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Staphylococcus Aureus and Pseudomonas Aeruginosa Biofilms in Ear-Set Associated Infections

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Abstract **Background and objectives:** Ear infections are a serious public health issue in developing countries. Gram-positive and gram-negative Bacteria are both capable of developing biofilms on medical equipment and earphones; however, Staphylococcus aureus, Staphylococcus epidermidis, E. coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, and Enterococcus faecalis are the most prevalent types and may involve in ear infections. Since there was no such study in our region about ear set association with biofilm formation in patients with ear infections, this study was accomplished. **The aim of the study:** Therefore, this study was suggested in order to evaluate the relationship between the continuous use of Ear set with biofilm formation and ear infections among handlers. **Patients, Materials and Methods:** This study was carried out in Kirkuk City (The urban region only) from the 15th of January 2023 to the 23rd of May 2023, done on a total number of (168) participants. The total number of the patients who were Ear set users enrolled for the current study was (120), divided into two groups including (60 patients with biofilm positive (including 27 male and 33 female patients respectively) and 60 patients with biofilm negative (including 23 male and 37 female patients separately), with age, ranged from 20 to 60 years old, who were attended at Kirkuk Teaching Hospital. The control group (non-Ear set users) of the presented study was a total number of (48) including (18) males and (30) females. The serological tests included: the estimation of Human GR, SOD, and MDA antioxidants levels through using of the ELISA technique for all the participants, and the biochemical tests included measuring Vitamin D3 levels by using the iChromaTM technique, while the microbial tests were done through using of VITEK 2 compact system. Biofilm formation was detected by using the Congo red agar method. All the steps of methods are conducted based on the manufacturer's instructions. The bacterial growth among the patients group was (22.5%) of Staphylococcus aureus and (14.16%) of Pseudomonas aeruginosa, while the control group revealed (8.33 %) of Staphylococcus aureus and (4.16%) of Pseudomonas aeruginosa. The age range (20-30) years showed the highest rates among the infected patients, with male patients being more vulnerable to the infections. The mean levels of vitamin D3, human GR, SOD, and MDA decreased significantly in patients in matching to control groups. **Conclusions:** As a conclusion, Staphylococcus aureus and Pseudomonas aeruginosa are more prevalent agents involved in ear infections. Male patients are more susceptible patients to contracting ear infections. Vitamin D3, Human GR, SOD, and MDA antioxidants levels decline in patients with ear infections.

Key Words Ear infections, Biofilm bacteria, Staphylococcus aureus, Pseudomonas aeruginosa Kirkuk

1. Introduction

Since it has a distinctive anatomy, the human Ear is considered a special ecosystem with its own microbiome, as the outer Ear is shielded by some physical elements, such as skin, and chemical barriers, primarily earwax, as defensive mechanisms that stop bacteria from penetrating the Ear and creating an infection [1]. One of the most prevalent infectious disorders in developing countries is ear infection [2]. Numerous types of ear infections, including acute otitis media, otitis media with effusion, chronic otitis media, and otitis externa, commonly result from bacterial invasion [3], [4]. The most common bacterial agents in ear infections include Pseudomonas aeruginosa, Staphylococcus epidermidis, and Staphylococcus aureus [3]. Clinically, microbial colonization and virulence appear to control the pathophysiology of infection and boost the development of biofilms in the middle ear mucosa. The two pathogens prevalent in chronic otitis media, drug-resistant and form biofilms in the ear canal,

are Pseudomonas aeruginosa and Staphylococcus aureus [5]. In addition, certain researchers pointed out the ability of some Gram-positive and Gram-negative bacteria to develop biofilms on medical equipment, including Ear sets [6], [7]. Such bacteria as Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, and Enterococcus faecalis are the most prevalent types [6].

