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Molecular identification and Control of some Gram negative bacterial isolates from Dental Unit Water Lines

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Abstract : The potency of biocides and disinfectants is very important for the prevention of diseases transmission through clinical pathogens In this Study, five bacterial strains were isolated on nutrient agar medium from a randomly selected dental unit, located in a public dental clinic in Giza governorate, Egypt. The obtained isolates were identified by molecular technical 16s rRNA as; *Pseudomonas monteilii* (DD2), *Pseudomonas monteilii* (DD3), *Pseudomonas aeruginosa* (DD4), *Stenotrophomonas sp* (DD5) and *Stenotrophomonas sp*. (DD7) respectively and were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers. The identified strains susceptibility towards different biocides was examined. The tested biocides were; SDS, H_2O_2, TW_20, EDTA, NaOCl and phenol in the concentrations of 25%, 50% and 75%. Results obtained clearly indicated that; the largest inhibition zone observed was 5.9 cm in case of using 75% NOCl for (DD7) Followed by the effect of 75% H_2O_2 in case of (DD5) giving inhibition zone of 4.9cm. Phenol gave 4.5 cm inhibition zone in the concentration of 75% with (DD3). The most resistant strain was (DD4) showing the largest inhibition zone of 3.1 cm with 75% phenol and the smallest inhibition zone of 1.3 cm with 25% H_2O_2.

Key word : Dental unit water lines, Gram negative bacteria, culture characteristics, 16S rRNA, biocides.

Introduction

Bacteria in Aquatic environment tend to form biofilms when coming to contact with solid surfaces, bacteria use this form for life preservation and easy uptake of nutrients. Isolation of bacteria from biofilm through Dental unit water lines (DUWL) was performed. It is not easy to mechanically remove and disinfect this biofilm from surfaces in most cases. Also, the presence of a biofilm in DUWL make as continues source of infection[1].

The water input is not thought to be the infection source, but the back flow from oral cavity may support the presence of biofilm in the water lines.[2] The back pressure from the dental machine and inhalation of microbial spore cause of dental-unit water pollution[3].

In the process of dental machine hygiene and disinfection, if dental staff are not applying the disinfectant according to the manufacturer's instructions [3].Bacterial fragments separated from the biofilm coating the inner surface of the dental tubing causes spread of bacterial biofilm infection inside the machine.

There are many approaches to contamination control of DUWL including: Filtration, Flushing, Biocides, chemical disinfectants, Chlorination, Peroxide, ozone and ultraviolet light, and use of autoclavable systems. Chlorine as sodium hypochlorite is the most commonly employed biocide in water treatment plants and has proven efficacy in cold water hospital systems, in particular in controlling Legionella [4]. A wide range of disinfectant products are now being developed for use in DUWS, and these have been evaluated using a variety of approaches, although rarely have these products been compared in a general dental practice setting [4].

This study aims to determining the bacterial contamination of patient suction tubings in DUWL that can be of possible hazard to dental staff and patients. Also testing some biocides for antibacterial activity against those pathogens to verify the susceptibility of those pathogens towards biocides. Contamination was determined by microbial isolation and identification of some bacteria by 16S rRNA.

Materials and Methods

Collection of water samples

The study material included 15 ml water samples taken from the patient suction tubings of a dental unit located in randomly selected dental private clinics and public health centers in the governorate of Giza, Egypt. Water samples were obtained in sterile, airtight test tubes and transported to the laboratory immediately after sampling in an insulated container at the temperature not exceeding 6°C. The samples reached the laboratory within 3 h and were inoculated into the media on the same day.

Bacterial isolation from DUWS

Conventional microbiological methods were used. Mesophilic bacteria were cultured on nutrient agar.

The examined water samples were inoculated on media simultaneously, using the plate dilution method with surface inoculation. A quantity of 0.1 ml of the water samples of dilution 10^{-3} in sterile phosphate buffer pH 6.5-7 were inoculated in each Petri-dish. The dishes were incubated at 35 °C for 48 h. After incubation, the initial identification of microorganisms was performed. The assessment of the growth of bacteria included their macroscopic morphological characteristics, such as the size and form of colonies, surface and margin, color, opacity and texture. Next, considering the previously described characteristics, the number of morphological types was determined, as well as their concentration, expressed in colony forming units in 1 ml of water (CFU/ml) according to the formula $x = a \times r/0.1$, where: 'x' is the concentration of bacteria in water expressed as CFU/ml; 'a' is the average number of colonies on a plate; and 'r' is the reverse of the dilution.

