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Molecular detection of some pathogenic bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*) from human saliva

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ABSTRACT

Saliva is thought to have a significant impact on the colonization of microorganisms in the oral cavity, the present study aimed to determine and confirming molecularly presence of the pathogenic saliva-bacterium, in addition to elaborating the effect of triclosan washing material on them. The study includes 42 saliva samples which were collected from patients in a private dental clinic from the area facing the lower front teeth. The samples were diagnosed by culture, microscopic and biochemical tests. Microbiologically 20 *Klebsiella pneumoniae*, 16 *Pseudomonas aeruginosa* and 6 *Escherichia coli* isolates confirmed. The sensitivity to the triclosan were varies and *P. aeruginosa* isolates were 80% and followed by 70% and 50% for *K. pneumoniae* and *E. coli* respectively. The results of the PCR reaction showed that *K. pneumoniae* bacteria with a size of 130 bp, *P. aeruginosa* bacteria with a size of 500 bp and *E. coli* with a size of 147 bp. Nitrogenous base sequences results for 6 bacterial isolates were compared with similar standard strains from the Gene bank available on the NCBI website and showed a 100% similarity between the bacterial isolates and those recorded in the gene bank.

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Introduction

The human oral cavity environment from birth is colonized with a complex community of microbiota associated with the oral mucous membranes (Deo & Deshmukh 2019). People who can't maintain good oral hygiene will be subjected to oral diseases like periodontal disease and dental caries as the normal flora have the ability to stick to the teeth surfaces and the gum which is difficult to be removed and become microbial population (Sharma et al. 2018).

Saliva in the oral cavity with other environmental factors like acidity, temperature, oral hygiene, and the diet make an open preferable environmental system for different types of microbes like the Gram-negative bacteria including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* with ill-defined complex relationship (Zawadzki et al.

2017). In the case of people with debilitating diseases or weakened immune systems these bacteria could cause infections with complications. As the resistance among Gram-negative bacteria is more rapid than Gram-positive bacteria so their treatment become more difficult with increasing resistance to antimicrobial agents (Sara et al. 2017).

Many environmental and genetic factors affect the development of oral pathology, especially among people who have difficulty to maintaining good oral health, as well as healthy social conditions, systemic disorders, and congenital malformations among are factors that affect oral hygiene and play an effective role in the emergence of symptoms of periodontal disease and dental caries (Mahdi et al. 2016).

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Nowadays, the natural products have been studied and evaluated to be used in most of the mouth washes due to the effectiveness of these extracted materials as antimicrobial agents against different types of oral microorganisms. Many studies review the effects of antibacterial properties of the Triclosan, Sage and Oak fruit in the mouthwash. In dentistry, these materials have been used effectively in the treatment of inflammations in the oral cavity (Schönknecht et al. 2021).

The effective oral defense mechanisms against Gram-negative rods bacteria make it unusual to be isolated normally. *K. pneumoniae* is a gram-negative, encapsulated, non-motile bacterium found in the oral environment. *P. aeruginosa* is a Gram-negative rod-shaped microorganism that is pathogenic to humans, animals, and plants (Diggle & Whiteley 2020). *E. coli* is anaerobic, single straight rod-shaped bacteria, can be motile or nonmotile and have the ability of both a respiratory metabolism and fermentation and do not produce the oxidase enzyme (Min et al. 2021).

This study aimed to isolate, tested bacteriologically, biochemically and confirming molecularly the pathogenic saliva-bacterium isolates. In addition, the sensitivity of the isolates will be tested by using mouthwash containing triclosan material. Finally, to analysis the nitrogenous bases sequence of the detected bacteria and compare them with the sequencing of the slandered sequence of that in the genomic bank in order to determine the infectious microorganisms that are risk factors for human oral health.

Materials & Methods

Ethical approval

For this study, ethical approval was obtained from Duhok Directorate of Health regarding the process of obtaining the saliva samples and personal data from the patients (Reference number: 18052022-3-3).

Sample collection and Isolation of bacteria

The oral swab samples of the 42 patients were collected of different age groups and both gender attending the private dental clinic from the area facing the lower front teeth taking by a sterile cotton swap (Table 1) in supplementary materials. The swabs were cultured on Nutrient agar, with a total of (42 samples), different types of microbes found in saliva were obtained, the mostly repeated bacteria in the samples were selected of three types (*K. pneumoniae*, *P. aeruginosa*, and *E. coli*) to be repeated in most of the samples. Bacterial isolates were cultured on a Nutrient agar medium and Nutrient broth medium to develop the bacterial isolates and keep them in the refrigerator until use. The media were prepared according to the instructions of the supplied company.

