IN VITRO ANALYSIS OF ANTIBIOTIC-LOADED BONE CEMENT EFFECTIVENESS IN PREVENTING BIOFILM FORMATION

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(Orthopaedic)

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- Colleagues and all the staff in the Department of Orthopedic, HUSM.
- My beloved wife, Dr Nadiah Mohd Nawawi and my daughter for their constant support and prayers.

CONTENTS

ABSTRAK

Latar Belakang:

Jangkitan berkaitan dengan implan adalah komplikasi yang membimbangkan dalam pembedahan ortopedik dan trauma kerana boleh mengakibatkan kegagalan implan, hasil fungsi yang buruk, osteomielitis kronik, sepsis dan kematian. Pembentukan biofilem pada permukaan prostesis menyukarkan untuk menghilangkan jangkitan yang disebabkan oleh mikroorganisma kerana adanya persekitaran perlindungan oleh glikokaliks yang menyumbang kepada ketahanan mereka terhadap mekanisme pertahanan tuan rumah dan terapi antimikrobial. Penggunaan simen tulang yang digabungkan dengan antibiotik sebagai salutan implan telah menjadi kaedah utama dan paling praktikal untuk memberikan antibiotik tempatan dalam keadaan klinikal untuk penggantian sendi total. Dalam kajian ini, keberkesanan simen tulang yang dimuatkan dengan antibiotik untuk mencegah pertumbuhan bakteria dan pembentukan biofilem disiasat menggunakan *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa,* polimetilmetakrilat (PMMA) silinder dan dua jenis simen tulang yang dimuatkan dengan antibiotik untuk menangani interaksi bakteria-biomaterial.

Kaedah:

Kajian ini melibatkan dua jenis simen tulang yang mengandungi antibiotik dan kumpulan kawalan. Pertama, simen disediakan dengan menuangkannya ke dalam acuan silinder (5mm x 2mm). Tiga spesies mikroorganisma digunakan untuk menyelidik keberkesanan simen tulang yang dimuatkan antibiotik untuk mencegah pertumbuhan bakteria dan pembentukan biofilem iaitu *S. aureus, S. pyogenes* dan *P. aeruginosa.* Kesan simen tulang yang dimuatkan dengan antibiotik pada setiap mikroorganisma yang diuji ditentukan dalam satu siri selang waktu. Simentara itu, model zon penghambatan digunakan untuk membandingkan keberkesanan antibakterial antibiotik terhadap bakteria dari waktu ke waktu. Akhirnya, analisis data untuk keberkesanan antibakteria dan pencegahan pertumbuhan bakteria dilakukan dengan menggunakan Analisis Varians (ANOVA) dan ujian *post hoc* Tukey. Sebagai tambahan, Saringan Mikroskopi Elecktron (SEM) digunakan untuk memeriksa pembentukan biofilem pada spesimen. Pengiraan ukuran sampel tidak diperlukan kerana kajian menggunakan analisis deskriptif.

Keputusan:

1. Keberkesanan simen tulang yang dimuatkan dengan antibiotik untuk mencegah pertumbuhan bakteria (Model zon penghambatan).

Gentian tulang yang mengandung gentamicin dan vancomycin menghalang pertumbuhan *S. aureus* dan *S. pyogenes* dalam masa 72 jam pendedahan. Sebaliknya, kedua-dua rawatan tidak menghalang pertumuhan *P. aeruginosa* pada mana-mana selang masa.

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2. Penjajahan bakteria (CFU / cm2) simen tulang yang dimuat, simen tulang gentamicin dan vancomycin.

Tulang yang dimuatkan dengan gentamicin dan vancomycin telah menghalang pertumbuhan *S. aureus* dan *S.pyogenes* berdasarkan kiraan CFU/cm^2 yang rendah. Walau bagaimanapun, kesan ini hanya bertahan sehingga 72 jam. Gentamicin dan vancomycin tidak menunjukkan pencegahan/ pengurangan kolonisasi *P.aeruginosa* yang signifikan pada mana-mana selang waktu berbanding dengan kumpulan kawalan.

3. Pembentukan biofilm.

Berdasarkan penilaian imbasan mikroskopi elektron, pembentukan biofilem oleh *S. aureus, S. pyogenes* dan *P. aeruginosa* dilihat pada simen tulang antibiotik gentamicin dan vancomycin selepas 72 jam. Semua mikroorganisma yang melekat membentuk koloni di permukaan simen tulang. Terdapat beberapa koloni yang diselaputi dengan filem berlendir manakala beberapa koloni lain dihubungkan dengan bebenang berlendir nipis.

Kesimpulan:

Simen tulang yang dimuatkan dengan antibiotik harus dipertimbangkan dalam pencegahan pencemaran langsung pada waktu operasi, atau dalam tempoh selepas operasi ketika luka sedang sembuh.

ABSTRACT

Background:

Implant-related infection is a feared complication in orthopaedic and trauma surgery as it may result in implant failure, poor functional outcome, chronic osteomyelitis, sepsis and death. Biofilm formation on the prosthesis surface makes it difficult to eliminate infections caused by microorganisms due to the presence of a protective environment created by the glycocalyx contributing to their resistance towards the host's defence mechanism and antimicrobial therapy. The use of antibiotic-loaded bone cement as an implant coating has been the primary and only practical method of delivering local antibiotics in the clinical setting of total joint replacement. In this study, the efficacy of antibiotic-loaded bone cement at preventing bacterial adherence and biofilm formation was investigated using *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa,* cylindrical polymethylmethacrylate (PMMA) and two types of antibiotic-loaded bone cement as biomaterials to address the bacteria-biomaterial interactions.

Methods:

This study involves two types of antibiotic-loaded bone cement and a control group. Firstly, the cement was prepared by pouring it into a cylindrical mould (5mm x 2mm). Three microorganism species were used to investigate the efficacy of antibiotic-loaded bone cement at preventing bacterial adherence and biofilm formation, namely *S. aureus, S. pyogenes* and *P. aeruginosa*. The effect of antibiotic-loaded bone cement on each tested microorganism was determined in a series of time intervals. Meanwhile, the zone of inhibition model was utilized in comparing the antibacterial efficacy of antibiotics against selected bacterial strains over time. Finally, the data analysis for antibacterial efficacy and prevention of bacterial adherence was performed using Analysis of Variance (ANOVA) and post hoc Tukey test. In addition, the Screening Electron Microscopy (SEM) was used to examine biofilm formation on the specimens. No sample size calculation was required since this is a descriptive analysis study.