Moreover, it was suggested that the usage of an Ear set might contribute to serious ear hygiene issues and ear canal infections [7]. Wearing Ear sets that are not properly maintained and cleaned frequently might serve as a reservoir for bacterial colonies that can enter the ear canal [8]. Furthermore, when Ear sets are shared, bacteria can spread between persons, causing a widespread infection [9]. Meanwhile, it was found that numerous ear infections, including otitis media, have been linked to vitamin D deficiency [10]. This association may be explained by vitamin D's ability to assist the immune system by upregulating antimicrobial peptides that are effective against antipathogens and the development of biofilms, promoting a less inflammatory immunological response, or encouraging good commensal Bacteria [10]. In the same context, antioxidants also significantly contributed to the pathogenesis of otitis media and ear infections, according to reports; when oxidative stress increases or the antioxidant defense system is deficient, elevated reactive oxygen species levels may have an impact on the chronicity of middle ear inflammation and the etiopathogenesis of chronic otitis media [11].

2. Patients, Materials and Methods

Patients

A total of 168 individuals from the community of Kirkuk city were volunteered in this study during the period from January 2023 to May 2023. They were divided into two groups; Group A n=(120) were Ear set users and Group B n=(48) non-users (were considered as the control group) and were all subjected to the study questionnaire according to the methodology cited in [12]. Ear infection patients were referred by Consultant Otolaryngologist (Dr. Tunjay Namiq Faiq)/ Kirkuk Teaching hospital.

Sample Collection

Blood Samples

Seven and a half ml of blood was collected by vein puncture using 5 ml disposable syringes from each patient and control group enrolled in this study. Blood samples were placed into sterile gel tubes for 15 minutes at room temperature; then they were centrifuged at 3000 rounds per minute (rpm) twice for 10 minutes. The clear serum was aspirated from the top of the gel tube using 100-1000 μl micropipette and transferred to the two sterile plane test tubes; one of them was for biochemical tests (Detection of total 25 (OH) D2/D3 levels), and the second, and third ones were stored at $-20(^{\circ}C)$ for late serological tests; for detecting of antioxidants including; Human MDA (Malondialdehyde), human GR (Glutathione Reductase), and Human SOD(Superoxide Dismutase).

Ear Swabs

Ear swabs were collected from both study groups (A and B) and were immediately cultivated on culture media according to reference in [13]. Regarding bacterial isolation and identification, a cotton swab was gently inserted into the ear canal to collect an ear discharge. The samples were then cultured on MacConkey Agar and Blood Agar culture media. Moreover, the VITEK 2 compact system is used for species identification and antibiotic sensitivity on the same culture media. Finally, the colonies were cultured on Congo red agar to identify biofilm; if the colonies turned black after 24 hours of incubation at $37^{\circ}C$, they were biofilm positive.

Ear Swabs Collected From Ear Set Devices

Cotton swabs were wiped gently on ear set devices being evaluated in this study and swabs were cultivated immediately on selective culture media in a procedure especially designed for this study.

Microbiological Examination of the Isolates

Collected ear swabs from participants and ear set devices were cultivated on selective culture media. If any growth appeared on the culture media, it was isolated and stained with Gram's stain to determine whether the microorganisms were Gram positive or Gram negative by microscopic examinations.

Biofilm Formation Detection

The biofilm formation ability by the isolated bacteria was distinguished using the Congo red agar method according to the methodology cited in [14].

Serological Tests

This study involved certain serological test including: the estimation of Human GR, SOD, and MDA antioxidants levels through using of the ELISA technique for all the participants, and the biochemical tests included measuring Vitamin D3 levels by using the iChromaTM technique.

Calculation of Results

Averaging the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Constructing a standard curve with the human GR concentration on the y-axis and absorbance on the x-axis, and drawing a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the standard curve had to be multiplied by the dilution factor. Using some plot software.

3. Results

Since it has distinctive anatomy, the human ear can be thought of as a special ecosystem with its microbiome.

Ear set types	No. of users	%
Wireless Ear sets	90	75
Wired Ear sets	30	25
Total	120	100

Table 1: Numbers of ear set users enrolled in the current study according to the device type.