Identification and Diagnosis of Isolates

The bacterial isolates were diagnosed based on the culture, microscopic and biochemical characteristics. The

bacteria characteristics were studied by testing their ability to grow in the culture media including the MacConkey agar medium and the blood agar medium. The samples under study were planted on the agar medium using the planning method. The plates were incubated at a temperature of 37°C for 24 hours to know the ability of bacteria to grow and to isolate the bacteria that have the ability to produce Hemolysin enzyme, which is also a rich media for bacterial growth. The medium was prepared according to the manufacturer's instructions (Forbes et al. 2007). The colonies were then transferred to MacConkey agar medium to diagnose bacteria that are capable of fermenting lactose sugar from those that are without fermentation. Cultivational characteristics of colonies play an important role in the diagnosis of bacteria, including colony shapes, sizes, textures, and odor (Jawetz, et al. 2019).

Microscopic gram staining

The microscopic examination of the bacterial isolates cells was carried out using an inoculation loop to transfer a part of a young colony, mixing it with a drop of water and spreading it on the surface of the slide and leaving it to dry, then fixing it with heat over a gentle flame and stained with gram stain and examined under the microscope, where the shapes and arrangement of bacterial cells were observed. As well as examine the colors that results from the interaction of bacteria with the dye (Jawetz, et al, 2019).

Biochemical tests

Biochemical tests were done, such as the catalase test to determine the ability of the isolates to produce the catalase enzyme that breaks down hydrogen peroxide into water and oxygen gas, and the oxidase test to test the ability of bacterial colonies to produce the oxidase enzyme, and the hemolysis test and test the ability of bacteria to produce The enzyme urease, which works on the decomposition of urea to ammonia and carbon dioxide, and the gelatin dilution test, these tests were conducted according to method (Forbes et al, 2007).

The effect of Triclosan on the growth of isolates test

The effect of triclosan in mouthwash on the bacterial growth was tested, the nutrient agar medium was sterilized, cooled and then 40 mg /L of sterilized Triclosan material by filtration method was added and leaving the medium to solidify. Twenty-one isolates were selected (S1, S2, S5, S7, S8, S15, S16, S19, S20, S21, S3, S4, S6, S10, S12, S13, S17, S18, S9, S11, S14) from each bacterial type under study were cultured on the medium and incubated at 37°C for 24 hours, the results were obtained by colony counting method.

Genomic DNA extraction

The genomic DNA of the bacterial isolates were isolated for molecular diagnosis using the steps and instructions attached to the Promega Wizard TM Genomic

Table 1. Specific primers used in PCR reaction in this study.

Bacterial isolates	Primers (5'-3')	PCR product size (bp)	Reference
<i>E. coli</i>	F(AAAACGGCAAGAAAAAGCAG) R(ACGCGTGGTTACAGTCTTGCG)	147	(Taha and Yassin, 2019)
<i>K. pneumonia</i>	F(Pf: ATT TGA AGAGGT TGC AAA CGA T) R(Pr1: TTC ACT CTGAAG TTT TCT TGT GTT C)	130	(Ranjbar <i>et al.</i> , 2016)
<i>P. aeruginosa</i>	F(ATG GAA ATG CTG AAA TTC GGC) R(CTT CTT CAG CTC GAC GCG ACG)	500	(Xu <i>et al.</i> , 2004)

DNA Purification Kits for the isolation of bacteria (Abdulmir *et al.*, 2010).

Agarose gel electrophoresis

This method was used to separate DNA molecules of different sizes and electrophoresis was carried out as it was mentioned in (Magdeldin 2012). Agarose gel was prepared at a concentration of 1% by dissolving 1 g of agarose in 50 ml of Tris-Acetate- EDTA-1 X buffer solution 3µl of red safe dye, which works on staining nucleic acids, then quietly poured into the agarose backing plate and fixed the special comb in the plate to form the holes and left to solidify for 20 minutes. The comb was gently lifted from the hardened Agarose and the plate was fixed in the electrolyte tank basin, and the basin was filled with 1X (TAE) solution to cover the entire surface of the Agarose gel. Each sample was loaded into the pits for transporting 2 µl of buffer solution. dye 5 microliters of DNA, then run a current of 100V for 90 minutes and exposing the gel to Transilluminator-UV source (Magdeldin 2012).

Polymerase chain reaction (PCR)

The PCR reaction was performed at Duhok Research Centre, college of medicine, university of Duhok. Using the specific primers for each bacterial isolate, as shown in (Table 1).

The PCR mixture of the 25 µL was consist of 12.5 µL of hot start premix (Genedirex, Taiwan), 1µL of each reverse and forward primer (10 pmol), 4 µL of sample DNA (30-100 ng/µL), the remainder was filled with double distilled water (Qiagen, Germany).

The PCR reaction was conducted for DNA samples isolated from *P. aeruginosa*, and the PCR machine was programmed according to (Hanoon *et al.*, 2020) using the protocol: Initial denaturation DNA 94°C for 5 minutes followed by 35 cycles that included the transcription stage at 94°C for 45 seconds and the initiator binding stage at 50°C for 5 minutes and the elongation phase at 72° C for 1 minute followed by the final elongation phase at 72° C for 5 minutes.

