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11- Use only SI standard abbreviations in the title and abstract. The full term for which the abbreviations stand should precede its first use the text.

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Evaluation of Apelin, Elabela, and Certain Biomarkers in Patients with Metabolic Disease Associated with Obesity

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Abstract

Background: A novel peptide known as Elabela was recently discovered; it functions similarly to apelin and acts through apelin receptors. This research is to compare the characteristics and biological roles of apelin and Elabela in patients with metabolic syndrome who are obese and those who are not.

Patients and Methods: A cross-sectional study included 90 participants, ages 20–45, whose samples were collected from Al-Yarmouk-Hospital/Al-Karkh/Baghdad, from April–July/2024. Classified into three groups: group1 was obese metabolic syndrome patients (N=30), group 2 was metabolic syndrome patients without obesity (N=30), and group 3 was control (N=30). Anthropometrics and parameters were assessed for all study groups. Fasting blood glucose levels and lipid profiles were determined using an enzymatic process with spectrophotometric methods, while insulin, apelin, and Elabela were evaluated by enzyme-linked immunosorbent assay.

Results: The result shows a significant difference in Body Mass Index, Waist to Hip Ratio, Lipid profile, FBS, apelin, insulin, and HOMA-IR, while there was no significant difference between the study groups in Elabela levels. In addition, there is a positive correlation between apelin and BMI, FBS, and insulin. apelin levels have a higher diagnostic value for obesity than metabolic syndrome, respectively.

Conclusion: This study suggests that apelin and Elabela are powerful modulators of the metabolism of adipose cell and highlights the crucial role of apelin in MetS in both non-obese and obese MetS, as well as in clinical and biochemical markers associated with obesity.

Keywords: Apelin, APJ, Elabela, Metabolic Syndrome, Obesity.

Introduction

The global health community is concerned about the rising prevalence of obesity since being overweight raises the risk of developing a number of illnesses, including cancer, diabetes, and cardiovascular diseases (1). There is significant variation in the prevalence of obesity among different populations due to the interaction between local environmental factors and genetic factors, as well as

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as well as the drivers of the global food system. According to epidemiology, middle-aged adults are primarily affected by obesity in low-income countries, but people of all ages and both genders are affected in high-income countries. However, rising obesity rates have a significant negative impact on people's health and economies globally (2).

A collection of metabolic illnesses, including abdominal obesity, hypertension, dyslipidaemia, and impaired glycemia, is collectively referred to as metabolic syndrome (MetS). It is very common everywhere in the world and has two main definitions. More than one-third of adults with MetS were reported by the National Health and Nutrition Examination Survey (NHANES), and this number increased by more than 35% between 1988–1994 and 2007–2012. All things considered, the prevalence of MetS increases in tandem with the obesity rate. In ten extensive European cohorts comprising 163,517 individuals, the age-standardized proportion of obese subjects with Metabolic Syndrome varied between 24 and 65% for females and between 43% and 78% for males (3).

Due to their widespread prevalence throughout the world and link to a higher risk of developing chronic illnesses, obesity and MetS are serious public health concerns (4). Apelin is the name of the peptide that binds to the G-protein-coupled receptor APJ. There are numerous active apelin forms, including apelin-13, apelin-36, apelin-17, and apelin-13's pyro-glutamate form. In particular, the hypothalamus and numerous peripheral tissues in the central nervous system express apelin and APJ. Studies have demonstrated the involvement of apelin in the control of angiogenesis, food intake, cardiovascular and fluid homeostasis, and cell proliferation. Apart from its widespread presence, apelin is classified as an adipokine since it is generated and released by adipocytes (5). This adipokine has been shown to play a significant role in the development of metabolism and eating behavior during storage, as well as in maintaining physiological balance and preventing obesity-related diseases, such as high blood pressure and type II diabetes. Although apelin was found in the white adipose tissue of rats, the cells secreting the substance were unknown. The identification of apelin transcripts may be the result of several cell types present in this tissue, aside from the adipocytes themselves (6).

Elabela is a peptide hormone found after apelin and acts by binding to purinergic receptors. It was first described by the riversides' group as the first ligand of APJ in zebrafish embryos and shown to have effects on endodermal differentiation and carcinogenesis. Elabela is primarily present in embryonic stem cells, vascular endothelium, kidney, prostate tissue, and placenta. Elabela is similar to apelin, both bind to the same receptor APJ and cause similar effects. Although they have similar effects, these two peptides differ from each other in that they employ distinct signaling pathways and induce diverse biological effects. Additionally, there have been reports indicating that Elabela produces its effects via a receptor other than the APJ receptor (7).

Patients and Methods

Study design and blood sample collection: The cross-sectional study was conducted from April to July 2024 at the Department of Chemistry, College of Science, Al-Nahrain University. Ninety selected participants, ages 20-45, collected from Al-Yarmouk Hospital, Al-Karkh, Baghdad, Iraq, classified into three groups: group1 patients diagnostic with obesity (≥30 kg/m2) and MetS (score ≥3) (obese metabolic syndrome patients) (N = 30), group 2 patients diagnostic only with MetS (score ≥3) without obesity (normal weight (<25 kg/m2) (non-obese metabolic syndrome patients) (N = 30), and control group (N = 30). Blood samples were taken from all individuals in the study, who were

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fasting between 7:00 and 9:30 AM. After being placed in gel tubes, the venous blood samples were centrifuged for 10 minutes at 3000 rpm in to extract serum, and they were allowed to clot for 30 minutes. Serum was examined for standard laboratory parameters. The remaining serum samples were kept at -20 °C until the day of analysis for the Apelin and Elabela measurement.

Inclusion criteria: Healthy men and women without metabolic syndrome, and obese or non-obese patient with metabolic syndrome. All parse participants aged (45-20).

Exclusion criteria: Diabetes mellitus patients with thyroid disorders (hyperthyroidism or hypothyroidism), and gastrointestinal diseases.

Biochemical assays: Measurement of glucose and Lipid profile (Cholesterol, triglycerides, HDL, LDL, VLDL) using a spectrophotometer instrument (Human kit, Germany).

Insulin measurement and HOMA-IR calculation: Insulin levels were determined using VIDAS KUBE, an immunoassay autoanalyzer, according to FISH methods (Durham, North Carolina 27712, USA). Using the following formula, the homeostatic model assessment of insulin resistance (HOMA-IR) was determined:

[HOMA-IR = (mg/dL of glucose x mU/L of insulin)/405].

Apelin and elabela quantification: A Human Apelin and Elabela ELISA kit, using the sandwich method (My BioSource, USA), was used to measure serum apelin and Elabela levels. This kit is a sandwich kit used for the quantitative determination of parameters in serum.

Anthropometric and metabolic syndrome assessment: Body mass index (BMI) was classified into three categories: normal weight (less than 25 kg/m^2), overweight (between 25 and 30 kg/m^2), and obesity (more than 30 kg/m^2). For metabolic patients who are obese, the following criteria must be met: more than 150 mg/dL of triglycerides, more than 110 mg/dL of fasting glucose, and more than 102 cm of abdominal obesity in women and 88 cm in men, respectively; HDL cholesterol ranges between 40 and 50 mg/dL for both sexes. additionally evaluated the elements that comprise the metabolic syndrome (MetS) and computed the MetS score. Individuals were categorized as presenting with MetS if their score was ≥3. The MetS score ranges from 0 to 6 (3). The waist-to-hip ratio (WHR) was calculated by dividing the waist circumference (measured in cm) by the hip circumference (measured in cm).

Statistical analysis

A program, SPSS version. 29, was used for analysis. The variables were reported as means ± standard deviations, with one-way ANOVA and correlation coefficients. To identify the risk factors for MetS, multiple logistic regression analyses were carried out. Apelin and Elabela's ideal threshold values for MetS diagnosis prediction were calculated using a receiver operating characteristic (ROC) curve. Statistical significance was defined as a P value of ≤ 0.05.

Results

Glucose and lipid profile levels assessment: A total of 90 individuals, 30 obese with metabolic syndrome patients, 30 non-obese with metabolic syndrome patients, and 30 healthy controls, were included in this study. The mean BMI values of obese Mets patients and non-obese Mets patients compared to control groups were 36.24±2.22, 23.47±1.7, vs. 22.25±1.9 kg/m^2, respectively. The obesity with MetS was significantly greater than non-obesity patients with MetS, and healthy groups among participants ($p<0.05$), as appears in Table 1.

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Table 1. Characteristics of patients from the study groups with and without obesity, MetS and control.

Groups Parameters	Obese Patient with MetS group No. (30)	Non-Obese Patient with MetS group No. (30)	Control group No. (30)	p-value
BMI (kg/m^2)	36.24±2.22	23.47±1.7	22.25±1.9	≤ 0.05
W/H ratio	0.93±0.09	0.90±0.1	0.82±0.05	≤ 0.05

The mean values of fasting blood glucose and lipid profile (triglycerides, cholesterol, HDL, VLDL, and LDL) for each of the research groups were included in Table 2. The mean standards in this table revealed that there were important variances in triglycerides, HDL, and LDL among the obese and non-obese patients with MetS groups, and the control group ($p \leq 0.05$), while there were no important variances in fasting blood glucose and cholesterol among all study groups ($p \geq 0.05$).

Table 2. The FBS and lipid profile of the study groups with and without obesity, MetS, and control groups.

Groups Parameters	Obese Patient with MetS group No. (30)	Non-Obese Patient with MetS group No. (30)	Control group No. (30)	p-value
FBG (mg/dL)	97.20±1.33	94.75±1.31	83.42± 0.95	NS
TC (mg/dL)	189.45±30.45	180.57±27.41	172.32±25.30	NS
TG (mg/dL)	269.99±63.11	249.12±56.14	132.6±50.4	≤ 0.05
HDL-C (mg/dL)	40.11±0.61	45.32±0.59	60.4±1.5	≤ 0.05
LDL-C (mg/dL)	186.8±28.66	90.6±1.22	85.4±0.99	≤ 0.05

Apelin, elabela, insulin, HOMA IR levels assessment: Table 3 presents the values of Apelin (pg/ml), Elabela (ng/mL), Insulin (μIU/mL), and HOMA IR for every group under study in the current research. The table demonstrated a statistically important variance among the obese MetS patients, non-obese MetS patients, and control group with ($p \leq 0.05$).

Table 3. Biochemical parameters of the study groups.

Groups Parameters	Obese Patient with MetS group No. (30)	Non-Obese Patient with MetS group No. (30)	Control group No. (30)	p-value
Apelin (pg/mL)	367.18±140.33	221.42±126.94	203.89±117.28	≤ 0.05
Elabela (ng/mL)	0.78±0.44	0.75±0.43	0.73±0.42	NS
Insulin(μIU/mL)	32.7±14.98	9.10±0.95	7.2±0.64	≤ 0.05
HOMA IR	7.8 ±0.62	2.1±0.05	1.5±0.03	≤ 0.05

Correlation analysis: Table 4 presents the outcomes of the correlation analysis conducted among numerical variables.

Table 4. Correlation Analysis between Variables.

		BMI	WHR	TC	TG	HDL	LDL	FBS	Insulin	HOMA-IR	Apelin	Ela
BMI	R	1										
	P											
WHR	R	.460	1									
	P	.041										
TC	R	.429	.176	1								

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	P	.059	.457									
TG	R	.457*	.525*	.309	1							
	P	.043	.017	.185								
HDL	R	.084	-.260	-.098	-.288	1						
	P	.726	.268	.682	.218							
LDL	P	.306	.127	.958*	.161	-.293	1					
	R	.190	.594	.000	.499	.210						
FBS	P	.227	.287	-.172	.398	-.237	.200	1				
	R	.335	.220	.467	.082	.315	.397					
Insulin	P	.093	.144	-.207	.042	-.067	.199	.090	1			
	R	.697	.545	.382	.859	.779	.400	.705				
HOMAIR	P	.078	.280	-.265	.268	-.172	.281	.675**	.793**	1		
	R	.743	.232	.259	.253	.469	.231	.001	.000			
Apelin	P	.760*	.348	-.038	-.286	.169	.070	.304*	.234	.005	1	
	R	.000	.133	.872	.221	.476	.771	.035	.321	.985		
Ela	P	.171	-.154	.195	-.156	-.120	.192	.011	.524	-.416	.327	1
	R	.471	.516	.409	.511	.615	.417	.965	.199	.068	.159	

The ROC curve analysis: According to the results of association analysis, the ROC curve analysis was used to examine whether apelin has the potential effect on obesity and metabolic disease. The area under curve (AUC) was 0.937 in Figure 1 and 0.711 in Figure 2. The area under curve for apelin showed higher values as shown in Figure 1, more than in Figure 2. Therefore, a high level of apelin expression in adipose tissue is associated with obesity and a metabolic syndrome (Table 5).

Table 5. ROC curve analysis of apelin in the MetS in both the obesity and non-obesity groups.

Area Under the Curve					
Test Result Variable(s): Apelin					
	Area	Std. Errora	Asymptotic Sig.b	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Figure 1	.93	.049	.000	.841	1.000
Figure 2	.71	.111	.046	.493	.928

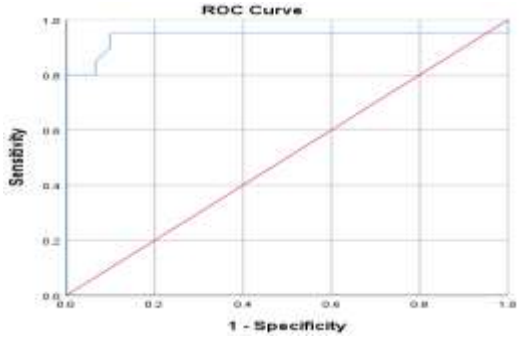

Figure 1. ROC curve of Apelin in obese-MetS patients.

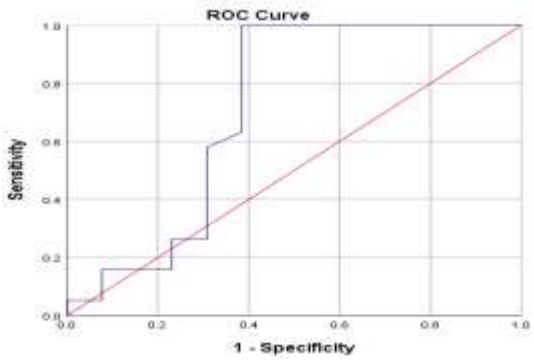

Figure 2. ROC curve of Apelin in non-obese- MetS patients.

The analysis of ROC curves was used to determine the efficacy of apelin in the MetS in both the obesity

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and non-obesity groups. Serum apelin performed better in the obese-MetS group (AUC = 0.937) than in the non-obese-MetS group (AUC =0.711), with a higher cut-off value (388 pg/mL) in the obese-MetS group than in the non-obese-MetS group (239 pg/mL, respectively). Furthermore, apelin is a more accurate indicator of obesity in the obese-MetS group than in the non-obese MetS group, according to our observations.

Discussion

The conditions insulin resistance, hyperglycemia, dyslipidemia, and hypertension are linked to obesity, especially excess visceral adiposity. These conditions are collectively referred to as the "metabolic syndrome." Type 2 diabetes mellitus (T2DM) and cardiovascular diseases are made more likely to occur by these metabolic disorders, which also lead to elevated rates of morbidity and mortality (8). According to the data from this study, BMI and WHR increase more in the obese MetS group than in the non-obese MetS group and the healthy group. This result agrees with Masquio et al., (9) who found that obesity subjects with MetS have shown significant differences in body weight, BMI, and WHR compared to non-obese subjects with MetS. Adiponectin is associated with central obesity and is produced by adipocyte tissue, indicating that its levels are correlated with adipocyte mass (10). Since all patients with metabolic syndrome had central obesity, the levels were significantly increase than in subjects with healthy group.

The result of this study was in agreement with study by Gallagher EJ et al,(11) according to which low HDL and elevated TG are components of the metabolic syndrome's dyslipidemia profile. There was a 2.5 relative risk of coronary disease between people with TG levels in the top third of the population and people with TG levels in the bottom third of the population, according to a meta-analysis of prospective studies. Lipid profiles are significantly impacted by the onset of IR. With the loss of its inhibition of hormone-sensitive lipase, IR causes an increase in the amount of FFA produced by adipocytes. Furthermore, there is a decrease in endothelial lipoprotein lipase activity, which both add to the rise in free fatty acids in circulation (12). Hepatic TG increased as a result of increased FFA inflow into the liver and insulin-stimulated hepatic lipogenesis. Due to the liver's increased influx of free fatty acids (FFAs) and insulin's stimulation of hepatic lipogenesis, the liver produced more triglycerides, which were stored in the liver and resulted in steatosis and very low-density lipoprotein. Because the TG is stored in the liver, it is produced as VLDL and steatosis (13). Transferring cholesterol esters from HDL to VLDL is made possible by the cholesterol ester transferase protein produced by adipocytes (14). The low HDL and elevated TG levels associated with the metabolic syndrome are caused by the liver's absorption of HDL and subsequent production of VLDL due to increased HDL clearance by the kidney (15).

As the information in Table 3 showed, there was a highly significant difference in Apelin, insulin, and HOMA IR in the Obese Patient with MetS group compared to the Non-Obese Patient with MetS group and the Control group. While no significant difference levels found in Elabela concentration between patients them self and with control group. This result agrees with the findings of Mutlak, S.S., et al., (16), which discovered that the group with MetS had higher serum apelin levels than the group without MetS .

Apelin is thought to be a significant predictive biomarker for metabolic disorders. Additionally, the outcome concurs with research conducted by other scientists (17). Which demonstrated that MetS had higher apelin levels than age-matched controls. According to their findings, apelin levels and IR were positively correlated. Furthermore, the outcome is consistent with Angelova, P., et al., (18).

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A comparison of the apelin levels in the obese patients and the control group in their study indicates a significant increase in apelin levels. Moreover, apelin levels decrease following insulin sensitizer therapy in individuals with low body mass index. Apelin synthesis occurs in other locations, such as the vascular endothelium, which may conceal less secretion from fat tissue. This is most likely the cause of the observed phenomenon. However, following weight loss, apelin expression is shown to be reduced (19). Increased apelin levels in humans and animals have been linked to a number of metabolic disorders, according to some research, but not all of them. Apelin is now known to be a useful adipokine that has anti-diabetic and anti-obesity properties, which makes it a potentially useful therapeutic target for a number of metabolic disorders. When compared to normal controls, apelin levels have been shown in certain studies to be higher in insulin-resistant individuals and morbidly obese people with type 2 diabetes (20). Human endocardial endothelial cells and vascular endothelial cells have been found to contain apelin. It has been demonstrated that apelin significantly increases heart rate and contraction in the heart. apelin produces peripheral vasodilatation through a mechanism that is dependent on nitric oxide (NO). Apelin inhibits the electrical activity of vasopressin-releasing neurons in the hypothalamus, indicating a potential role in the control of vascular tone (21). An elevated fat diet in rats causes an increase in the expression of apelin in the subcutaneous adipose tissue (SAT). Both significantly obese patients having gastric banding and mice fed a high-fat diet had higher plasma apelin levels. ApeLIN levels have recently been shown to decrease in obese women following a 12-week period of diet-induced weight loss (22).

Apigenin-angiotensin receptor-like 1 (APJ) has two ligands. There are two: apelin and elabela/toddler (ELA) (23). APLNR and APJ are apelin receptors that control the biological activity of the peptide hormones APELIN (APLN) and ELABELA (ELA, Toddler, apela). ELA and APLN, two agonists of APLNR, have the ability to alter a number of intracellular signaling pathways, including PKA (protein kinase A) and adenyl cyclase (AC).

Toddler, also referred to as apelin or elabela, is a peptide consisting of 54 amino acids, which includes a secretory signal. Two research groups have recently discovered a mature form of elabela, which comprises 32 amino acids (24). The function of this peptide in relation to adipose tissue metabolism remains unclear. But given the information in the literature and how it affects metabolic pathways, like the control of SIRT3-mediated oxidative stress inhibition through Foxo3a deacetylation or the inverse relationship between blood glucose level and ELA, it is reasonable to believe that APLN and ELA are also involved in the metabolism of adipose tissue (25). The findings of this study concur with the first one conducted by Yeniel N. et al., (24). Which discovered that the Elabela concentrations in the obese and control groups did not differ significantly. Despite the fact that Elabela levels did not differ between obese participants in this study, the paucity of research on Elabela's function in metabolism or obesity makes it incorrect to draw firm conclusions. We think that more thorough research will provide more important details regarding Elabela's involvement in obesity.

The two primary underlying risk factors for metabolic disturbances are obesity and insulin resistance (IR), which also contribute to the rise in other risk factors like dyslipidaemia, hypertension, and hyperglycaemia (26). Another indicator of insulin resistance is an increase in hepatic fat brought on by an excess of free fatty acid influx to hepatocytes as a result of dysfunctional lipolysis brought on by insulin resistance. Visceral or hepatic fat cannot be accurately estimated by BMI or waist

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circumference alone. Selective loss of body fat, severe insulin resistance, and non-alcoholic fatty liver disease are characteristics of genetic or acquired lipodystrophies, suggesting that obesity in and of itself is not the primary cause of the metabolic syndrome. Rather, the cluster of abnormalities associated with the metabolic syndrome may be primarily caused by lipotoxicity and ectopic fat deposition. The outcome concurred with Widjaja, Nur Aisiyah, and others (27). Insulin resistance occurs when insulin is unable to inhibit lipolysis and Fox01, which influences how insulin signalling regulates gluconeogenesis and glycogenolysis, but instead triggers rapamycin complex-1 (mTORC1). The overproduction of VLDL and decreased clearance of VLDL are the results of insulin's inability to suppress Fox01, which raises the expression of apoCIII and microsomal triglyceride transfer protein (MTTP) (28).

