Research article

Isolation and characterization of bacteriophage targeting *Enterococcus faecalis* isolated from root canal infection (*in vitro* study)

Maha F. Almelan[1], Uroba Khalid Abbas[2], Al-Zubidi M.[3]

[1]Department of Basic Science, College of Dentistry, Baghdad University, Baghdad, Iraq
[2]Department of Microbiology, College of Medicine, Mustansiriyah University, Baghdad, Iraq
[3]Department of POP, College of Dentistry, Mustansiriyah University, Baghdad, Iraq

(Received: April 2023 Revised: May 2023 Accepted: June 2023)

Corresponding author: **Maha F. Almelan**. Email: mahaestephan2017@gmail.com

ABSTRACT

Introduction and Aim: *Enterococcus faecalis*, a Gram-positive bacterium inhabits the human and animal intestinal tracts and the environment. *E. faecalis* in addition to being implicated in a wide variety of infections in humans is considered the most common threat associated with recurrent root canal treatment failures. In this investigation we aimed to characterize *E. faecalis* strains isolated from chronic endodontic infection and evaluate the effect of a bacteriophage for its capability to eliminate *E. faecalis* biofilm formation.

Materials and Methods: This study involved 65 chronic endodontic infection samples which were studied for the prevalence of *Enterococcus faecalis*. The identification of *E. faecalis* was accomplished by a combination of biochemical testing and the sequencing of the 16S rRNA gene. Phages isolated from cow dung samples were purified and evaluated based on their capability of preventing the development of biofilms by *Enterococcus faecalis* on biotic surfaces.

Results: Of the 65 chronic endodontic infection samples studied nineteen tested positive for the presence of *E. faecalis*. *E. faecalis* strain K3 was found to be vancomycin resistant. Biofilm development on abiotic surfaces by *E. faecalis* strain K3 was shown to be greatly reduced after exposure to the enterococcal phage BAG1.

Conclusion: This investigation shows that the *E. faecalis* bacteriophage BAG1 significantly decreases the growth of *E. faecalis* biofilm on abiotic surfaces.

Keywords: Enterococcus faecalis; bacteriophage; biofilm; chronic endodontic infection; antibiotic resistance.

INTRODUCTION

Enterococcus faecalis, a Gram positive facultative anaerobic bacterium, is usually known to establish itself in failed root canals, capable of surviving in a wide range of temperature and pH. Long-lasting root canal infection allows *E. faecalis* to colonize the dentinal tubules and lateral branches of a tooth's root canal. *E. faecalis* has been implicated in 63% of root canal treatment failures, mostly due to reinfection by this bacterium (1). The production of biofilms is one of the factors that allows *E. faecalis* to persist in root canals (2). *E. faecalis* can form biofilms in infected root canals as a monoinfection, independent of other species from where it can enter the root canal wall through the dentinal tubules and persist there, thus rendering it more resistant to standard endodontic disinfection procedures (3).

Alternate strategies to combat infections caused by antibiotic-resistant bacterial strains are being addressed, the most promising among them being "phage therapy". Phage therapy involves the use of a bacteriophage, a virus that can infect the bacterium and lyse them. The use of a phage with broad lytic activity against clinical isolates of *E. faecalis* has been previously demonstrated (4). Against this background, we aim to isolate phage and evaluate its activity against biofilm formation by *E. faecalis* isolated from root canal infection patients.

MATERIALS AND METHODS

E. faecalis isolation and identification

This study was carried out at the Department of Microbiology, College of Dentistry at Baghdad University between January 2021 to February 2022. Collection of chronic endodontic infection samples was performed according to Al-Huwaizi *et al.,* (5) with some modification. The selected teeth for sampling were first cleaned with pumice, and then separated with the aid of a rubber dam. The operating field was disinfected by 35% hydrogen peroxide (H_2O_2) and 5.25% sodium hypochlorite (NaOCl) respectively. All the coronal restorations and carious lesions were removed by large new diamond and carbide round burs. The root canal was accessed using a sterile diamond fissure bur with care not to go deep inside the pulp chamber to avoid disturbing the bacterial flora. Thereafter, the operating field was disinfected again with 5.25% NaOCl (5). ISO type k-files of sizes 15 and 20 were introduced into the canal with a working length 1.0 mm shorter than the apex and confirmed by X ray, then initial instrumentation of the canal was done by sterile rotary file size 20# without any irritants. The canal was flooded with

sterile saline solution and agitation vertically with the last file was done to form a microbial suspension inside the root canal. Paper points were introduced into the canal and kept for one minute. Thereafter, they were transferred immediately to a tube containing sterile transport media (AMIES) and transported to the laboratory for isolation and identification (6).