The PCR reaction was also conducted for the DNA samples isolated from the bacteria *K. pneumoniae* and *E.*

coli, and the PCR machine was programmed according to (Taha and Yassin, 2019) using the protocol: the initial denaturation DNA at a temperature of 95°C for 5 minutes and then followed by 35 cycles that included DNA replication at a temperature of 95°C for 1minute, bonding stage at 58°C for 1 minute, elongation stage at 72°C for 1 minute, followed by final elongation stage at 72°C for 5 minutes. After the end of the PCR reaction the results were taken and runed in 2% agarose gel prepared from (1× Tris-acetate-EDTA (TAE) buffer and stained by red safe DNA staining solution (GeNetBio, Korea) for 1.15 hours at 85 V).

DNA sequencing

The DNA sequence was determined by the method known as Sanger sequencing and by using the Genetic Analysis 3130 device of the Japanese company Hitachi at the MacroGen biotechnology company, where the sequences of nitrogenous bases were obtained. After that, these sequences were analyzed and compared with the sequences of the nitrogenous bases of the standard strains registered in the gene bank to find similarities and affinity between them.

Statistical analysis

Statistical analyses were performed using the computational software SPSS version 25. Significant difference test was used to compare the detection frequency of each species, in addition to compare the effect of triclosan on bacterial colonies growth. A P value < 0.05 was considered statistically significant.

Results and discussion

Isolation and identification of samples

The isolated bacteria recovered from the saliva of 42 patients were pathogenic bacteria 20 isolates belong to *K. pneumoniae*, 16 isolates belong to *P. aeruginosa* and 6 isolates belong to *E. coli*. The isolated bacteria were diagnosed based on their characteristics obtained from culture, microscopic, and biochemical tests. On a blood agar medium colony of bacteria *K. pneumoniae* appeared non-hemolytic (γ-hemolytic type). While the colony on MacConkey agar medium appeared large, mucoid smooth

and pink color due to the bacteria's ability to ferment lactose in the middle (Fig 1).

On the nutrient agar medium *P. aeruginosa* isolates appeared as large colonies with a high, flat edges, and a grape-like smell, while the colonies were pale on the MacConkey agar medium because they were not fermented to sugar lactose, the colonies also appeared mucoid of β -hemolytic type on the blood agar medium due to complete hemolysis of the blood, and this agrees with (Al-Ammari, 2018) (Fig 2). The bacterial isolates on the gelatin medium were caused hemolysis to the gelatin due to the production of the gelatinase enzyme (Baron et al, 2007). The microscopic examination showed cells of gram-negative bacilli, these results were in agreement with (Al-Tikriti, 2021).

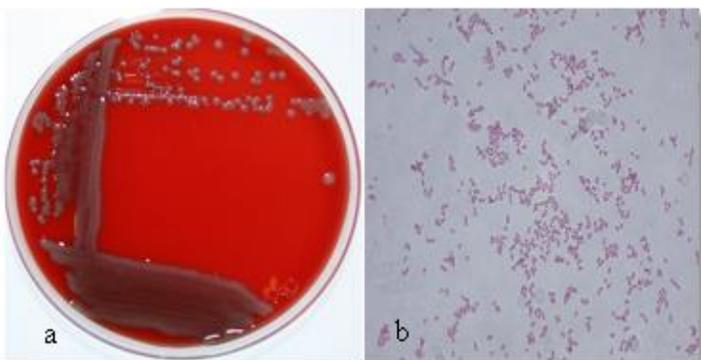


Fig 1. Colonies of *K. pneumoniae* (a) on blood agar medium, (b) Gram stain.



Fig 2. Colonies of *P. aeruginosa* (a) on blood agar medium, (b) Gram stain.

The results of isolates bacteria *E.coli* on the blood agar medium showed that the bacteria were not hemolytic type because they were unable to produce the enzyme Hemolyticase, while on the MacConkey agar medium the bacterial isolates appeared as pink colonies with sharp edges due to their ability to ferment the lactose sugar and produce acids. The MacConkey agar medium is considering a selective medium that allows the growth of gram-negative bacteria and prevents the growth of gram-positive bacteria because it contains yellow salts that inhibit the growth of gram-positive bacteria and therefore it is considered

negative bacteria for gram stain. Microscopic examination of the gram stains showed short rod-shaped cells that were negative for gram stain, as shown in the (Fig 3), these results agree with (Al Tikriti, 2021).