The present study, demonstrated a positive correlation between serum plasma apelin levels and BMI. Comparable results were found in another study conducted by Zaki, Moushira, et al., (29) .Indicating that apelin plays a part in the etiology of obesity. Furthermore, additional research (30). The apelin levels were considerably higher in obese individuals than in control subjects, and they showed a positive correlation with BMI. Therefore, it seems that apelin concentration is significantly influenced by obesity. Furthermore, the significantly elevated apelin and insulin levels in obese MetS raise the possibility that apelin homeostasis is compromised. It could also imply that elevated insulin levels lead to an increase in apelin blood concentrations, as another study has also suggested. The results also showed a negative correlation between apelin and glucose, which is consistent with a study by Saadi, H.A.H., et al. (20). This shows that apelin may have significant therapeutic implications for metabolic syndrome. According to a study, apelin levels and fasting blood glucose (FBG) were negatively correlated. There was no correlation found between any of the parameters and Elabela levels. Further investigation is necessary to clarify the relationship between Elabela, insulin resistance, and the metabolism of glucose in individuals who do not have diabetes. This finding is consistent with a study conducted by Yeniel N. et al., (24). Which reported that there was no correlation between Elabela levels and other numerical variables. This might be because Elabela cannot sufficiently penetrate the blood-brain barrier to affect human nutritional centers.

Conclusions

The metabolic syndrome should be considered when assessing patients who are overweight or obese, as it may help identify those who are at a higher risk of developing type 2 diabetes and cardiovascular disease (CVD) in the future. The current study highlights the crucial role of apelin in MetS in both non-obese and obese MetS, as well as in clinical and biochemical markers associated with obesity. When it comes to MetS in obesity, serum apelin exhibits a high degree of predictive accuracy, outperforming matched non-obese individuals with MetS. This implies that serum apelin might be useful in MetS and obesity-related comorbidity settings, both clinically and therapeutically. Taking everything into account, the data points to APLN and ELA as potent adipocyte metabolism modulators. Nevertheless, since the effects of ELA are still unclear, more investigation is required.

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Ethical clearance: The Research Ethics Committee of AL-Nahrain University's Department of Chemistry, College of Sciences, approved this study, and in accordance with the ethical guidelines of the Declaration of Ethical Committee of the College (2024NC690). Written consent was obtained from all patients before inclusion.

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تقييم الأبلين والإيلابيلا وبعض المؤشرات الحيوية لدى المرضى المصابين بأمراض التمثيل الغذائي المرتبطة بالسمنة

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الملخص

الخلفية: تم اكتشاف ببتيد جديد يُعرف باسم ايلابيلا ؛ يعمل بشكل مشابه للأبيلين ويعمل من خلال مستقبلات الابلين.

الأهداف: يهدف هذا البحث إلى مقارنة خصائص وأدوار الأبلين والايلابيلا البيولوجية لدى مرضى متلازمة التمثيل الغذائي الذين يعانون من السمنة وأولئك الذين لا يعانون من السمنة.

المرضى والطرق: شملت الدراسة المقطعية ٩٠ مشاركًا تتراوح أعمارهم بين ٢٠ و ٤٥ عامًا، تم جمع عيناتهم من مستشفى اليرموك / الكرخ / بغداد، من أبريل إلى يوليو / ٢٠٢٤. تم تصنيفهم إلى ٣ مجموعات: المجموعة الاولى تشمل مرضى متلازمة التمثيل الغذائي المصابين بالسمنة (٣٠)، والمجموعة الثانية تشمل مرضى متلازمة التمثيل الغذائي غير المصابين بالسمنة (٣٠)، والمجموعة الثالثة تشمل مجموعة التحكم (٣٠). تم إجراء القياسات الأنثروبومترية والمعايير لجميع مجاميع الدراسة، والتي تتضمن: قياس نسبة الكلوكوز في الدم الصائم ومستوى الدهون بواسطة عملية إنزيمية باستخدام طرق قياس الطيف الضوئي، في حين تم تقييم الأنسولين والأبلين والإيلابيلا بواسطة اختبار الممتز المناعي المرتبط بالإنزيم.

النتائج: تظهر النتيجة فرقًا كبيرًا في مؤشر كتلة الجسم ونسبة الخصر إلى الورك ومستوى الدهون ونسبة مصل الأبلين والأنسولين و-HOMA IR، في حين لم يكن هناك فرق كبير بين مجموعات الدراسة في مستويات الإيلابيلا. أيضًا وجد ارتباط إيجابي بين الأبلين ومؤشر كتلة الجسم ونسبة مصل الابيلين والأنسولين. مستويات الأبلين لها قيمة تشخيصية أعلى للسمنة من متلازمة التمثيل الغذائي على التوالي.

الاستنتاج: تشير الأدلة إلى أن الابيلين والايلابيلا من المنظمات القوية لعملية التمثيل الغذائي للخلايا الدهنية. ومع ذلك، هناك حاجة إلى مزيد من البحث لأن تأثير الايلابيلا لا يزال غير معروف.

الكلمات المفتاحية: الأبلين، **APJ**، الإيلابيلا، متلازمة التمثيل الغذائي، السمنة.

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Study of Gene Expression Variation of Heat Shock Factor 1 and Estimates of the Concentration of Epithelial- Derived Neutrophil-Activating Protein-78, and Ability for Bacterial Biofilm Formation in Patients with Tonsillitis

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Abstract

Background: Tonsillitis is an inflammation of the tonsils, often spreading to the adenoids and lingual tonsils. It is influenced by immune and genetic factors. This study aimed to evaluate the gene expression of *Heat Shock Factor 1(HSF1),* a protein that regulates cellular responses to stress, especially heat stress, and to measure the concentration of epithelial-derived neutrophil-activating protein-78, a chemokine involved in neutrophil activation and recruitment during inflammation. The study also sought to explore the relationship between these molecules and their roles in inflammation and stress responses. Additionally, it aimed to isolate bacterial species responsible for tonsillitis and assess their ability to form biofilms.

Patients and Methods: Sixty blood samples from tonsillitis patients and 30 from healthy individuals were collected, along with 200 throat swabs from patients, between December 2023 and March 2024. Blood samples were processed for ELISA to quantify ENA-78 and for RNA extraction to analyze *HSF1* gene expression. Bacterial cultures were grown on Congo Red Agar to detect biofilm production.

Results: Out of 120 positive cultures, 70% were Gram positive, 23% were Gram negative, and 7% mixed. ENA-78 levels were significantly higher in patients (619.2±123.4) than in controls (490.1±145.8) pg/mL ($P \leq 0.0001$) *HSF1* gene expression was also significantly elevated in patients (9.913) ($P = 0.0001$).

Conclusion: Gram positive bacteria, particularly *Staphylococcus aureus*, were the predominant biofilm producers, while *Escherichia coli* was common among Gram-negative isolates. Significant differences in ENA-78 levels and *HSF1* expression suggest their potential as diagnostic markers and targets for immune - based therapies.

Keywords: Tonsillitis, ENA-78, *HSF1,* Biofilm formation.

Introduction

Tonsillitis is the inflammation of the tonsils, which typically spreads to the adenoid and lingual tonsils. The tonsils are part of Waldeyer's ring, which consists of lymphatic tissue (1). The tonsil is an autonomous organ composed of Mucosal-Associated Lymphoid Tissue (MALT), As the initial line

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of defense against invading infections, the tonsil has the ability to selectively modulate immunological responses throughout the body (2).

The palatine tonsil, situated near the pharyngeal opening, is a suitable location for exposure to foreign substances, including bacteria and viruses, and their subsequent transfer to lymphoid cells. The immune system depends on the creation of lymphoid cells, such as T and B cells, which is continuously stimulated by healthy palatine tonsils (3). Although it affects people of all ages and genders, school–aged children are especially more likely to experience this, particularly during the fall and winter months. A tonsillar infection may be hypertrophic, recurring, chronic, or acute (4).

The inflammation of the palatine tonsils, known as tonsillitis, is brought on by bacteria, viruses and other immunologic causes (5). Although tonsillitis is typically treated symptomatically with good clinical outcomes, complications such as, rheumatic fever, abscesses, acute glomerulonephritis, and scarlet fever, can arise due to the fact that many organisms inhabit the oropharynx, including *Streptococcus pyogenes*, *Staphylococcus aureus*, *Hemophilus influenza*, and *Streptococcus pneumoniae* (6). Tonsillitis refers to a variety of infectious and inflammatory disorders that can be bacterial or viral. Since most viral tonsillitis episodes resolve on their own, it is crucial to distinguish between the two etiologies for treatment purposes. Nonetheless, bacterial tonsillitis patients can benefit from treatment by experiencing a shorter illness duration and fewer sequelae (7). In healthy tissue, epithelial cells are the crucial source of the CXC chemokine, well-known as epithelial cell-derived neutrophil-activating peptide 78 (ENA-78). In inflammatory tissue, however, inflammatory cells that penetrate the submucosa are the most significant cellular source of this chemokine. The body's initial line of defense, neutrophils, respond swiftly to invading microorganisms and tissue injury (8). This chemokine is composed of 78 amino acids and comprises four cysteines; it seems to be a homologue of IL-8 (9).

Most tissue and cell types express *Heat Shock Factor 1*, a key transcription factor that is activated by heat. It is dormant when no stimuli are present (10) .An increase in temperature is necessary for *HSF1* activation because heat separates the *HSF1* inhibitory complex in the cytosolic compartment and creates a DNA-binding competent homotrimer complex (11).

The current study aimed to study gene expression variation of *Heat Shock Factor1*, to estimate the concentration of epithelial-derived neutrophil- activating protein- 78 in patients compared to healthy individuals, and to isolate bacterial species causing bacterial tonsillitis and producing biofilm.

Patients and Methods

Study design: This study is a cross-sectional study at Tikrit Teaching Hospital. Swab samples were obtained from patients admitted to the ear, nose, and throat unit at Tikrit Teaching Hospital, in Salah AL Din Governorate between December 2022 to March 2023. Patients were divided into two groups: acute infection into 32 and chronic infection 88.

Swabs were removed from the tonsil surface without touching any neighboring surfaces, and all samples were collected in a way that prevented contamination. The swabs were then closed immediately until transferred to the microbiology laboratory of Tikrit Teaching Hospital and cultured on various media for 24 hours at 37 °C, followed by Gram staining and biochemical tests for bacterial identification. Transport media were utilized for delayed cases (12).

Detection of biofilm-producing bacteria: Congo red agar plates were prepared, inoculated with the bacterial isolates, and then incubated in an incubator at 37°C for 24-48 hours. After incubation, the color of the growing bacterial colonies was observed, with black colonies

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indicating that they were biofilm producers. Red colonies did not produce biofilms (13).

Immunological and genetic evaluation: For immunological and molecular detection, five milliliters of venous blood were obtained from each patient and control individual's, transferred into a gel tube, centrifuged at 4500 rpm for 8 minutes, and the serum was collected in sterile Eppendorf tubes in two copies and kept frozen at -20 ℃ for ELISA testing to limit the level of ENA-78, using the ENA-78 ELISA kit made by Sun Long (China). Additionally, 250 ml of whole blood was added to Eppindr of tube containing 750 µl of TransZol and mixed well to use for gene expression variation, The primers were designed specifically for this study (F: ATCTTCCGTGGACACCCTCT, R: GCTACG CTGA GGCACTTTTC)

Gene expression analysis: After collecting the blood sample, 250 microliters were combined with 750 microliters of Trizol. RNA was extracted from Trizol-preserved whole blood samples from 30 control subjects and 60 tonsillitis patients. The mRNA was extracted using the Transzol up Plus RNA kit from TRANS Company(china). To measure gene expression using the two-step qPCR technique, during the two-step thermal cycling, single strands of RNA were converted into complementary strands of cDNA using the Easyscript First-Strand cDNA Synthesis Diagnostic Kit prepared by TRANS Company. At Tikrit University, College of science, Department of Biology-Molecular Laboratory, the RT-qPCR reaction was performed. the reaction mixture was prepared using Biolab's Luna® Universal qPCR Master Mix extraction kit.

Statistical analysis

Microsoft Excel 2010 and the statistical program for social science (SPSS) version 23 and ROC curve were used to gather, compile, analyze, and present the data. When means and standard deviation data were analyzed using the t-test, a P-value of 0.05 was deemed significant. The Δct value and the ΔΔct value were utilized to perform statistical evaluations during qPCR, and to determine the relative fold change in gene expression of the sample.

Results

Distribution patterns of tonsillitis based on infection type: A total of 120 swab specimens were obtained for the current investigation from patients with tonsillitis of both genders in various age groups between December 2023 and March 2024. Additionally, blood samples were taken from both healthy individuals and those who were sick.

Acute and chronic tonsillitis were separated into two categories based on the specialist doctor's assessment, with varying numbers and percentages. As seen in Figure 1, the majority of tonsillitis patients with chronic infection, 88(73.3%), emerged first, followed by acute infection 32(26.6%).

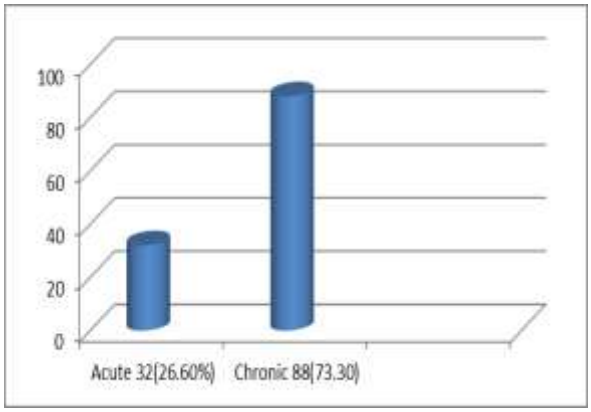

Figure 1. Distribution of Tonsillitis cases according to type of infection.

Gender differences in tonsillitis incidence: The distribution of patients in this study, according to their gender, was as follows: male (115) and female (85). These sample size represent the number of people from each gender who were tested or diagnosed with tonsillitis in our study and showed that males were more likely than females to have tonsillitis, as shown in Figure 2.

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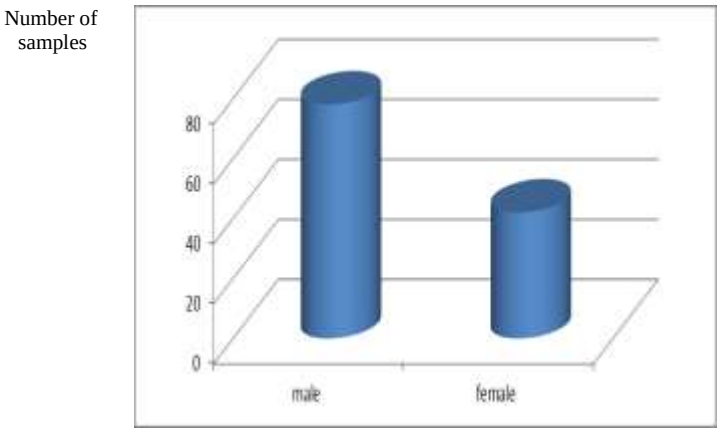

Figure 2. Distribution of tonsillitis cases according to gender.

Distribution of tonsillitis cases according to age: Patients were divided into four age categories: The group from 1 to 10 years had the highest infection rate at 48%. This indicates that nearly half of the patients in this group were affected by the infection. The infection rate for the 11–20-year-old group was 33%, which is lower than that of the 1–10-year-old age group but still represents a significant proportion of patients. The 21-30 years group had an infection rate of 13%, which is much lower than both of the younger groups, indicating that fewer patients in this age range were infected. The infection rate of group 31- 40 years was 7%, which is lower than all groups, as shown in Figure 3.

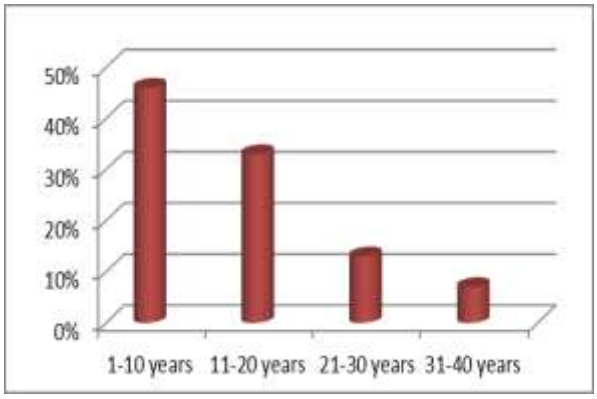

Figure 3. Distribution of Tonsillitis cases according to the Age.

Bacterial isolation and identification: Biochemical tests and culture characteristics were used to diagnose the bacterial isolates. The Vitek 2 technique was also used to diagnose several isolates at the species level. Culture was the major method used to characterize the bacterial isolates. All of the isolated bacteria were then subjected to morphological, microscopic, and biochemical testing using blood agar, Mannitol salt agar, MacConkey, Eosin Methylene Blue agar, and Gram stain (14).

According to the bacteriological study, numerous bacteria were found in the tonsil swabs of patients who were examined; of these, 70% were found to be Gram positive, 28% to be Gram negative, and 7% to be mixed growth. The Gram-positive bacteria were *Staphylococcus aureus and Streptococcus pyogenes*, while the Gram negative were *Esherichia coli*, *Klepsiella Pneumoniae*, and *Seratia fonticola.* The results revealed a predominance of Gram-positive bacteria, with the most common isolates being *S.aureus* and *S.pyogenes*, there was also a significant presences of Gram-negative bacteria, with *E.coli* and *K.pneumoniae* being the most notable pathogens, as shown in Figure 4.

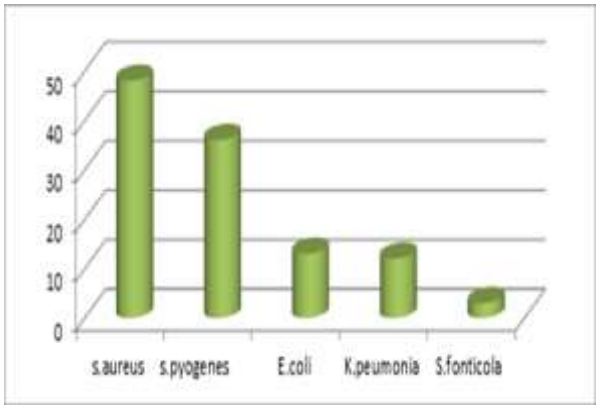

Figure 4. Bacterial species isolated from tonsil swabs.

Biofilm Formation of Bacterial Isolates: As shown in Figure 5, the study demonstrated that

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S. aureus isolates were biofilm producers at a percentage of 38 (79%), *S. pyogens* at 25 (70%), *E. coli* at 8 (62), *K. pnemoniae* at 7 (58%), while *S. fonticola* was at 0 (0%). The ability of 114 isolates to produce biofilm was examined, as shown in Table 1. The results indicate that *S. aureus*, *S. pyogenes*, *E. coli*, and *K. pneumoniae* all exhibit varying abilities to produce biofilms, with *S. aureus* and *S. pyogenes* being the most prominent biofilm producers. Conversely, *S. fonticola* does not produce biofilms under the tested conditions, which might have implications for its role in infections. These findings highlight the potential importance of biofilm formation in the pathogenicity and persistence of certain bacterial species.

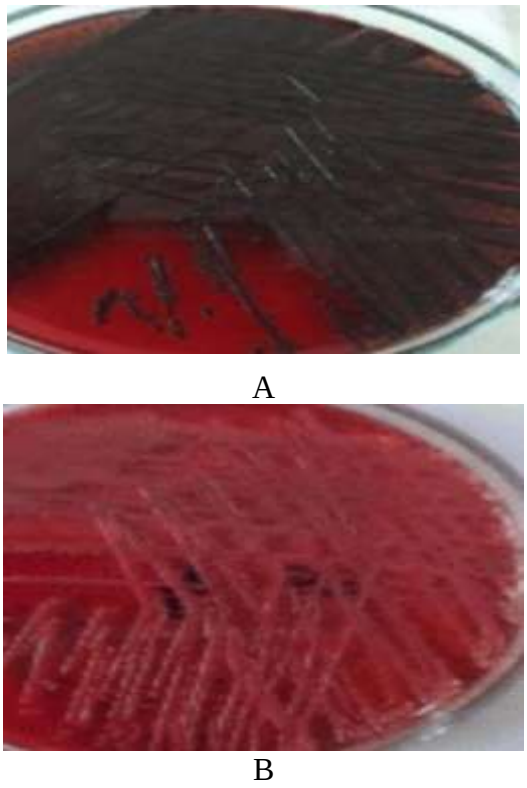

Figure 5. Biofilm production on Congo red agar: A: Positive result, B: Negative result.

Table 1. Comparative Analysis of *H. pylori* Infection in Benign and Malignant Lesions. N.S.: not significant (p > 0.05).