The samples obtained from chronic endodontic infections, were subjected to preliminary identification of bacterial using a series of microbiological tests (growth in bile – esculin and BHI agar medium containing 6.5% salt, Gram stain, catalase, Vitek 2 test) and molecularly confirmed by16 S rRNA sequencing.

Antibiotic susceptibility test

The antibiotic susceptibility of the *E. faecalis* strain to different antibiotics was determined by Kirby-Bauer disc diffusion technique. Testing was done for antibiotics tetracycline (10 µg), ampicillin (10 µg), erythromycin (15µg) ciprofloxacin (5µg), vancomycin (30µg), and gentamicin (10µg), using standard antibiotic discs (HiMedia Laboratories, Mumbai, India). *E. faecalis* was used as a reference, according to Clinical and Laboratory Standards Institute 2020 (CLSI 2020) (7).

Bacterial DNA extraction

DNA was extracted from overnight grown bacterial cultures by first harvesting the cells by centrifugation at 10000 x g followed by resuspension of the pellets in sterile phosphate buffer saline with the volume adjusted to 300 µl. to this 300 µl lysis buffer (AL kit, QIAAmp DNA mini kit column, Qiagen) was added and incubated for 10 min at 56°C. Genomic DNA was extracted from the cell suspension using a QIAAmp DNA mini kit column (Qiagen, UK) as per the manufacturer's instructions.

PCR, gel electrophoresis and sequencing

GoTaq Green Master Mix (2x) polymerase was used in conjunction with the manufacturer's instructions in 25 µl reaction volumes. Typically, this consisted of 12.5µl Go Taq Green polymerase 2x master mix, 1µl of forward primer 27F (5nM), 1µl of reverse primer 1492R (5nM), 1µl DNA and 9.5µl distilled nuclease free sterile water. The primers (27F 5`-AGAGT TTGATCCTGGCTCAG-3`and 1492R 5`-TACGGTT ACCTTGTTACGACTT-3`) were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100 pmol/µl stock solution. A working solution of these primers was prepared by adding 5 µl of primer stock solution (stored at freezer -20 C°) to 95 µl of nuclease free water to obtain a working primer solution of 5 pmol/µl. PCR reactions were analyzed via gel electrophoresis. 1 % agarose was added to 1x TAE buffer and heated until all the agarose powder had dissolved. Ethidium bromide solution (PROMEGA, USA) was added to a concentration of 1 µg/ml to the gel before it was poured into the cast and the comb placed to create the wells. Once set, the gels were run in a 1 x TAE buffer in a (Hoefer, Belgium). The samples were loaded with 5 µl of 50 bp DNA Ladder (PROMEGA, USA) used as a size standard for comparison and run at 100 V until the gel had migrated sufficiently. DNA separation was visualized under a UV light source and photographed.

The PCR products were excised and sequenced using Sanger automated DNA sequencer ABI3730XL (Macrogen Corporation, Korea). The sequencing results obtained were analyzed using Geneious software.

Bacteriophage isolation

The bacteriophage was isolated from cow dung collected in a plastic container. Approximately 100 gm of cow dung was suspended in 50 ml of SM buffer (1M Tris-HCl Buffer pH 7.4 with 5 M NaCl, 1 M MgSO4, and 1% gelatin), vortexed, and filtered using filter paper with pore sizes of 4-7 m (Schleicher & Schuell, Germany). The samples were then centrifuged at 6000 xg for 15 minutes, and the supernatant from each tube was filtered through a 0.45 m syringe filter (Sartorius, Germany) before being utilized for bacteriophage isolation.

Phage clearance was first identified by spotting assay against *E. faecalis* clinical strain. 10 µl of processed phage samples were dispensed on sterile filter papers with 6 mm in diameter (Filter paper Whatman No. l thickness 1 mm, UK) that were already placed on the surface of BHI double layer agar with the (5%) top agar containing 100 µl of *E. faecalis* indicator bacteria as shown in Fig.6. Simultaneously, 50µl of processed samples were mixed with 200 µl of mid log phase of the indicated clinical strain of *E. faecalis*; left for 10 min to allow the phage particles to adsorb into the bacteria. The resulting suspension was mixed with 5-6 ml (5%) BHI soft top agar (47°C) containing 1 M of $MgSO_4$ and poured onto a previously solid BHI bottom agar.