Identification of Bacterial isolation from DUWS

Gram staining

The smear was prepared from positive culture and flooded with crystal violet solution for two minutes. Then the slide was washed with distilled water and Gram's Iodine was applied for one minute. After that 95% alcohol was applied until the colour runs off. Finally dilute fuchsin solution was applied for about one minute. Then the slide was washed with distilled water and microscopically examined under oil immersion [5].

Molecular identification

Isolates were characterized by 16S rRNA sequencing analysis. Chromosomal DNA was extracted with Qiagen kit according to the manufacture instruction. The 16S rRNA gene (~ 1500 bp) was amplified using universal primers F:- AGA GTT TGA TCC TGG CTC AG, R:- GGT TAC CTT GTT ACG ACT T . The PCR reaction of 16S rRNA was in a volume of 50 µl containing 1x green Taq PCR Buffer, 200 mM of each dNTPs, 100 mg BSA, 10 pmole of each oligonucleotide primer, 2.5U of green Taq DNA polymerase (Sigma) and 10 ng of DNA extract. PCR was performed by the following conditions: 1 min at 98 ^{o}C followed by 35 cycles of 1 min at 94 ^{o}C, 30 sec at 55 ^{o}C, and 1 min at 72 ^{o}C. The 16S rRNA product was eluted, purified by (Qiagen elution kit) and sequenced in Promega company laboratory (Cairo, Egypt).

The sequence was matched with previously published bacterial 16S rRNA sequences in the NCBI databases using BLAST. Selected sequences of other microorganisms with greatest similarity to the 16S rRNA

sequences of bacterial isolate were extracted from the nucleotide sequence databases and aligned using MEGA6. Multiple Sequence Alignment generating the phylogenetic tree. The 16S rRNA gene sequences of the bacterial isolates which reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers: LC200433 (DD2), LC200432 (DD3), LC200431(DD4), LC200430(DD5) and LC200429 (DD7) .

Biocides used for controlled the bacterial growth

Biocides	Biocides concentration (%)
Sodium dodecyl sulphate (SDS)	1.0
hydrogen peroxide (H_2O_2)	35.0
Tween 20 (Tw 20)	4.0
Ethylene di-amino tetra acetic acid (EDTA)	1.0
Sodium hypochlorite (NaOCl)	5.2
Phenol (Phe)	1.0

The biocides used were; SDS, H_2O_2, TW20, EDTA, NaOCl and phenol. Different biocide concentrations 25%, 50% and 75% were prepared in sterile distilled H_2O. Susceptibility to biocides. The isolates under test were grown on agar slants for 24 h. Swab sticks containing the different bacterial cultures were swirled into different test tubes containing 10 ml of sterile water. The content of each of the tubes was properly homogenized before the inoculation. Another set of sterile swab sticks were dipped into each of the bacterial solution and were used to inoculate the solidified Nutrient agar plates ensuring that the plates were completely covered for uniform growth.

Using sterile forceps, different sterile filter paper discs impregnated with 0.1 ml of different disinfectant dilutions were placed on each of the plates inoculated with the bacterial isolate under test. The plates were incubated at 37°C for 24 hours. The zones of inhibition were measured. A zone of inhibition is indicative of anti-microbial activity against the organism.