Bacteria	Positive to biofilm production	Negative to biofilm production	Characteristic of the participants
S.aureus	12(25%)	38(75%)	88 chronic and 26 acute
S.pyogenes	11(30%)	25(70%)	
E.coli	5(38%)	8(62%)	
K.pnemoniae	5(41%)	7(58%)	
S.fonticola	3(100%)	0(0%)	

ENA-78 levels for tracking inflammatory signals from epithelial cells: The findings demonstrated a notable difference in ENA-78 levels between patients and control individuals. The Mean ±SD were (619.2 ± 123.4) and (490.1 ± 145.8) pg/mL respectively, as shown in Figure 6, which visually presents this difference in the form of a box plot, where the patients group has a higher average ENA-78 level than the control group, with variation shown by the error bars.

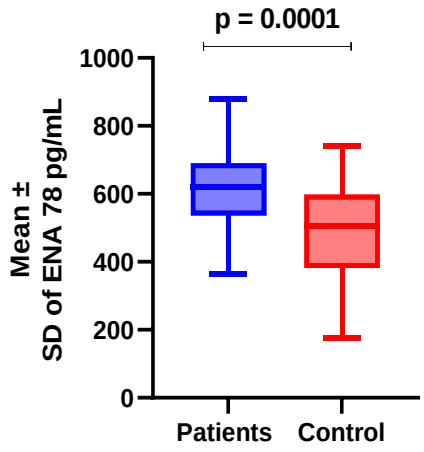

Figure 6. The level of ENA-78 in patients and in healthy people.

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The expression of HSF1: Figure 7, Table 2, and ROC curve (Figure 8) demonstrate that the variation reaches a significant value when comparing the gene expression data between the patient and healthy groups (9.913). The values of ΔCT (mean ± SD) were (-0.7615±0.452) (7.975±4.342), respectively. These were the Roc curve readings (AUC = 0.9607) (sensitivity% = 93.33) (specificity%=85.56), (P=0.0001).

The variation reaching a significant value refers to a notable difference in the gene expression data when comparing the patient group to the healthy group, the P value indicates statistically significant results, meaning the observed differences are highly unlikely to have occurred by chance. ΔCT refers to the difference in the threshold cycle between the target gene and a reference gene (GAPDH) in the PCR analysis. This indicates that the patients show a lower CT value compared to the healthy group, suggesting a higher expression of the gene in patients compared to healthy individuals, a lower CT value typically corresponds to higher gene expression.

Table 2. Expression of *HSF1* in patients and control.

Gene	ΔCT of patients (mean ± SD)	ΔCT of control (mean± SD)	P value	Expression fold ($2^{-\Delta\Delta ct}$)
HSF1	-0.7615± 0.452	7.975± 4.342	0.001	9.913

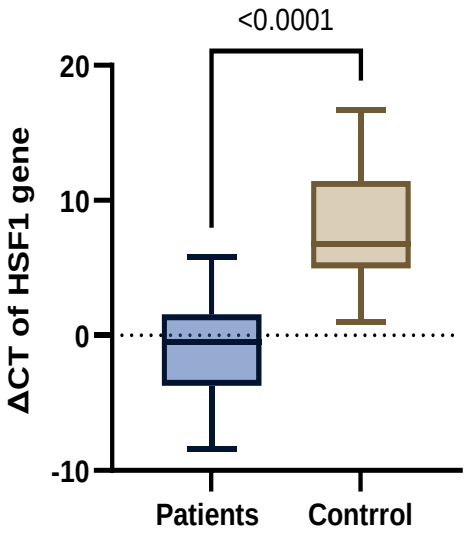

Figure 7. Relative expression levels of *HSF1* in patients compared with controls, ΔCT = Delta cycle threshold.

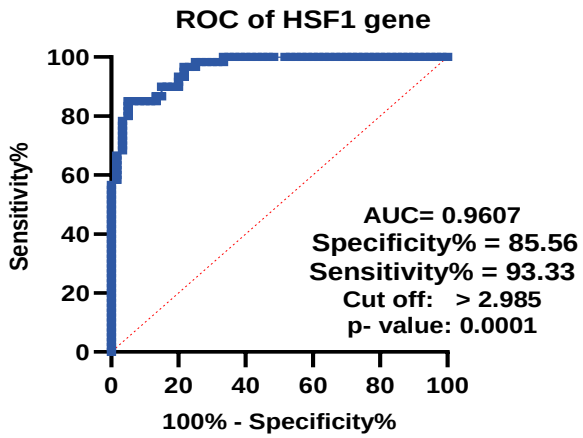

Figure 8. ROC curve of *HSF1*.

Discussion

This study separated patients into two types of infection. The high percentage of tonsillitis appeared with chronic tonsillitis. This study differed from a study by (14), which revealed that the proportion of acute infections was higher than that of chronic infections. The synthesis of β-lactamase and resistance in numerous organisms resulted in failed medical therapy and chronic or recurrent forms of tonsillitis. The influence of various types of food kinds is one of the risk factors for chronic tonsillitis since children often eat artificial sweeteners with a lot of preservatives and neglect their dental hygiene

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(15).

This study showed that males had a higher prevalence of infection compared to females. This finding is consistent with previous studies by (16, 17). Theoretically, gender differences in disease have been linked to genetic, biochemical, environmental, or psychosocial factors [19], Males are more likely to be exposed to infectious microorganisms and come into contact with ill individuals due to their environmental interactions (18), Woman have higher immune response to self and foreign antigens than men, resulting in gender disparities in autoimmunity and infectious disease. Males are more prone to bacterial infections than females, both in animals and humans (19).

This study separated patients into four age groups. The highest percentage of infection appeared in 1-10 age group. These findings were similar to other research, which has found that the majority of tonsillitis patients were children (5). Children increased activity at this age may enhance their susceptibility to infection compared to other age groups. In addition, this is the school age when children mingle and speak in the classroom (20). In children, the immune system is still developing to recognize and protect against germ. However, in adolescents and adults, the immune system quickly recognizes and attacks germs (21).

The bacteriological study revealed the presence of various bacteria in the tonsil swabs of the examined patients, illustrating that Gram-positive bacteria are the most common cause of bacterial tonsillitis. *S. aureus* was frequently found, followed by *S. pyogenes*. This result is similar to a study done by (22); gram-positive bacteria are the most common and most virulent cause of tonsillitis. They can also live as normal colonies on the skin and in other oral cavities, so that they may be more numerous than the Gram-negative bacteria (23).

Biofilm production by bacteria is highly relevant to tonsillitis, a condition characterized by inflammation of the tonsils, as it significantly impacts both the pathogenesis and treatment of the disease. The biofilm matrix, which is made up of extracellular polymeric substances (EPS), is essential for shielding bacteria from environmental stresses. It functions as an effective diffusion barrier, preventing dangerous substances from entering the biofilm (24). The host's immune system, antibiotics resistance, and other environmental factors have made bacterial biofilms a significant contributor to global health issues; the persistence of bacteria in biofilms can complicate the effectiveness of standard antibiotics. Antibiotics often struggle to penetrate the biofilm, and bacteria within it may have a slower growth rate, reducing the antibiotics' efficacy. In some cases, the biofilm can even cause bacteria to become more tolerant to the antibiotic treatment, leading to treatment failure or relapse of the infection (25, 26). A research (27) pointed out that a *S.aureus* is one of the most gram-positive bacteria forming biofilms, while another study (28) mentioned that *E.coli* is the most gram-negative bacteria forming biofilms, which agrees with our current study. Researchers' (29) showed that *S.aureus* and *E.coli* bacteria have the ability to form biofilms in tonsillitis patients, and this result similar to our current study, This is because of the crypt tissue structure and direct, repetitive exposure to respiratory bacterial pathogens, such as *S.aureus*, which cause chronic infections in the upper respiratory tract, including chronic tonsillitis, the tonsillar tissue and adenoids are prone to biofilm formation. Therefore, understanding biofilm production is crucial for improving the treatment of tonsillitis. Strategies to disrupt biofilms or develop drugs that can penetrate biofilm layers are areas of active research and could significantly enhance treatment outcomes, reducing the chances of chronic or recurrent infections and minimizing the need for frequent or prolonged antibiotic use.

Inflammation-activating peptide-78, a CXC chemokine, is produced by epithelial cells and attracts neutrophils (30).

The CXCL5 gene encodes, the inflammatory CXC chemokine ENA-78, and many inflammatory disease have elevated amounts of this chemokine (8). ENA-78 is involved in leukocyte recruitment and activation in autoimmune illnesses and inflammatory diseases. It can speed up the exodus of monocytes and macrophages and granulocyte from the

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bloodstream through the endothelium, this can lead to a significant increase in chronic inflammation (31). An elevated ENA-78 gradient in the blood leads to increased accumulation of neutrophils surrounding the lesions, and generating severe clinical symptoms (32).

There was a substantial difference in the relative expression of *HSF1* between patients and healthy individuals (-0.7615±0.452) (7.975±4.342), respectively, ($p=0.0001$), as shown in Figure 7 and ROC curve (Figure 8). Numerous studies have shown that changes in *HSF1* function impact protein homeostasis and are strongly linked to disease. *HSF1* is a member of the heat shock factor (*HSF*) family, which is activated under a variety of stressors and subsequently causes the upregulation of heat shock genes such as Hsp27 (33). The majority of tissues and cell types constitutively express *HSF1*, which has been shown to support the immune system's proper function (34). A basic and well- preserved cellular mechanism, the stress response, is also referred to as heat shock gene expression, shielding organisms from a variety of physical and chemical stresses, such as high temperatures, heavy metals, oxidants, and toxins, as well as bacterial and viral infections, is crucial. Anti-inflammatory prostaglandins and the high temperatures found in fever and inflammatory tissues can both cause *HSF1* (35).

One important metabolic mechanism that influences the resolution of inflammation, particularly in situations involving proteotoxic stress, is the heat shock response. Heat shock transcription factor 1, which is necessary for the expression of heat shock proteins and other chaperones, mediates this potent anti-inflammatory mechanism (36). Similar effects are seen in microbes, where *HSF1* activation causes an increase in HSPs in response to stress or injury. The association between ENA 78 and *HSF1* in tonsillitis may indicate that elevated ENA- 78 levels may lead to an increased inflammatory response in immune cells, which may stimulate increased expression of *HSF1*, furthermore, *HSF1* may play a role in regulating the immune response against pathogens such as bacteria or viruses during inflammation, and thus may influence inter-individual variation in the extent of immune cell response to inflammation (30, 37). This study is an extension of research focused on understanding the body's responses to chronic or acute infections; it aligns with current literature investigating the role of genetics and immune factors in the development of tonsillitis. Previous research focuses on the association of chemokines and genetic factors with disease progression, which may contribute to the development of more personalized treatment strategies. These finding are expected to help open up a deeper understanding of how chemokines, such as ENA-78, influence the immune system response in the tonsils. Studying *HSF1* gene expression may also provide insights into how to improve treatment response or predict treatment responses in patients. For example, this research may contribute to improved immunotherapies or gene modification strategies that target specific pathways in immune cells.

Conclusions

The results indicated that percentage of Gram-positive isolates was higher than Gram-negative isolates, in patients with tonsillitis, *S.aureus* was the most abundant biofilm-forming gram-positive bacteria, while *E.coli* was the most abundant biofilms- forming gram-negative bacteria. Males had a higher prevalence infection compared to females. The highest percentage of infection appeared in 1-10 age group, and there was a notable variation in ENA-78 levels between patients tonsillitis and healthy individuals. There was also a significant variation in the relative expression of *HSF1* between patients and controls. This study reveals potential links between increased ENA-78 concentrations and elevated *HSF1* gene expression and the severity of tonsillitis, offering a new path to understanding the immune dynamics of the disease, this could significantly impact improving immunotherapy for tonsillitis

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patients, as well as contributing to the development of targeted therapeutic strategies based on identifying the genetic factors involved. It can be used as a diagnostic marker. It was recommended to do more genetic studies to identify host factors required for successful bacterial tonsillitis infection.

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Ethical clearance: The Ethics Committee for this investigation at Tikrit Teaching Hospital, Salah Al Din, Iraq, approved the study with No. 2157 on 26 December 2022.

Conflict of interest: None.

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دراسة تباين التعبير الجيني لعامل الصدمة الحرارية ١ وتقدير تركيز البروتين المنشط للعدلات المشتق من الخلايا الظهارية والقدرة على تكوين الأغشية الحيوية البكتيرية لدى مرضى التهاب اللوزتين

[١] نمارق عطاء الله محيميد، [٢] سهى ماهر عبد

الملخص

الخلفية: التهاب اللوزتين هو التهاب يصيب اللوزتين يتأثر بعوامل مناعية ووراثية.

الأهداف: هدفت الدراسة الحالية الى تقييم التعبير الجيني لعامل الصدمة الحرارية ١,وهو بروتين ينظم الاستجابات الخلوية للإجهاد, وخاصة الاجهاد الحراري, وقياس تركيز البروتين المنشط للعدلات المشتق من الخلايا الظهارية -٧٨,وهو كيموين يشارك في تنشيط العدلات , كما سعت الدراسة الى استكشاف العلاقة بين هذين الجزيئين ودورهما في الاستجابة الالتهابية الضغوط الخلوية, بالإضافة الى ذلك, هدفت الى عزل الانواع البكتيرية المسببة لالتهاب اللوزتين وتقييم قدرتها على تكوين الاغشية الحيوية.

المرضى والطرق: تم جمع ٦٠ عينة دم من المرضى و٣٠ عينة من اشخاص اصحاء, بالإضافة الى ٢٠٠ مسحة من حلق المرضى خلال الفترة من كانون الاول ٢٠٢٣ الى اذار٢٠٢٤,تم معالجة عينات الدم باستخدام تقنية الاليزا للاختبار المناعي وتم استخلاص الحامض النووي الرايبي من عينات الدم كما تم زرع العزلات على وسط الكونغو الاحمر للكشف عن قدرتها على تكوين الاغشية الحيوية.

النتائج: من بين ١٢٠ مزرعة ايجابية , كانت ٧٠٪ ايجابية لكرام , و٢٣٪ سلبية لكرام, و٧٪ مختلطة, وكانت مستويات البروتين المنشط المشتق من العدلات أعلى بشكل ملحوظ لدى المرضى (١٢٣,٤±٦١٩,٢) مقارنة بالأصحاء (١٤٥,٨ ±٤٩٠,١) بيكوغرام/مل قيمة (P =(0.0001 كما كان تعبير الجين النسبي لعامل الصدمة الحرارية ١ مرتفعا لدى المرضى (٩,٩١٣) قيمةP=0.0001.

الاستنتاج: كانت نسبة البكتريا الايجابية لجرام اعلى من نسبة السلبية والمختلطة , ووجد تباين ملحوظ في مستويات البروتين المنشط للعدلات المشتق من الظهارية, و تباين كبير في التعبير الجيني النسبي لعامل الصدمة الحرارية بين المرضى والاصحاء, يمكن ان يكون لهذا تأثير كبير على تحسين العلاج المناعي لمرضى التهاب اللوزتين فضلا عن المساهمة في تطوير استراتيجيات علاجية مستهدفة تعتمد على تحديد العوامل الوراثية المعنية ويمكن استخدامها كعلامات تشخيصية للمرض.

الكلمات المفتاحية: التهاب اللوزتين, البروتين المنشط للعدلات المشتق من الخلايا الظهارية – ,٧٨ عامل الصدمة الحرارية ١ , تكوين الاغشية الحيوية.

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Investigation of *Ascaris lumbricoides* Egg Morphology and Transmission Dynamics in the Iraqi Population

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Abstract

Background: *Ascaris lumbricoides* remains a significant public health concern, especially in settings with poor sanitation. Prevalence, transmission dynamics, and knowledge at the population level can inform the implementation of enhanced control. This study establishes the infection rate of *A. lumbricoides* in the Iraqi population, investigates factors associated with sociodemographics contributing to infection, and explores diversity in egg morphology

Patients and Methods: A total of 110 participants were involved in this cross-sectional study. Stool samples collected from each participant were examined microscopically for the presence of *A. lumbricoides* eggs. The sociodemographic data collected include age, gender, residence, educational status, occupation, hand-washing habits, and household size, as determined through structured questionnaires. Associations of these variables with infection rates were analyzed using various statistical tools. Egg morphology- decorticated and mamillated eggs- was investigated.

Results: The overall prevalence of *A. lumbricoides* was 14.64%, with a higher rate amongst rural residents, 9.09%, compared to urban people, 4.54% ($p=0.0067$). The infection rates were also strongly inversely related to education level; the highest rate was among those with no education, 7.27% ($p<0.00001$). A low infection rate was significantly associated with washing hands before meals ($p<0.00001$). The morphology varied, with the fertilized eggs showing both mamillated and decorticated morphology, while the eggs from the unfertilized females were larger, with disorganized contents internally.

Conclusion: This study reflects that high infection burdens with *A. lumbricoides* are significantly associated with sociodemographic variables, particularly education, hygiene practices, and rural residency. The diversity observed for egg morphology underlines further complications in the parasite's transmission and adaptation to environments. Sanitation improvement and extension programs may reduce the burden of infections caused by *A. lumbricoides* in Iraq.

Keywords: *Ascaris lumbricoides*, Infection, Parasites, Education, *Ascaris eggs*.

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Introduction

Intestinal nematodes, predominantly *Ascaris lumbricoides*, afflict one-third of the global population. Infection may result in pulmonary and severe gastrointestinal manifestations; nevertheless, the majority of patients are asymptomatic. Inadequate sanitation is the primary source of *Ascaris lumbricoides* infection, which can lead to malnutrition, deficiencies in vitamins and minerals, and cognitive and developmental impairments. Mass pharmaceutical treatment programs for Ascaris and other neglected tropical diseases have increased in prevalence (1). This parasite induces a detrimental

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infection in the human gastrointestinal tract, and research indicates that it can persist within the human body for one to two years. Extra-intestinal ascariasis (EIA) is a condition resulting from the aberrant migration of *Ascaris lumbricoides*, potentially affecting the hepato-biliary-pancreatic (HBP) system or other extra-gastrointestinal (EGI) organs (2).

Ascaris lumbricoides impacts children's health and growth. Farm kids who eat raw vegetables and wander barefoot are more likely to have ascariasis. No symptoms indicated ascariasis. Clean water, sanitation, and facilities can prevent ascariasis (3). This disease is more common in poorer nations like Brazil due to poor infrastructure and sanitation. These places lack public health care, which helps the parasite spread, especially among the economically underprivileged and insecure, especially children (4).

The transmission route involves the intake of faecal waste, usually resulting from insufficient personal hygiene and sanitation standards. Additional significant elements encompass elevated temperatures and increased humidity levels. Infection arises from the oral ingestion of eggs, potentially leading to respiratory and gastrointestinal complications. Infection is contracted via the consumption of eggs, commonly found in soil or food. The mature worms inhabit the lumen of the small intestine, where the female lays eggs that are excreted with the faeces. Throughout the incubation phase, the eggs progress through three distinct developmental stages before being exposed to the external environment. When consumed by a human host, the parasite's eggs incubate in the small intestine. After a short travel, the larvae return to the small intestine to mature and mate. (5).

Female worms generate approximately 200,000 eggs. Notably, *A. lumbricoides* eggs may be unfertilised, corticated, or decorticated. Unfertilised eggs are larger than fertile ones (~90 µm), possess a thinner shell, and exhibit varied mamillated layers, with or without protuberances (6).

Utilizing wastewater in agricultural contexts may promote the spread of parasite infections. The utilization of recycled wastewater in agricultural environments seems to contaminate the soil and crops with detrimental parasites, thus elevating the likelihood of health complications for individuals who come into contact with the procedure. Utilizing wastewater for irrigation poses possible health hazards, which can be mitigated by implementing multi-barrier protective measures (7).

The interaction between children who are infected with parasites and those who are more vulnerable, whether in a home or school setting, combined with their frequent activities involving handling soil and the habit of putting dirty hands in their mouths, are factors that significantly raise the risk of infection in children aged 1 to 12 years (4). Although adults typically do not exhibit symptoms of specific parasitic diseases, possibly due to alterations in hygiene practices or acquired immunity from childhood, children are still highly vulnerable to intestinal parasitic infections since they can be exposed to these pathogens from early infancy. Children who do not have established hygiene routines are the most vulnerable category and are at a considerably higher risk of infections. The conducive settings for the proliferation of parasitic infections are mostly attributed to the high level of interaction among children and the prevalent unsanitary surroundings, often stemming from insufficient training of personnel (8, 9).

The primary clinical manifestation identified in persons diagnosed with Ascaris lumbricoides was reported abdominal discomfort. Intestinal obstruction, intussusception, cholangiohepatitis, pancreatitis, and acute appendicitis are potential

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acute abdominal consequences resulting from a severe helminthic infection (10).

Commonly utilised anthelminthic agents for preventing parasite migration in ascariasis patients include 400 milligrams of albendazole administered once daily, 100 milligrams of mebendazole taken twice daily for three days, or 500 milligrams once daily, and 11 milligrams per kilogram of pyrantel pamoate given once daily, with a recommendation for pregnant women to take up to one gramme of this medication (11).

Patients and Methods

Study design: This is a cross-sectional study that includes 110 subjects from Thi-Qar Province, Iraq, including different age groups. The study was conducted in 3 months, starting from the first of December 2023 to the end of February 2024. This study aims to explore and evaluate the genetic diversity of *Ascaris lumbricoides in the* Iraqi population and also investigate the dynamics of transmission of this common parasite.