All plates were incubated in a 37°C incubator for 24 hr, following which the plates were visualized for clearance around the filter paper or clear plaque. Clear plaques were picked using sterile 1 ml pipet tips. The pipet tips immediately suspend in 200 µl SM buffer plane tube and left in 4°C overnight to allow the phage to diffuse in the SM buffer. 50 µl of SM buffer samples containing phage were used in double layer agar to infect the indicator *E. faecalis.* This process was repeated 3 times to ensure that only a single phage is obtained. Plaque from the 3 third round was picked and stored at 4°C until use.

Concentration of bacteriophage using Polyethylene Glycol (PEG 8000)

Phage concentration was achieved to produce a working suspension that was appropriate for DNA extraction and genomic digestion. This was accomplished by precipitation with PEG 8000 as described by Green and Sambrook, (9) In short 200 ml of exponential growth phase of *E. faecalis* (5 x 10^6 CFU/ml) were infected with phage suspension stock at MOI (multiplicity of infection) of 0.05 for 3h. 1M NaCl was added with incessant mixing using a magnetic stirrer at 4C° for 1h. followed by gradual addition of 10% PEG 8000 (w/v) with continuous mixing and left overnight in 4C° to precipitate phage particles. The mixture was centrifuged at 10,000 xg for 30 minutes to deposit the precipitated phage. The resulting pellets were suspended in 1 ml of SM buffer and stored at 4°C.

Phage DNA extraction

The PEG 8000 concentrated phage stock was treated with DNase and RNAse enzymes (5µg/ml) to eradicate contaminating DNA and RNA. The mixture was incubated at 37°C for 30 minutes. A final concentration to break down the enzymes, 100 µg/ml of proteinase K was added to the mixture and incubated for 45 minutes at 45°C. Next, 80 µl of isopropanol were added to each 1 ml of sample volume. The NORGEN Phage DNA Extraction Kit was used to isolate the phage DNA (NORGEN, Canada) according to manufacturer's instructions. In brief, 500 µl of binding solution was added to each collecting tube after vortexing. 600µl of the phage mixture was applied to the column and centrifuge at 8000 rpm for 1 min and the flow throw was discarded. After that, 300µl of washing solution of the kit was added to the column and centrifuge at 8000 rpm for 3 min. To thoroughly clean the column, this process was repeated twice more. To elute the DNA, 50 µl of sterile water was added to the column and centrifuged at 2000 rpm for two minutes. The phage DNA from each column was collected and stored at -20 C° to be used later.

Assay of biofilms on polystyrene plates

The capability of the *E. faecalis* strains to form a biofilm on an abiotic surface was quantified based on a previously described method (10) with suitable modifications. Briefly, *E. faecalis* strains were grown overnight in BHI broth at 37°C. 100 µl from overnight culture was added to 900 µl fresh BHI broth and inoculated to sterile flat-bottomed 24-well polystyrene microtiter plates (Thermo Fisher Scientific, China) followed by the addition of 900 µl of fresh BHI broth

Biofilm formation was obtained after 72 hours of static aerobic incubation at 37 C˚ with fresh Brain Heart Infusion broth added every 24 hours. After a period of incubation lasting four hours, the broth was removed from the wells in a very cautious manner and replaced with one milliliter of freshly prepared broth containing 10^6 PFU/ml of the separated phage or Brain Heart Infusion alone (controls) in three of the six wells of each group. Then, one millilitre of phosphate-buffered saline (PBS) was used to thoroughly rinse each well three times. After drying the plates upside down on a paper towel, they were stained with 1% crystal violet for 15 minutes. This process was repeated three times. After a third washing, 500 µl of an ethanol-acetone (80:20, vol/vol) mixture was used to dissolve the crystal violet, and the wells were rewashed. The optical density at 570 nm was measured with the Universal Microplate Reader (EL 800). Each test was repeated three times.

Statistical analysis

The GraphPad Prism ver. 6 for Windows (GraphPad Software, La Jolla, CA, USA) was used in statistical analysis of data.