Results:

1. The efficacy of antibiotic-loaded bone cement at preventing bacterial colonization (Zone of inhibition model).

Gentamicin and vancomycin-loaded bone cement prevented the colonization of both *S. aureus* and *S. pyogenes* within 72h of exposure. Conversely, both treatments did not suppress the colonization by *P. aeruginosa* at any time interval.

2. Bacterial colonization (CFU/cm^2) of unloaded bone cement, gentamicin and vancomycin-loaded bone cement.

Gentamicin-loaded bone and Vancomycin-loaded bone cement inhibited *S. aureus* and *S. pyogenes* based on the low CFU/cm^2 count. However, this effect only lasted up to 72h. Gentamicin and vancomycin showed no significant prevention/ reduction of *P. aeruginosa* colonization at all time intervals compared to the unloaded bone cement.

3. Biofilm formation.

Based on the scanning electron microscopy evaluation, biofilm formation by *S. aureus*, *S. pyogenes* and *P. aeruginosa* were observed on gentamicin and vancomycin-loaded antibiotic bone cement after 72h. All the adhering organisms formed colonies on the bone cement surfaces. Some colonies were covered with a slimy film while others were connected by thin slimy threads.

Conclusions:

Antibiotic-loaded bone cement should be considered to prevent direct contamination at the time of surgery or during the postoperative period as the wound seals.

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Implant-related infection is a feared complication in orthopaedic and trauma surgery as it may result in implant failure, poor functional outcome, chronic osteomyelitis, sepsis and death. Consequently, implant removal and multiple debridements become necessary to eradicate the infection from the affected bone. Therefore, a low risk of infection estimates (0.5% - 5%) must be considered to prevent this unfavourable situation (1).

In view of this, bacterial infection remains a significant complication following a total hip replacement, with implant infection, primarily with Staphylococcus spp. (2). As the bacteria grow predominantly within a confluent biofilm on the prosthesis surface, it renders them resistant to currently employed antibiotics; hence, eradicating infection with systemic antibiotics usually fails. The surface adherence property of some bacteria may be associated with a higher risk of infection (3,4,5). When bacteria adhere to biomaterials, they encase themselves in a hydrated biofilm matrix of polysaccharide and protein on the prosthesis surface, making it difficult to eradicate the infection.

The resistance of periprosthetic infection to host defence mechanism and antimicrobial therapy is related to the protective environment of the glycocalyx (6,7). Furthermore, the incubation of different bacterial strains on multiple biomaterials revealed that free-floating (planktonic) bacteria are more susceptible to antibiotics than the bacteria (sessile) encased within the biofilm (8). Sessile microorganisms are highly resistant to antimicrobial agents via multiple mechanisms (9), including delayed penetration of the antimicrobial into the biofilm's extracellular matrix (10).

In the last few decades, perioperative infection prevention methods have seen tremendous improvement. Buchholz and Engelbrecht (11) incorporated gentamicin in polymethylmethacrylate (PMMA) to treat infections in prosthetic joints in 1970. Until today, antibiotic-loaded bone cement as an implant coating has been the primary and only practical method of delivering local antibiotics in the clinical setting of total joint replacement. It is not known to what extent the presence of local antibiotics delivered from antibiotic-loaded bone cement reduces infection by interfering with biofilm formation or simply by eradicating planktonic bacteria that may be adjacent to the prosthesis (12). In this regard, the elution behavior of acrylic bone cement has been studied extensively (13), while gentamicin is the most common additive used in combination with bone cement (14,15).

The use of antibiotic-loaded acrylic cement in joint replacement provides short-term to medium protection against prosthetic infection. It aims to overlap with, and then replace, the prophylaxis provided by perioperative intravenous antibiotics. Tunney et al. (2006) (16) reported a combination of gentamicin-loaded bone cement and cefuroxime were given pre and postoperatively prevented biofilm formation when a low bacterial inoculum was present. There is valid evidence to support the prophylactic use of antibiotic-loaded acrylic cement (17), which remains a standard practice in the United Kingdom, Western Europe and Scandinavia, and is in transition in the United States of America (USA).

In this study, the efficacy of antibiotic-loaded bone cement in preventing bacterial adherence and biofilm formation was investigated. These studies use *S. aureus, S. pyogenes, P. aeruginosa*, cylindrical PMMA and two types of antibiotic-loaded bone cement as the biomaterials to address bacteria-biomaterial interactions.

Chapter 2

RESEARCH OBJECTIVES

2.1 GENERAL OBJECTIVE.

To determine the effectiveness of antibiotic-loaded bone cement in preventing biofilm formation.

2.2 SPECIFIC OBJECTIVES.

1. To compare the antibacterial efficacy of two different antibiotic-loaded bone cement versus control in vitro.

2 To compare the bacterial adherence load of two different antibiotic-loaded bone cement versus control.

3. To compare biofilm formation of two different antibiotic-loaded bone cement versus control.

Chapter 3

MANUSCRIPT

3.1 TITLE: *IN VITRO ANALYSIS OF ANTIBIOTIC-LOADED BONE CEMENT EFFECTIVENESS IN PREVENTING BIOFILM FORMATION*

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In this study, the efficacy of antibiotic-loaded bone cement in preventing bacterial adherence and biofilm formation was investigated. The study utilized *S. aureus, S. pyogenes, P. aeruginosa*, cylindrical PMMA and two types of antibiotic-loaded bone cement as the biomaterials to address bacteria-biomaterial interactions.

3.4 METHODOLOGY

Preparation of Cement and Antibiotics

Firstly, the bone cement was loaded with 40g of PMMA Simplex P. Then, two types of antibiotics were added to the polymer powder: gentamicin 500 mg (12.5 mg per gram) and vancomycin 1.0 g (25 mg per gram) and mixed in a bowl with a spatula for 60s at a constant temperature (23°C ± 5°C) and humidity (50% ± 10%). After that, the cement was poured into a mould (5mm x 2mm) containing holes with a diameter of 5mm, using a 20ml needle-nose syringe and allowed to harden for 15 min. Later, the samples were removed from the mould and stored in a dark, sterile condition at room temperature. The weight of each disc is 100mg with a surface area of 78mm^2. The unloaded Simplex P bone cement was used as a control in all experiments.

Preparation of Bacteria.

Three bacteria species were used in this study: *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC19615) and *P. aeruginosa* (ATCC 27853*).* The microorganisms were grown in the Muller-Hinton broth for 24 hours at 37 . Then, the inoculum for each of the bacterial strain was prepared by adjusting the turbidity of an overnight broth culture to an optical density of 550 nm, which is equivalent to ~ $1x10^3$ CFU/ml. A low bacterial inoculum of $1x10^3$ CFU/ml was used to represent the number of bacteria present at the operative site as the result of skin contamination (16)

Antibacterial efficacy.