This is a two-pronged investigation. The first is a well-structured questionnaire that includes questions regarding demographics, sanitation facilities, the presence of waste sources and waste collection, different hygiene practices, and the previous infection or symptoms of *Ascaris lumbricoides*. The second approach is the practical part, in which stool samples were collected from all participants using a noninvasive method. The samples were transported to the lab in an ice pack for examination.

Inclusion criteria: Regarding the current study, the inclusion criteria include individuals from different age groups and educational backgrounds. Participants shouldn't have severe or chronic health conditions that may affect the results.

Exclusion criteria: On the other hand, individuals who live outside the selected city and who have taken antiparasitic medication in the last six months are excluded from taking part in this study. Pregnant women and individuals with severe health conditions are also excluded. The exclusion criteria also include unwillingness and inability to complete the study procedure.

Sample examination: Direct microscopic examination was performed to identify eggs of *Ascaris lumbricoides* to estimate the presence of the eggs using the Kato-Katz technique (12). The isolation of *Ascaris lumbricoides* eggs from the samples was conducted using the flotation method, which is characterized by a clearer view and accuracy. Direct microscopic examination was done for the eggs to make morphological analysis, including shape, size, and appearance under a microscope (16). Morphological analysis is important to assess genetic diversity.

Statistical analysis

All the data obtained from the questionnaire and samples were organized and statistically analyzed using the Social Package for Social Science (SPSS) version 25.0. Evaluation of genetic diversity and transmission dynamics in Iraqi population was conducted. Moreover, Descriptive statistics will summarize demographic data, educational level, occupation and hygiene practices. Also, the F-test is used to assess the correlation between morphological variations, epidemiological factors, and parasite prevalence.

Results

Prevalence of *Ascaris lumbricoides*: Figure 1 demonstrates that the prevalence of *Ascaris lumbricoides* is 14.64%, as 15 participants tested positive for the infection, and 95 (86.36%) were found to be negative for the infection.

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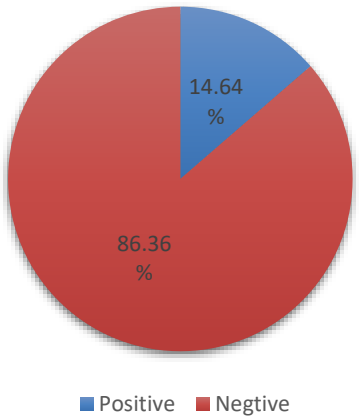

Figure 1. Prevalence of *Ascaris lumbricoides* among participate.

Sociodemographic distribution and its correlation with the prevalence of *Ascaris lumbricoides*: Table 1 provides significant insights by elucidating the correlation between sociodemographic characteristics and the prevalence of *Ascaris lumbricoides* infection. Firstly, the examination of the age groups reveals no statistically significant correlation with the prevalence of infection. This is supported by a p-value of 0.065177, somewhat higher than the conventional threshold of 0.05. Although the infection rate is most significant among those under 18 years (83%), it decreases considerably in older age groups. There are no reported cases in the 36-45 age group, and the prevalence is only 2.73% in those over 45.

Conversely, the geographic location of one's residence demonstrates a robust and statistically significant association with the occurrence of infections, as indicated by a p-value of 0.0066545. The statistics indicate that people living in rural areas have a far greater level of infection (9.09%) than those in urban areas (4.54%).

Regarding gender, the obtained p-value of 0.215548 indicates that there is no statistically significant relationship between gender and the prevalence of infection. While females exhibit a somewhat greater infection rate (9.09%) than males (4.54%), the numerical disparity is not statistically significant, suggesting that both genders are equally vulnerable to infection.

Table 1. Correlation of sociodemographic characteristics to Ascaris lumbricoides prevalence.

			prevalence				p. Value
			No		Yes		
			%	No.	%	No.	
Age	15.45%	17	c	6	23	Less than 18	0.065177
	50.91%	56	5.45%	6	62	18-35	
	11.82%	13	0%	0	13	36-45	
	8.18%	9	2.73%	3	12	More than 45	
Residence	30.91%	34	9.09%	10	39	Rural	0.0066545
	60%	66	4.54%	5	71	Urban	
Gender	43.64%	48	4.54%	5	53	Male	0.215548
	42.73%	47	9.09%	10	57	Female	
Significant difference between two independent variables at 0.05 level.							

Educational level and occupation among participants and their correlation to prevalence: The analysis of educational attainment and employment status, and their association with the occurrence of *Ascaris lumbricoides* infection, uncovers significant trends as shown in Table 2. The p-value of <0.00001 indicates a strong and statistically significant correlation between

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educational level and infection prevalence. The infection rate is highest among participants who lack formal education, with 7.27% of persons being infected. This incidence exhibits a progressive decline with higher levels of education. Within the population with primary education, the infection rate decreases to 0.91%, whereas those with secondary education exhibit a somewhat elevated rate of 5.45%. Significantly, individuals who have completed a university education or higher have not reported any instances of infection (0%).

When analyzing occupation, the p-value of 0.986607 suggests that there is no statistically significant relationship between occupation and the prevalence of infection. The prevalence rates of infection among students, employed persons, and the unemployed are reported to be 4.54%, 5.45%, and 3.64%, respectively.

Table 2. Educational level and occupation among participants and correlation to prevalence.

			prevalence				p. Value
			No		Yes		
			No.	%	No.	%	
Educational level	No formal education	14	8	7.27%	6	5.45%	<0.00001
	Primary education	20	1	0.91%	19	17.27%	
	Secondary education	28	6	5.45%	22	20%	
	University or higher	486	0	0%	48	43.64%	
occupation	Student	35	5	4.54%	30	27.27%	0.986607
	employed	46	6	5.45%	40	36.36%	
	unemployed	29	4	3.64%	25	22.73%	
Significant difference between two independent variables at 0.05 level.							

Habits of washing hands, the number of people in the house, and sanitation are correlated with the prevalence of *Ascaris lumbricoides*: A statistically significant correlation is found between handwashing practices and infection rates, with a p-value of less than 0.00001 for both handwashing after using the toilet and handwashing before and after meals. Individuals who regularly followed hand hygiene practices after using the toilet did not have any documented infections, whereas those who only occasionally or seldom washed their hands had higher infection rates of 2.73% and 9.09%, respectively. In a similar vein, individuals who consistently engage in handwashing before and after meals also did not experience any infections. Conversely, those who adhered to this
practice only occasionally or seldom had infection rates of 3.64% and 6.36%. Regarding sanitation, the statistics indicate a little rise in the occurrence of infections among individuals who use outdoor toilets as opposed to indoor toilets. However, this finding lacks statistical significance, as evidenced by a p-value of 0.508644. The statistical analysis reveals a significant correlation between household size and infection prevalence, as indicated by a p-value of 0.002157. Higher infection rates were observed in larger households, especially those with more than six members, at 11.82%, compared to smaller households, where infection rates were significantly lower or absent, as shown in Table 3.

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Table 3. Correlation between hand washing habits, sanitation and number of people living in home and prevalence of Ascaris lumbricoides.

			prevalence				p. Value
			Yes		No		
			No.	%	No.	%	
Washing hands after the toilet	Always	67	0	0%	67	60.91%	<0.00001
	Sometimes	28	3	2.73%	25	22.73%	
	Rarely	13	10	9.09%	3	2.73%	
	Never	2	2	1.82%	0	0%	
Washing hands before and after meals	Always	67	0	0%	47	42.73%	<0.00001
	Sometimes	29	4	3.64%	2	1.82%	
	Rarely	10	7	6.36%	3	2.73%	
	Never	4	4	3.64%	0	0%	
sanitation	Indoor toilet	84	13	11.82%	71	64.55%	0.508644
	Outdoor toilet	26	2	1.82%	24	21.82%	
	No toilet	2	0	0%	2	1.82%	
Number of people living in house	1-3	18	0	0%	18	16.36%	0.002157
	4-6	44	2	1.82%	42	38.18%	
	More than 6	51	13	11.82%	38	34.55%	
Significant difference between two independent variables at 0.05 level.							

Source of drinking water, regular waste collection and presence of source of contamination and correlation with *Ascaris lumbercoides* prevalence: The statistical analysis reveals a significant correlation between the source of drinking water and the prevalence of infection, as indicated by a p-value of 0.0196869 as elucidated in Table 4. The infection rate was highest (9.09%) among those who depend on tap water, but significantly lower infection rates of 2.73% and 1.82% were observed among those who use well water and bottled water, respectively. There is a significant association between regular waste collection and infection prevalence, as shown by a p-value of <0.00001. Among regions that implement routine waste collection, the infection rate is notably low at 0.91%, compared to a significantly higher rate of 12.73% in regions lacking regular waste collection. Furthermore, the existence of a contamination source, such as adjacent sewage or stagnant water, is strongly linked to a greater occurrence of infection, as indicated by a p-value of 0.00182. Within regions where a source of pollution exists, the prevalence of infection is 9.09%, compared to 4.54% in regions without such contamination.

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Table 4. Source of drinking water, regular waste collection, and sources of contamination percentages, and correlation to Ascaris lumbricoides prevalence.

			prevalence				p. Value
			No		Yes		
			No.	%	No.	%	
Drinking water source	Tap water	50	10	9.09%	40	36.36%	0.0196869
	Bottled water	29	2	1.82%	27	24.55%	
	Well water	31	3	2.73%	28	25.45%	
Regular waste collection	Yes	76	1	0.91%	75	68.18%	<0.00001
	No	34	14	12.73%	20	18.18%	
Presence of source of contamination	Yes	35	10	9.09%	25	22.73%	0.00182
	No	75	5	4.54%	70	63.64%	
Significant difference between two independent variables at 0.05 level.							

History and symptoms associated with *Ascaris lumbricoides* infection: The analysis of the data investigating the association between familial infection history, prior parasite treatment, symptomatology, and *Ascaris lumbricoides* infection uncovers several significant connections as shown in Table 5.

A p-value of 0.00021 indicates a robust correlation between a family history of prior infections and the prevalence of Ascaris lumbricoides. Within the group of participants who had a familial background of infection, 9 individuals (8.18%) were indeed infected, but 16 individuals (14.55%) were not. Conversely, the infection rate among individuals without a familial background was comparatively lower, at 5.45%. These findings suggest that a familial background of parasitic infection may be a predisposing factor, possibly resulting from common environmental circumstances or individual genetic vulnerability.

The analysis of prior medication usage for parasites does not exhibit a statistically significant association with infection, as indicated by a p-value of 0.258298. Although there is a greater infection rate (10.91%) among individuals who had previously used medication for parasites, this correlation is not sufficiently robust to establish a definitive protective or risk factor. This implies that re-infection may still occur even after previous treatments, or that access to treatment does not entirely prevent future infections.

Symptoms exhibit a strong and statistically significant association with the prevalence of infection, as indicated by a p-value of <0.00001. The infection rate among asymptomatic individuals was remarkably low at 0.91%, whereas those who reported symptoms, such as fatigue (2.73%), diarrhea (3.64%), and abdominal pain (6.36%), had relatively higher rates of infection.

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Table 5. Source of drinking water, regular waste collection, and sources of contamination percentages, and correlation to Ascaris lumbricoides prevalence.

			prevalence				p. Value
			No		Yes		
			No.	%	No.	%	
Previous infection in family	Yes	25	9	8.18%	16	14.55%	0.00021
	No	85	6	5.45%	79	71.82%	
Previous medication of parasites	Yes	74	12	10.91%	62	56.36%	0.258298
	No	36	3	2.73%	33	30%	
symptoms	No symptoms	69	1	0.91%	68	61.82%	<0.00001
	Fatigue	14	3	2.73%	11	10%	
	Diarrhea	7	4	3.64%	3	2.73%	
	Abdominal pain	20	7	6.36%	13	11.82%	
Significant difference between two independent variables at 0.05 level.							

Diversity of egg morphology of *Ascaris lumbricoides* among participants: Figure 2, A and B show a fertilized *Ascaris lumbricoides* egg without the outer mammillated coat. The egg is smooth, with a round to oval shape. The loss of the outer layer may be an environmental phenomenon or may have occurred during handling. The lack of a mammillated coat does not preclude the presence of a thick chitinous layer, and the embryo remains well-preserved. The size ranged from 45-60 mm for Figure 2A, where in Figure 2B, it is larger, approximately 50-70 mm long. Where Figure 2C shows the typical fertilized *Ascaris lumbricoides* egg, which is oval to round, and about 60-70 micrometers long. The outer protein layer of the egg is well-defined and mammillated, which is characteristic of freshly excreted eggs. The shell, comprising an outer protein layer, a chitinous middle layer, and an innermost lipid layer, provides resilience in various environmental conditions.

Figure 2D represents an unfertilized egg that has lost the outer mammillated layer, leaving it smooth and much thinner. The internal contents of the egg are disorganized, with granular material filling the egg. This would be consistent with an unfertilized egg. This egg is larger than the usual fertilized eggs due to a lack of embryonic development, and it has a thinner, less developed shell. The size range observed is 60-80 mm long. All the eggs showed viability in size, fertilization, and being mamillated or decorticated. Sixty percent of the eggs were decorticated, while forty percent were milled.

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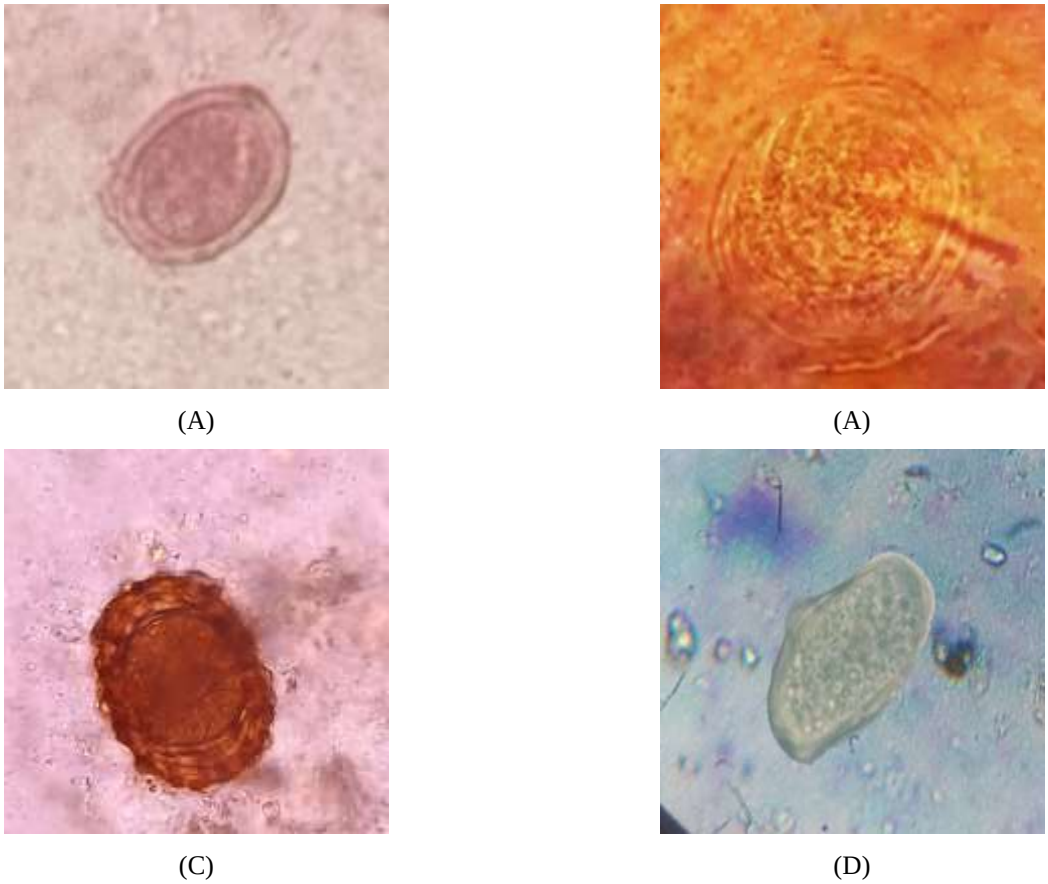

(A) (A)

(C) (D)

Figure 2. The eggs of Ascaris lumbricoides from participants, fertilized decorticated (A), fertilized decorticated (B), fertilized mamillated (C), and unfertilized decorticated (D).

Discussion

The present study indicated the prevalence of *Ascaris lumbricoides* at 14.64%. A study at Al-Mustaqbal University College in Iraq showed a marginally higher prevalence of 18.48% (13). A global meta-analysis determined the overall prevalence of A. lumbricoides to be 11.01%, exhibiting considerable regional disparities.

The current study deviates from this trend, indicating the highest prevalence among individuals aged 18-35. This contrasts with findings from Venezuela, where *A. Lumbricoides infections were markedly lower in all age groups above 16 than in* children under 5 years old (14). The disparity in prevalence among age groups may be attributed to variations in public health strategies, especially mass drug administration (MDA) programs.

This study indicates that the prevalence is greater in females than males, although no significant correlation was identified (15). A survey in District Karak, Pakistan, confirmed infection prevalence was higher in females than males, with rates of 53% for females and 45% for males (16). While in Venezuela, the prevalence was markedly lower in females than males (14). Conversely, the results from the United States and earlier studies indicated no notable gender-based disparities (17). The disparities in gender-related prevalence may be affected by regional behavioral factors, occupational exposure, or cultural practices that differentially impact sanitation and hygiene for males and females.

The results of the present study indicate a significantly higher prevalence of *A. lumbricoides* infection in rural regions compared to urban areas. (14). A recent study found that 69% of the infected

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individuals resided in rural areas, whereas only 31% were from urban regions (16). The increased incidence in rural areas is likely attributable to various factors, particularly the use of night soil (human excrement) as fertilizer, a prevalent practice among farmers in certain regions (18). The current study revealed that the highest prevalence of A. lumbricoides infection occurred among participants lacking formal education. These results align with a study conducted in Honduran communities, which demonstrated a significant correlation between education and *A. lumbricoides* infection (p = 0.01) (19).

Conversely, occupation did not exhibit a significant correlation with prevalence in the present study. This does not correspond with prior research indicating a substantial correlation between *A. lumbericoides* infection and occupation, especially among individuals engaged in agricultural labor (20). A robust correlation has been documented between the prevalence of *A. lumbricoides* and occupation in previous studies, with food handlers and farmers exhibiting the highest infection rates (21). The notable correlation between *A. lumbricoides* prevalence and occupation observed in this study may be ascribed to the substantial population of rural workers engaged in agriculture, thereby heightening their exposure to contaminated soil and inadequate sanitation practices. The present study revealed a significant correlation between handwashing practices and household size with Ascaris lumbricoides infection, while sanitation showed no correlation. Previous studies, including in Sri Lanka, have noted a greater prevalence among children who failed to wash their hands before meals (41.5%) or following defecation (52.6%) (22). An additional study in Indonesia identified substantial correlations between handwashing before eating (p = 0.004), post-eating (p = 0.027), and following defecation (p = 0.04) and intestinal parasitic infections (23).

The present study revealed a significant correlation between household size and the risk of Ascaris lumbricoides infection. These results align with prior research. In Brazil, residents of densely populated households were more susceptible to severe Ascaris infection than those living in less crowded environments (24). A recent study in Ethiopia found that children from households with two or more children under five years of age had a higher likelihood of infection compared to those from households with fewer children (25). A positive correlation was identified in Nigeria between the *Ascaris lumbricoides* parasite load and family size (26), drinking water source, waste disposal practices, and proximity to pollution sources, which were significantly associated with Ascaris prevalence in the present study.

In Iraq, the incidence of ascariasis was demonstrated to rise with unsafe drinking water, and contamination sources near residential areas were identified as significant factors in the proliferation of *Ascaris lumbricoides* infections in Iraq (27, 28). The findings emphasize the importance of environmental hygiene in transmitting *Ascaris lumbricoides* and other parasitic infections. Efficient sanitation and access to clean water are crucial in mitigating the risk of infections in impacted areas. This study emphasizes the influence of familial *Ascaris lumbricoides* infections, indicating that individuals with a family history of *Ascaris* exhibit increased susceptibility to the infection. This corresponds with earlier research, which identified the familial history of *Ascaris* as a risk factor for infection prevalence (29). Abdominal pain, diarrhea, and fatigue were the most frequently reported symptoms in infected participants (30, 31). Regarding egg morphology, 40% of the eggs in this study exhibited mammillation, whereas 60% were decorticated. The findings align with another study, which reported that 56.3% of eggs exhibited mammillation (16).

The variation may be attributed to environmental

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exposure of eggs, genetic differences among *A. lumbricoides* populations, and varying egg size, which may indicate genetic diversity in parasite development. The lack of embryonic development or reduced shell thickness may result in larger eggs. Decorticated and mamillated eggs exhibit prolonged viability, demonstrating the parasite's adaptability. Morphological variations may be associated with genetic factors influencing parasite transmission and survival across diverse environments (32, 33). This capacity enables the parasite to survive and continue the parasite life cycle.