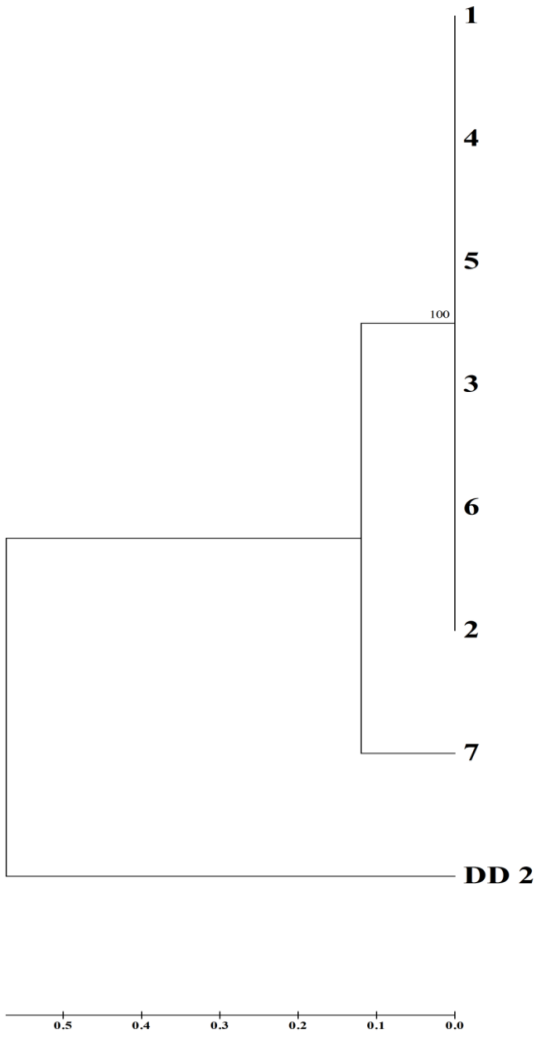
Results and Discussion

The isolation, morphological examination and counted on solid agar medium were carried out. Gram staining was performed and the results showed that the majority of isolates were Gm –ve (62.7%). Five Gram negative strains were chosen for further identification by 16s rRNA (table 1).

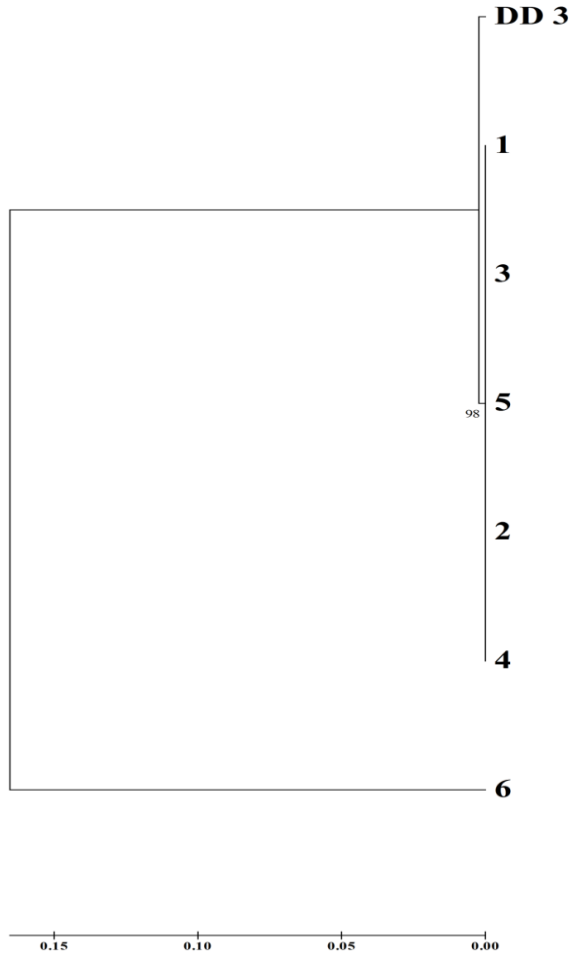
For identification, 16S rRNA encoding gene of DD2, DD3, DD4, DD5, DD7 was PCR-amplified and sequenced. The nucleotide sequence was compared to existing sequences in the databases. A dendrogram showing the results of 16S rRNA analysis is shown in Figure 1,2,3. The results showed highest matching of *Pseudomonas monteilii , Pseudomonas monteilii , Pseudomonas aeruginosa, Stenotrophomonas sp* and *Stenotrophomonassp* respectively. The isolates were recorded in gene bank with accession no as new strains LC200432 (DD3), LC200431 (DD4), LC200430 (DD5) and LC200429 (DD7) .

Table (1): The identified Gram negative strains and their share percentage value.