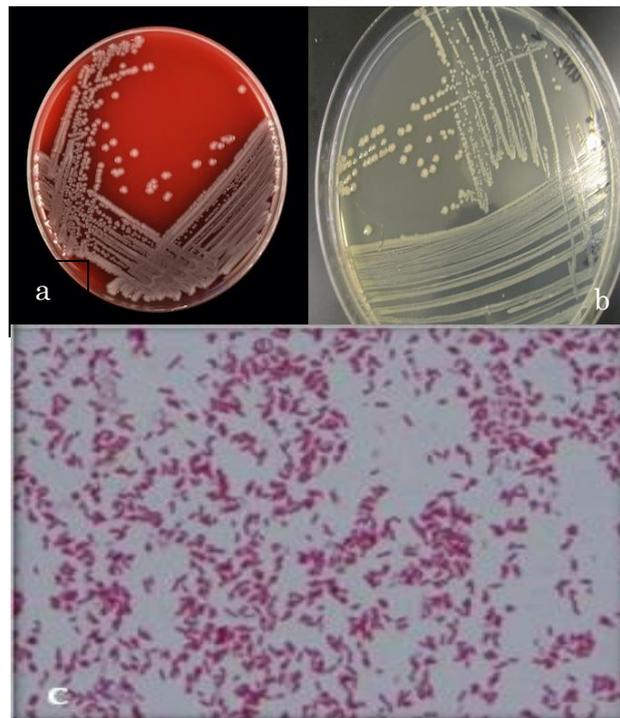


Fig 3. *E. coli* colonies on: a) blood agar, b) nutrient agar, and c) Gram stain.

Biochemical tests

Biochemical tests were performed on all isolates under study as shown in (Table 2), (supplementary files), and it was found that the positive results of the Catalase test appeared in all bacterial species (*K. pneumoniae*, *P. aeruginosa*, and *E. coli*) where the oxygen gas bubbles appeared on the test slide this result from bacteria's ability to produce the catalase enzyme that breaks down hydrogen peroxide into water and oxygen gas. While the positive result of the Oxidase test appeared only in *P. aeruginosa*, where the violet color of the test appeared within 5-10 seconds on a filter paper saturated with oxidase reagent which indicates the ability of the bacteria to produce the oxidase enzyme. As for the positive results of the urease production test were in the bacteria *K. pneumoniae*, as the color of the medium changed from yellow to pink, indicating the ability of the bacteria to produce the urease enzyme, which works on the decomposition of urea into ammonia and carbon dioxide, and the positive results appeared to gelatin liquefaction test in *P. aeruginosa* bacteria where the bacteria produce the hydrolyzed gelatinase enzyme, these results were agree with (Al-Tikriti, 2021, Al-Mamari, 2018, Forbes et al., 2007).

The effect of Triclosan on the growth of isolates test

The results showed a discrepancy in the sensitivity of *pneumonia* showed sensitivity to this material, namely bacterial isolates to triclosan, as the bacterial isolates of *K.*

Table 2. Diagnostic and biochemical tests results used in the diagnosing of the isolated bacteria from the saliva.

Bacteria	Isolates	Gram Stain	β -hemolysis	Biochemical tests				
				MacConkey agar	Catalase test	Oxidase test	Urease test	Gelatinase test
<i>K. pneumoniae</i>	S1	-	-	+	+	-	+	-
	S2	-	-	+	+	-	+	-
	S5	-	-	+	+	-	+	-
	S7	-	-	+	+	-	+	-
	S8	-	-	+	+	-	+	-
	S15	-	-	+	+	-	+	-
	S16	-	-	+	+	-	+	-
	S19	-	-	+	+	-	+	-
	S20	-	-	+	+	-	+	-
	S21	-	-	+	+	-	+	-
	S22	-	-	+	+	-	+	-
	S23	-	-	+	+	-	+	-
	S26	-	-	+	+	-	+	-
	S28	-	-	+	+	-	+	-
	S29	-	-	+	+	-	+	-
	S36	-	-	+	+	-	+	-
	S37	-	-	+	+	-	+	-
S40	-	-	+	+	-	+	-	
S41	-	-	+	+	-	+	-	
S42	-	-	+	+	-	+	-	
<i>P. aeruginosa</i>	S3	-	+	-	+	+	-	+
	S4	-	+	-	+	+	-	+
	S6	-	+	-	+	+	-	+
	S10	-	+	-	+	+	-	+
	S12	-	+	-	+	+	-	+
	S13	-	+	-	+	+	-	+
	S17	-	+	-	+	+	-	+
	S18	-	+	-	+	+	-	+
	S24	-	+	-	+	+	-	+
	S25	-	+	-	+	+	-	+
	S27	-	+	-	+	+	-	+
	S31	-	+	-	+	+	-	+
	S33	-	+	-	+	+	-	+
S34	-	+	-	+	+	-	+	
S38	-	+	-	+	+	-	+	
S39	-	+	-	+	+	-	+	
<i>E. coli</i>	S9	-	-	+	+	-	-	-
	S11	-	-	+	+	-	-	-
	S14	-	-	+	+	-	-	-
	S30	-	-	+	+	-	-	-
	S32	-	-	+	+	-	-	-
S35	-	-	+	+	-	-	-	