Conclusions

The present study has consequently highlighted the prevalence, sociodemographic distribution, and some key environmental factors associated with the infection of *Ascaris lumbricoides* among the population in Iraq. The parasitic infection rate in this study suggests that this infectious disease is still a public health problem. Sociodemographic variables such as rural residence, lack of formal education, and poor hygiene practices, particularly handwashing, are significantly associated with infection rates. Analysis also underlines the main determinants of transmission: household size, source of drinking water, and waste collection. Besides, there is genetic variability in egg morphology in decorticated and mamillated varieties. This paper highlights that sanitation, education, and the delivery of public health initiatives are crucial in reducing the transmission of infections. It was recommended that further studies are needed to understand the morphology of eggs transmitted from humans to distinguish the ways of transmission and the environmental factors involved in the exposure to the parasite.

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دراسة مورفولوجيا بيض دودة الاسكارس اللومينية وديناميكيات انتقالها في السكان العراقيين

[1] محمد جاسم شاكر

الملخص

الخلفية: دودة ***Ascaris lumbercoides*** تشكل مصدر قلق كبير للصحة العامة، وخاصة في البيئات ذات الصرف الصحي السيئ. وفي هذا المجال، يمكن أن تشكل معدلات الانتشار وديناميكيات الانتقال والمعروفة على مستوى السكان أساسًا لتطبيق تدابير للمكافحة المعززة.

الأهداف: تهدف هذه الدراسة إلى تحديد معدل الإصابة بدودة ***Ascaris lumbercoides***بين سكان العراق، ودراسة العوامل المرتبطة بالظروف الاجتماعية التي تساهم في الإصابة، واستكشاف التنوع في اشكال البيض.

المرضى والطرق: شارك في هذه الدراسة ما مجموعه ١١٠ مشاركًا. تم أخذ عينات البراز التي تم جمعها من كل مشارك للفحص المجهري بحثًا عن بيض دودة ***Ascaris lumbercoides***. تشمل البيانات الاجتماعية التي تم جمعها العمر والجنس والإقامة والحالة التعليمية والمهنة وعادات غسل اليدين وحجم الأسرة من خلال استبيانات منظمة. تم تحليل ارتباطات هذه المتغيرات بمعدلات الإصابة باستخدام تحاليل إحصائية مختلفة. تم دراسة في اشكال البيض - البيض المقشر والمخصب.

النتائج: كان معدل انتشار A. lumbricoides الإجمالي ١٤,٦٤٪، مع معدل أعلى بين سكان الريف، ٩,٠٩٪، مقارنة بسكان المدينة، ٤,٥٤٪ (ص = ٠,٠٠٦٧). كانت معدلات الإصابة مرتبطة عكسيا بمستوى التعليم؛ كان أعلى معدل بين أولئك الذين ليس لديهم تعليم، ٧,٢٧٪ ($p<0.00001$). وجد أن معدل الإصابة المنخفض مرتبط بشكل كبير بغسل اليدين قبل الوجبات ($p<0.00001$). اختلفت الاشكال، حيث أظهرت البيض المخصبة اشكالا مخاطية ومقشرة، في حين كانت بيض الإناث غير المخصبة أكبر، مع محتويات غير منظمة داخليًا.

الاستنتاج: تعكس هذه الدراسة أن الأعباء العالية للإصابة بـ A. lumbricoides مرتبطة بشكل كبير بالمتغيرات الاجتماعية والديموغرافية، وخاصة التعليم وممارسات النظافة والإقامة الريفية. إن التنوع الملحوظ في اشكال البيض يؤكد على المزيد من التعقيدات في انتقال الطفيليات وتكيفها مع البيئات. إن برامج تحسين الصرف الصحي والإرشاد قد تقلل من عبء العدوى التي تسببها ***Ascaris lumbercoides***في العراق.

الكلمات المفتاحية: ***Ascaris lumbricoids***، العدوى، الطفيليات، التعليم، بيض الاسكارس.

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Estimation of miRNA-21, miRNA-422, miRNA-142-3p, and miR-181c Gene Expression as Potential Biomarkers of Osteoporosis in Premenopausal Women

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Abstract

Background: MicroRNAs (miRNAs) regulate several biological pathways in osteoporosis patients. The study aimed to estimate the correlation of miRNA-21, miRNA-422, miRNA-142-3p, and miR-181c with osteoporosis in premenopausal women.

Patients and Methods: This study was performed on 60 osteoporosis premenopausal and 30 healthy women. Determination of osteocalcin (OC), deoxypyridinoline (DPD), and bone-specific alkaline phosphatase (BAP) were done by ELISA, and estimation of miRNA gene expression was done by using qRT-PCR.

Results: OC, DPD, and BAP levels indicated a significant decrease in osteoporosis women. In addition, our data showed that miRNA-21 (p=0.0001, r=-0.5585) and miRNA-422 (p=0.0035, r=-0.3715) have a high expression and negative correlation with BMD. Meanwhile, the miRNA-142-3p (p=0.0136, r=0.3089) and miRNA-181c (p=0.0401, r=-0.2685) have decreased expression and positive correlation with BMD value.

Conclusion: The results of this study indicated a clear association of miRNA-21 and miRNA-422 in osteoporosis thus they may be useful as biomarkers for osteoporosis.

Keywords: Osteoporosis, miRNA-21, miRNA-422, miRNA-142-3p, and miR-181c.

Introduction

Osteoporosis is a systemic disease characterized by bone loss, resulting in deterioration of bone tissue and low bone mineral density (BMD). Thus, the risk of fragility-related fractures increases in women and worsens with age (1). Osteoporosis prevalence is estimated at 23.1% for women and around 11.7% for men (2). Osteoporosis leads to severe complications such as fractures. It was projected that by 2050, the worldwide incidence of hip fracture in women would increase by 240% (3). Osteoporosis can be classified into two types: primary osteoporosis, which is related to menopause and aging, and secondary osteoporosis, which is caused by medical conditions or medications (4). It affects a significant portion of the global human population, with a prevalence rate of 18.3%. women are particularly impacted, experiencing a prevalence of 23.1%. Furthermore, Africa has the highest

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prevalence at an alarming 39.5% (2).

Premenopausal osteoporosis can be attributed to various factors, for example, anorexia nervosa, hyperprolactinemia, and drug-induced amenorrhea resulting from GnRH analog (5). chronic inflammation, lack of physical activity, and insufficient sex steroids (6–7). Genetic conditions, such as osteogenesis imperfecta, can predispose individuals to primary bone fragility disorders (6). Idiopathic osteoporosis is one of the conditions that can affect premenopausal women (7).

Biochemical bone markers, such as osteocalcin (OC), deoxypyridinoline (DPD), and bone-specific alkaline phosphatase (BAP), are increasingly recognized for their critical role as osteoporosis biomarkers (8). Genome-wide association studies (GWAS) have identified numerous susceptibility loci related to osteoporosis and bone mineral density (BMD). BMD and osteoporosis share many common susceptibility loci. The corresponding susceptibility genes are significantly enriched in biological pathways associated with bone health (9).

In silico approaches for small non-coding RNAs, particularly microRNAs, are crucial for predicting their interactions with target molecules and regulatory functions. Therefore, researchers can gain vital insights into the binding mechanisms and biological roles of microRNAs, thereby enhancing our understanding of their impact on post-transcriptional regulation and advancing this important field (10). Research shows that epigenetic changes may link genetic factors and environmental influences, increasing the risk of osteoporosis. Among these changes, certain RNA types, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), play crucial roles in regulating gene expression and are essential to bone metabolism (11). MicroRNAs play a crucial role in osteoporosis by influencing bone metabolism, particularly bone resorption and synthesis, which leads to an understanding of the disease and potential therapies (12). Osteoporosis is a complex disease influenced by multifactorial risk, and its underlying causes can differ based on factors such as race, geographic location, and age. Therefore, this study aimed to estimate the correlation among miRNA-21, miRNA-422, miRNA-142-3p, and miR-181c with osteoporosis in a sample of premenopausal Iraqi women.

Patients and Methods

Selection of the study population: The present study included 60 women with osteoporosis, aged 19 to 50 years, and 30 matched-age healthy women who served as a control group. All subjects were selected from the Tikrit/Iraq population. The inclusion criteria focused on women with osteoporosis who had not yet reached menopause. In contrast, the exclusion criteria ruled out women who exhibited clinical signs of poor general health unrelated to osteoporosis. Bone mineral density (BMD) was assessed using a dual-energy X-ray absorptiometry (DEXA) device. A T score of -2.5 or lower indicates the presence of osteoporosis.

Analysis of bone biomarkers: Venous blood samples were drawn from patients and controls in the morning after a 8- to 12-hour fast. To separate the serum from the blood components, the samples were centrifuged at 3000 RPM for 15 minutes. Once the serum was separated, it was divided into three smaller replicates and stored at -80°C to preserve its integrity for biochemical analysis. Finally, Serum levels of OC, DPD, and BAP were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sunlong, China).

Quantitative real-time PCR (qRT-PCR): In this study, to evaluate the expression levels of microRNA (miRNA) using quantitative qPCR, RNA was first extracted from blood samples

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collected from both patient and healthy groups (QIAGEN, Hilden, Germany). The extracted RNA molecules were converted into complementary DNA (cDNA). For the quantitative RT-PCR, we utilized an ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA) along with SYBR Green QPCR Master Mix, which ensured accurate and sensitive detection of the RNA molecules. The primer sequences used in the current study are listed in Table 1. The $2^{-\Delta\Delta CT}$ method was used to estimate relative expression (13).

Table 1. primer sequences of miRNA genes**.**

Genes	Nucleotides sequence		Source
miRNA-21-5p	F	5′-AACACGCTAGCTTATCAGACTGATG-3′	Current study
miRNA-422a-5p	F	5′-AACACGCACTGGACTTAGGGT -3	
miR-142-3p	F	5′-AACACGCTGTAGTGTTTCCTACTTT-3′	
Universal	R	5′-CAGTGCAGGGTCCGAGGT-3′	
miR-181c	F	5′-GTGTGGGAACATTCAACCTGTCGGTG-3′	
	R	5′-CCAGTCTCAGGGTCCGAGGTATTC-3′	
U6	F	GTGCTCGCTTCGGCAGCA	
	R	CAAAATATGGAACGCTTC	

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 software. A Student's T-test was strategically employed to reveal significant associations between crucial clinical and pathological osteoporosis-related factors. Furthermore, the Receiver Operating Characteristic (ROC) test was implemented to accurately establish osteoporosis markers risk. An in-depth investigation using Pearson correlation analysis shed light on the correlation between miRNA and BMD degree, as the correlation coefficient (r) effectively measures these associations. A probability value of less than 0.05 was applied to signify the presence of statistically significant differences, reinforcing the validity of our findings.

Results

Demographic data: The mean age of osteoporosis women is 43.45 years, with a standard deviation of 7.303 years. The mean BMI is 30.92 kg/m2, with a standard deviation of 2.96. Their average Body Mass Index (BMI) stands at 30.92 kg/m², accompanied by a standard deviation of 2.96, emphasizing the association between weight and bone health. Furthermore, the mean bone mineral density (BMD) is -2.043 g/cm², with a standard deviation of 0.9241 g/cm², underscoring the critical need for early detection in this demographic age to promote better health outcomes. Table 2 presents more details about demographic data.

Table (2). The mean and standard deviation of the demographic in patients and control.

Categories	Osteoporosis group		Control group	
	Number	Mean ± SD	Number	Mean ± SD
Age (years)	60	43.45 ± 7.303	30	41.93± 6.664
BMI (kg/m^2)	60	30.92 ± 2.96	30	32.56± 3.56
Normal weight	32	22.8 ± 4.28	15	19.21± 7.11
Overweight	20	28.29 ± 2.53	11	26.81± 5.23
Obesity	8	34.13 ± 2. 5	4	35.12± 8.45
BMD	60	-2.043 ± 0.9241		

Biochemical profile: The osteoporosis group's mean ± standard deviation osteocalcin is 21.81± 2.093 ng/ml, while 13.51± 3.269ng/ml in the control group with p-value = 0.0001 (highly

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significant). The mean± standard of DPD was 24.68± 6.143 nmol/l in the osteoporosis group and 22.16± 3.517 nmol/ in healthy women with p-value = 0.0405 (significant). The osteoporosis group's mean ± SD BAP is 37.67± 9.223 U/L, while 33.37± 6.906 U/L in the control group with p-value = 0.0373 (significant). Table 3 shows all the details of biochemical markers.

Table 3. The mean, standard deviation (SD), and p-value of biochemical data in patients and control.

Categories	Osteoporosis		Control		P value
	Median	Mean ± SD	Median	Mean ± SD	
Osteocalcin (ng/ml)	21.70	21.81± 2.093	14.48	13.51 ±3.269	0.0001
DPD (nmol/l)	27.39	24.68± 6.143	24.83	22.16 ±3.517	0.0405
BAP (U/L)	36.38	37.67± 9.223	40.56	33.37 ±6.906	0.0373

miRNA expression: According to the Figure 1A, the osteoporosis group exhibits a considerably greater Mean ± SD of miRNA-121 expression than the control group (1.435±0.5429 vs 0.6415±0.3246, p value= 0.0001). The osteoporosis group's median value is higher than the control group's (1.956 vs0.7849). The ROC curve shows an AUC of 0.8539, indicating a high diagnostic accuracy of miRNA-121. Thus, miRNA-121 may successfully discriminate against osteoporosis cases. There is 76.67% sensitivity and 86.66% specificity. It can therefore detect about 76.67% of osteoporosis cases and 86.66% of healthy cases by miRNA-121 expression (Figure 1B).

Figure 1. (A) Box plot of the Relative miRNA-21 expression, T-test, significant at $p \leq 0.05$, **(B)** ROC curve of the Relative miRNA-21 expression, significant at $p \leq 0.05$.

As can be observed in the Figure 2A, the osteoporosis group has a significantly higher miRNA-422 expression than the control group (Mean ± SD=1.767± 0.5599 vs 1.034± 0.2996, p value= 0.0001). Furthermore, the median of the osteoporosis group is higher than that of the control group (2.136 vs 1.270). According to ROC curve analysis in Figure 2B, the miRNA-422 has a strong diagnostic accuracy, as indicated by its area under the curve (AUC) of 0.9033. Therefore, miRNA-422 can correctly detect the majority of osteoporosis cases and exclude the healthiest individuals, with a sensitivity of 73.33% and a specificity of 93.33%.

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Figure 2. (A) Box plot of the Relative miRNA-422 expression. T-test, significant at $p \leq 0.05$, **(B)** ROC curve of the Relative miRNA-422 expression, significant at $p \leq 0.05$.

The relative expression of miRNA-142-3p in the osteoporosis group appeared to be considerably lower (1.824±0.5680) than that in the healthy group (3.712±0.8056), with a p-value of less than 0.0001, indicating a statistically significant difference, as shown in Figure 3A. The ROC Curve for the expression of relative miRNA-142-3p is illustrated in Figure 3B. The AUC of 0.9711 indicates excellent discrimination between the two groups. Sensitivity (88.33%) refers to the percentage of all volunteer women who have been correctly diagnosed with osteoporosis.

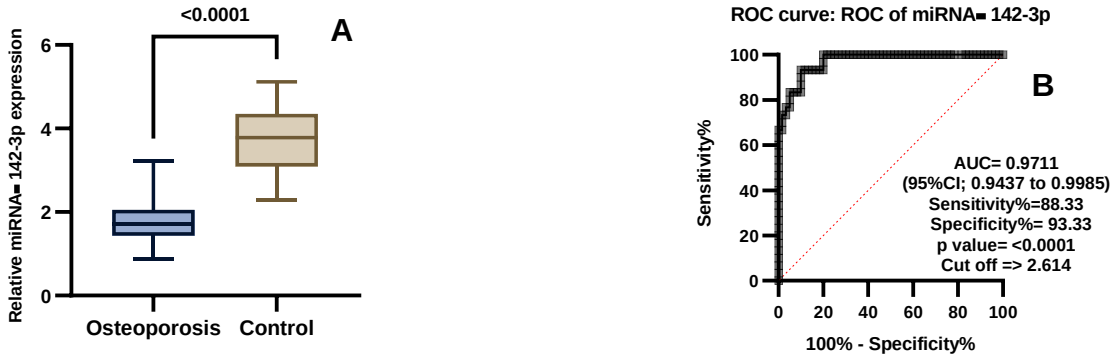

Figure 3. (A) Box plot of the Relative miRNA-142-3P expression, T-test, significant at $p \leq 0.05$, **(B)** ROC curve of the Relative miRNA-142-3P expression, significant at $p \leq 0.05$.

At 1.396, the median expression of miRNA-181C level for the osteoporosis women is lower than that of the healthy women at 4.091. Compared to the healthy women, the osteoporosis group's gene expression of miRNA-181C seems to be significantly lower (1.589± 0.6601 vs 4.100± 1.160, p value= 0.0001) as seen in Figure 4A. Figure 4B displays the ROC curve analysis of two study groups, as indicated by the AUC of 0.9728. Sensitivity at 88.33% is the percentage of osteoporosis women who had their condition correctly identified out of all those patients with osteoporosis. Specificity at 96.66% is the percentage of healthy controls that were accurately recognized out of all healthy volunteers. As well as for sensitivity and specificity, the AUC results indicate that miRNA-181C gene expression may be an excellent biomarker for differentiating between healthy and osteoporosis women.

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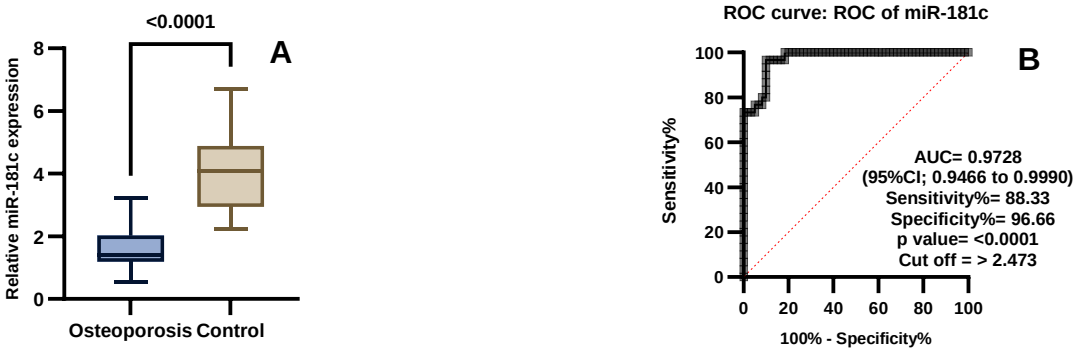
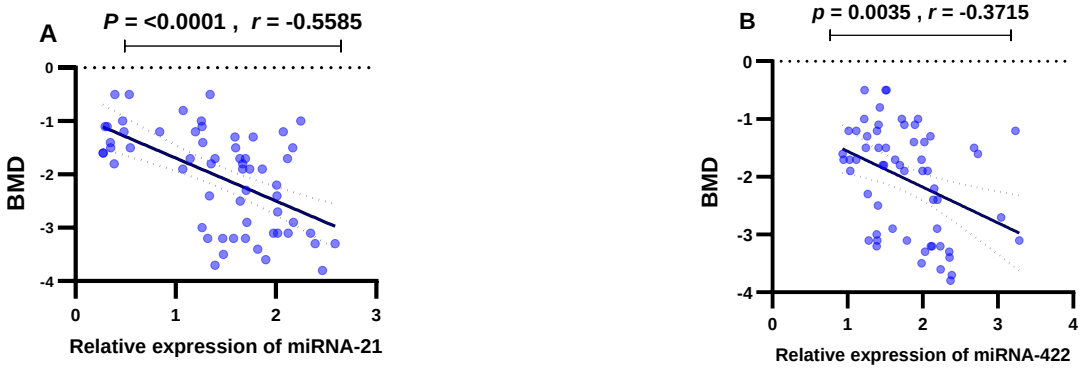


Figure 4. (A) Box plot of the Relative miRNA-181C expression, T-test, significant at $p \leq 0.05$. **(B)** ROC curve of the Relative miRNA-181C expression, significant at $p \leq 0.05$.

The correlation between bone mineral density (BMD) and the gene expression of four types of microRNAs (miRNAs) miRNA-21, miRNA-422, miRNA-142-3p, and miRNA-181c is shown in scatter plots with regression lines and statistical data in Figure 5.

Figure 5A (miRNA-21) shows a strong negative correlation (r = -0.5585) with high statistical significance ($p < 0.0001$). This implies that higher gene expression of miRNA-21 is correlated with lower BMD value in osteoporosis women. On the other hand, a moderately negative correlation (r = -0.3715) and statistical significance (p = 0.0035) are seen in Figure 5B (miRNA-422). This means that although the correlation between increased gene expression of miRNA-422 and lower value of BMD is smaller than that of miRNA-21, it still exists in osteoporosis women.

The results of the correlational analysis are presented in Figure 5C and show a weak positive correlation (r = 0.3089) with statistical significance (p = 0.0163) between miRNA-142-3p gene expression and BMD. Although the correlation is weak, higher expression of miRNA-142-3p is correlated to higher value of BMD. From Figure 5D, it can be seen that a weak positive correlation (r = 0.2658) and statistical significance (p = 0.0401) are observed for miRNA-181c gene expression with BMD value. This implies that there is a weak association between BMD value and miRNA-181c gene expression in women with osteoporosis.