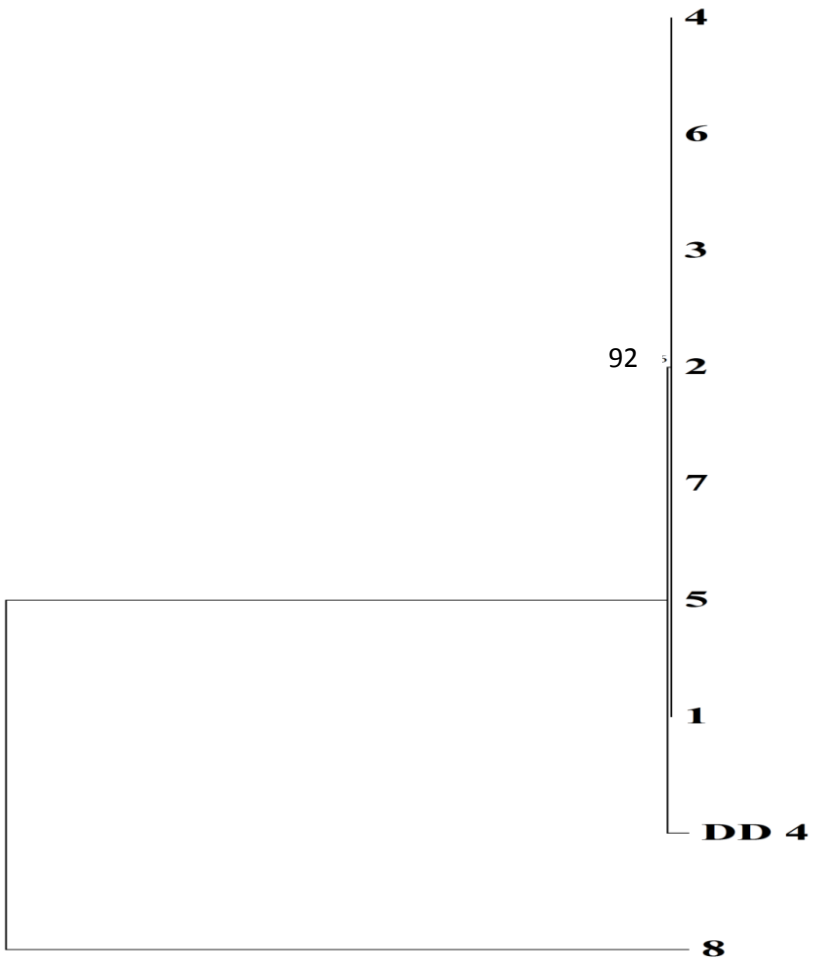
Genus/Species	CFU/ml	Share percentage (%)
Pseudomonas sp. (DD2)	21	12.0
Pseudomonas sp. (DD3)	18	10.6
Pseudomonas aeruginosa (DD4)	9	5.3
Stenotrophomonas sp. (DD5)	28	16.5
Stenotrophomonas sp. (DD7)	31	18.3
Total		62.7



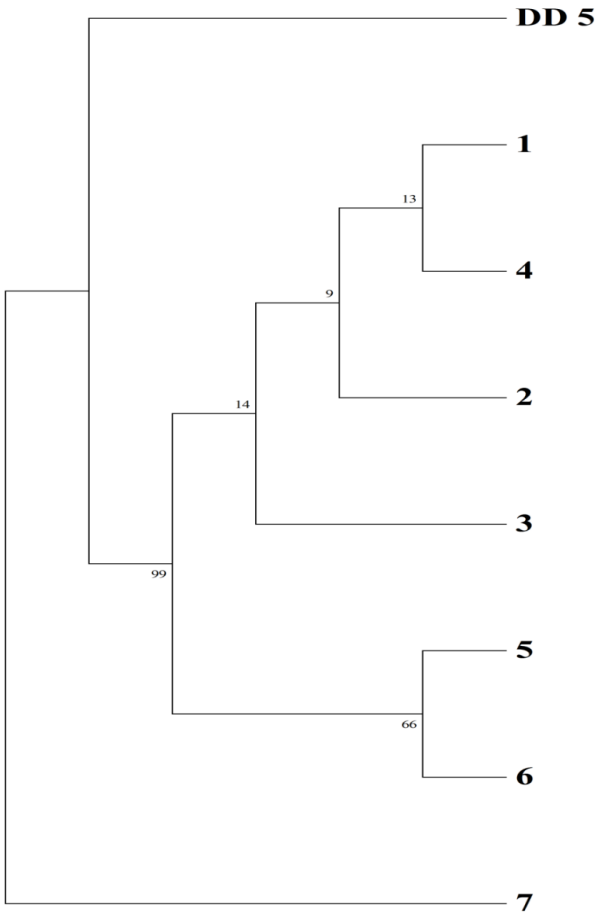
(AB739622)DD2 (LC200433),1- Pseudomonas monteilii (KU550170), 2- Pseudomonas monteilii (KU550155), 3- Pseudomonas monteilii (KU550143),4- Pseudomonas monteilii (KT881478),5- Pseudomonas sp. ,(KT354245),6- Endophytic bacterium (KP757639), 7- Bacillus subtilis (GQ482982)