RESULTS

Identification of *E. faecalis*

There was a total of 65 samples from patients with persistent endodontic infections, 19 tested positive to identify as *E. faecalis*. The *E. faecalis* examined under the microscope appeared to be gram-positive cocci that were organized in pairs as well as in short and long chains. *E. faecalis* colonies appeared as white gray colonies on the blood agar with no hemolysis, while on Bile Esculin agar the colonies appeared as having black halos around the colonies. *E. faecalis* colonies were catalase negative. The Vitek2 system showed the presence of *E. faecalis* with 99% identification probability.

One among the 19 isolates showed positive for vancomycin resistance and this clinical *E. faecalis* strain was named as K3. Analysis of the 16s rRNA partial gene sequencing of the K3 strain showed it to be 100% identical to *E. faecalis* strain 2358. The 16s rRNA sequence of the *E.faecalis* K3 strain has been deposited in GenBank and provided with the accession no. OM250466.

Antibiotic sensitivity test for *E.faecalis* isolates

Results for each antibiotic were recorded as sensitive, intermediate, and resistant based on a validated protocol. K3 clinical strain isolates was tested against the antibiotic disks selected for the current study (6 mm) in a diameter included Vancomycin (30 µg), Erythromycin (15µg), Ciprofloxacin (5µg), Tetracycline (30µg), Gentamicin (10µg), and Ampicillin (10 µg) (Liofilchem, Italy).

The sensitivity of *E. faecalis* strains to various antibiotics is presented in Table 1. We evaluated a total of six different antibiotics. Each drug was given a reading of three possible outcomes: sensitive, moderate, or resistant. The diameter of inhibition zones observed for *E. faecalis* antibiotic susceptibility

agrees as mentioned by the Clinical and Laboratory Standards Institute 2020. The result is resistant to vancomycin, erythromycin, ampicillin, and gentamicin but sensitive to tetracycline and highly sensitive to ciprofloxacin as shown in Fig. 1.

Table 1: Antibiotic susceptibility testing of *E. faecalis* strains

Antibiotics	Antibiotic concentration	Sensitivity	Intermediate	Resistant	Diameter of inhibition zone
Vancomycin	30 µg	≥ 17 mm	15-16 mm	≤ 14 mm	13mm
Erythromycin	15 µg	≥ 23 mm	14-22 mm	≤ 13 mm	15mm
Tetracycline	30 µg	≥ 19 mm	15-18 mm	≤ 14 mm	**22mm**
Ciprofloxacin	5 µg	≥ 21 mm	16-20 mm	≤ 15mm	**28mm**
Ampicillin	10 µg	≥ 17 mm	-	≤ 16 mm	14mm
Gentamicin	10 µg	≥ 15 mm	-	13-14 mm	11mm

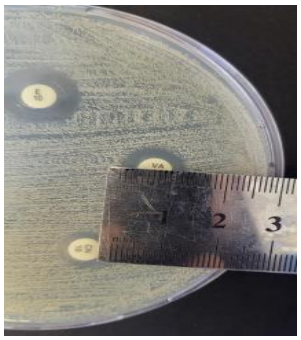

Fig. 1: Resistance of K3 clinical strain to Vancomycin antibiotic disc reflected by diameter of the inhibition zone (VA.) Vancomycin.

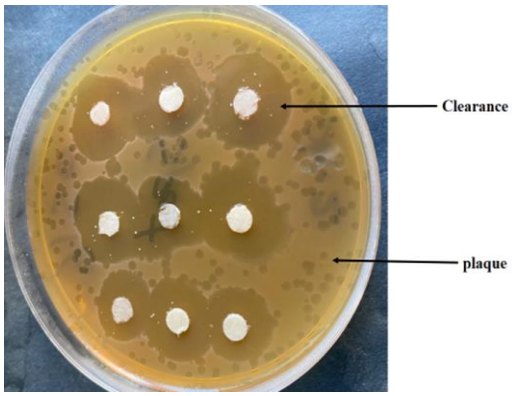

Fig. 2: Plate showing *E. faecalis* K3 clearance zone by bacteriophage

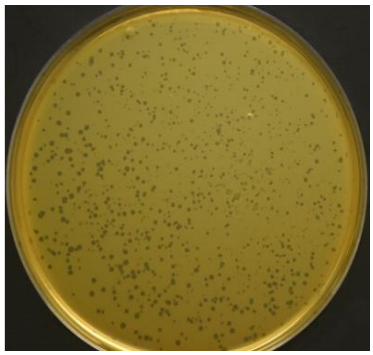

Fig. 3: Isolation of bacteriophages targeting K3 bacteria and plaque morphology.