isolates (S1, S2, S5, S7, S8, S15, S16, S19, S20, S21), as the number of colonies were decreased by 70%. While the sensitivity of *P. aeruginosa* isolates was more sensitive, namely isolates (S3, S4, S6, S10, S12, S13, S17, S18,), the colonies number decreased by 80%, while the bacterial isolates of *E. coli* (S9, S11, S14) were sensitive by 50%. These results agree with (Shrestha et.al. 2020) in terms of the ability of triclosan to inhibit the growth of bacterial

isolates *P. aeruginosa* and *E. coli* at a concentration of 40 mg/L. (Fig 4).

Triclosan is an antimicrobial and is found in some mouth care products, including toothpaste, mouthwash treatments. It is considered a therapeutic and preventive agent for the care of the oral cavity as it has anti-inflammatory effects. It is a soothing, regenerating and astringent substance for diseases of the gums and the mucous membrane of the oral cavity. It also prevents the

growth of bacteria (Slater-Radosti et al.,2001). Its effect comes through the inhibition of the synthesis of fatty acids

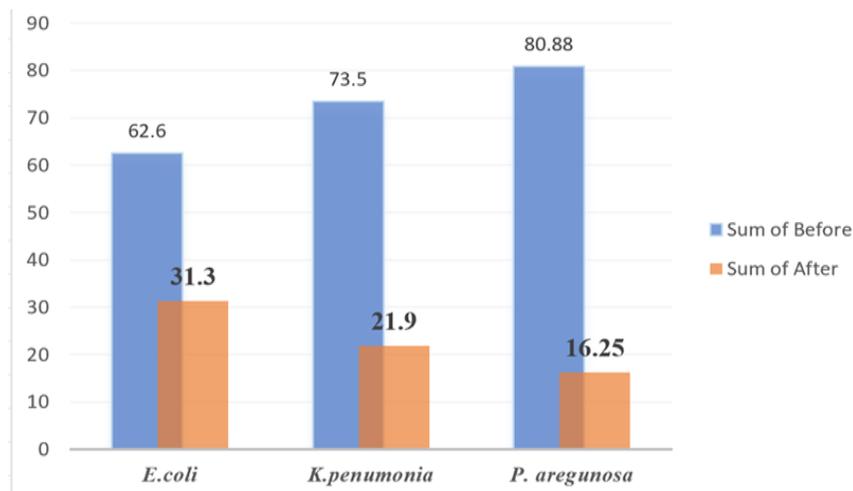


Fig 4. The results of the sensitivity of bacterial isolates to Triclosan, at concentration of 40 mg/L.

in all bacterial strains, as well as the inhibition of membrane. Both *K. pneumoniae* and *P. aeruginosa* have the ability to get protection from oxidant, phagocytes, and antibiotic as they utilize the biofilm lifestyle, this biofilm contains high bacterial population that even in exposure to antibiotic provide mutation especially in immune selective patients. In the case of *K. pneumoniae*, could change to different phenotypes that are important and related to human disease, including both colistin resistance and the hypervirulent phenotype (Riquelme et al. 2018).

Microscopic examination after gram stain showed long cells of gram-negative bacilli, these results were agreed with that reported by (Al-Tikriti, 2021).

Molecular confirmation

A DNA extraction process was carried out for the genomic material of the isolated bacteria from the saliva. The electrophoresis process was carried out on agarose gel to obtain the Genomic DNA. The DNA extraction results showed a clear band of all the bacterial types, (Figs 5-7) the same results were reported by (Green & Sambrook 2017).

PCR reaction

The PCR was carried out for purified DNA samples of each bacterial species isolated from saliva. In (Fig 8) appeared that 20 bands of purified DNA are from isolates *K. pneumoniae* of one size (130bp), which are the isolates (S1, S2, S5, S7, S8, S15, S16, S19, S20, S21, S22, S23, S26, S28, S29, S36, S37, S40, S41, S42), these results agree with (Lery et al. 2014) depending on the DNA bands size.

The PCR results of purified DNA samples from *P. aeruginosa* showed the presence of 16 bands from DNA of one size (500bp), which are isolates (S3, S4, S6, S10, S12, S13, S17, S18, S24, S25, S27, S31, S33, S34, S38, S39)

(Fig 9). These results are in agreement with the results of (Gad et al., 2007) when 107 pathological and 57 environmental isolates of *P. aeruginosa* were collected and molecularly detected using PCR technique and were of a size (500bp) as well as in agreement with the results of (Amutha & Kokila, 2014) when diagnosing isolates of *P. aeruginosa* using the same primers.