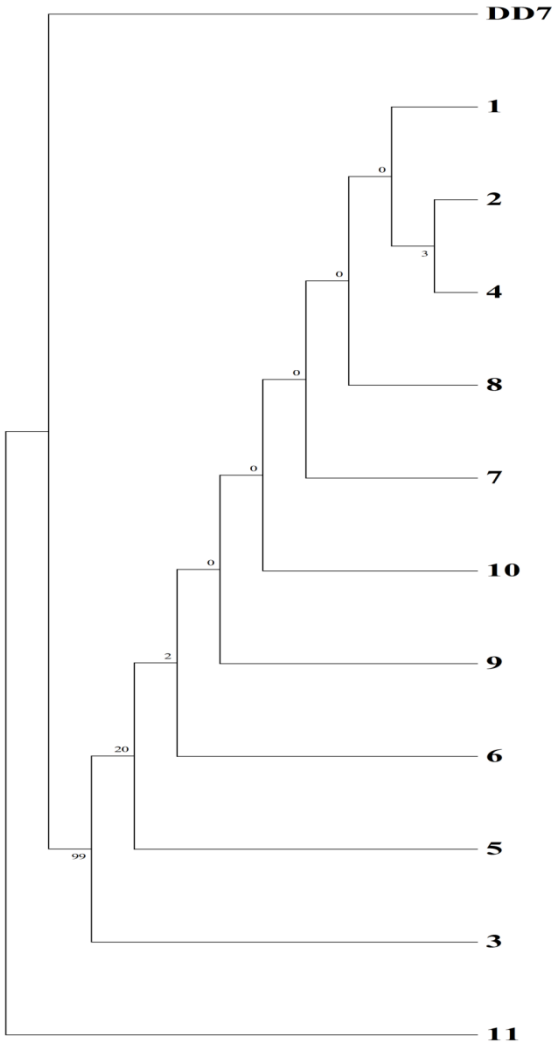
DD3 (LC200432),1- Pseudomonas monteilii (KU550170),2- Pseudomonas monteilii (KU550155),3- Pseudomonas monteilii strain (KU550143),4-Pseudomonas sp.(KU869716),5- Pseudomonas (KU512626),6- Bacillus subtilis (GQ482982(



DD4 (LC200431),1-Pseudomonas sp. RH-7 (KT715742), 2- Pseudomonas aeruginosa strain TBH2 (KU708862), 3- Pseudomonas aeruginosa strain NAL24 (KU550208),4- Pseudomonas aeruginosa strain W1 (KX353803), 5- Pseudomonas sp. SP-3.1 (KX390661),6- Pseudomonas sp. BAB-5365 (KX168032.1),7- Pseudomonas sp. H117 (KU194211),8- Streptomyces pseudogriseolus NRC-15



DD5(LC200430), 1-Stenotrophomonas sp. 329P5R (KR611641), 2-Stenotrophomonas sp. PA44May (KJ482759),3-Stenotrophomonas rhizophila isolate P3F12(HF936880),4-Stenotrophomonas rhizophila GL5(HF545317), 5-Stenotrophomonas sp. RYC16 (KX450459.1),6-Stenotrophomonas sp. strain H14G3 (KU534303) and 7-Streptomyces pseudogriseolus NRC-15



DD 7(LC200429),1-Stenotrophomonas sp. HH10 (KC857480),2-Stenotrophomonas sp. Es35 (JQ977442),3-Stenotrophomonas rhizophila strain TRJB_41(KX774590),4-Stenotrophomonas sp. strain RC5 (KU697294),5-Microbacterium sp. JCM 28702 (LC133733),6-Stenotrophomonas sp. JCM 28697 (LC133728),7-Bosea sp. JCM 28691(LC133722.1),8-Streptomyces sp. JCM 28690 (LC133721),9-Olivibacter sp. JCM 28686 (LC133717),,10-Stenotrophomonas sp. JCM 28723 (LC133754) and 10-Streptomyces pseudogriseolus strain: NRC-15 (AB739622(

Stenotrophomonas spp. is an aerobic Gm -ve bacillus, found in different environments. Risk factors associated include malignancy, immunosuppressant therapy procedures, cystic fibrosis, and exposure to broad-spectrum antibiotics[6].

Pseudomonas aeruginosa is considered a major cause of hospital infections. It is frequently found in water lines supplying dental units [7,8].

Pseudomonas monteilii is a Gram-negative, rod-shaped, motile bacterium isolated from human bronchial aspirate. Based on 16S rRNA analysis, *P. monteilii* has been placed in the *P. putida* group [9].

The 5 identified strains were tested against the selected biocides as shown in fiures (1-5). Many studies reported that, Bloodstream infections, wound or surgical site infections, pneumonia and meningitis are diseases caused by bacterial contamination from DUWL.

Results clearly demonstrate that the most effective screened biocides for the antibacterial effect were, sodium hypochlorite, hydrogen peroxide and phenol respectively. While, sodium dodecyle sulphate (SDS), tween-20 and EDTA had a moderate to weak effect especially in the first concentration 25% where they all showed no inhibition zones on tested bacteria.

Concerning the inhibition zones produced with (DD2) it was found that 75% SDS had a very close effect to 75% H_2O_2 and NaOCl and the resulting inhibition zones measuring; 3.0, 3.2 and 3.5 cm respectively.