Isolation of virulent bacteriophage towards K3

Several attempts were made to isolate bacteriophage by spot pipetting on DLA to target microorganisms related to chronic endodontic infection. The isolated clinical strain K3 was used as the host for these experiments. Bacteriophages were sourced from cow feces as shown in Fig.2.

Characterization of the isolated phages

The bacteriophage DNA digested with Hind III restriction enzymes yielded fragments of sizes 1400 bp to 100000 bp (Fig. 4). The isolated phage was named BAG1.

Effect of phage BAG1 on *E. faecalis* biofilm formation

We investigated whether phage BAG1 could successfully eliminate K3 biofilms that had already developed in vitro. Phage was administered to the biofilm both after 24 hours and after 72 hours, and the results showed that BAG1 phage is capable of almost eliminating *E. faecalis* endodontic clinical strain mature or immature biofilm. ($p<0.0001$; Fig. 5).

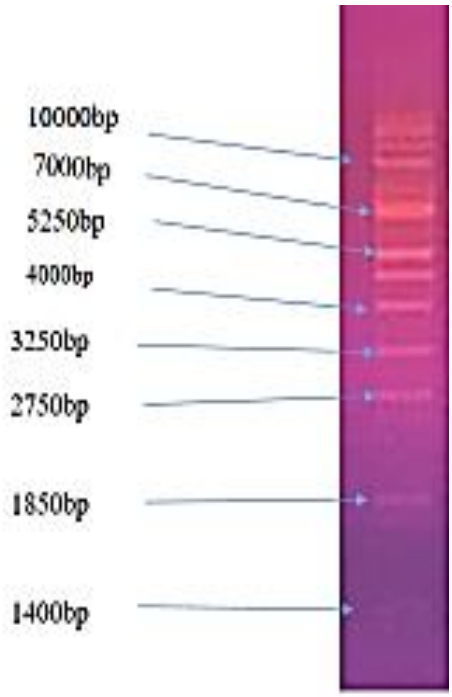

Fig. 4: Restriction fragment length polymorphism of BAG1 phage digested with endonuclease *Hind*III.

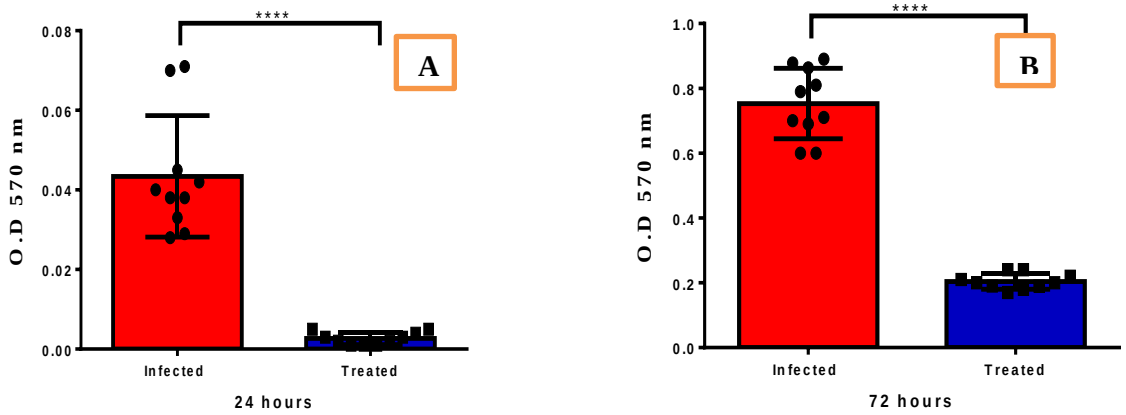

Fig. 5: Graph showing *E. faecalis* biofilm formation at 24 h (A) and 72 h (B) after treatment with phage BAG1

DISCUSSION

Secondary endodontic infection can occur because of inadequate root canal system disinfection during root canal therapy or bacterial leakage after root canal therapy is completed. Polymicrobial endodontic infections are characterized by the coexistence of various facultative and stringent anaerobe bacteria, both gram-positive and gram-negative. One of the most prevalent bacteria found in root canals after endodontic treatment is *E. faecalis* (11). In this study, 19 out of the 65 (29.2%) of endodontic infection samples tested positive for the presence of *E. faecalis*, indicating its association with chronic endodontic infection as reported in previous studies (12-14).