Each antibiotic-loaded bone cement section (gentamicin and vancomycin) and the control (Simplex P) were placed in 10ml of sterile water and stored in the dark at 37 ± 5˚C to measure the antibacterial efficacy of the treatments. At every designated time interval, 10µL water sample from each group was removed and applied on agar plates and incubated with *S. aureus*, *S. pyogenes* and *P. aeruginosa* in triplicates. The plates were incubated at 37̊C, and antibacterial efficacy was evaluated at 30 min, 1h, 2h, 4h, 8h, 12h, 16h, 24h, 48h and 72h by measuring the inhibition zones.

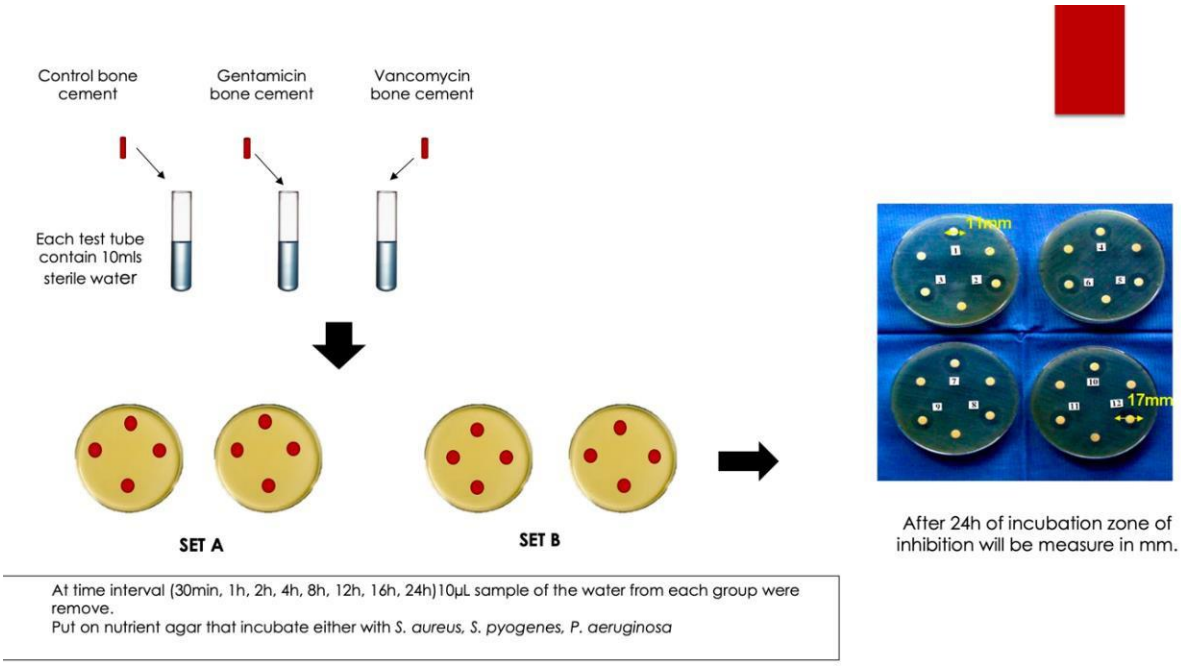

Figure 1 Antibacterial efficacy test for Simplex P, gentamicin-loaded bone cement and vancomycin-loaded bone cement against *S. aureus*, *S. pyogenes*, *P. aeruginosa*

Bacterial adhesion on the implant.

Biofilms are allowed to form for each bacterial strain *(S. aureus, S. pyogenes, P. aeruginosa*) in triplicates on control (Simplex P), gentamicin-loaded bone cement and vancomycin-loaded bone cement. A total of 15 bone cement samples for each bone cement were placed aseptically in separate sterile test tubes. Two bone cement samples were used for every time interval; 10 ml of final inoculum was added to sterile test tubes, and the samples were incubated with constant agitation at 37˚C. After 6 hours of the incubation period, three samples were removed with sterile forceps and gently washed for 20sec in 10ml sterile isotonic saline to remove any non-adherent bacteria and allowed to dry for 30 min. After that, the samples were transferred into microcentrifuge tubes containing 200µl MH and vortexed for 2 min to dislodge the bacteria. This step was repeated at 6h, 24h, 48h and 72h. A 10-fold serial dilution was performed and viable bacterial counts were estimated. Then, the agar plates were incubated at 37˚C for 24h before the number of colonies forming units (CFU) on each blood agar will be counted and expressed relative to bone cement surface area (CFU/cm^2).

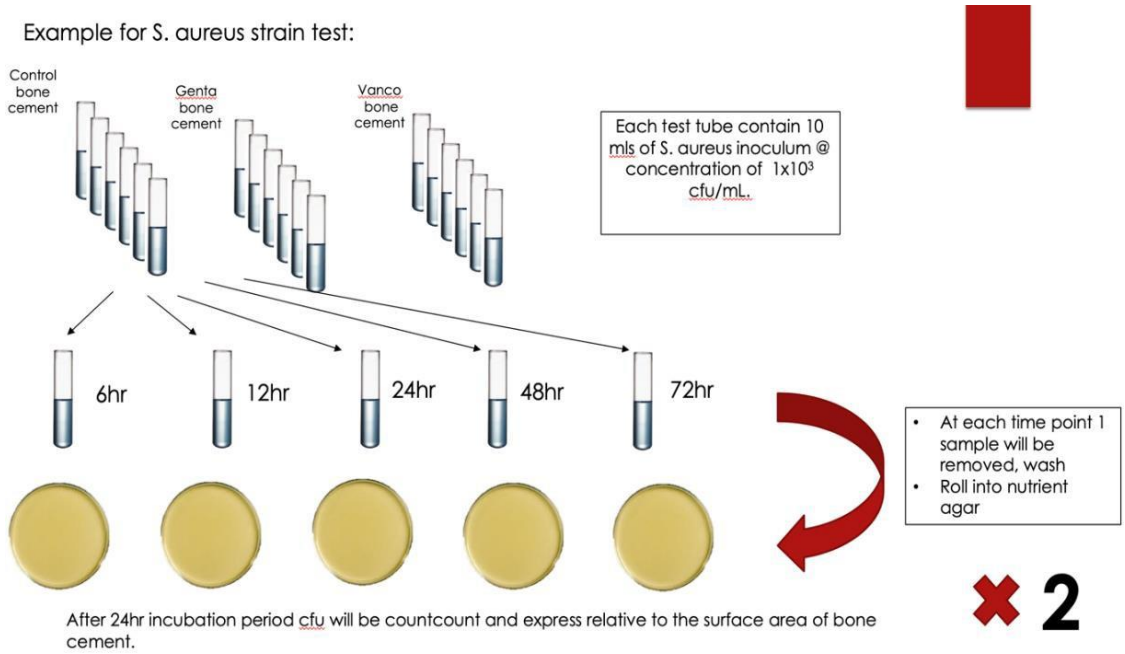

Figure 2 Inhibition of biofilm formation by Simplex P, gentamicin-loaded bone cement and vancomycin-loaded bone cement treatment against *S. aureus, S. pyogenes, P. aeruginosa*