The smallest inhibition zone after using H_2O_2 in the concentration of 25% was with (DD3) measuring 1.5 cm while the largest inhibition zone was observed when using 75% phenol measuring 4.5 cm.

For (DD5), there was a close range for the difference between the inhibition zones of different concentrations of the same biocide. The measured inhibition zones were; 4.2, 4.5 and 4.9 cm for 25%, 50% and 75% H_2O_2 while for NaOCl the results were; 4.3, 4.8 and 5.3 cm for the concentrations 25, 50 and 75% respectively.

The inhibitory effect of every biocide compound under test increases by increasing its concentration. The largest inhibition zone was observed in (DD7) when the concentration of NaOCL was increased to 75% where it reached 5.9 cm. on the other hand when, H_2O_2 was used in the same concentration showed similar effect with an inhibition zone of 5.4 cm .

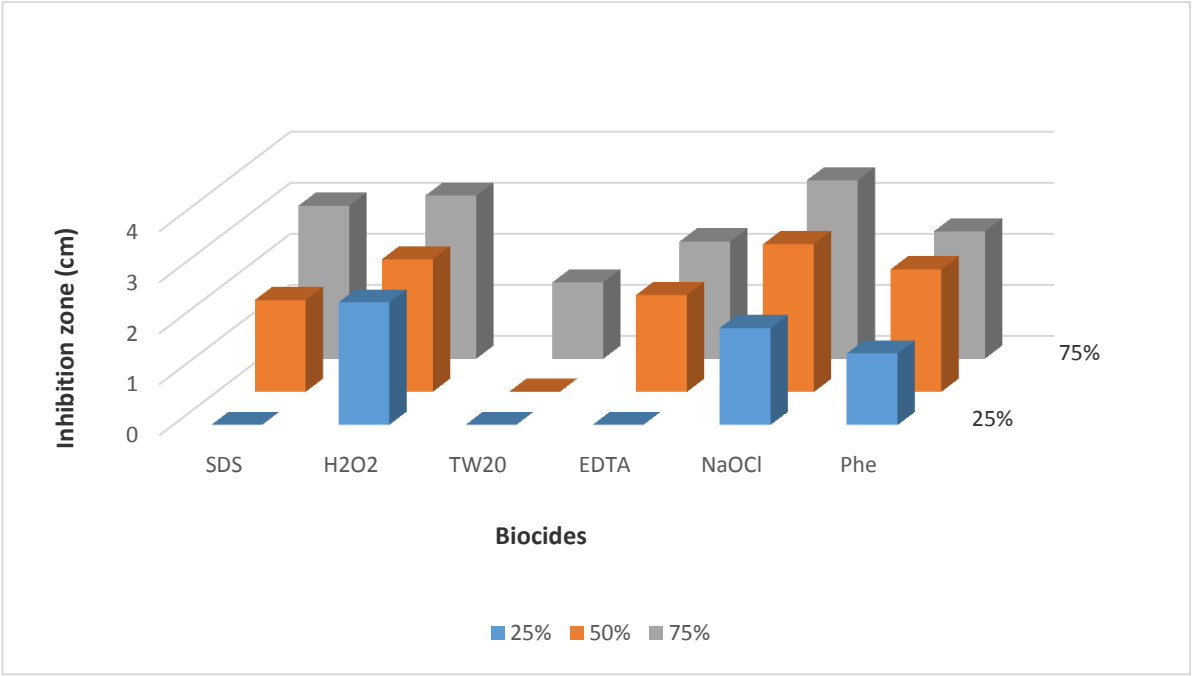


Fig. 1. Effect of tested biocides on (DD2)