Types of Bacteria	Group A n=120 Patients		Group B Control n=48 Participants	
	No.	%	No.	%
S. aureus	27	22.5	4	8.33
P. aeruginosa	17	14.16	2	4.17
K. pneumonia	8	6.67	1	2.08
E. coli	8	6.67	1	2.08
Positive bacterial cultures	60 +ve	50	8 +ve	16.66

Table 2: Numbers of isolated Bacteria from group A and B.

Naturally, the outer ear is shielded by some physical elements, such as skin and chemical barriers, primarily earwax; these organic defenses stop bacteria from penetrating the ear and creating an infection [1]. Accordingly, many types of ear infections, including acute otitis media, chronic otitis media, and otitis externa, commonly resulted from bacterial invasion [3], [4], where the most common bacterial agents involved included Pseudomonas aeruginosa, Staphylococcus epidermidis, and Staphylococcus aureus which were associated with the external environment of the ear [3]. Thus, this study evaluated the association between ear infections, certain bacteria, and some physiological factors.

Samples were collected from two groups, group A (120 patients, ear set users) and group B (48 controls, non-users of ear sets). Different types of ear sets were used, where the wireless ear set was one of the most commonly used ear sets, as shown in Table 1.

Then, ear swabs were collected from the participants and their devices, cultured on selective media where different types of bacteria were isolated as shown in Table 2, Figure 1 and Figure 2.

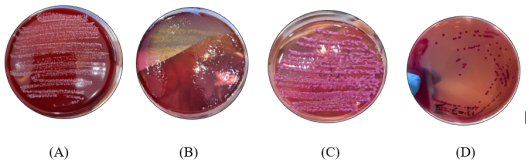

Figure 1: The colony appearance of: (A): Staphylococcus aureus on Blood agar, (B): Pseudomonas aeruginosa on MacConkey agar, (C): Klebsiella pneumoniae on MacConkey agar, (D): Escherichia coli on MacConkey agar Isolated from Group (A) participants of the study.

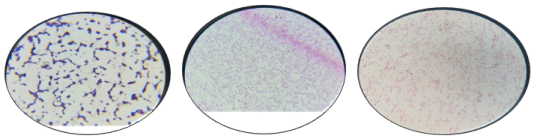

Figure 2: Gram's stain photos of the isolated bacteria under microscope (100x) showing: (A): Staphylococcus aureus, (B): Pseudomonas aeruginosa, (C): Klebsiella pneumoniae Isolated from Group (A) participants of the study.

Bacterial Growth Among Groups (A) and (B) of the study

Results of Table 2 demonstrate the numbers and ratios of bacterial growth from both groups (A) and (B) of the current study and showed 27 (22.5%) of S. aureus strains were isolated from 120 patients who used ear sets in comparison to 4 (8.33%) strains isolated from group (B). On the other hand, the current study also displayed that 17 (14.16%) of P. aeruginosa were detected among the ear sets users patients in comparison to 2 (4.16 %) found among the control group. Additionally, the current study revealed that 8 (6.66%) of K. pneumonia were isolated from the patients, and this was also applicable for the number of E. coli within the same group, while the control group showed 1 (2.08 %) of K. pneumonia and 1 (2.08%) of E. coli respectively. Furthermore, the presented study revealed the isolation of a total of (66.66%) bacteria isolated from the study participants where (50%) of these isolates were collected from ear set users. In a study conducted in Maysan, Iraq, they found that patients with otitis media had Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, Klebsiella pneumoniae, and Staphylococcus epidermidis bacteria isolated from them, furthermore they detected that Staphylococcus aureus was the most prevalent bacterial agent causing otitis media, which is by our results (15). The presented study revealed that the ratios of bacterial growth in Group A were (22.5%) for Staphylococcus aureus, (14.16%) for Pseudomonas aeruginosa, (6.66%) for Klebsiella pneumonia, and (6.66%) for Escherichia coli. In contrast, in Group B, the ratios of bacterial growth were (8.33%) for Staphylococcus aureus, (4.16%) for Pseudomonas aeruginosa, (2.08%) for Klebsiella pneumonia, and (2.08%) for Escherichia coli. The current study likewise was near to a study conducted in Ramadi city, Iraq, where they found that the two most frequently isolated species were Pseudomonas aeruginosa in (57.5%) of the patients and Staphylococcus aureus in (16.8%) of the cases, this study also showed some disagreement to this study in regards to the percentage of bacterial growth, where they showed as mentioned before the growth proportion of Pseudomonas aeruginosa was higher than the one for Staphylococcus aureus, these variances may be attributed to the differences of sample sizes of both studies and the differences in the age groups, where about 20% of the patients were lower than 10 years old. Which their response