Screening electron microscopy

A bone cement sample for each treatment group was placed in a separate sterile test tube containing 10ml of final inoculum and incubated at 37 ± 0.5℃ for 72 hours. Then, the samples were removed and rinsed with 10ml isotonic saline before being placed in a sterile container. After that, the samples were subjected to screening electron microscopy (SEM) at the Department of Pathology, HUSM.

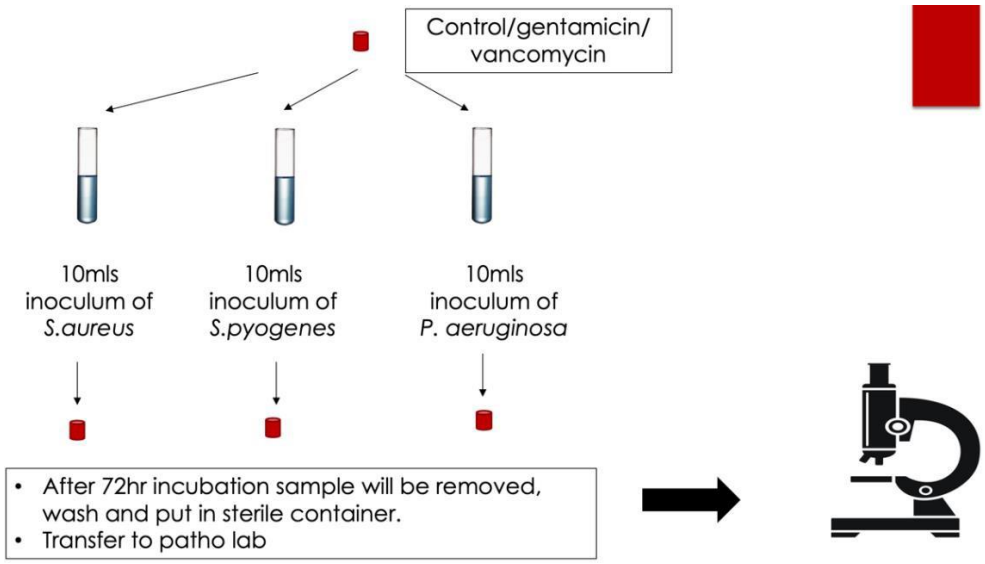

Figure 3 SEM of Simplex P, gentamicin-loaded bone cement and vancomycin-loaded bone cement treatment against *S. aureus*, *S. pyogenes*, *P. aeruginosa*

3.5 RESULTS

The efficacy of antibiotic-loaded bone cement against the colonization of three bacterial strains was determined by measuring the inhibition zone around the disc plates. In the present study, gentamicin-loaded bone cement prevented the colonization of *S. aureus,* beginning at 30min and achieved its maximum effect between 4h to 12h before declining slowly. Besides, the *S. pyogenes* colonization was also inhibited by gentamicin-loaded bone cement at all time intervals, with the maximum effect observed between 12h to 16h and slowly regressed after that. Interestingly, the progression in inhibition activity is slower against *S. pyogenes* compared to *S. aureus,* but the effect lasted longer. Meanwhile, vancomycin-loaded bone cement prevented the colonization of *S. aureus* at 30 min and achieved its maximum inhibition between 4h to 12h. Similarly, vancomycin had the same effect on S. *pyogenes*. On the other hand, neither gentamicin nor vancomycin-loaded bone cement prevented the colonization by *P. aeruginosa* at any time interval.

Table 1 Zone of inhibition (mm) around Gentamicin-loaded bone cement and Vancomycin-loaded bone cement vs Simplex P (control) against *S. aureus, S. pyogenes and P. aeruginosa*

	Inhibition zone (mm)									
Treatment	30 m	1h	2h	4h	8h	12h	16h	24h	48h	72h
S. aureus										
Simplex P	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]
Gentamicin	6.0±0.00[b]	7.0±0.00[a]	6.0±1.41[a]	8.0±0.00[a]	8.0±0.00[a]	8.5±0.71[b]	6.0±1.41[a]	4.5±0.71[a]	5.5±0.71[a]	5.0±1.41[a]
Vancomycin	6.5±0.71[b]	6.0±0.00[a]	7.0±0.00[a]	8.0±0.00[a]	8.0±0.00[a]	7.5±0.71[b]	5.5±0.71[a]	4.0±0.00[a]	6.5±0.71[a]	4.5±0.71[a]
S. pyogenes										
Simplex P	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]
Gentamicin	5.5±0.71[a,b]	5.0±1.41[a]	5.5±0.71[a,b]	6.0±0.00[a]	6.0±0.00[a]	8.0±0.00[a]	8.5±0.71[b]	5.5±0.71[a]	9.0±0.00[a]	6.5±0.71[b]
Vancomycin	6.0±0.00[b]	6.0±0.00[a]	7.0±0.00[b]	8.0±0.00[a]	9.0±0.00[a]	5.0±0.00[a]	5.0±0.00[a]	8.0±0.00[b]	12.0±0.00[a]	6.5±0.71[b]
P. aeruginosa										
Simplex P	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]
Gentamicin	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.5±0.71[a]	4.0±0.00[a]
Vancomycin	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]	4.0±0.00[a]

*Mean±standard deviation values followed by same letters in a given column are not significantly different at $p<0.05$ according to post hoc Tukey test.