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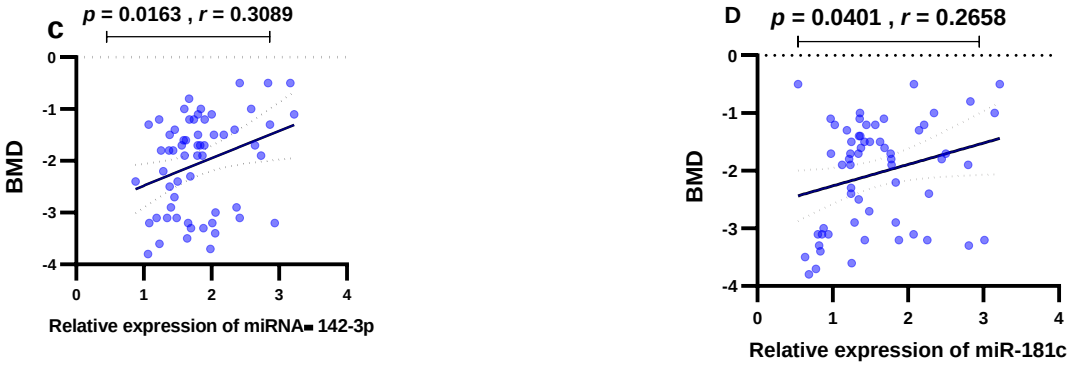

Figure 5. Correlation between BMD and relative expression of the following: (A) miRNA-21, (B) miRNA-422 (C) miRNA-142-3P and (D) miRNA-181C , significant at $p \leq 0.05$.

Discussion

Osteoporosis is typically common in postmenopausal women with low estrogen levels decreases dramatically with progress age and bone mineral density (BMD) typically decreases. There is very important in initiating discussions and encouraging people to take proactive steps to increase bone mass and lower fracture risk far earlier in life, especially when bones are still growing (14). Numerous biochemical markers have been confirmed to help evaluate bone metabolism, bone loss, and osteoporosis. These include deoxypyridinoline (DPD), which is associated with the process of bone resorption, and osteocalcin (OC) and bone-specific alkaline phosphatase (BAP), which is correlated with bone growth (15, 16, 17, 18, 19, 20). However, the specificity of these indicators can differ and cannot fully represent bone metabolism. Therefore, more research is needed to standardize them (20, 21).The diagnosis of osteoporosis, the individual prognosis of bone loss, fracture, or the choice of pharmacological treatment are areas in which bone turnover markers are not helpful. In clinical trials, the turnover markers have helped clarify the pharmacodynamics and efficacy of osteoporosis medicines. Bone turnover markers could help track the stages of osteoporosis treatments as an alternative to BMD testing (23). The common cause of low bone mineral density (BMD) is increased bone resorption by osteoclasts, as opposed to bone synthesis, which is carried out by osteoblasts (24). Therefore, Bone mineral density (BMD) is an established and active measure to diagnose osteoporosis in patients (25-27). MicroRNAs (miRNAs) have grown in significance for the research of osteoporosis pathogenesis because they are the main regulators of gene expression and can alter processes associated with bone homeostasis (27-30). This study's results observed an increase in miRNA-21 expression and miRNA-422 expression in women with osteoporosis compared to healthy women. Some studies reported higher expression of miR-21 in osteoporotic fractures (31), whereas others noted a decrease in miR-21 expression (32). Some authors found exogenous miR 21 has demonstrated the ability to speed up the formation of new bone, indicating that it may find use in osteoporosis bone regeneration therapy (33). On the other hand, increased fragility fracture risk is linked to upregulation of miR-21, suggesting that miR-21 may be used as a biomarker to predict fractures caused by osteoporosis (34). Also, in previous meta-analysis which included and examined 27 trials with 2,263 individuals in total. According to the findings, miR-21-5p was significantly upregulated (WMD 0.88, 95% CI: 0.22 to 1.55) and may be a useful biomarker for

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osteoporosis diagnosis (35). Previous studies found a negative correlation between miR-422a levels and T- and Z-scores in patients with osteoporosis. These results indicate that miR-422a contributes to hBMSC adipogenesis through downregulating MeCP2 and that the loss of bone density in primary osteoporosis is associated with its circulating levels (36). MiR-422a was significantly upregulated in the low BMD group compared to the high BMD group. Nevertheless, it is still unknown how miR-422 a lowers BMD (37,38). Both miRNA-21 and miRNA-422 a showed significant inverse correlations with the BMD-lumbar spine, and miRNA-21 and miRNA-422 at showed inverse correlation with the BMD-femoral neck (38).

According to the current study, women with osteoporosis have lower levels of miRNA-142-3P and miRNA-181C than women in good health. A comparison of the findings with those of other studies confirms that miRNA-142-3P was significantly downregulated in osteoporosis and showed a strong correlation with bone mineral density (15,39). miR-142-3p inhibits Human periodontal ligament stem cells (hPDLSC) osteogenic development by downregulating SGK1 expression (40). A previous study indicated that miR-142-3p gene expression was down-regulated in osteoprotegerin through animal experiments and analysis of blood miRNA validation in osteoporosis cases (41). On other hand, bone Marrow-Derived Mesenchymal Stem Cells' miR-142-5p Inhibits Cell Migration and Targets the Adhesion Molecule VCAM-1 to Promote Osteoporosis (42). MiR-181c-5p has an important role in bone metabolism and is associated with progressive bone loss in osteoporosis patients (43). On other hand, the MiR-181c-5p inhibits Foxo1 from adversely controlling the osteogenic development of bone marrow mesenchymal stem cells (BMMSCs) in osteoporosis (44). MiR-181c-5p has a crucial role in bone formation and mineralization by upregulating Runx2 expression and downregulating Notch2 to improve osteogenic differentiation and mineralization of osteoblastic cells generated from the human jawbone (45). MiR-181c-5p contributes to bone loss by encouraging cell cycle arrest, mainly through the downregulation of cyclin B1 expression (46).

These data suggest that miRNA-21 and miRNA-422 may play a role in bone mineral density, with higher gene expression leading to lower BMD. The correlation with miRNA-142-3p is less clear and may require further research. The expression of miRNA-181c does not appear to be significantly correlated with BMD value. When the correlation value is moderate to weak, it indicates that other factors may also contribute to BMD value. It is important to remember that correlation does not always imply causation. In addition, according to the available data on miRDB, there are 469 predicted target genes for hsa-miR-21-5p, 418 predicted for hsa-miR-142-3p, and 1409 predicted for hsa-miR-181c-5p. This indicates the multiple functions expected for each of the study miRNA genes. Although there have been global studies on the role of miRNA-21, miRNA-422, miRNA-142-3p, and miR-181c genes in patients with postmenopausal osteoporosis, this is the first study to investigate these genes in premenopausal osteoporosis patients. It is the first study conducted on osteoporosis patients in the Iraqi population. The findings of this study have numerous essential implications for future practice, particularly in the development of early diagnosis methods and the treatment of osteoporosis.

Conclusions

The present study aimed to investigate the expression of miRNA in osteoporosis. This study has identified increased expression of miRNA-21 and miR-422a, along with decreased gene expression of hsa-miR-

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142-3p and hsa-miR-181c-5p, in women withosteoporosis. The evidence from this study suggests that miRNA plays a role in determining bone mineral density (BMD) values. Although this current study is limited by a relatively small sample from one ethnic group, our data may be considered possible markers for diagnosis and future therapy for osteoporosis. Additionally, it is recommended to evaluate the role of microRNAs *in vivo* and silico, alongside other forms of epigenetics. This may help identify potential diagnostic or therapeutic targets for women patients with osteoporosis.

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Ethical clearance: Before conducting the study, ethical approval was obtained from the College of Education for Women at Tikrit University in Iraq (No. 3\7\4768, 7\11\2023), in accordance with the ethical guidelines outlined in the Declaration of Helsinki (1975).

Conflict of interest: None.

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تقدير التعبير الجيني لـ miRNA-21 و miRNA-422 و miRNA-142-3p و miR-181c كعلامات حيوية محتملة لهشاشة العظام لدى الاناث قبل انقطاع الطمث

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الملخص

الخلفية: تنظم microRNAs (miRNAs) العديد من المسارات البيولوجية لدى مرضى هشاشة العظام.

الأهداف: تهدف الدراسة إلى تقدير ارتباط miRNA-21 وmiRNA-422 وmiRNA-142-3p وmiR-181c بهشاشة العظام قبل انقطاع الطمث.

المرضى والطرقأجريت هذه الدراسة على ٦٠ امرأة مصابة بهشاشة العظام قبل انقطاع الطمث و ٣٠ امرأة سليمة. تم بواسطة فحص إليزا بتحديد أوستيوكالسين (OC) وديوكسي بيريدينولين (DPD) والفوسفاتيز القلوي الخاص بالعظام (BAP). قدر التعبير الجيني لـ miRNA باستخدام تقنية الـ qRT-PCR.

النتائج: أشارت مستويات OC وDPD وBAP إلى انخفاض كبير لدى النساء المصابات بهشاشة العظام. كما تظهر بياناتنا أن miRNA-21 (p=0.0001, r=-0.5585) و miRNA-422 (p=0.0035, r=-0.3715) لهما تعبير عالي وارتباط سلبي مع كثافة المعادن في العظام. التعبير المنخفض والارتباط الإيجابي مع miRNA-142-3p (p=0.0136, r=0.3089) و miRNA-181c (p=0.0401, r=-0.2685) مع قيمة كثافة المعادن في العظام.

الاستنتاج: أشارت نتائج هذه الدراسة إلى وجود تأثير واضح لـ miRNA-21 وmiRNA-422 في هشاشة العظام وبالتالي قد يكونان مفيدين كعلامات حيوية لهشاشة العظام.

الكلمات المفتاحية: هشاشة العظام، **miRNA-21، miRNA-422، miRNA-142-3p، وmiR-181c**.

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Evaluation of Biofilm Formation in Molecular Identification *E. coli* Strains that Cause Urinary Tract Infection in Children and Antibiotic Resistance

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Abstract

Background: *Escherichia coli* (*E. coli*) is the primary causative agent of urinary tract infections (UTIs), one of the most common human illnesses, which frequently occurs in children.

Objective: This study aimed to identify *E. coli* strains that cause UTIs in children and determine the correlation between biofilm formation and antibiotic resistance.

Patients and Methods: A total of 290 cases of UTI patients were collected from Al-Batoul Teaching Hospital in Diyala, Iraq. The ages of these patients ranged from 1 day to 12 years, from February 2023 to January 2024. The strains of *E. coli* that cause UTIs were identified using polymerase chain reaction (PCR) and sequencing methods. Antimicrobial susceptibility was evaluated, and a microtiter plate assay was used to assess biofilm production.

Results: The predominant bacteria responsible for UTI in children were *E. coli* (40%), and it was noted that the lowest percentage of bacteria causing UTI in this study were *Klebsiella oxytoca* and *Pseudomonas aeruginosa*, as they appeared in 5% of cases. The strains of *E. coli* that cause UTIs in the current study are *E. coli* Y8-2 (14.8%), *E. coli* 106K88 (19.3%), *E. coli* UA32 (11.4%), *E. coli* RM11911 (20.5%), and *E. coli* EC1704-1 (34%). *E. coli* EC1704-1 showed multidrug resistance (MDR) to ciprofloxacin (100%), sulfamethoxazole-trimethoprim (100%), cephalosporins and penicillin (100%), and aminoglycosides (93.3%). *E. coli* Y8-2, *E. coli* 106K88, and *E. coli* UA32 appeared less resistant to antibiotics than *E. coli* EC1704-1 and *E. coli* EC1704-1. Additionally, it was demonstrated that biofilm formation and antimicrobial resistance were negatively correlated among the isolates.

Conclusion: This study demonstrated a clear link between biofilm formation and antibiotic resistance, suggesting that this bacterium with reduced resistance may depend on biofilms to enhance its survival.

Keywords: *16SrRNA*, *Escherichia coli* strains, Antibiotic resistance, Urinary tract infection, Biofilm formation.

Introduction

Urinary tract infections (UTIs) are the most common bacterial infections, affecting approximately 150 million people worldwide each year, and are often seen in children. Understanding the underlying

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infections and their antibiotic resistance patterns in certain regions are crucial for providing the best possible care (1, 2). UTIs might cause short-term complications, including fever, painful urination, lower abdominal discomfort, and may lead to irreversible kidney scarring (3). Urinary tract infection is more prevalent in women than in men due to the close physical proximity of the urethra to the end of the gastrointestinal tract (4). Screening for bacterial susceptibility in each city is critical for generating essential data on antibiotic resistance (5, 6). UTI problems mostly involve kidney failure caused by severe renal damage and sepsis, which results from the infection spreading beyond the lower urinary tract to other areas of the body. These infections are often treated with antibiotics. Antibiotic susceptibility testing is crucial for doctors to select appropriate medications for patients with urinary tract infections (5). The frequency of antibiotic resistance in *E. coli* causing UTIs in children in primary care is significant. Therefore, it is essential to examine the treatment usage and antibiotic resistance patterns of bacteria in patients with UTIs. Various risk factors for UTIs in children were identified, such as sex, ethnicity, vesicoureteral reflux, neurogenic bladder, phimosis, structural anomalies of the lower urinary tract, constipation, and delaying voiding have been linked to UTIs (7). In newborns, urinary tract infections might present with nonspecific symptoms such as poor feeding, diarrhea, failure to grow, vomiting, moderate jaundice, lethargy, fever, and hypothermia. In certain instances, UTI can progress to neonatal sepsis. Infants under 2-years old with UTIs may have non-specific symptoms, including fever, gastrointestinal issues such as vomiting, diarrhea, abdominal discomfort, or urine with a strong odor. In children older than 2-years, cystitis or pyelonephritis may present with typical symptoms. (8). *Escherichia coli* is the predominant bacterium responsible for causing urinary tract infections in children. *E. coli* strains possess several genes encoding various virulence factors that significantly contribute to the bacteria's pathogenicity. The severity of urinary tract infection depends on the virulence gene characterization of the invading *E. coli* strain (9, 10). In addition, antimicrobial resistance (AMR) has become a major threat to world health. It is expected that AMR will cause 10 million deaths by the year 2050 (6, 7). The broad distribution of antibiotics to urinary tract pathogens with resistance, such as *E. coli*, is driven by the widespread use of antibiotics to treat UTIs (8). Afterward, the course of treatment of UTIs has been increasingly complex as a result of the multidrug-resistant (MDR), particularly in those suffering from repeated UTIs (9-11). One characteristic that hinders bacterial removal during antibiotic therapy is the development of biofilms on biological surfaces. The 3-dimensional multicellular biofilms are arranged in groups that have the ability to adhere to both abiotic and biological surfaces and are coated with an extracellular polymeric substance (12, 13). *E. coli* cells may produce biofilms inside and on the surface of catheters (3, 14,15). An in-depth investigation into the connection between biofilm and antibiotic resistance reveals incongruous results regarding its forming capability. Biofilm generation has been shown to improve the resistance of bacteria by a number of methods, including decreased expansion and reduced spreading rate of antimicrobials (14, 16). Biofilms might work independently of the process, mostly used for bacterial resistance by sensitive isolates as a method of surviving (17-20). A significant distinction in the sensitivity between implanted biofilm cells and planktonic cells that are part of the reports of the same strain exists (21, 22). When collectively, these Observations emphasise how crucial it is to consider biofilm formation capacity as an essential bacterial factor in the treatment strategy for UTIs (23, 24). Hence, this

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study aimed to identify *E. coli* strains that cause UTIs in children and study the correlation between biofilm formation and antibiotic resistance of *E coli* isolates.

Patients and Methods

Bacterial isolation and identification: It was collected 290 urine samples of children for cultures and sensitivity tests from February 2023 to January 2024. Information on age and gender was recorded; the ages of these patients ranged from 1 day to 12 years. The study was conducted at Al-Batoul Teaching Hospital in Diyala Province, Iraq. The clinical urine specimens were obtained from midstream and catheter-aspirated urine samples of individuals diagnosed with urinary tract infections, prior to the initiation of antimicrobial therapy. The positive urine cultures were defined as having a bacterial count of at least 105 CFU/mL (25). The colonial morphology of the isolated bacteria on various culture media, including blood agar, MacConkey agar (Oxoid, USA), and Cysteine Lactose Electrolyte Deficient (CLED) agar (Oxoid, USA), was initially used to identify the bacteria. Gram staining was also employed in this process. Several common biochemical tests (methyl red test, KIA test, Voges-Proskauer test, indole test, citrate test, and urease test) were used further to validate the identification of *E. coli* (26). For long-term preservation, the isolates were then kept in tryptic soy broth (TSB) supplemented with 20% glycerol at -80 °C.

Antimicrobial susceptibility testing (AST): The susceptibility tests of bacterial isolates for the antibiotics were done by following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (27). It was determined that the susceptible isolates to nineteen different antimicrobial drugs across thirteen different categories. Azithromycin, nitrofurantoin, tetracycline, chloramphenicol, imipenem, meropenem, cefozolin, cefuroxime, cefotaxime, ciprofloxacin, ceftazidime, gentamicin, amikacin, trimethoprim-sulfamethoxazole, aztreonam, ampicillin, and amoxicillin-clavulanic acid were among the agents employed in the Kirby-Bauer disc diffusion method. Classified isolates as multi-drug resistant (MDR) if they resisted at least three antimicrobial classes. The method previously outlined (28) was used to calculate the multiple antibiotic resistance (MAR) indexes.

Molecular methods for *E. coli* detection: Using 16S-R and 16S-F primers, *16S rRNA* was amplified by polymerase chain reaction (PCR), and molecular detection was applied to all 88 *E. coli* isolates. DNA was extracted from *E. coli* isolates and then subjected to PCR analysis using the identified gene. The gene was amplified by polymerase chain reaction (PCR) using primers specific to the *16SrRNA*. F (AGAGTTTGATCCTGGCTCAG) and R (TACGGTTACCTTGTTACGACTT) were the primers used. This was followed by verifying the amplified target genes using agarose gel electrophoresis (10, 11).

DNA sequencing: The amplified gene product was purified using a Sanger sequencing ABI 3730XL for DNA sequencing before being sent to Macrogen Corporation in Korea for further study. After receiving the data via email, powerful software was used to analyse it and identify the different strains of *E. coli* (12, 13).

Biofilm formation assay: As previously mentioned, the 96-well microtiter plate assay assessed the isolates' ability to produce biofilms (29). Luria Bertani (LB) broth was used as the medium, and bacterial suspensions were shaken and cultured at 37 °C for 18 to 24 hours. Subsequently, the suspensions were diluted 1: 100 to a final level of 200 µL in M63 medium containing 0.25% glucose. The inoculated microtiter plates were then incubated for 48 hours at 30 °C without shaking. After the culture was removed, sterile phosphate-buffered saline (PBS)

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was used to wash the wells. Biofilms were dried at 65 °C for ten minutes, then cleaned with PBS and dyed with 2% crystal violet (CV). Thereafter, 33% glacial acetic acid was used to dissolve the adhering CV, and to calculate the biofilm production capacity, the optical density at 580 nm (OD580) was measured using a microplate reader (SunriseTM, TECAN, Switzerland). The medium that was not inoculated acted as a negative control. The experiment was run in duplicate. The cutoff value (ODc) was determined as three standard deviation units over the average absorbance of the sterile media, as previously mentioned (30). The isolates were then divided into four categories: moderate biofilm-producing isolates ($2ODc \geq OD \leq 4ODc$), strong biofilm-producing isolates ($OD \geq 4ODc$), weak biofilm-producing isolates ($ODc \geq OD \leq 2ODc$), and non-biofilm-producing isolates ($OD \leq ODc$) (31).

Statistical analysis

In order to do statistical analysis, the Graphpad Prism program (Graphpad, California, United States) was utilized. Analysis of variance (ANOVA) was performed in either a one-way or two-way fashion in order to compare the groups for the investigations.

Results

Isolation and identification of *E. coli*: The results of this study showed male and female patients were suspected to the urinary tract infection, 93 (32.1 %) and 197 (67.9 %), respectively. According to the current study's findings of infections were significantly greater in female patients than in male patients (Figure 1).

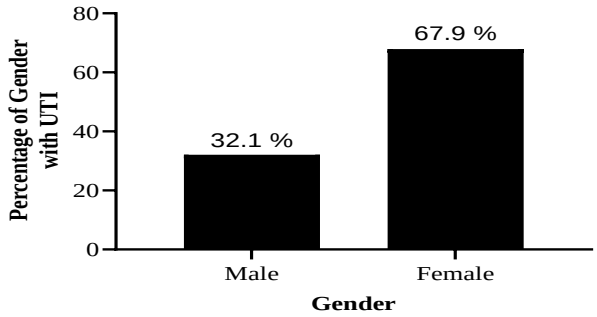

Figure 1. *Distribution of UTI patients according to their gender.*

In addition, the patients were divided into four age groups, and the results showed that the percentage of infection increased in the age group of 8 to 12 (49.7%) and decreased within the first year of age. It was shown that both the gender and age of the patients had a significant impact on the prevalence of the condition (Figure 2).

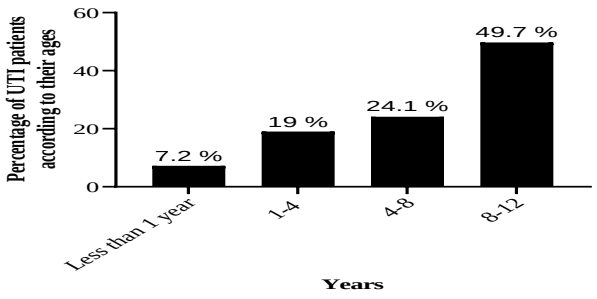

Figure 2. Distribution of UTI patients according to their ages.