Figure 3: Biofilm formation ability of the isolates from group (A) on Congo Red agar

Biofilm formation	Group A		Group B	
	No.	%	No.	%
Positive biofilm				
S. aureus	27	22.5	-	-
P. aeruginosa	17	14.16	-	-
K. pneumonia	8	6.66	-	-
E. coli	8	6.66	-	-
Negative biofilm				
S. aureus	-	-	4	8.33
P. aeruginosa	-	-	2	4.16
K. pneumonia	-	-	1	2.08
E. coli	-	-	1	2.08

Table 3: The ratio of biofilm formation in group A and B.

to the bacteria is different since their immune system are not well-developed and respond differently to Bacteria [15]. Further studies have revealed that Staphylococcus aureus is the most prevalent cause of otitis media [16], [17]. The presented study also displayed an agreement with a study performed in Duhok city, Iraq, where they found that both Staphylococcus aureus and Pseudomonas aeruginosa were the most frequent Bacteria involved in otitis media [18]. The biofilm phenotypic trait of Staphylococcus aureus, which permits entrance to the middle ear through the external canal, may be associated with its dominating isolation rate in this investigation. In accordance, the biofilm formation ability of the isolated strains was identified by Congo Red agar, as shown in Figure 3.

The current study showed that 60 (50%) of the samples showed biofilm positive within group (A). Whereas bacterial strains isolated from group (B) showed negative biofilm results as demonstrated in Table 3.

Since ear sets (Bluetooth and headphones) were the most widely used electronic ear devices among the general public [19], where the devices are placed at the opening of the ear canal increasing the possibility of ear infections [20]. In the same context and in a study in 2018, it was declared that earphones harbored bacteria in the form of biofilms [19].

Ear Set Association with Biofilm Formation

So, based on the high possibility of forming biofilm in ear canals of ear set users, swabs were collected from different types of ear sets (wireless and wired ear sets) and different types of bacteria were isolated as shown in Table 4.

Types of ear sets	Types of Bacteria		
	Bacteria	No.	%
Wireless	Staphylococcus aureus	18	30
	Pseudomonas aeruginosa	10	16.66
	Klebsiella pneumoniae	6	10
	Escherichia coli	5	8.33
Wired	Staphylococcus aureus	9	15
	Pseudomonas aeruginosa	7	11.66
	Klebsiella pneumonia	2	3.33
	Escherichia coli	3	5
Total		60	100

Table 4: Proportion of Bacteria isolated from different types of ear sets.

Ear set types	Bacteria types	Biofilm	
		No.	%
Wireless	Staphylococcus aureus	18	30
	Pseudomonas aeruginosa	10	16.66
	Klebsiella pneumonia	6	10
	Escherichia coli	5	8.33
Wired	Staphylococcus aureus	9	15
	Pseudomonas aeruginosa	7	11.66
	Klebsiella pneumonia	2	3.33
	Escherichia coli	3	5
Total		60	100

Table 5: Proportion of biofilm of Bacteria isolated from different types of ear sets.

Then, testing for biofilm formation had been performed on the isolated Bacteria, where they revealed the ability of the same types of isolated bacteria from the ear canals of infected patients (Group A) to form biofilm as shown in Table 5.

According to that, utilizing ear sets may lead to infection with different types of bacteria and increase the possibility of biofilm formation. These results agreed with an earlier study in Libya in 2021, where they found that many bacteria could spread through ear sets, sharing earphones, and using them frequently and continuously, which might promote bacterial growth in the ear, leading to serious illness [21].