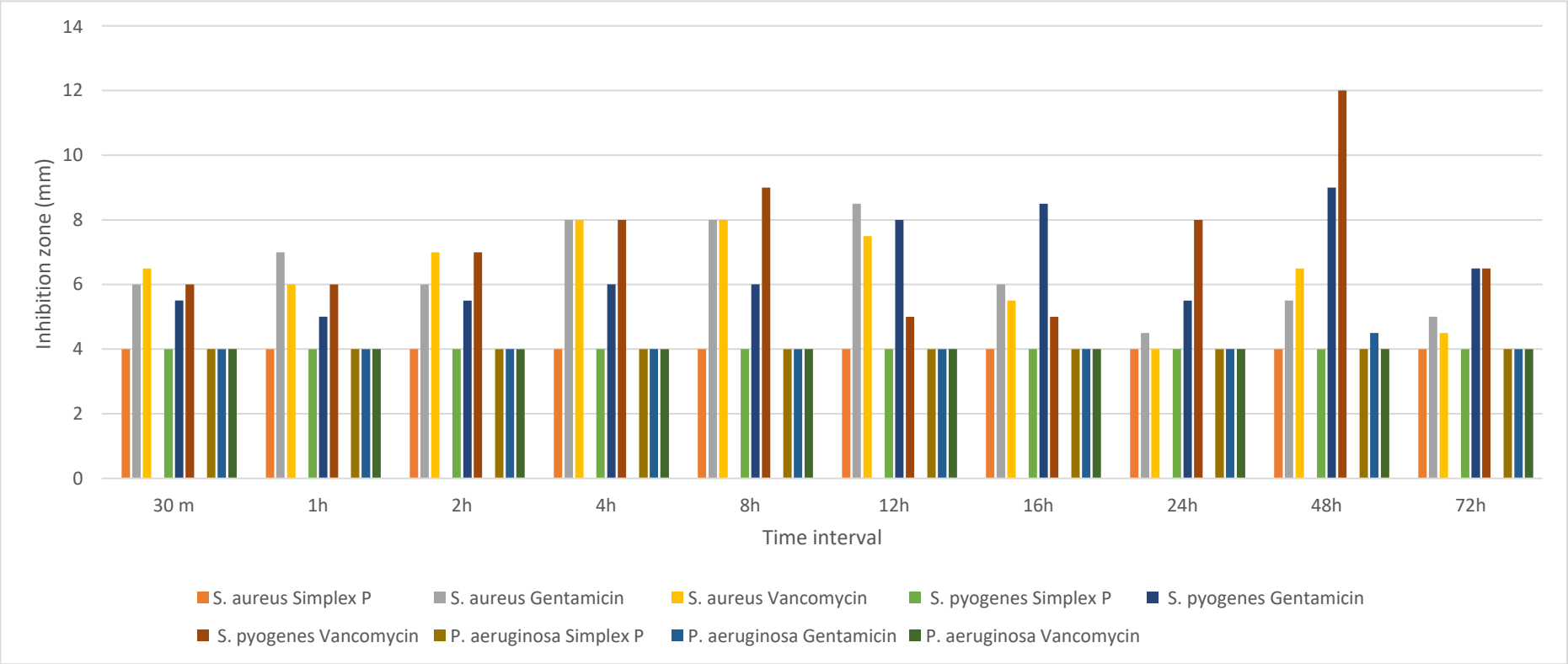

Figure 4 Inhibition zone (mm) of Simplex P, Gentamicin and Vancomycin against three bacteria strains

3.5.2 Bacterial adhesion on the implant.

Table 2 Bacterial colonization (CFU/cm^2) of *S. aureus*, *S. pyogenes and P. aeruginosa* treated with unloaded bone cement, Gentamicin-loaded bone cement and Vancomycin-loaded bone cement

	Bacterial colonization (CFU/cm^2)			
Treatment	6h	24h	48h	72h
S. aureus				
Simplex P	107.03±6.220^{b}	5.033±0.459^{b}	821.05±0.00^{c}	11561.27±363.336^{b}
Gentamicin	4.46±0.180^{a}	0.29±0.005^{a}	99.40±2.686^{b}	99.37±0.895^{a}
Vancomycin	13.84±2.060^{a}	17.95±0.876^{c}	8.68±0.409^{a}	152.16±14.624^{a}
S. pyogenes				
Simplex P	0.03±0.00	0.52±0.00^{a}	5.91±0.06^{a}	5.60±0.312^{a}
Gentamicin	N/A	0.86±0.053^{b}	6.32±0.181^{a,b}	22.33±4.003^{a}
Vancomycin	N/A	0.82±0.0245^{b}	6.50±0.153^{b}	164.67±12.257^{b}
P. aeruginosa				
Simplex P	48.33±1.905^{a}	101.37±3.724^{a}	1456.94±190.779^{a}	96.67±8.439^{c}
Gentamicin	77.71±3.827^{a}	157.58±22.285^{a,b}	1035.34±67.876^{a}	73.95±1.487^{b}
Vancomycin	172.45±18.760^{b}	217.00±37.835^{b}	1430.00±183.848^{a}	51.18±1.661^{a}

*Mean±standard deviation values followed by same letters in a given column are not significantly different at $p < 0.05$ according to post hoc Tukey test

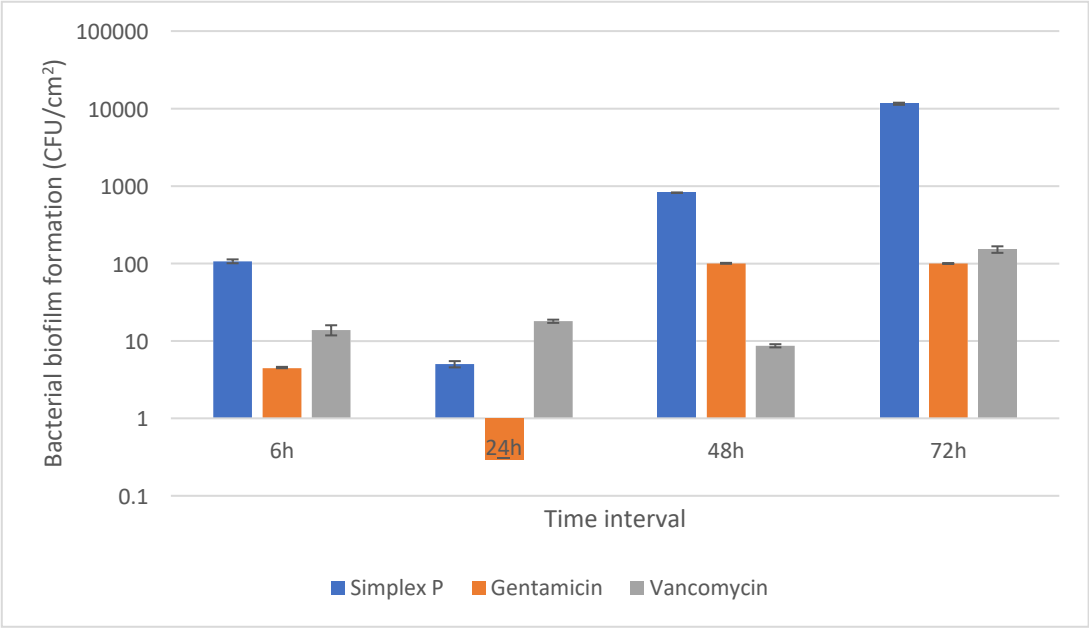

Figure 5 Bacterial colonization for Gentamicin and Vancomycin vs Simplex P (control) for *S. aureus*

Gentamicin- loaded bone cement exhibited significant reduction/prevention of *S. aureus* colonization at 6h, 24h compared to the control treatment. In contrast, the inhibition was not significant at 48h and 72h. Meanwhile, vancomycin-loaded bone cement demonstrated significant inhibition of *S. aureus* at 6h and 48h, but no significant effect was observed after 72h.