The low susceptibility of enterococci to antibiotic resistance is a contributing factor that makes the problem of endodontic failure caused by enterococci even more severe (13). *E. faecalis* isolates from this study were evaluated for antibiotic sensitivity against routinely used antibiotics in clinical practice such as vancomycin, erythromycin, ampicillin, gentamicin, tetracycline, and ciprofloxacin. Strains were mostly seen to be resistant to vancomycin, erythromycin, ampicillin, and gentamicin. Increased resistance to antibiotics has been associated with biofilm production which contributes to the bacteria's long-term survival in root canals (15).

The spread of antibiotic-resistant strains among harmful bacteria has highlighted the critical need for the development of new, alternative bacterial infection management methods. Phage treatment, or the use of pathogenic bacterial viruses to infect and kill host cells, is one such option. Bacteriophages are most widespread on earth, playing an important part in the dynamics and development of microbial populations (16, 17). In this study, we were able to isolate bacteriophage (BAG1) from cow dung, which showed high virulence activity and was significantly effective in controlling biofilm formation by *E. faecalis* K3 endodontic clinical strain. Prior research has documented the existence of bacteriophages that specifically target biofilm formation in *E. faecalis* isolated from both oral and abiotic surfaces (18). On the other hand, there are alternate strategies to investigate antibacterial effects by applied nanoparticles, plant ethanolic extraction and other products (19-23). However, none of them used a clinical strain of *E. faecalis* isolated directly from a root canal infection. Bacteriophages are known to infiltrate biofilms via water channels or destroy the biofilm matrix by producing depolymerases (24). Further, lysins, which are derived from phages, are effective at disrupting biofilms by destroying the bacterial cell wall (25). Hence, the exact mechanism by which the bacteriophage isolated in this study exerts its anti-biofilm effect needs further investigation.

CONCLUSION

Our findings suggest that *E. faecalis* bacteriophage BAG1 considerably reduces *E. faecalis* biofilm formation on abiotic surfaces.

CONFLICT OF INTEREST

Authors have no conflicts of interest.

REFERENCES

1. Fisher, K., Phillips C. The ecology, epidemiology, and virulence of *Enterococcus*; Microbiology. 2009; 155: 1749-1757.
2. Hussein, H.H., AlhelaL, A.G., Abood, F.M., Molecular study on *Enterococcus faecalis* isolated from primary endodontic infection. International Journal of Pharmaceutical Research. 2020; 12(3):385-390.
3. Tinoco, J.M., Buttaro, B., Zhang, H., Liss, N., Sassone, L., Stevens, R. Effect of a genetically engineered bacteriophage on *Enterococcus faecalis* biofilms; Archives of Oral Biology. 2016; 71: 80-86.
4. Lee, D., Im, J., Na, H., Ryu, S., Yun, C.H., Han, S.H. The novel Enterococcus phage vB_EfaS_HEf13 has broad lytic activity against clinical isolates of *Enterococcus faecalis*. Frontiers in Microbiology. 2019; 10: 2877.
5. Mahdi, A., Al-Huwaizi, H., Abbas, I. A comparative evaluation of antimicrobial activity of the ethanolic extract of *Cinnamomum zeylanicum* and NaOCl against oral pathogens and against swabs taken from nonvital teeth- An *in vitro* study; International Journal of ChemTech Research. 2018; 39-47.
6. Al-Badr, R. J., Al-Huwaizi, H.F. Antimicrobial evaluation for novel solution of iron oxide nanoparticles functionalized with glycine and coated by chitosan as root canal final irrigation. Systematic Reviews in Pharmacy. 2020; 11(6): 633-642.