The results of this study showed that there are six different types of bacteria were caused UTI in children and the most common bacteria that cause this infection are *E coli* 88 (40 %), *Klebsiella pneumoniae* 60 (27.3 %) and *Proteus* spp 38 (17.3 %). It appeared that *Staphylococcus saprophyticus*, *Kebsiella oxytoka* and *Psuedomonas aureginosa* have the lowest percentage of bacteria caused UTI infections among children as appeared in 12 (5.4 %), 11 (5 %) and 11 (5 %), respectively of cases (Figure 3).

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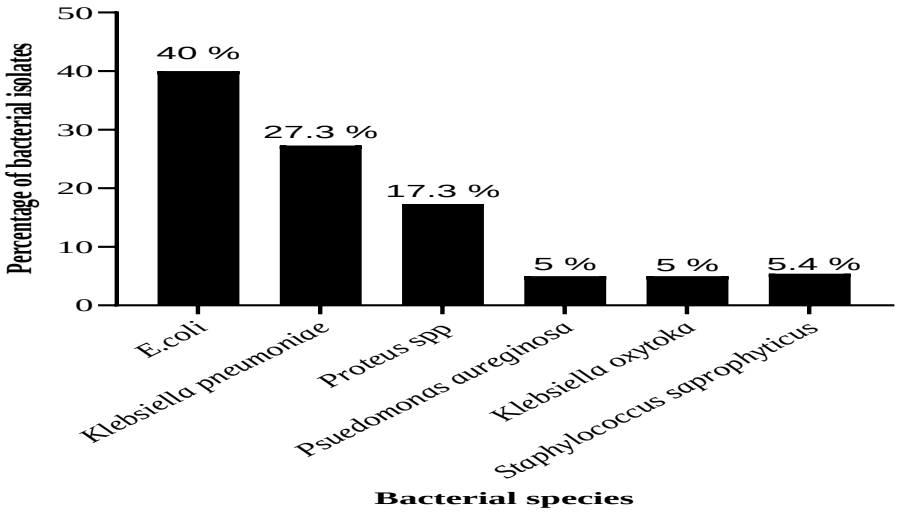

Figure 3. The types of bacteria that cause UTIs in children.

Molecular detection of *E. coli*: In this study, the molecular identification of *E. coli* was done using *16SrRNA* that was amplified via PCR by using the specific primers, and the results showed an amplified 1500 bp PCR product of the same size as the target gene, as shown in Figure 4.

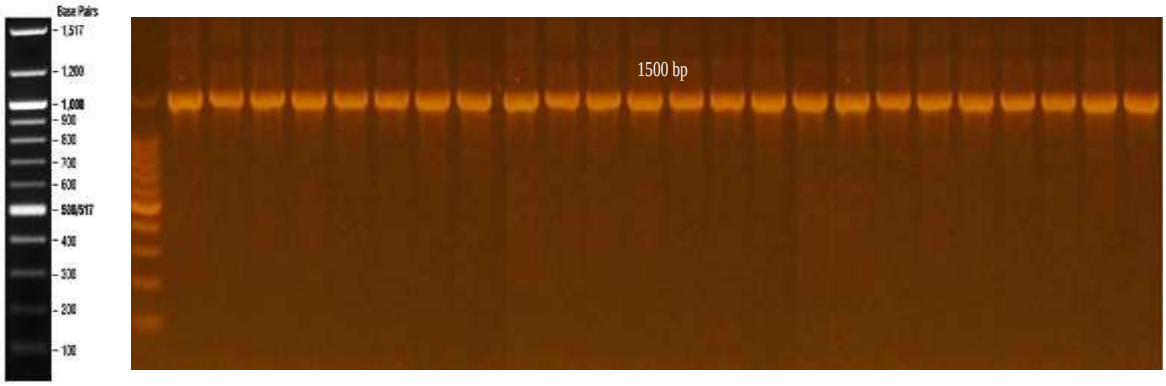

Figure 4: Agarose gel electrophoresis of PCR products. The results appeared to amplify 1500 bp fragments, which is the size of *16SrRNA* as compared to the molecular ladder, which is between 1500 and 100 base pairs.

Sequencing results: In order to determine the strains of *E. coli* that are responsible for urinary tract infections (UTIs), the PCR products were purified and then sent for sequencing. According to the analysis of DNA sequencing alignment findings, it was shown that several strains of this bacterium that cause urinary tract infections in children, which are *E. coli* Y8-2 13 (14.8 %), *E. coli* 106K88 (19.3 %), *E. coli* UA32 (11.4 %), *E. coli* RM11911 (20.5 %), and *E. coli* EC1704-1 (34 %) as shown in Figure 5.

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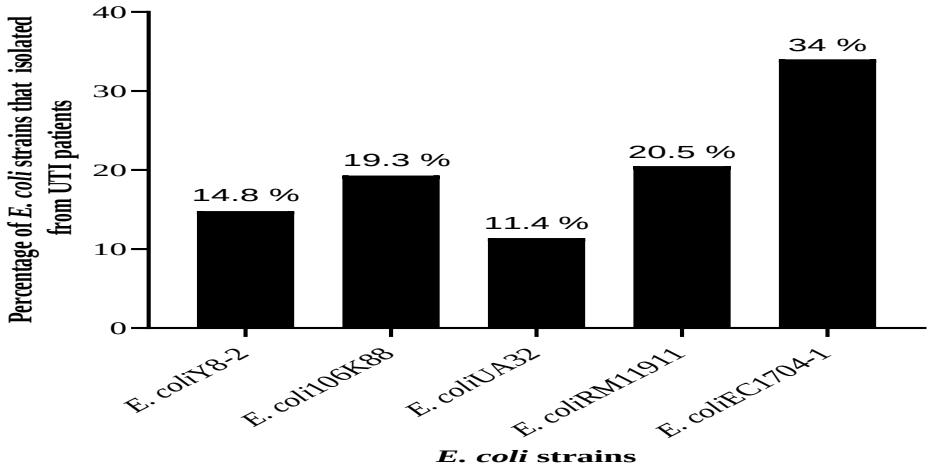

Figure 5. The percentages of *E. coli* strains, which they identified according to the DNA sequencing alignment results.

Antibiotic susceptibility and biofilm formation of E. coli strains: The results of this study showed that *E. coli* EC1704-1 and *E. coli* RM11911 were multi-drug resistant, which showed resistance against most of the tested antibiotics. However, *E. coli* Y8-2, *E. coli* 106K88, and *E. coli* UA32 appeared less resistant against the antibiotics that were used in this study compared with the first two strains, as shown in Table 1. The antibiotic sensitivity test was done for *E. coli* strains, and the results showed that *E. coli* EC1704-1 and *E. coli* RM11911 were multi drug resistant, which showed resistant against most of the tested antibiotics. However, *E. coli* Y8-2, *E. coli* 106K88, and *E. coli* UA32 appeared less resistant against the antibiotics that were used in this study.

Table 1. Percentages of antibiotic resistance (%) of *E. coli* strains that were isolated from UTI patients against the tested antibiotics.

Antibiotic category	Antimicrobial agent	E.coli Y8-2	E. coli 106K88	E. coli UA32	E. coli RM11911	E. coli EC1704-1
Non-Extended spectrum cephalosporin	Cefazolin cefuroxime	0.00 0.00	5.9 47	0.00 10	77.8 83.3	100 100
Extended spectrum cephalosporin	Cefotaxime ceftazidime	7.7 0.00	11.8 11.8	10 10	83.3 72.2	100 93.3
Fluoroquinolones	ciprofloxacin	0.00	0.00	10	100	100
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	30.8	41.2	0.00	100	100
Aminoglycosides	Gentamicin Amikacin	15.4 7.7	47.1 5.9	40 10	83.3 100	93.3 93.3
Carbapenems	Imipenem Meropenem	0.00 0.00	5.9 0.00	20 0.00	83.3 44.4	83.3 50
Monobactams	Aztreonam	15.4	5.9	0.00	55.6	66.7
Penicillins	Ampicillin	23.1	41.2	70	100	100
Penicillin-Betalactamase inhibitor	Amoxicillin-clavulanic acid	23.1	5.9	0.00	100	100

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Phenicols	Chloramphenicol	0.00	0.00	20	33.3	50
Tetracycines	Tetracycine	7.7	29.4	20	55.6	16.7
Macrolide	Azithromycin	7.7	11.8	0.00	11.1	33.3
Nitrofurans	Nitrofurantoin	0.00	5.9	10	22.2	16.7

Biofilm formation was done for *E. coli* strains identified in this study. The 96-well microtiter plate assay, the gold standard for estimating the biofilm formation capacity, was used to assess the biofilm formation capacity of *E. coli* strains in M63 broth. The strains were then separated into three groups including, weak, moderate, and strong. The results of this study showed that the strains, *E.coli* EC1704-1 and *E. coli* RM11911 created OD 0.5 and 0.7, respectively (weak biofilms), *E. coli* UA32 produced OD 1.1 (moderate biofilms), and *E. coli* 106K88 and *E. coli* Y8-2 produced OD 1.4 and 1.6, respectively (strong biofilms). This correlation was found to be statistically significant ($P < 0.0001$) (Figure 6).

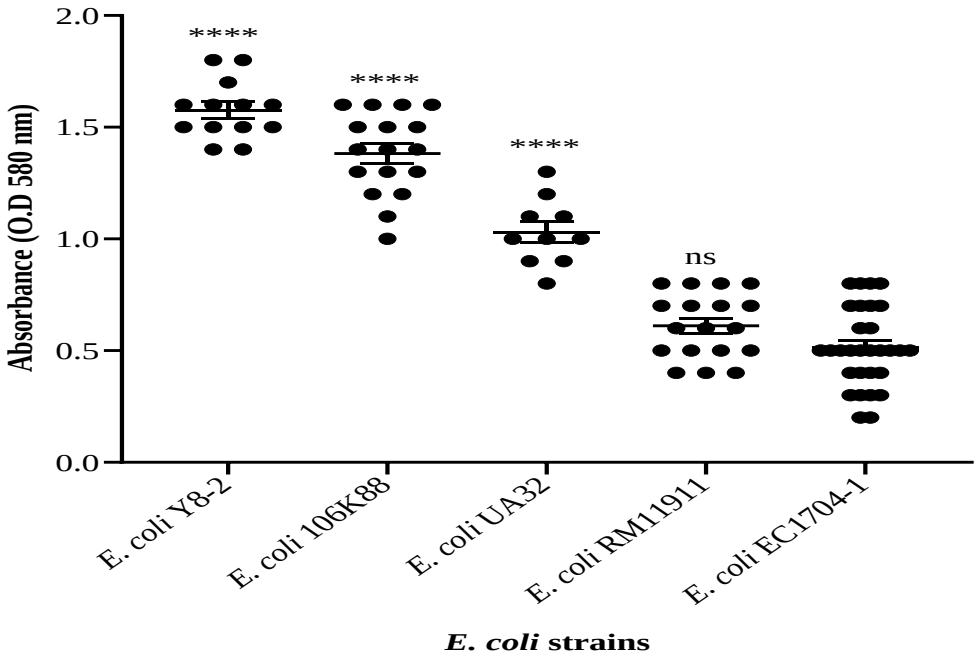

Figure 6. Biofilm formation capacity among *E. coli* strains. The experiment was repeated using three replicates of four independent biological samples. Significant differences were observed when comparing the biofilm formation of E. coli EC1704-1 with that of other strains using one-way ANOVA and Dunnett's multiple comparisons test. (**** $p<0.0001$, ns= non-significant, relative to the *E. coli* EC1704-1).

Discussion

Several bacteria cause UTIs in children, and *E. coli* was chosen for antimicrobial susceptibility testing and molecular identification due to the high percentage of this bacterium that causes UTIs. Both the gender and age of the patients had a significant impact on the prevalence of the condition.

According to the findings of the current study, the incidence of infections was significantly greater in female patients compared to male patients. Similarly, it was reported that the same prevalence in males and females within the first year of age increased the UTI infection in females compared with males after the first year of life (14). In addition, this study demonstrated that the risk of UTI increases after the age of 8. This data, similar to that of other studies,

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reports that UTIs are believed to be caused by a shorter distance between the anus (the typical source of uropathogens) and the urethral meatus, the longer length of the male urethra, and the antibacterial activity of prostatic fluid in males (15). This condition is more prevalent in the female population, particularly among young and middle-aged individuals. In accordance with the findings of earlier research, which revealed that *E. coli* was present in 75–90% of UTI isolates, it was demonstrated that *E. coli* was the bacterium that caused the majority of UTIs, and molecular identification for the *E. coli* isolates was done using *16S rRNA* that is the housekeeping gene of most types of bacteria (21- 23). Thus, these findings are crucial for identifying the strains of this bacterium, which in turn leads to the control and prevention of this infection in children. The antibiotic sensitivity test was done for *E. coli* strains, and the results showed that *E. coli* EC1704-1 and *E.coli* RM11911 were multidrug-resistant, which showed resistance against most of the tested antibiotics. However, *E. coli* Y8-2, *E. coli* 106K88, and *E. coli* UA32 appeared less resistant against the antibiotics that were used in this study. This may be due to the fact that the route of medication administration is simple, as well as the fact that bacteria in the juvenile population are sensitive to antibiotics. Because of the nature of antibiotics and treatment recommendations for the route of drug administration in children, almost all of the medications were given intravenously (16, 17). This may have been the basis for the medication administration. Before deciding on a treatment plan for urinary tract infections (UTIs), it is strongly recommended to do an antibiotic sensitivity test. It is the only method to ensure that the treatment plan remains on track, and it should be carried out on a regular basis in order to monitor the development of antibiotic resistance in the various clinical settings used.

Target gene mutations and the acquisition of resistance genes by mobile genetic elements such integrons and plasmids, which can confer co-resistance to many antimicrobial agents (32, 33), are the primary causes of antimicrobial resistance. Additionally, biofilm formation provides a further defense mechanism that enables the encased bacterial cells to evade harsh ambient conditions and the damaging effects of antimicrobial agents (34). A potential link between acquired antimicrobial resistance and virulence has been suggested by the fact that both virulence and antimicrobial resistance genes can be transferred together through plasmids or other transferable genetic elements, in addition to the ability of acquired resistance, such as fluoroquinolone (FQ) resistance, to influence gene expression among resistant isolates (16). This study demonstrated that biofilm formation is negatively correlated with antibiotic resistance. It has previously been documented that the biofilm-forming ability of uropathogenic *E. coli* is negatively impacted by acquired antibiotic resistance (35). Similarly, Poursina and colleagues (31) found that multidrug-resistant (MDR) isolates were present in negative and weak biofilm-producing UPEC isolates. In contrast, non-MDR isolates comprised 69.2% of the strong biofilm-producing isolates. The biofilm architecture uses a number of surface appendages, including fimbriae, as well as additional non-fimbrial proteins, as a supporting framework. The expression of these organelles may be impacted by the development of antibiotic resistance, which would be detrimental to the ability to create biofilms (31, 36). Similarly, it has been previously observed that the acquisition of genes producing ESBL enzymes negatively affects the ability of *E. coli* and *Pseudomonas aeruginosa* build biofilms (37). This implies that biofilm formation is a method that helps bacteria to get better survival, especially with bacteria that are less antibiotic-resistant, and this may be due to the reduced exposure to multiple

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antibiotics (31, 38). All of these findings support the theory that uropathogenic to *E. coli* isolates' ability to form biofilms, which is negatively impacted by the development of antibiotic resistance.

Conclusions

E. coli strains that cause UTIs include *E. coli* Y8-2 (14.8%), *E. coli* 106K88 (19.3%), *E. coli* UA32 (11.4%), *E. coli* RM11911 (20.5%), and *E. coli* EC1704-1 (34%). This study demonstrated a negative correlation between antibiotic resistance and biofilm formation. The findings of this study contribute to a better understanding of the pathogenic potential of *E. coli* strains that can lead to severe cases of urinary tract infections. Additionally, it was recommended that identifying the expression of biofilm formation genes in this bacterium in the presence of antibiotics be crucial.

Source of funding: No source of funding.

Ethical clearance: The study protocol was approved by the Ethics Committee of Al-Batoul Teaching Hospital, a healthcare facility in Diyala, Iraq. The study was conducted, and samples were collected after receiving approval from the University of Diyala/College of Medicine's Research Ethics Committee (No.2024ASM878).

Conflict of interest: None.

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تقييم تكوين الأغشية الحيوية في التعرف الجزيئي على سلالات الإشريكية القولونية المسببة لعدوى المسالك البولية عند الأطفال ومقاومة المضادات الحيوية

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الملخص

الخلفية: تُعد الإشريكية القولونية (**E. coli**) العامل المسبب الرئيسي المسبب لألتهاب المسالك البولية، وهي من أكثر الأمراض شيوعًا، لا سيما بين الأطفال.

الأهداف: تهدف هذه الدراسة إلى تحديد السلالات من **E. coli** المسببة لألتهاب المسالك البولية لدى الأطفال، وتقييم العلاقة بين تكوين الأغشية الحيوية ومقاومة المضادات الحيوية.

المرضى والطرق: تم جمع ٢٩٠ حالة من مرضى التهاب المسالك البولية من مستشفى البتول التعليمي في محافظه ديالى ، العراق. تراوحت أعمار هؤلاء المرضى من يوم واحد إلى ١٢ عامًا ، من فبراير ٢٠٢٣ إلى يناير ٢٠٢٤. تم تحديد سلالات **E. coli** التي تسبب التهاب المسالك البولية باستخدام تفاعل سلسلة البلمرة (**PCR**) وطرق التسلسل. تم تقييم حساسية مضادات الميكروبات ، واستخدم اختبار لوحة **microtiter** لتقييم إنتاج الأغشية الحيوية.

النتائج: كانت البكتيريا السائدة المسؤولة عن التهاب المسالك البولية في الأطفال هي E. coli (٤٠ %) ، ولوحظ أن أدنى نسبة من البكتيريا التي تسبب التهاب المسالك البولية في هذه الدراسة كانت klebsiella oxytoca و pseudomonas aeruginosa ، كما ظهرت في ٥٪ من الحالات الاخرى. سلالات E. coli التي تسبب التهاب المسالك البولية في الدراسة الحالية هي E. coli Y8-2 (14.8%)، E. coli 106k88 (19.3%)، E.coli UA32 (11.4%)، E. coli RM11911 (٢٠,٣%)و E.coli EC1704-1 (٣٤%). اظهرت E. coli EC1704-1 مقاومة متعددة للادوية إلى سيبروفلوكساسين (١٠٠٪) ، سلفاميثوكسازول تريميثوبريم (١٠٠ %) ، السيفالوسبورين والبنسلين (١٠٠ %) ، والأمينوغليكوسيدات (٩٣,٣ %). ظهر E. Coli Y8-2 و E. Coli 106k88 و E. coli UA32 أقل مقاومة للمضادات الحيوية من E. coli EC1704-1 و E. Coli EC1704-1. بالإضافة إلى ذلك ، ثبت أن العلاقه بين تكوين الأغشية الحيوية ومقاومة مضادات الميكروبات كانت سلبيه بين العزلات.

الاستنتاج: أظهرت هذه الدراسة وجود صلة واضحة بين تكوين الأغشية الحيوية ومقاومة المضادات الحيوية ، مما يشير إلى أن هذه البكتيريا مع انخفاض المقاومة قد تعتمد على الأغشية الحيوية لتعزيز بقائها.

الكلمات المفتاحية: ***16SrRNA***، سلالات الإشريكية القولونية ، مقاومة المضادات الحيوية ، التهاب المسالك البولية ، تكوين الأغشية الحيوية.

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c-MYC Levels and Metabolic Parameters in Triple-Positive and Triple-Negative Breast Cancer

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Abstract

Background: Breast cancer is a complex, heterogeneous disease and the most common malignancy among women. It is classified based on the presence of estrogen receptors (ER), progesterone receptors (PR), and HER2. Rapid proliferation of cancer cells increases their energy demands leading to enhanced glycolysis and lactate accumulation. Lactate dehydrogenase (LDH) is pivotal in this process, particularly in tumors with anaerobic metabolism. The transcription factor c-Myc (cellular myelocytomatosis oncogene) promotes aerobic glycolysis by increasing glucose uptake and lactate production are a hallmark of the Warburg effect.

Objectives: This study was aimed to evaluate c-Myc expression levels in patients with triple-positive breast cancer (TPBC) and triple-negative breast cancer (TNBC), comparing them to healthy control.

Patients and Methods: The study included 80 women (ages 35–66): 20 with TNBC, 20 with TPBC, and 40 individual healthy as a control, matched for age, sex, and Body Mass Index (BMI). Serum levels of c-Myc, CA 27-29 was measured using an ELISA sandwich technique. Additionally, lactate dehydrogenase (LDH), glycated hemoglobin (HbA1c), liver enzymes (ALT and AST), and lipid profiles were assessed using spectrophotometric techniques.

Results: c-Myc levels were significantly higher in breast cancer patients (4.71 ± 3.75 ng/ml) compared to controls (0.81 ± 0.44 ng/ml, $p = 0.001$). LDH and CA 27-29 levels were significantly elevated ($p = 0.001$). Metabolic parameters, including HbA1c%, ALT, AST, and lipid profiles (except HDL), showed significant changes, with reduced HDL levels in cancer patients. Notably, TNBC patients exhibited higher c-MYC and HbA1c levels compared to TPBC patients.