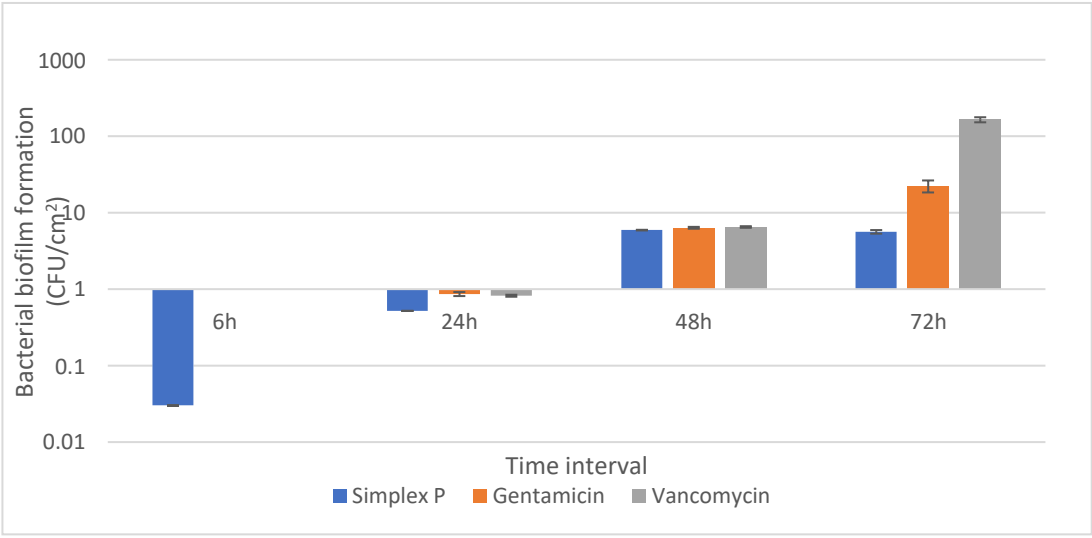

Figure 6 Bacterial colonization for Gentamicin and Vancomycin vs Simplex P (control) for *S. pyogenes*

Both Gentamicin and Vancomycin showed significant reduction/ prevention against *Streptococcus pyogenes* colonization at 6h and 24 h compared to unloaded bone cement. However, this beneficial effect was not significant at 48h and 72h.

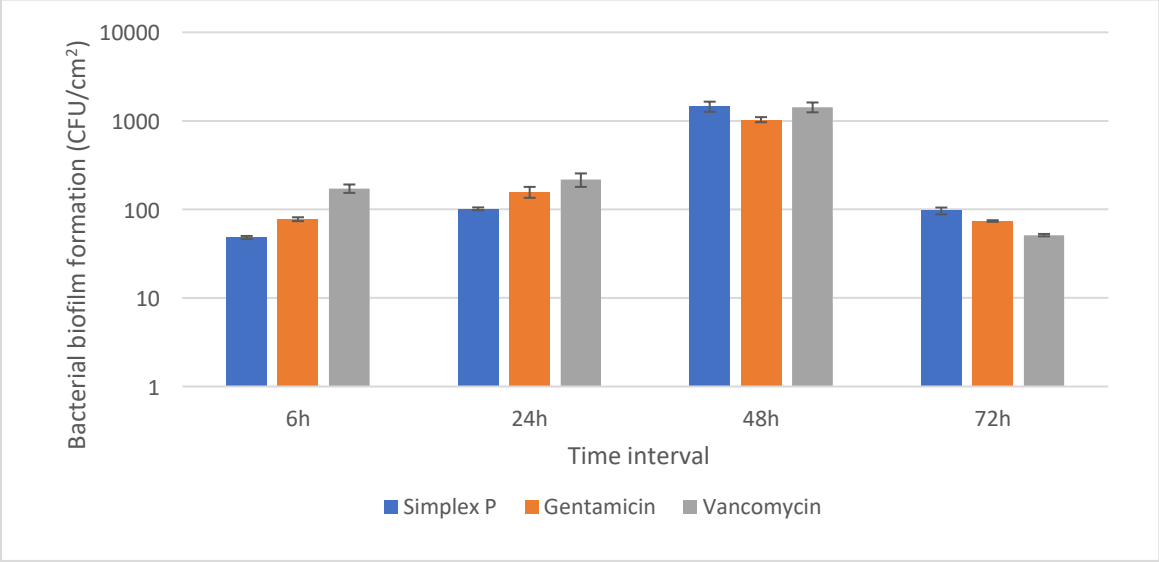

Figure 7 Bacterial colonization for Gentamicin and Vancomycin vs Simplex P (control) for *P. aeruginosa*

Gentamicin and vancomycin showed no significant prevention/ reduction of *P. aeruginosa* colonization at all time intervals compared to unloaded bone cement.