7. Clinical and Laboratory Standards Institute: Performance Standards for antimicrobial susceptibility testing, 30th Edition. 2020; M100: S29.
8. Endo, M.S., Signoretti, F.G., Kitayama, V.S., Marinho, A.C., Martinho, F.C., Gomes, B.P.F.A., Culture and molecular detection of *Enterococcus faecalis* from patients with failure endodontic treatment and antimicrobial susceptibility of clinical isolates. Braz Dent Sci. 2014; 17(3): 83-91.
9. Green, M.R., Sambrook, J., Preparation of single-stranded bacteriophage M13 DNA by precipitation with polyethylene glycol. Cold Spring Harbor Protocols. 2017;11:093419.
10. Afonina, I., Lim, X. N., Tan, R., Kline, K. A. Planktonic interference and biofilm alliance between aggregation substance and endocarditis-and biofilm-associated pili in *Enterococcus faecalis*. Journal of Bacteriology. 2018; 200(24): e00361-18.
11. Barbosa-Ribeiro, M., De-Jesus-Soares, A., Zaia, A.A., Ferraz, C.C., Almeida, J.F., Gomes, B.P. Microbiological analysis of endodontically treated teeth with apical periodontitis before and after endodontic retreatment. Clinical Oral Investigations. 2021; 25(4): 2017-2027.
12. Nobrega, F.L., Vlot, M., de Jonge, P.A., Dreesens, L.L., Beaumont, H.J. Molecular identification of cultivable bacteria from infected root canals associated with acute apical abscess. Braz. Dent. 2016; 27: 318-324.
13. Manoil, D., Al-Manei, K., Belibasakis, G.N. A systematic review of the root canal microbiota associated with apical periodontitis: lessons from next generation sequencing; Proteomics Clin. Appl. 2020; 14(3): 1900060.
14. Sudrik, R.U., Gulve, M., Ahiwale, T., Poster 5: *Enterococcus faecalis*: The endodontic enigma; Journal of Dental Sciences & Oral Rehabilitation. 2015; 4(1): 1-2.
15. Asmah, N. Pathogenicity biofilm formation of *Enterococcus faecalis.* Journal of Syiah Kuala Dentistry Society.2020; 5(1): 47-50.
16. Martha R.J. Clokie., Andrew D. Millard., Andrey V. Letarov., Shaun Heaphy. Phages in nature; Bacteriophage. 2011; 1(1): 31-45.
17. Wardani, A.K., Nurbayu, I.R., Qodriyah, N.L. Isolation of lytic bacteriophages and their potential to control *Cronobacter* spp.-opportunistic food-borne pathogens. IOP Conference Series: Earth and Environmental ScienceIOP Conf. Series: Earth and Environmental Science. 2020; 475: 012086. 2020.
18. Loganathan, A., Manohar, P., Leptihn, S., Ramesh, N. Effect of phage lifestyle on the plaque morphology. International Journal of Bacteriophage Research. 2021; 1(1):10-15.
19. Al-Saidi, M.H., Al-Bana, R. J. A., Hassan, E., Al-Rubaii, B.A.L. Extraction and characterization of nickel oxide nanoparticles from Hibiscus plant using green technology and study of its antibacterial activity. Biomedicine (India). 2022; 42(6):1290-1295.
20. Saleh, T.H., Hashim, S.T., Malik, S.N., AL-Rubaii, B.A.L. Down-regulation of flil gene expression by Ag nanoparticles and TiO2 nanoparticles in pragmatic clinical isolates of Proteus mirabilis and Proteus vulgaris from urinary tract infection. Nano Biomedicine and Engineering. 2019; 11(4):321-332.
21. Jalil, I. S., Mohammad, S. Q., Mohsen, A.K. and Al-Rubaii, B.A.L. Inhibitory activity of *Mentha spicata* oils on biofilms of *Proteus mirabilis* isolated from burns. Biomedicine. 2023; 43(2):748-752.
22. Mohsin, M.R., AL-Rubaii, B. A. L., Bacterial growth, and antibiotic sensitivity of *Proteus mirabilis* treated with anti-inflammatory and painkiller drugs. Biomedicine. 2023; 43(2):728-734.
23. Ali, M.A., Al-Rubaii, B.A. Study of the Effects of Audible Sounds and Magnetic Fields on *Staphylococcus aureus* Methicillin Resistance and mecA Gene Expression. Tropical Journal of Natural Product Research. 2021; 5(5): 825-830.
24. Shende, R.K., Hirpurkar, S.D., Sannat, C., Rawat, N., Pandey, V. Isolation, and characterization of bacteriophages with lytic activity against common bacterial pathogens. Veterinary World. 2017; 10(8): 973.
25. Rai, S., Tyagi, A., Kumar, B.N., Singh, N., Optimization of plaque forming conditions for an *Aeromonas hydrophila* lytic bacteriophage. Int. J. Curr. Microbiol. Appl. Sci. 2020; 9: 3764-3768.