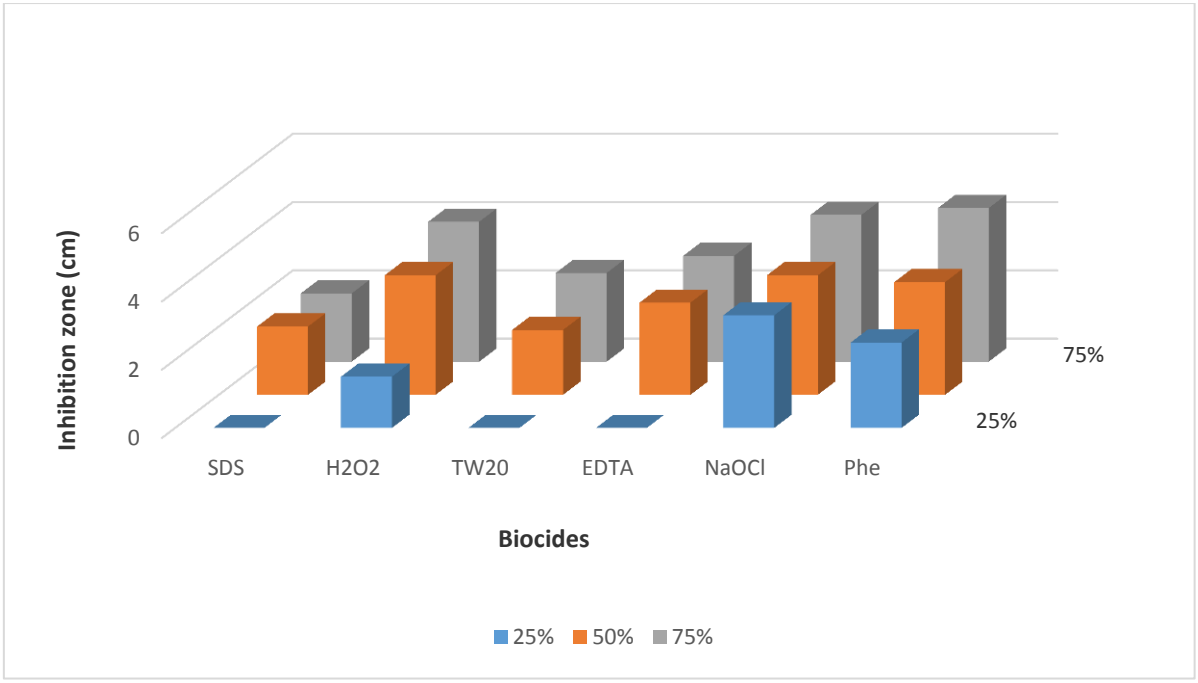


Fig. 2. Effect of tested biocides on (DD3)

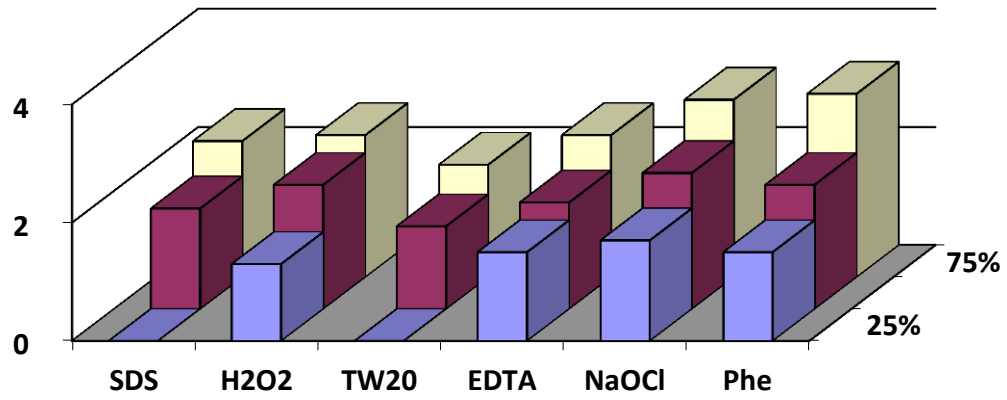


Fig.3. Effect of tested biocides on (DD4)