3.5.2 Scanning Electron Microscopy (SME) evaluation.

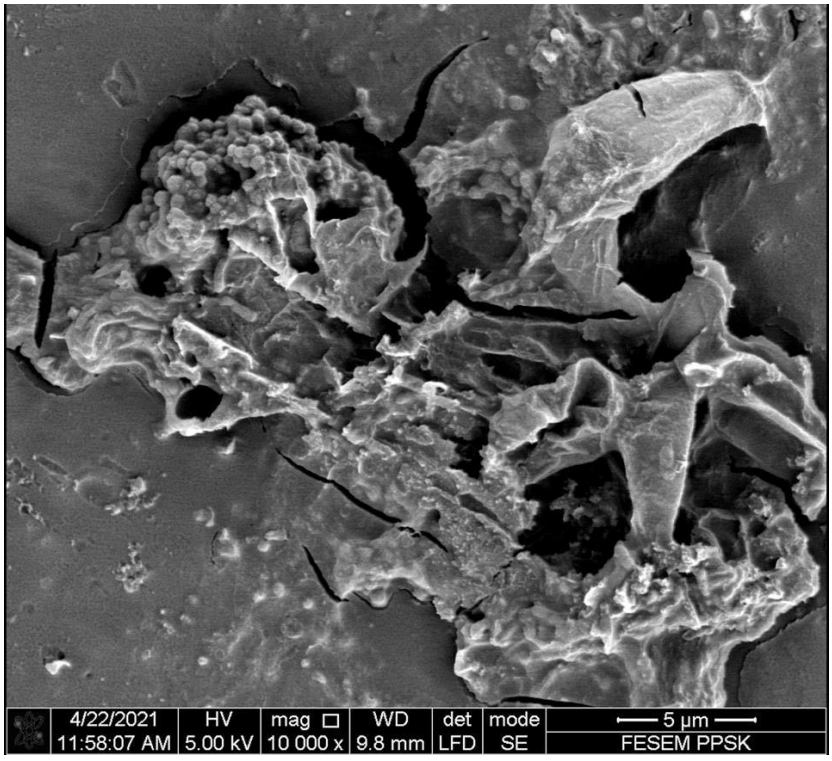

Figure 8: *S. aureus* biofilm characterized by chains of cocci-coating in the matrix on gentamicin-loaded bone cement surface

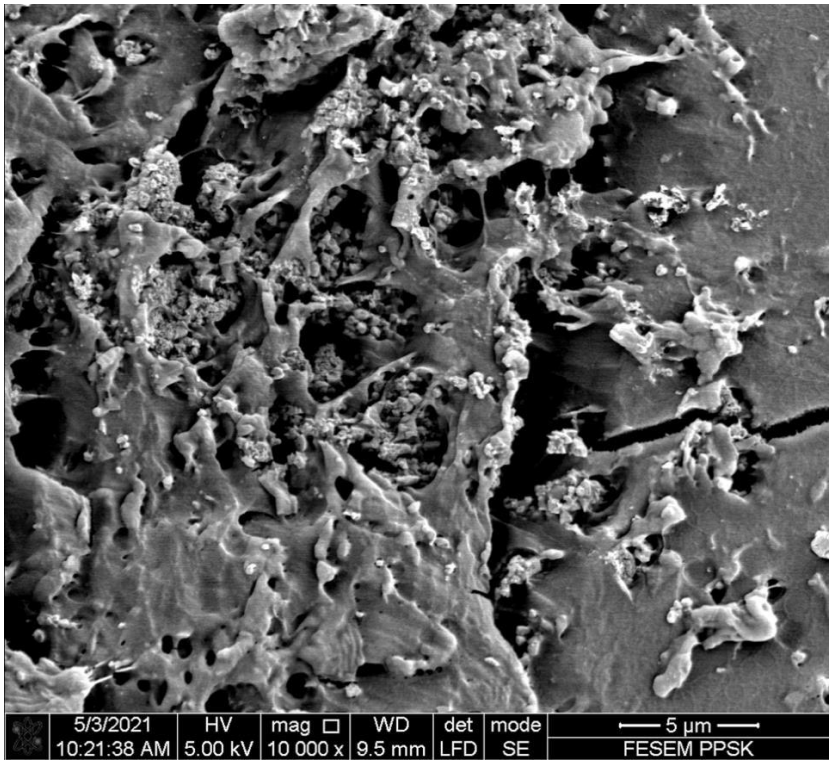

Figure 9: *S. pyogenes* characterized by chains of adherent cocci organized into biofilm on gentamicin-loaded bone cement surface

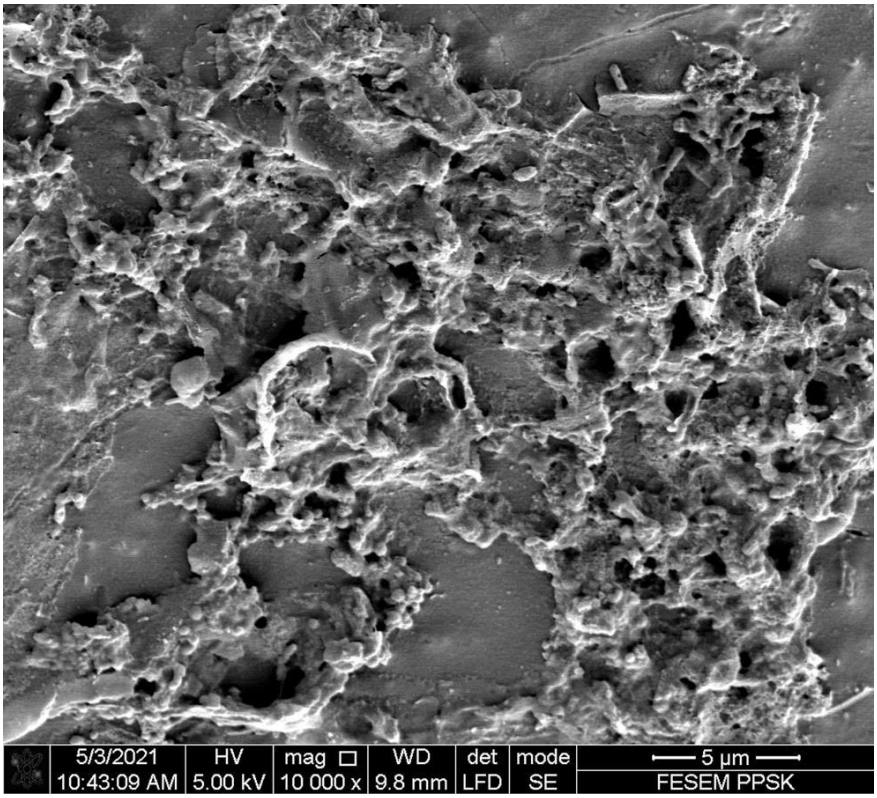

Figure 10: *P. aeruginosa* biofilm characterized by rod-shaped chains on gentamicin-loaded bone cement surface

