Effectiveness of disinfecting K-file contaminated with *Staphylococcus aureus* using alcohol, chlorhexidine, and sodium hypochlorite in different concentrations and durations

Efektivitas desinfeksi K-file yang terkontaminasi *Staphylococcus aureus* menggunakan alkohol, klorheksidin, dan natrium hipoklorit dengan konsentrasi dan durasi yang berbeda-beda

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ABSTRACT
This study evaluated the effectiveness of K-files contaminated with *Staphylococcus aureus* and immersion in alcohol, chlorhexidine, and sodium hypochlorite through CFU counting. Bacteria were identified through Gram staining and dilution to obtain an initial 300 CFU, then the K-files were contaminated with bacteria. The samples were divided into three groups and repeated several times in a time series. For alcohol, the groups were divided into 70% and 96% concentrations. For chlorhexidine and sodium hypochlorite, each concentration was divided into three groups, and CFU counting was performed manually. The CFU results after immersion in 70% alcohol for 5, 10, and 15 minutes were 28 CFU, 5 CFU, and 0 CFU; 96% alcohol was 5 CFU, 1 CFU, and 0 CFU; 0.5%, 1%, and 2% chlorhexidine for 5 minutes and 10 minutes were 0 CFU; 0.25% sodium hypochlorite was 3 CFU, and for 10 minutes was 1 CFU; 0.5% and 1% sodium hypochlorite for 5 minutes and 10 minutes were 0 CFU. It was concluded that the antibacterial effectiveness of alcohol and sodium hypochlorite against *S.aureus* increased with increasing disinfectant concentration and K-file immersion time, while chlorhexidine was effective in killing *S.aureus* at a concentration of 0.5%. All disinfectants were effective for K-file disinfection.
Keywords: K-file disinfection, alcohol, chlorhexidine, sodium hypochlorite, *Staphylococcus aureus*

ABSTRAK
Penelitian ini mengevaluasi efektivitas alat K-file yang terkontaminasi *Staphylococcus aureus* dan perendaman dalam alkohol, klorheksidin, dan natrium hipoklorit melalui penghitungan CFU. Identifikasi bakteri melalui pewarnaan Gram dan proses pengenceran untuk mendapatkan 300 CFU awal, kemudian proses kontaminasi bakteri dilakukan pada K-file. Sampel dibagi menjadi 3 kelompok dan diulang beberapa kali dalam seri waktu. Untuk alkohol dikelompokkan menjadi konsentrasi 70% dan 96%. Untuk klorheksidin dan natrium hipoklorit, masing-masing konsentrasi dibagi menjadi 3 kelompok, kemudian penghitungan CFU dilakukan secara manual. Hasil CFU setelah perendaman dalam alkohol 70% selama 5, 10, dan 15 menit adalah 28 CFU, 5 CFU, dan 0 CFU; alkohol 96% adalah 5 CFU, 1 CFU, dan 0 CFU; klorheksidin 0,5%, 1%, dan 2% selama 5 menit dan 10 menit adalah 0 CFU; natrium hipoklorit 0,25% 3 CFU, selama 10 menit 1 CFU; natrium hipoklorit 0,5% dan 1% selama 5 menit dan 10 menit adalah 0 CFU. Disimpulkan bahwa efektivitas antibakteri alkohol dan natrium hipoklorit terhadap *S.Aureus* meningkat seiring dengan peningkatan konsentrasi desinfektan dan waktu perendaman K-file, sementara klorheksidin efektif membunuh *S.Aureus* pada konsentrasi 0,5%. Semua disinfektan efektif untuk desinfeksi K-file.
Kata kunci: desinfeksi K-file, alkohol, klorheksidin, natrium hipoklorit, *Staphylococcus aureus*
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INTRODUCTION

In the practice of dentistry, blood and saliva are considered as dominant contaminants that contain 750 millions of microorganisms which causes cross infection among health care workers.[1] In the last two decades, infection control is the main concern with strict protocol in order to protect the workers and patients. On the other hand, limited space of practice room increase the risk of biologic agent transmission due to air shared. Patients inhale 700 liters of air per hour and significantly increase when they are in anxiety during dental treatment.[2,3]

In endodontic treatment the success depends on the eradication of microorganism of root canal and prevention of reinfection. It is of utmost important to build and maintain aseptic during treatment. K-file instruments are repeated use during root canal preparation that might increase the risk of treatment failure.[4] The result of Merdad dan Alghamdi study found 9 of 25 new K-files are contaminated with microorganisms.[5] Tonello, et al reported some types of microorganisms usually contaminate instruments of dental clinics are *Bacillus subtilis, Streptococcus viridans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Staphylococcus epidermidis*.[6]

Data from local dental hospital showed *S.aureus* is one of the microorganism frequently found to contaminate dental instruments and materials. *S.aureus* is Gram positive microorganism that can cause inflammation, infection or necrosis to host tissues. To avoid the potency of aseptic chain breakage and treatment failure, the instruments should be disinfected prior to their use in the root canal.[7]

Various measures have been used to disinfect endodontic instruments before sterilization including sodium hypochlorite, chlorhexidine, alcohol, povidone iodine.[7,8]

Alcohol is a stable desinfectant, is indicated to clean small surface, does not injure the skin or materials, biodegradable and mostly used in concentrations of 70% or 96%.[9] Chlorhexidine is a disinfectant of bactericidal activity, and the concentration usually used is 0.5%, 1%, and 2%[10], while NaOCl has strong antibacterial disinfectant which can dissolve pulpal tissue and biofilm. There is still no consensus of the concentration used that can vary 0.5-6%.