The PCR results of the purified DNA samples from *E. coli* isolates appeared bands of one size (147bp), which are the isolates (S9, S11, S14, S30, S32, S35) (Fig 10), showed that these results are in agreement with the findings of (Taha & Yaseen, 2019), who was performed a PCR reaction on *E. coli* samples isolated from different sources, using the same primers, and all isolates were 147bp in size.

The appearance of these bands from the DNA was due to the presence of common and similar sequences in their nitrogenous bases in the genomic DNA of these isolates, which were able to complete those in the primers and starts the PCR reaction and produce DNA bands of similar sizes. The PCR is considered more accurate in diagnosis than other diagnostic tests because the pathogen is isolated from the human body, as it detects and diagnoses bacteria based on its gene even when the bacteria are dead or weak and the appearance of DNA bands of one size proves that the DNA belongs to an organism of the same species (Hanoon et al, 2020). The PCR technology is one of the main and widely popular methods for detecting pathogenic bacteria in clinical laboratories, although this technology is relatively new.

Among the gram-negative bacteria, *K. pneumoniae*, *P. aeruginosa*, and *E. coli* are considered the common causes of various infections as concluded in a molecular and clinical study by (Yungyuen et. al. 2021). Among all

species isolated, *K. pneumonia* and *E. coli* were the commonly identified as reported by (Hayajneh et. al., 2015). These results indicated that the organized genes

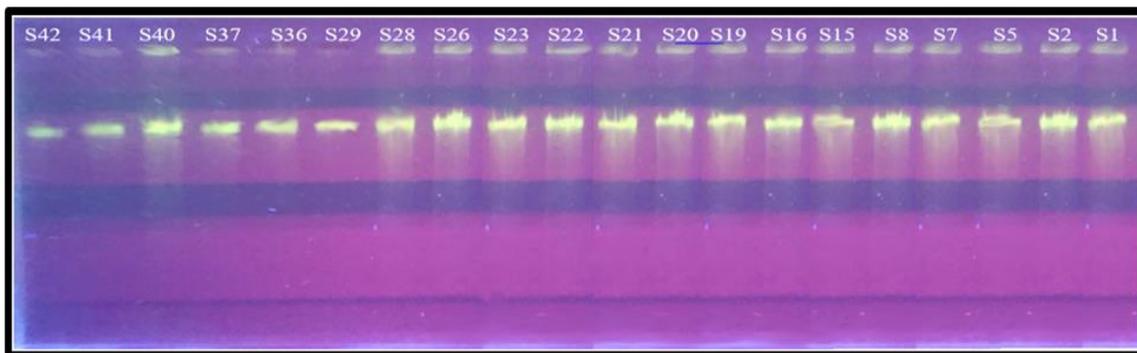


Fig 5. Electrophoresis results of DNA isolated from *K. pneumonia* isolates.

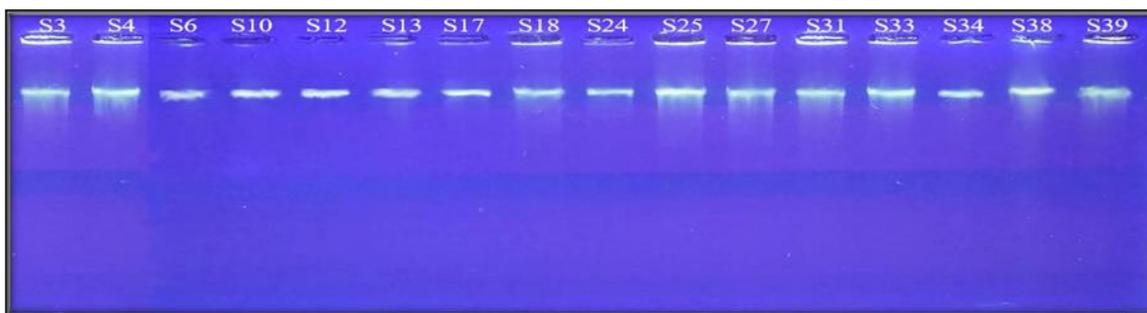


Fig 6. Results of electrophoresis of DNA isolated from *P. aeruginosa* isolates.