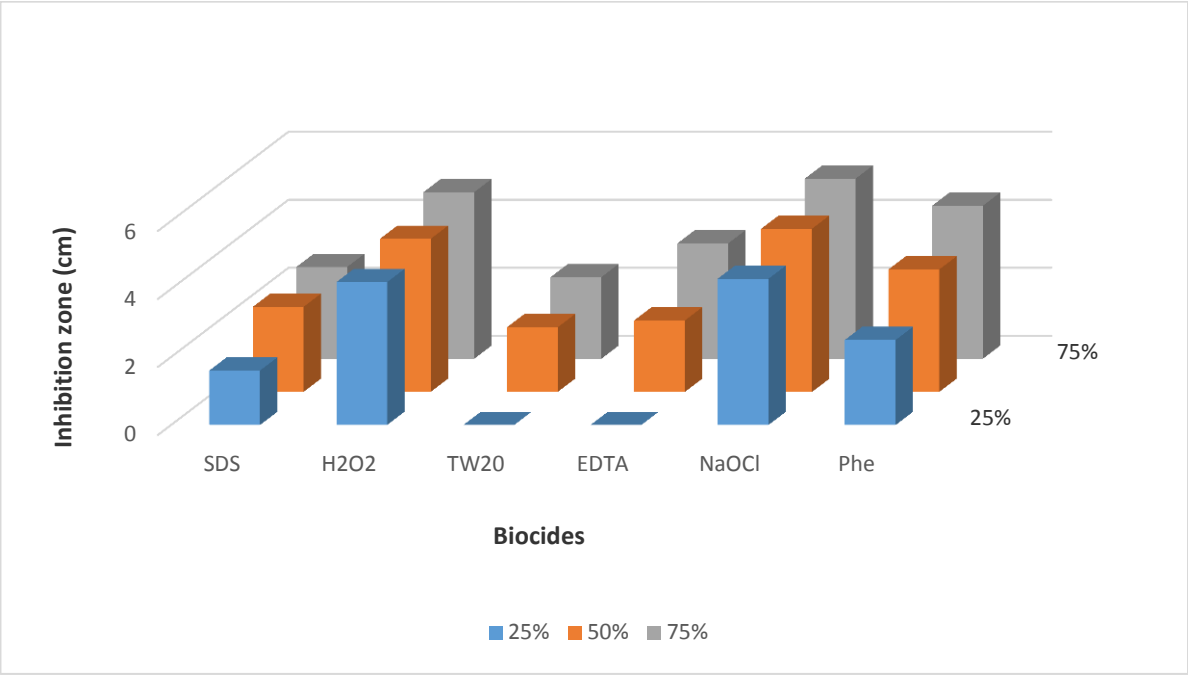


Fig.4. Effect of tested biocides on (DD5)

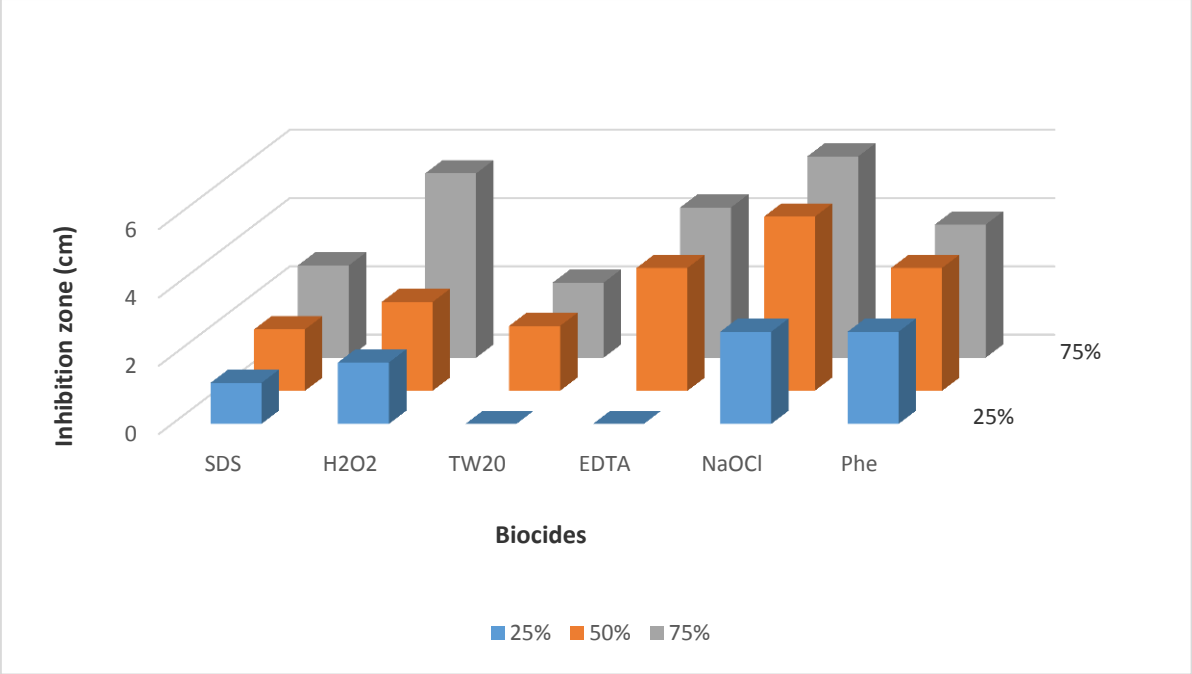


Fig. 5. Effect of tested biocides on (DD7)

Conclusion

The presence of potentially pathogenic microorganisms among the isolated bacteria with relatively high concentrations and the tissue invasive procedures used in dental clinics makes it inevitably important for the daily use of effective disinfectants and biocides to reduce dental unit contamination and associated health risk. Disinfectant products containing NaOCl, H_2O_2 and phenol were found to be most effective among the screened biocides. The concentrations used in such products should be carefully monitored in order not to cause skin irritation or corrosion to surfaces.

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