This study aims to evaluate the disinfection efficacy of K-files contaminated with *S.aureus* following immersion with alcohol, chlorhexidine, and sodium hypochlorite at various concentrations and exposure times.

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METHODS

This study was conducted following the approval of the Ethical Exemption of Dental Faculty, Hasanuddin University, Makassar (No.059/KEPK FKG-RSGMP UH/EE/XI/2024) on November 13th, 2024. This laboratory study was designed with a pre and posttest study to evaluate the number of CFU of bacteria found on K-file following immersion in disinfectants alcohol, chlorhexidine, and sodium hypochlorite.

The instruments used are inoculating loop, preparation glass, reaction tube, micropippete, incubator, vortex mixer, pincet, stirring rod triangle, bunsen burner, K-file size 25, length 25 mm.

S.aureus is obtained from bacterial culture ATCC 29737; blood agar plate is obtained from Microbiology Laboratory, Medical Faculty, Hasanuddin University. Chemicals used are 3% KOH solution, 70% and 96% alcohol, 0,5%, 1%, and 2% chlorhexidine, 0,25%, 0,5%, 1%, sodium hypochlorite, physiologic solution, crystal violet solution, mordant solution and methyl alcohol.

Working procedures

Bacterial identification was conducted using the Gram staining method. One drop of 3% KOH solution was placed on a glass slide using an inoculation loop and close to a Bunsen burner to maintain sterility. A culture of S.aureus was then smeared onto the KOH drop and left to dry. Once dried, the slide was stained evenly with crystal violet and kept for 1 minute. The slide was then tilted and rinsed gently with running water. A mordant solution was applied and left for 1 minute, followed by another gentle rinse. The slide was then ready for microscopic examination under 40X magnification for bacterial identification.

Identified *S.aureus* was inoculated into a test tube containing physiological saline using an inoculation loop. Serial dilution was performed until initial CFU count reached 300 CFU. The K-File was then placed into the test tube containing the *S.aureus* suspension using tweezers and left for 5 minutes to allow for bacterial contamination. Following contamination, 1.5 mL of the bacterial suspension containing the K-file was transferred to a blood agar plate (BAP) using a pippete. The solution was evenly spread using a sterile triangular stirring rod and stored for incubation as a negative control.

Contaminated K-files were placed into labeled tubes containing 70% and 96% alcohol for the disinfection process. The tubes were subjected to vibration to ensure that alcohol reached all grooves of the K-files evenly. The vibration was followed by immersion for predetermined durations of 5, 10, and 15 minutes. At each time interval, 1.5 mL of alcohol solution was transferred to BAP using a pippete and spread with a triangular stirring rod. This step was repeated at 10- and 15-minute intervals. The BAPs were then stored as positive control samples for incubation.

The chlorhexidine disinfection procedure followed the same protocol as the alcohol method. The concentrations used were 2%, 1%, and 0.5% with exposure times of 5 and 10 minutes. Thus, transfer of the chlorhexidine solution to BAPs was performed only at these two time points. The BAPs were then stored as positive controls for incubation.

The disinfection procedure using sodium hypochlorite was also similar to that used for alcohol. Concentrations of 0.25%, 0.5%, and 1% sodium hypochlorite were used with exposure times of 5 and 10 minutes. Transfer of sodium hypochlorite to BAPs was done at both time intervals. The BAPs were then stored as positive controls for incubation.

All BAPs containing negative and positive control samples were incubated in an incubator for 48 hours, and the bacterial colonies formed on the BAPs were directly counted to determine the number of viable bacteria.

Reduction of CFU of *S.aureus* following 5 minutes im-mersion in 70% alcohol was 90.6 % while reduction of CFU of *S.aureus* following 5 minutes immersion in 96% alcohol was 98.3 %. Reduction of CFU of *S.aureus* following 10 minutes immersion in 70% alcohol was 98.3% while reduction of CFU of *S.aureus* following 10 minutes immersion in 96% alcohol was 99.6% (Table 1). Reduction of CFU of S.aureus following 5 minutes or 10 minutes immersion in 0.5%, 1% and 2% in chlorhexidine was 100%. Reduction of CFU of *S.aureus* following 5 minutes