Age and Gender Distribution Among Study Groups

In the same context, studying of the association of bacterial biofilm with age range and sex of the patients was determined as shown in Table 6.

Regarding the patient group, we enrolled (49 male and 71 female) patients, while for the control group, we enrolled (18 male and 30 female) participants, as shown in Table 6. The greatest rates (42.85 vs. 45.07%) of male and female patients, respectively, were within the age group (20-30) years old in comparison to the rest of the age groups within the same study group, while the lowest rates (10.20vs. 5.63%) of male and female respectively was found within the age range (50-60) years old patients. The control group of the current study demonstrates 7 (38.88 %) males and 12 (40%) females within the age range (20-30) years old; the lowest rate of 2 (11.11

Age range	Group (A) Gender No. (%)			Group (B) Gender No. (%)		
	Male 49 patients No. (%)	Female 71 patients No. (%)	Total 120 Patients No. (%)	Male 18	Female 30	Total No. (%)
20-30	21(42.85)	32(45.07)	53(44.16)	7(38.88)	12(40)	19(39.58)
30-40	12(24.48)	20(28.16)	32(26.66)	6(33.33)	8(26.66)	14(29.16)
40-50	11(22.44)	15(21.12)	26(21.66)	3(16.66)	5(16.66)	8(16.66)
50-60	5(10.20)	4(5.63)	9(7.5)	2(11.11)	5(16.66)	7(14.58)
Total	49	71	120(100)	18(37.5)	30(62.5)	48(100)
An independent sample t-test was utilized to study the numerical variables. A probability (P) value lower than 0.05 is considered as significant, while P value greater than 0.05 considered as non-significant. P value <0.01 is highly significant. Mean ± Standard error.						

Table 6: Age and gender distribution among the study groups.

Parameters	Group A	Group B	P. Value
Vit. D3	19.42±0.80	36.32±1.24	0.000
Anti-oxidants			
GR	1124.86±239.11	3281.46±333.38	0.008
SOD	0.77±0.18	2.01±0.59	0.000
MDA	382.67±35.91	544.35±38.84	0.855
An Independent sample t-test was utilized to study the numerical variables. Probability (P) value lower than 0.05 considered as significant, while a P value greater than 0.05 is considered non-significant. P value <0.01 is highly significant. Mean ± Standard error.			

Table 7: Relationship between patients and control group on basis of vit D3 and anti-oxidants.

%) of male patients were within the age group (50-60) years old.

Comparison between Patients in regards to Vit. D3 and Antioxidant Levels

In accordance other physiological parameters were also taken into account in order to evaluated the effect of other factors in the patients to enhance biofilm formation capacity among ear set infecting bacteria. Thus, Vit. D3 and antioxidants were considered as parameter to compare between group A and B as shown in Table 7.

The mean value of vitamin D3 concentration level was (19.42 ng/mL) in group A patients, while the mean value of vitamin D3 levels was (36.32 ng/mL) in the (control group). The differences were highly significant, as revealed in Table 7. The current study revealed that there was a significant decrease in the concentration levels of Vit. D3 (19.42 ng/mL) among Group (A) patients in comparison to (36.32 ng/mL) within Group (B). The difference was highly significant, $P = 0.000$. These were stated, who found that patients with otitis media suffered from Vit. D3 deficiency, and adding Vitamin D3 to their treatment regimen would help them to relieve the infection [21]. In addition, the current study's findings were supported by Asher BF and Guilford FT, who found that Otolaryngology patients frequently have Vitamin D deficiency, which can be treated with supplementation. The etiopathology of ear disorders in adults and children was linked to Vitamin D3 deficiency [22]. Moreover, There was a significant dropping in GR antioxidant concentration level (1124.88 pg/mL) among (Group A) patients in matching to (3281.46 pg/mL) for (Group B). These were in line with what was reported earlier by, who revealed that GR antioxidant decreased significantly in patients with ear infections [23].