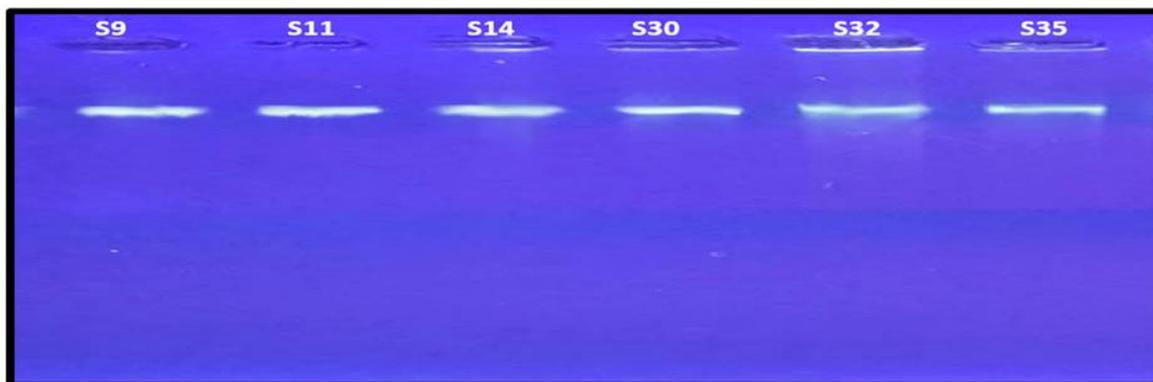


Fig 7. Electrophoresis results of DNA isolated from *E.coli* isolates.

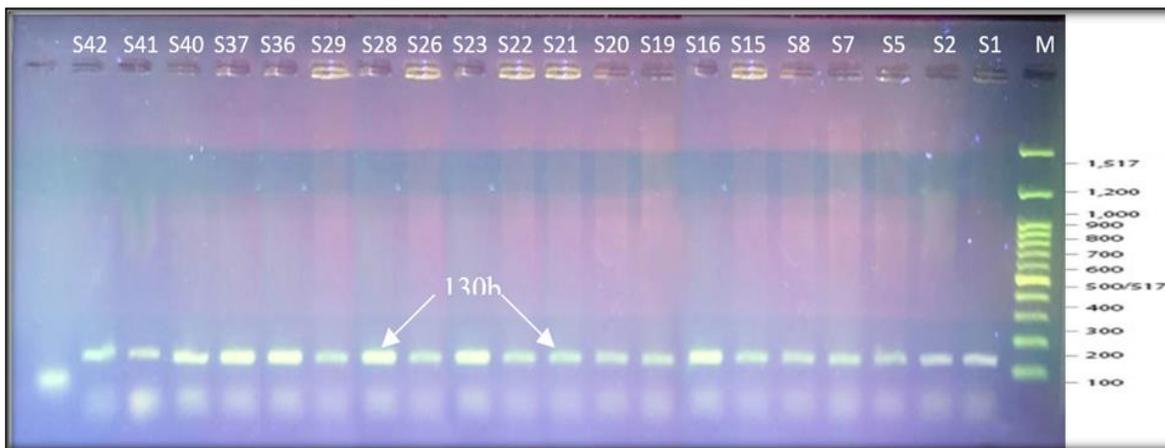
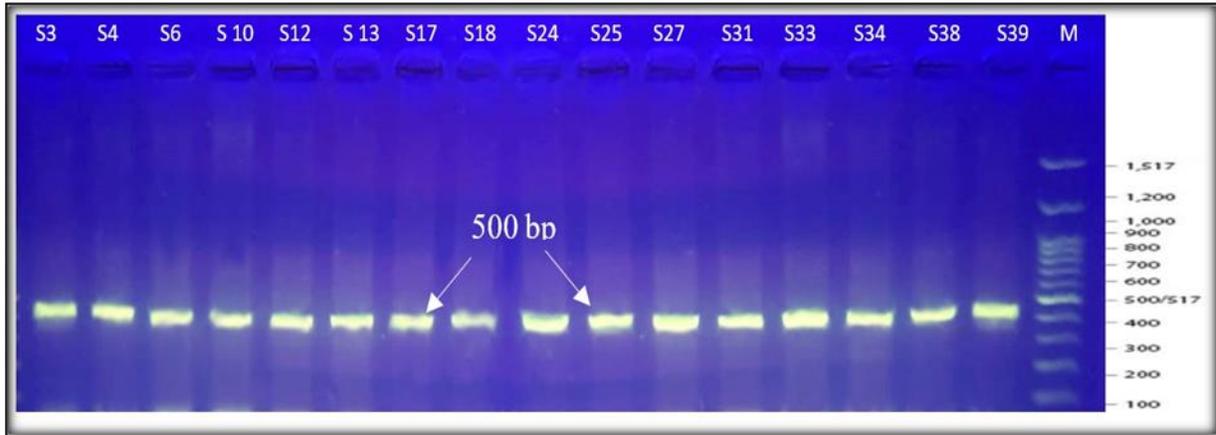
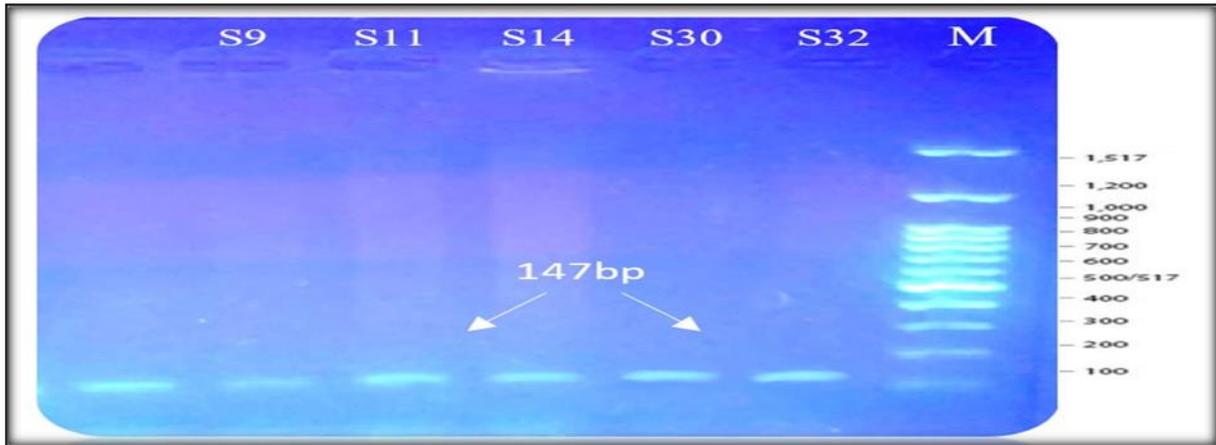