RESULTS

Table 1 Number of *S.aureus* following immersion with 70% and 96% alcohol

	Number of CFU				
Time	Control (-)	Alcohol 70%	Reduction (%)	Alcohol 96%	Reduction (%)
5 minutes	300	28	90.6	5	98.3
10 minutes		5	98.3	1	99.6
15 minutes		0	100	0	100

Table 2 Number of *S.aureus* following immersion with chlorhexidine

		Number of CFU					
Time	Control (-)	*Chlorhexidine* 0.5%	Reduction (%)	*Chlorhexidine* 1%	Reduction (%)	*Chlorhexidine* 2%	Reduction (%)
5 minutes	300	0	100	0	100	0	100
10 minutes		0	100	0	100	0	100

Table 3 Number of *S.aureus* following immersion with sodium hypochlorite

		Number of CFU					
Time	Control	Sod.hypochlorite 0.25%	Reduction	Sod.hypochlorite 0.5%	Reduction	Sod.hypochlorite 1%	Reduction
5 minutes	300	3	99%	0	100	0	100
10minutes		1	99.6%	0	100	0	100

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Table 4 Number of *S.aureus* following immersion with alcohol, chlorhexidine, and sodium hypochlorite

	Number of CFU						
Time	Control	Alcohol 96%	Reduction	Chlorhexidine 0.5%	Reduction	Sodium hypochlorite 0.5%	Reduction
5 minutes	300	3	99%	0	100	0	100
10 minutes		1	99.6%	0	100	0	100

or 10 minutes immersion in 0.25% sodium hypochlorite was 99% and 99.6% respectively, while reduction of CFU of *S.aureus* following 5 minutes or 10 minutes immersion in 0.5% and 1% sodium hypochlorite was 100 %.

DISCUSSION

Alcohol, typically ethanol or isopropyl alcohol is a stable disinfectant, has broad-spectrum antimicrobial activity, indicated for use on small surfaces, does not irritate the skin or materials, and is biodegradable, which suits its use for immersion instruments such as K-files. The concentrations commonly used for disinfection are 70% and 96%.[2,9] Alcohol denatures proteins that leads to loss of enzyme and structural protein function, causing cell death. In addition, alcohol can dehydrate cells that helps in preserving cell structure, however it contributes to cell lysis when denatured protein occurred. For optimal protein denaturation, water is required. Therefore 70% alcohol is assumed more effective than 95%.[9] In this study, the reduction of CFU is similar between 70% alcohol and 96% alcohol in each duration of immersion. At lower concentrations, disinfectants exhibit bacteriostatic activity, whereas higher concentrations, molecular kinetics increase and enhance inhibitory effects, leading to bactericidal activity. Limitations of alcohol is ineffective against bacterial endospores.[9]

Chlorhexidine is a disinfectant with bactericidal properties, capable of killing microorganisms Gram+ and Gram-. The concentrations commonly used for chlorhexidine as a disinfectant are 0.5%, 1%, and 2%.[10] In this study, the lower concentration (0.5%) of chlorhexidine has been able to eradicate 100% CFU of *S.aureus*. Chlorhexidine is a cationic bisbiguanide, binds to negatively charged phospholipids on bacterial membranes, increases membrane permeability that cause leakage of intracellular contents, and cell death. At higher concentration, it causes precipitation of cytoplasmic protein by penetrating the bacterial cell. It is slowly released, maintaining prolonged residual antimicrobial effect up to 12 hours. Chlorhexidine is commonly used as root canal irrigant and mouth wash.[10,11]

Meanwhile, sodium hypochlorite is effective due to its strong antimicrobial activity, ability to dissolve biofilms and pulp tissue, and its capacity to reduce bacterial virulence factors. NaOCl concentrations range 0.5-8%, although there is no consensus on the ideal concentration. NaOCl at 5.25% and chlorhexidine at 2% have been proven effective in killing various microorganisms, including *E.faecalis* and *C.albicans*.[12,13] However, NaOCl is superior in dissolving the organic tissue. The combination of NaOCl and chlorhexidine can enhance antimicrobial effectiveness, but it must be neutralized to prevent the formation of harmful precipitates.[14] NaOCl disintegrates cell membranes via saponification, causing cytoplasmic leakage, neutralizes amino acids which disrupt bacterial metabolism and proteins, kills bacteria through its oxidative action damaging DNA, enzymes, and lipids, dissolves necrotic tissue and biofilms, making it ideal for root canal irrigant.[12-14] Results in this study showed that at lowest concentration (0.25%) of NaOCl, almost total reduction of *S. aureus* was found.

It is concluded that 1) the antibacterial effectiveness of alcohol and sodium hypochlorite against *S.aureus* increases with higher disinfectant concentrations and longer durations of K-files immersion, 2) chlorhexidine has been effective at low concentration (0.5%) in killing *S.aureus* within 5 minutes immersion, and 3) all desinfectant are effective in 5 minutes K-files immersion at low concentration. So, it is recommended that further study need to be conducted to evaluate the physical characteristics of K-file following immersion into disinfectants 70% alcohol, 0.5% chlorhexidine, and 0.5% sodium hypochlorite.

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