The control group showed a greater mean level of GR (3281.46 pg/mL) in (the control group), matching (the 1124.86 pg/mL) that was recorded in the patients group. The variances were highly significant. In regards to the association of SOD antioxidant levels with group A, patients, and the control group demonstrate highly significant discrepancies. A minimal increment in the concentration level of SOD antioxidant (2.01 U/mL) was noticed within (Ear set users, biofilm negative) patients in matching to (0.77 U/mL) recorded among (Ear set users, biofilm positive) patients. The most potent detoxifying enzyme and antioxidant in a cell is superoxide dismutase (SOD). It is a crucial endogenous antioxidant enzyme that serves as a part of the body's primary defense mechanism against microbial agents, so the decrease in their concentrations leads to infections and makes the host more susceptible to contract infections, including ear infections [20].

Regarding the MDA concentration level, there was an insignificant lowering in their level (382.67 pg/mL) in Group (A), whereas (Group B) documented (544.35 pg/mL). This disagreed with, who noticed that patients with ear infections had higher levels of MDA in comparison to healthy control; these differences may be attributed to the variances in the lifestyle in regards to using headphones and the kinetic responses of MDA in these patients [24].

Relationship between Patients and Control Group on Basis of Vit D3 and Anti-Oxidants

The presented study also showed that the Vitamin D3 concentration level (19.42 ng/mL) was extremely lowering in (Group A), matching the (36.32 ng/mL) that was recorded for the (control group) as shown in Table 7. Likewise, it was noticed that vitamin D3 levels recorded a slight difference in their levels (19.42 vs. 36.32 ng/mL) in patients and control groups, respectively, and the P. Value was lower than 0.01, as shown in Table 4. These were close to the results of a case-control study done by Hosseini S et al., who detected that patients with otitis media had lower Vitamin D levels than healthy control [25]. The control group showed a greater concentration level of GR (3281.46 pg/mL) in matching the (1124.86 pg/mL) that was recorded among (Ear set users, the positive) patients group. The variances were highly significant, as shown in Table 3.

Moreover, GR antioxidants showed a higher level (3281.46 pg/mL) in the control group compared to the patient's group, which was Ear set users but with biofilm negative results, where they recorded (2872.07 pg/mL). In regards to the association of SOD antioxidant levels with the (Ear set users, biofilm-positive) patients, and control group demonstrate highly significant discrepancies. Furthermore, there was insignificant change in SOD concentration levels (1.25 vs.2.01 U/mL) in patients and the control group, respectively. There was a slight change in the concentration levels of serum MDA (382.67 vs. 544.35 pg/mL) documented among (Ear

set users, biofilm-positive) patients, and the control group, respectively. The difference was nonsignificant. The outcomes were statistically nonsignificant, which was applicable to the concentration levels of MDA, as shown in Table 4. These were in line with what was reported earlier by Ozek H et al., who stated that SOD and Glutathione levels were reduced in patients with ear complications in comparison to those with no ear infection [26]. At the same time, our results showed disagreement with Garça MF et al., who found that MDA levels were higher in patients with ear infections in comparison to those with no infection; these differences might be correlated to the differences of bacterial species that caused ear infection and resulted in increased levels of MDA in their study, from the bacterial species detected in our study, and as a result produced high MDA [27].

4. Conclusions

1) Staphylococcus aureus and Pseudomonas aeruginosa were more prevalent agents involved in ear infections.
2) Male patients with age range of (20-30) were more susceptible patients to contract ear infections than their female partners.
3) Vitamin D3 concentration levels significantly declined in patients with ear infections.
4) Human GR, SOD, and MDA antioxidants levels decreased in patients with ear infections.
5) The current study is one of the rare studies conducted at Iraq and international level.

5. Recommendations

1) Performing molecular studies on those bacterial isolates from ear infection.
2) Conducting more studies about the relationship of human GR, SOD, and MDA antioxidants levels and ear diseases among other ear set device users.
3) Involving larger sample size in the further researches to step on more risk factors associated with ear infections.

Conflict of Interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

Authors Contribution

All authors contributed equally in this paper.

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