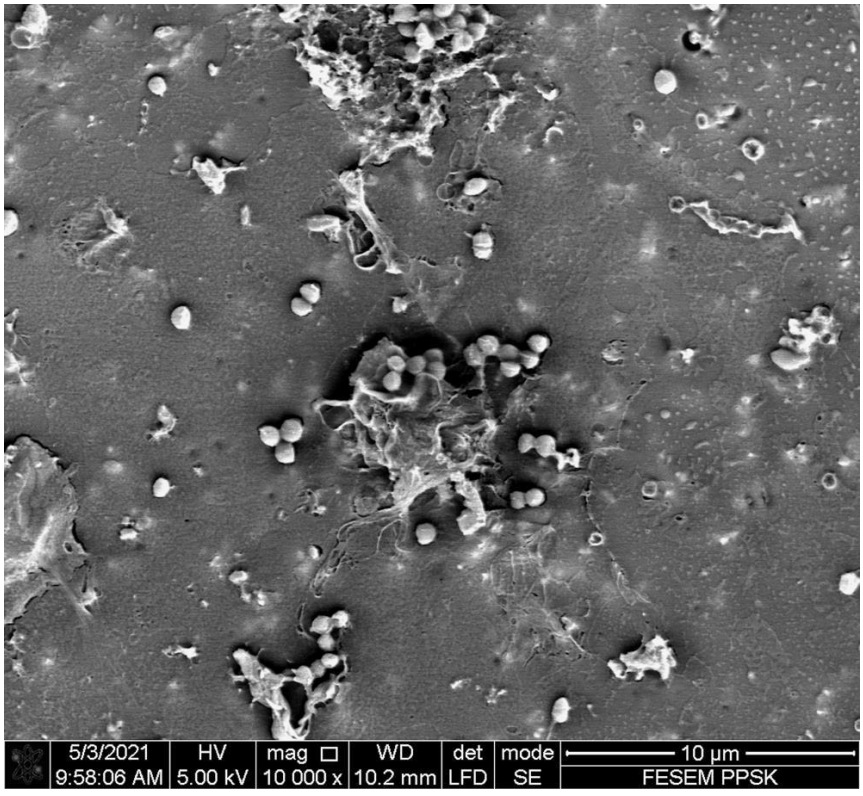

Figure 11: *S. aureus* biofilm characterized by chains of cocci connected by thin slimy threads on vancomycin-loaded bone cement surface

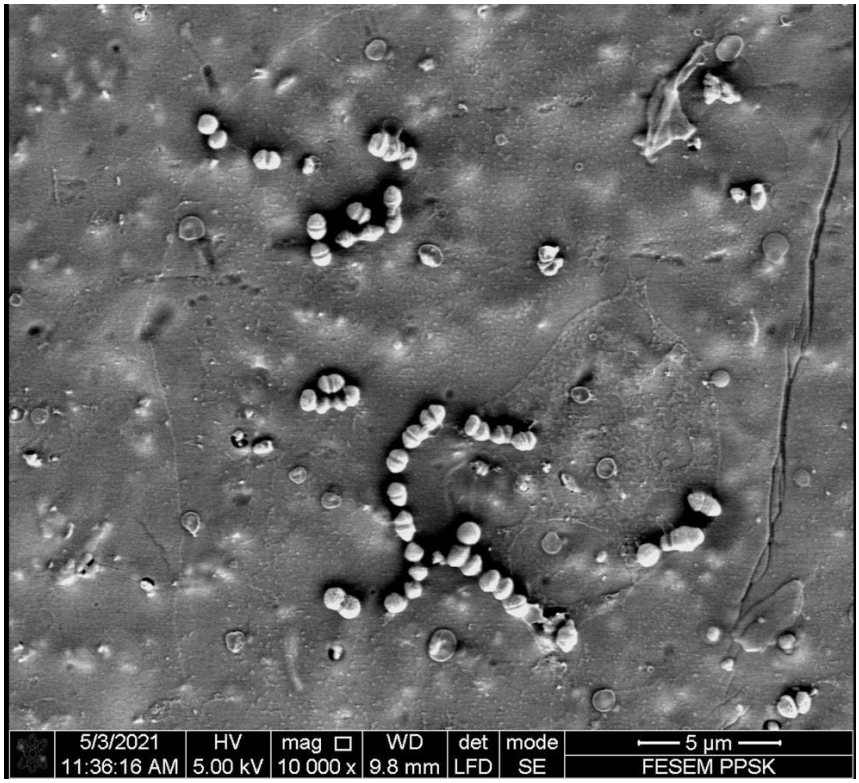

Figure 12: *S. pyogenes* biofilm characterized by colony formation with a slimy film-covering on vancomycin-loaded bone cement surface

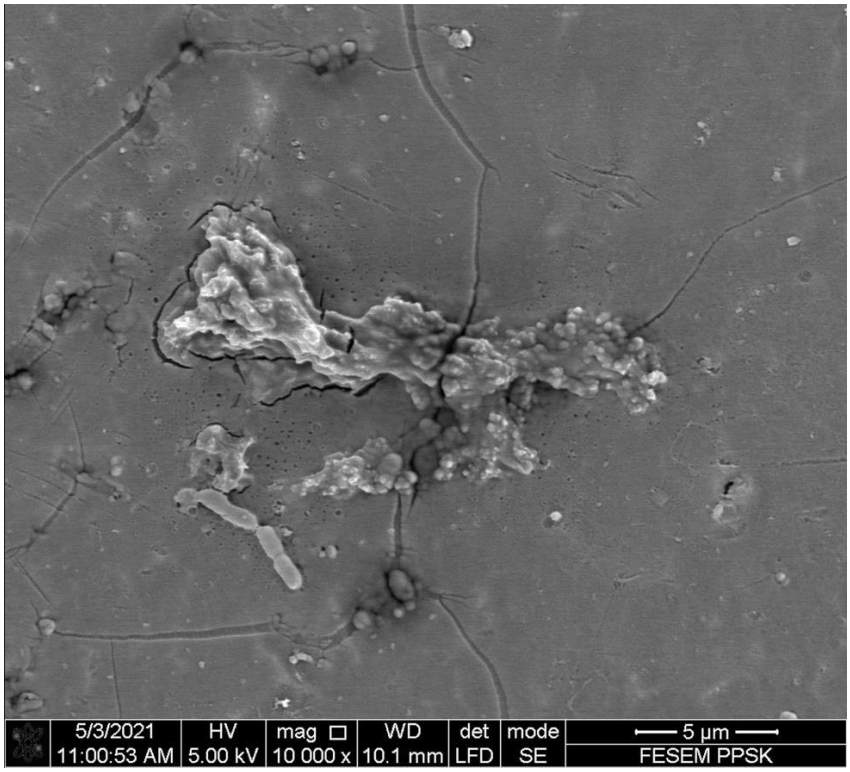

Figure 13: *P. aeruginosa* biofilm on vancomycin-loaded bone cement surface