>
<th></th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
<td>CZ</td>
</tr>
<tr>
<td>SA-Initial</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SA-Unexposed</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SA-AMP</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>SA-CZ</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>SA-Ka</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>SA-AgNPs</td>
<td>32</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 1. Susceptibility to antibiotics before and after long exposure to antimicrobials; Data show the mean (rounded the unit) of antibiotic inhibition zones for S. aureus ATCC 6538 and their analogues before and after antimicrobial exposure and represent the results for samples taken from two separate experiments each with three technical replicates.
biofilm community that have an altered physiology potentially equivalent to persister cells. Recent studies have directly linked the cellular ATP production by persister cells as the key feature of *S. aureus* and the basis for their tolerance of a range of antibiotics [29]. In addition, the changes in biofilm formation and grow rate may potentially be a consequence of changes in the expression of the intercellular polysaccharides, protein *PIA* and *Aap* or rather changes in the staphylococcal accessory regulator gene sarA, which controls several virulence determinants, including biofilm formation, hemolysins, and DNase [7]. These observations, combined with those above mentioned suggest that the use of Kanamycin at sub-therapeutic doses should be avoided under penalty of making non-pathogenic *S. aureus* pathogenic, that can easily colonize the host because of their increased growth rate and possibly causing biofilms-associated diseases [33].

Fig. 2. Cultures on Muller Hinton Agar of *S. aureus* ATCC 6538 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexp), strains exposed to Kanamycin (Ka), Cefazolin (CZ), Ampicillin (AMP) and Silver nanoparticles (AgNPs)

Fig. 3. Planktonic growth of *S. aureus* ATCC 6538 parent strain (white circles), strain passed 8 times in BHIB antimicrobials-free (white triangles), strains exposed to Kanamycin (black triangles), Cefazolin (white square), Ampicillin (black square) and silver nanoparticles (black circles)
3.4 Relative Pathogenicity of S. aureus after Long-Term Antimicrobials Exposure and Hierarchical Ascending Classification

A G. mellonella waxworm model was used to determine relative pathogenicity (Fig. 4). The data indicates that S. aureus exposed to Kanamycin were more pathogenic, followed by those exposed to ampicillin and those exposed to AgNPs. These 3 strains were more pathogenic than the control parent strains but, interestingly, the exposure to cefazoline seemed to have significantly attenuated the pathogenicity. The attenuation of the pathogenicity observed following exposure to Cefazoline could be explained by the ability of this antibiotic to reduce the expression of leucocidin ED (LukED) which is one of the most common virulence factors in S. aureus [5,34]. It is a bicomponent pore-forming toxin playing an important role in S. aureus pathogenicity and is present in 2/3 to 4/5 of S. aureus [5]. LukED is closely associated with bloodstream infection, impetigo, and antibiotic-associated diarrhea among others [5,35,36]. LukED leads to the lysis of cells such as neutrophils, dendritic cells, T cells, myeloid cells, macrophages, and erythrocytes by attaching to their membrane and eliciting β-barrel pores that span the lipid bilayer and lead to osmotic lysis of the host cell [5,37,38]. Although further investigations are needed, the result on the impact of cefazolin on S. aureus may constitute a line of research to be exploited so as to choose the appropriate agents to avoid promoting bacterial virulence in S. aureus LukED-positive infections. Otherwise, the increase in pathogenicity observed in the strain passed into Kanamycin could be associated with the production of carotenoid pigment staphyloxanthin as we have reported above (Fig. 2). Fig. 6 presents the ascending hierarchical classification (AHC) of all S. aureus having undergone exposure to antibiotics or nanoparticles. This AHC was obtained following a principal component analysis including inhibition diameter to antibiotics, and their variation compared to the parent strain, the biofilms formation, MIC and MBC to antimicrobials, growth rate and pathogenicity. As shown in Fig. 6, two large clusters were formed. The first cluster (in blue) consists of S. aureus strains exposed to ampicillin and Kanamycin, which confirms the similar variations observed in these strains in compared to the parent strain. The second cluster (in red) shows us that the strain exposed to AgNPs does not show much dissimilarity with the parent control strains.

Fig. 4. Biofilm formation of S. aureus ATCC 6538 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexp), strains exposed to Kanamycin (Ka), Cefazolin (CZ), Ampicillin (AMP) and Silver nanoparticles (AgNPs)
Fig. 5. Relative pathogenicity of S. aureus ATCC 6538 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexposed), strains exposed to Kanamycin (Ka), Cefazolin (CZ), Ampicillin (AMP) and Silver nanoparticles (AgNPs).

Fig. 6. Ascending hierarchical classification and dissimilarity between S. aureus ATCC 6538 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexposed), strains exposed to Kanamycin (Ka), Cefazolin (CZ), Ampicillin (AMP) and Silver nanoparticles (AgNPs).
4. CONCLUSION

Exposure to Ampicillin, Kanamycin, Cefazoline and silver nanoparticles led to increase in MIC and MBC of most of these antimicrobials. In addition, S. aureus exposed to Kanamycin and Ampicillin produced more biofilms and were more pathogenic on G. mellonella waxworm model while cefazoline attenuated the pathogenicity. In conclusion, more research should be carried out with other microorganisms considering not only conventional antibiotics but also antimicrobial alternative to antibiotics such as plant extracts and nanoparticles in order to anticipate the potential consequences of prolonged exposure of pathogenic bacteria (or not) to these antimicrobials.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ACKNOWLEDGEMENTS

This publication has been supported by the RUDN University strategic Academic Leadership Program.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


26. Pantosti A, Sanchini A, Monaco M. Mechanisms of antibiotic resistance in