Fig 8. PCR results for the DNA isolated from *K. pneumoniae* isolates, all of one size (130bp).**Fig 9.** PCR results for DNA isolated from the local isolates of *P. aeruginosa* all of one size (500bp).**Fig 10.** PCR results for DNA isolated from the local isolates of *E. coli* all of one size (147bp).

functioned regularly to control the genes expression that allowed the *K. pneumoniae* to respond to the environment and successfully infect its hosts (Tu et al. 2009). One of the common causes of infection is the *P. aeruginosa* that specially affecting the immunocompromised patients (Cabot et al. 2016).

Similar to our findings, another study showed that the Gram-negative bacteria responsible for the primary infections were *K. pneumoniae*, *E. coli*, and *P. aeruginosa* (Chelazzi et al., 2015), also as mentioned by (Lila et al. 2018) who said that the molecular detection observed that the *P. aeruginosa* is an important opportunistic pathogen.

DNA sequencing

The sequencing of the PCR products for DNA samples isolated from the saliva was carried out by NCBI BLAST website, and the results showed a 100% similarity between

the two local isolates (S16 and S37) and the standard strains recorded in gene bank with the number *K. pneumoniae* AP024918.1 and *K. pneumoniae* CP050334.1 respectively and these results are in agree with Lev et.al. (2018). Moreover, the *K. pneumoniae* have a high degree of stability of the environmental changes and can survive in the hospital despite to the continues decontamination procedures and cleaning (Moghnia & Al-Sweih 2022).

The results of the analysis also showed a 100% similarity between the two isolates (S18 and S39) and between the standard strains registered in the gene bank with the number *P. aeruginosa* CP050334.1 and *P. aeruginosa* CP050327.1, respectively. Although the sequencing technic of using the whole-genome is important clinically isolates but also may leads to the detection targets of both species together, which could be therapeutically accomplishment (Riquelme et al. 2018).

Data of sequences showed that there is a 100% similarity between the two local isolates (S14, S35) and similar standard strains from the gene bank *E. coli* CP056114.1 and *E. coli* CP054251.1, respectively. Another interesting finding in our study was detecting of gene sequencing, highlighting the presence of these gene in the gene bank in two isolates. Our findings parallel to a study that reported primarily in Gram-negative bacteria, including *E. coli* strains isolated worldwide (Moghnia & Al-Sweih 2022; Jiang et.al. 2020).

These results were in agreement with (Al-Tikriti, 2021), who diagnosed pathogenic bacterial isolates that had been isolated from different parts of human body using DNA sequence technology to determine bacteria of different types and species. It also agreed with the results of (Ibrahim et al., 2019) when using DNA sequence technology in diagnosing different species of *K. pneumoniae* bacteria isolated from different sources. Moreover, both bacteria *P. aeruginosa* and *E. coli* were also diagnosed and determined their species by DNA sequence technique as shown in the research results of (Le et. al. 2020) who used DNA sequencing method for the diagnosis of Gram-negative bacteria in the oral cavity.

Conclusion

In conclusion, the present work shows that the use of Triclosan materials in the mouth wash will protected the oral cavity from the common dental diseases as it decreases the number of bacterial colonies. three types of bacteria generally play a significant role in development of inflammation such as gingivitis. Thus, further studies using genome sequencing are needed to characterize the role of the pathogenic bacteria in different diseases of the oral cavity. DNA sequence technique as shown in the research results of (Le et al. 2020) who used DNA sequencing method for the diagnosis of Gram-negative bacteria in the oral cavity.

Research limitation

The main limitation in this study was the number of the samples, this due to difficulty in obtaining the patients agreements.

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