Conclusion: Elevated c-Myc levels are associated with metabolic reprogramming in breast cancer and may serve as a potential therapeutic target. The higher c-Myc expression in TNBC correlates with its more aggressive nature, suggesting c-Myc's role in tumor progression.

Keywords: Breast cancer, c-Myc, Lactate dehydrogenase, Triple-negative breast cancers, Triple-positive breast cancer.

Introduction

Breast cancer (BCa) is the most commonly diagnosed cancer in women (1), and has been the leading cause of death among women in Iraq for the past three decades (2). Risk factors include age, gender, genetics, lifestyle, and environmental toxins (3). BCa is categorized into four molecular subtypes based on the status of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) (4). Breast cancer subtypes can be classified by immunohistochemistry as follows: luminal A (ER+ and/or PR+ and HER2−), luminal B (ER+ and/or

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PR+ and/or HER2+), HER2- enriched (ER−, PR−, HER2+), and triple-negative (ER−, PR−, HER2−) (5). Triple-negative breast cancer (TNBC) is characterized by the absence of ER, PR, and HER2 expression, which occurs in 1 in 5 cases of women (6). TNBC tumors are more aggressive, have a poor prognosis, high recurrence rates, and low survival, with metastasis commonly affecting the brain and visceral organs (7). In contrast, TPBC is a distinct subtype within the HER2-positive luminal B category, characterized by the presence of all three hormone receptors (4).

c-Myc (cellular myelocytomatosis oncogene), the first member of the Myc family in mammalian cells (along with N-Myc and L-Myc), is a 62 kDa protein comprising 439 amino acids (8). It plays a crucial role in tumor metabolic reprogramming by regulating glucose and glutamine uptake, metabolism, and the invasive properties of cancer cells (9). c-Myc activation is associated with several key cancer hallmarks, including uncontrolled proliferation, metastasis, immune evasion, genomic instability, and metabolic reprogramming (10). Additionally, c-Myc regulates cell growth, division, metabolism, and apoptosis. Despite its central role in cancer, no approved clinical inhibitors of c-Myc exist, presenting a significant challenge for cancer therapy (11).

Lactate dehydrogenases (LDH, EC 1.1.127) are essential enzymes in anaerobic metabolism, catalyzing the reversible conversion of pyruvate to lactate during the final step of glycolysis, with NADH as the coenzyme (12). Lactate enhances tumor invasiveness by promoting key steps in metastasis: (I) angiogenesis, (II) immune evasion, and (III) extracellular matrix degradation and cell migration (13).

CA 27.29 is a carbohydrate-containing protein antigen, or Breast Carcinoma-associated antigen, released into the bloodstream by breast cancer cells (14).

This study aimed to evaluate the expression levels of the c-Myc gene and several other metabolic markers in patients with triple-positive breast cancer (TPBC) and triple-negative breast cancer (TNBC), and to compare these levels with those of healthy controls.

Patients and Methods

Study population and data collection: Blood samples were collected from patients with breast cancer at the Tumors Teaching Centre of the Medical City of Baghdad and the Oncology Unit at Al-Yarmook Hospital between August and November 2023. The study involved 80 participants, including 20 patients with triple-negative breast cancer (TNBC) and 20 with triple-positive breast cancer (TPBC), all of whom were undergoing hormone therapy, radiation, and chemotherapy. Additionally, 40 women without breast cancer served as controls. The majority of cancer patients were in stages II and III. The participants, aged 35 to 66, were matched for age and body mass index (BMI), and none had diabetes, polycystic ovary syndrome (PCOS), or other hormonally-related conditions affecting glucose metabolism. Each patient underwent a metastatic biopsy to confirm diagnosis and evaluate receptor status (HER2, PR, ER). Anthropometric and biochemical data were collected via questionnaires

Blood collection and laboratory analysis: Ten milliliters of venous blood were collected from each participant (patients and controls) using a disposable syringe. After 3 ml was extracted for HbA1c analysis, serum was separated into a gel tube, centrifuged at 3000 RPM for 10 minutes at room temperature, and stored in Eppendorf tubes at -20°C. Serum c-MYC concentration was measured using an ELISA sandwich technique by (ELK3329 Human Myc BP). The process involved adding samples or standards to pre-coated microtiter plate wells, followed by the addition of a biotin-conjugated antibody specific to human MYCBP and enzyme-

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linked avidin. After the TMB substrate was added, a color change occurred in the wells containing human MYCBP, which was measured spectrophotometrically at 450 nm. The c-MYC concentration was determined based on the color intensity. Human total serum CA 27-29 was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) method (Human Cancer Antigen (27-29). The micro Elisa strip plate, pre-coated with a CA 27-29-specific antibody, was incubated with samples or standards, followed by a horseradish peroxidase (HRP)-conjugated antibody. After adding the TMB substrate, a color change occurred in wells containing CA 27-29, which turned yellow upon addition of the stop solution. Optical density (OD) was measured at 450 nm, and CA 27-29 concentration was determined by comparing the OD values to a reference curve. Lipid profile, ALT, AST, and LDH were analyzed using Siemens Healthcare equipment, and HbA1c was measured by Siemens Healthineers. Body mass index (BMI) was calculated as weight (kg) / height² (m).

Statistical analysis

The correlation coefficient (r) between parameters, the T-test, and the differences between three independent variables were assessed using analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$, with $p > 0.05$ indicating no significant difference. Data were analyzed using SPSS version 26, and results are presented as mean ± SE.

Results

The demographic characteristics of patients and controls: The study involved 80 participants, including 40 patients with breast cancer and 40 control patients without breast cancer. All participants were women, aged 35 to 66 years, and were matched for age and body mass index (BMI), as detailed in Table 1.

Table 1. The demographic characteristics of patients and control.

Parameter	Control	Patients
Number	40	40
Age (year)	49.45±6.79	50.60±9.86
BMI (kg.m^2)	27.24±2.31	28.51±4.40
Type of tumor		
Triple -ve		20 (50%)
Triple +ve		20 (50%)
Family history		24 (60%)

The breast cancer patients were classified into two molecular subtypes: triple-positive breast cancer (TPBC) and triple-negative breast cancer (TNBC), with 20 patients assigned to each subtype. Notably, 60% of the breast cancer patients had a family history of the disease. These molecular classifications of breast cancer play a crucial role in treatment decisions and patient assessment.

Comparison of biochemical markers between patients and healthy controls: Table 2 presents various tests and examinations used to monitor disease progression and treatment response. Notably, the CA 27-29 and c-Myc markers are critical for assessing patient health. Additionally, variables such as HbA1c, LDH, ALT, AST, and lipid profiles were evaluated, with patients showing significantly higher values compared to controls.

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Table 2. Comparison of biochemical markers between patients and healthy controls.

Marker	Control	Patients	p-value
CA27-29(U/mL)	0.986±0.294	1.752±0.573	0.0001
C-MYC (ng/ml)	0.81±0.44	4.71±3.75	0.0001
HbA1c %	4.36±0.64	5.27±0.70	0.0001
LDH (IU/L)	84.38±19.68	300.86±169.69	0.0001
ALT (IU/L)	14.60±6.72	21.64±8.00	0.0001
AST (IU/L)	14.35±4.09	20.87±7.17	0.0001
TC (mg/dL)	161.83±13.73	174.51±24.27	0.0001
TGs (md/dL)	103.58±19.45	122.24±31.70	0.0001
HDL-C (mg/dL)	53.85± 6.09	50.51± 8.51	0.015
VLDL-C (mg/dL)	20.72± 3.89	24.45± 6.34	0.0001
LDL-C (mg/dL)	87.26± 15.56	99.55± 25.24	0.001

The results revealed significant differences ($p < 0.05$) in several biomarkers between breast cancer patients (TPBC, TNBC) and the control group. Specifically, CA 27-29 levels were higher in the patient groups with mean values of (1.752±0.573 and 0.986±0.294). Similarly, c-Myc levels were significantly elevated in breast cancer patients with mean values of (4.71±3.75 ng/ml) compared to controls (0.81±0.44 ng/ml). These findings highlight the relationship between breast cancer and metabolic/functional changes, offering insights that could enhance treatment strategies and deepen understanding of the disease's biological processes.

Additional metabolic markers, such as cumulative sugar analysis (%HbA1c), lactate dehydrogenase (LDH), ALT, and AST levels, were also significantly altered in breast cancer patients. HbA1c levels were higher in patients without diabetes (5.27±0.70) compared to controls (4.36±0.64), reflecting elevated blood glucose. LDH levels were significantly higher in breast cancer patients (300.86±169.69) than in controls (84.38±19.68). ALT and AST activities were elevated in patients undergoing chemotherapy, radiation, or hormonal therapy. Furthermore, lipid profiles showed a significant decrease in high-density lipoprotein (HDL) levels and an increase in cholesterol, triglycerides, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) levels in breast cancer patients. Notably, none of the patients in the study had obesity.

Biomarkers in breast cancer patients, categorized by tumor type: Table 3 presents the relationship between various biomarkers (CA 27-29, C-Myc, HbA1c, LDH, AST, ALT, and the lipid profile) in the two disease groups. This provides valuable insights into the aggressiveness and characteristics of the molecular subtypes of breast cancer, which can inform treatment decisions and enhance the understanding of this complex condition.

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Table 3. Biomarkers in breast cancer patients according to the type of tumor.

Parameter	Triple-ve	Triple+ve	p-value
CA27-29(U/mL)	1.90±0.61 ^	1.79±0.60 ^	0.132
C-MYC(ng/ml)	6.25±4.98 #^	3.66±2.19 ^	0.029
HbA1c %	5.46±0.57 #^	4.91±0.68 *^	0.048
LDH (IU/L)	323.95±172.49 ^	269.55±123.34 ^	0.096
ALT (IU/L)	21.55±7.04 ^	18.72±7.02	0.238
AST (IU/L)	23.35±6.69 ^	18.81±6.40	0.053
TC (mg/dL)	178.85±17.89 ^	174.40±22.96	0.260
TGs (md/dL)	133.70±28.99 ^	118.80±30.88	0.288
HDL-C (mg/dL)	46.55±7.07 ^	52.25±9.42	0.108
VLDL-C (mg/dL)	26.74±5.80 ^	23.76±6.18	0.288
LDL-C (mg/dL)	105.56±18.31 ^	98.39±25.73	0.322
* significant corresponding to triple -ve, # significant corresponding to triple +ve, , ^ significant corresponding to control.			

When studying the relationship between the two tumor groups concerning the biomarkers CA27.29 and c-Myc, no statistically significant difference was observed for the CA27.29 biomarker ($p > 0.05$). However, a significant difference was identified between the two groups with respect to c-Myc ($p < 0.05$).

This study compared HbA1c levels between two subgroups of breast cancer patients: triple-negative breast cancer (TNBC) and triple-positive breast cancer (TPBC). The results revealed HbA1c levels of 5.46±0.57 in TNBC and 4.91±0.68 in TPBC, with a statistically significant difference between the two groups ($p < 0.05$). However, no statistically significant differences were observed in lactate dehydrogenase (LDH) levels, lipid profiles, or liver function between the TNBC and TPBC groups. These findings suggest that these biomarkers are not strongly associated with tumor subtype, whether triple-positive or triple-negative.

c-Myc levels in breast cancer patient groups: Figure 1 illustrates the levels of c-Myc in breast cancer patient groups with the two subtypes examined in this study, as well as in the healthy control group.

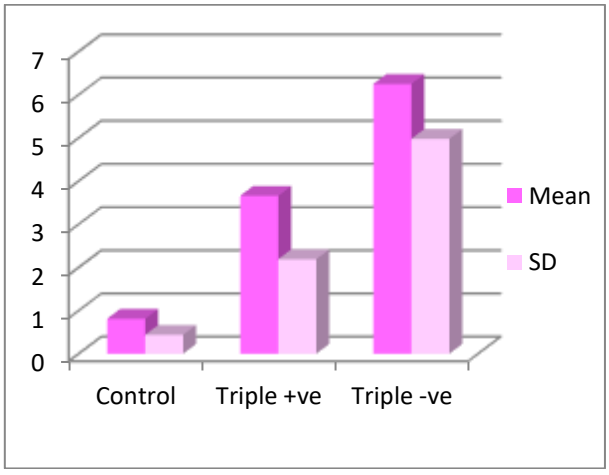

Figure 1. c-Myc level in breast cancer patients according to the type of tumor.

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Correlation of c-Myc with other biomarkers in breast cancer patients: The Correlation coefficient of c-Myc levels in ng/ml with (CA27-29, Age, BMI, HbA1c, LDH, ALT, AST, TC, TGs, HDL-C, VLDL-C,and LDL-C) as shown in Table 4.

Table 4. Correlation of c-Myc with other biomarkers in breast cancer patients.

Parameters	c-MYC					
	Breast cancer patients		Triple -ve breast cancer patients		Triple +ve breast cancer patients	
	r	p	r	p	r	p
CA27-29(U/mL)	-0.242*	0.031	-0.124	0.603	-0.379	0.100
Age (Years)	-0.306*	0.006	0.473*	0.035	-0.263	0.263
BMI(kg.m2)	-0.136	0.230	-0.145	0.541	-0.095	0.690
HbA1c%	0.101	0.374	-0.105	0.661	0.615*	0.004
LDH(IU/L)	0.091	0.420	0.209	0.377	0.267	0.255
ALT(IU/L)	0.279*	0.012	0.069	0.772	0.557*	0.011
AST(IU/L)	0.155	0.171	0.203	0.391	0.413	0.070
TC(mg/dL)	-0.023	0.842	0.201	0.396	0.292	0.212
TGs(md/dL)	-0.032	0.777	0.246	0.295	0.317	0.173
HDL-C(mg/dL)	0.011	0.923	-0.149	0.530	-0.181	0.444
VLDL-C(mg/dL)	-0.032	0.777	0.246	0.295	0.317	0.173
LDL-C(mg/dL)	-0.017	0.878	0.176	0.459	0.251	0.286

Discussion

The molecular classifications of breast cancer used in this study, with 20 patients assigned to the TNBC subtype and 20 patients assigned to the TPBC subtype, are outlined as noted by Saroglu et al. This classification aids in understanding tumor behavior and guiding treatment decisions (15). All groups were matched for age and BMI. Sixty percent of patients had a family history of the disease, in contrast to the study by Rihab Ibrahim Ahmed, where only 11% had a first- or second-degree relative with breast cancer (2).

In agreement with the study of Pekarek et al. (16), who reported an association between elevated CA 27-29 levels and tumor burden, metastatic spread, and potential disease progression, suggesting it could be an early marker of treatment failure or recurrence (16). In contrast, Kaur et al. noted that CA 27-29 levels increase progressively with advancing cancer stages, helping to stage new cases but not those with existing disease (17).

c-Myc expression was elevated in cancer patients compared to healthy individuals, consistent with findings by Gao et al., who emphasized a strong association between tumor growth and c-Myc expression (18). Similarly, Al-Hassany et al. reported c-Myc expression in various malignant tumors, highlighting its role in regulating cell proliferation and metabolism (19).

When blood markers, including %HbA1c and lactate dehydrogenase (LDH), were measured in breast cancer patients and compared to healthy controls, significant differences ($p \leq 0.05$) were observed in HbA1c levels. Even without diabetes, breast cancer

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patients exhibited higher blood glucose levels, which supports findings by Yoo et al. linking increased HbA1c levels, even within the nondiabetic range, to higher cancer-related mortality (20). Elevated LDH levels in patients align with Al-Daam et al.'s findings, which associate higher LDH levels with advanced cancer, tissue damage, and disease severity (21). Similarly, Barrak et al. highlighted high LDH levels as indicators of poor prognosis and chemotherapy resistance (22). Additionally, elevated ALT, AST, cholesterol, triglycerides, LDL, and VLDL levels were observed, while HDL levels were significantly lower. These findings are consistent with Ahmad et al., who reported significantly higher triglycerides (TG), LDL, ALT, AST, and cholesterol levels in breast cancer patients, with lower HDL levels (23). Notably, none of the patients were obese. However, in contrast, Hamid Ali et al. suggested that liver function tests may not serve as effective biochemical markers for monitoring breast cancer during treatment, as no significant differences were found between patient and control groups (24).This research underscores the importance of continuous patient assessment, regardless of breast cancer subtype, and emphasizes the need for using CA 27-29 within a broader set of tests to obtain a comprehensive understanding of the patient's condition. However, no studies have confirmed a direct association between CA 27-29 levels and molecular subtypes of breast cancer, nor can the subtype be determined based on this marker alone. Our analysis revealed elevated c-Myc levels in patients with triple-negative breast cancer (TNBC) compared to those with triple-positive breast cancer (TPBC). This is consistent with findings by Xiao-Ning Yuan et al., who noted that TNBC exhibits higher c-Myc levels, an oncogene transcription factor linked to aggressive cancer progression and metastasis (25). In contrast, Elena A. Dukhanina et al. reported that TPBC presents more aggressive clinical characteristics, with a lower overall survival rate compared to TNBC five years after treatment (26). Additionally, increased HbA1c levels were observed in TNBC patients compared to those with TPBC, which is more responsive to hormonal therapy. Although research on the impact of HbA1c across molecular subtypes is limited, studies, including one by Nehad M. Ayoub et al., suggest that TNBC is more aggressive and linked to poorer clinical outcomes (27). Their research also advocates for screening breast cancer patients for glycemic status at diagnosis and exploring therapies to manage hyperglycemia, potentially improving prognosis and clinical outcomes (27).

The findings suggest that LDH may not be a reliable biomarker for distinguishing between breast cancer subtypes, as it is not specific to any particular tumor type, a point also supported by Vladimir Jurišić et al. (28). Additionally, our study found no statistically significant differences between the two disease groups regarding ALT, AST, and lipid profile. This contrasts with Ameer Jawad Hadi et al.'s research, which indicated that estrogen may induce hyperlipidemia by altering lipid metabolism, increasing triglycerides (TG) and very-low-density lipoprotein (VLDL) (29).

Table 4 shows that c-Myc may be associated with factors such as age, liver function, and glucose levels, particularly in patients with triple-positive breast cancer. These findings suggest that c-Myc could play a role in multiple biological pathways influencing disease progression.

Conclusions

The study highlights the significant role of c-Myc in breast cancer pathogenesis, suggesting its potential as a key biomarker for distinguishing subtypes. c-Myc may also serve as a valuable molecular target for understanding tumor aggressiveness and developing tailored therapeutic strategies. Additionally, it was recommended for studying c-

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Myc in other cancer types that share similar cellular pathways could help to determine them.

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Conflict of interest: None.

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مستويات السي-مايسين والمعايير الايضية في سرطان الثدي الثلاثي الايجابي والثلاثي السلبي

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الملخص

الخلفية: سرطان الثدي مرض معقد ومتنوع وهو اكثر انواع الاورام الخبيثة شيوعا بين النساء. يتم تصنيفه على وجود مستقبلات هرمون الاستروجين, ومستقبلات البروجستيرون, ومستقبل عامل نمو البشرة. يؤدي الانتشار السريع للخلايا السرطانية الى زيادة احتياجها من الطاقة, مما يؤدي الى زيادة تحلل الجلوكوز وتراكم اللاكتات. يعتبر لاكتات ديهيدرزجينيز دورا محوريا في هذه العملية, وخاصة في الاورام ذات التمثيل الغذائي الهوائي. يعزز عامل النسخ سي-مايسين تحلل الكلوكوز الهوائي عن طريق زيادة امتصاص الكلوكوز وانتاج اللاكتات, وهي الصفة المميزة لتاثير واربورغ.

الأهداف: هدفت هذه الدراسة الى تقييم مستويات التعبير عن جين السي-مايسين لدى مريضات سرطان الثدي الثلاثي الايجابي وسرطان الثدي الثلاثي السلبي, ومقارنتهن بالمجموعة الضابطة الصحية.

المرضى والطرق: شملت الدراسة ٨٠ امراه (تتراوح اعمارهن بين ٣٥ و٦٦ عاما): ٢٠ مصابة بسرطان الثدي الثلاثي الايجابي, و٢٠ مصابة بسرطان الثدي الثلاثي السلبي, و٤٠ امراه سليمة كمجموعة ضابطة متطابقة من حيث العمر والجنس ومؤشر كتلة الجسم. تم قياس مستويات السي-مايسين وجين الورم ٢٧-٢٩ باستخدام تقنية الشطيرة (الاليزا). اما مستويات لاكتات الهيدروجين, السكر التراكمي, انزيمات الكبد, ومستويات الدهون فقد تم تقييمها بتقنيات القياس الطيفي الضوئي باستخدام معدات شركة سيمنز.

النتائج: كانت مستويات السكر اعلى بشكل ملحوظ لدى مريضات سرطان الثدي (٤,٧١+٣,٧٥ نانوجرام\مل), مقارنة بالضوابط (٠,٨١+٠,٤٤ نانوجرام\مل) ص=٠,٠٠٠١. كما ارتفعت مستويات لاكتات ديهيدروجيبيز وجين الورم ٢٧-٢٩ بشكل ملحوظ (ص=٠,٠٠٠١). اظهرت المعايير الايضية, بما في ذلك الهيموكلوبين السكري, وظائف الكبد, وملف الدهون (بأستثناء البروتين الدهني عالي الكثافة), تغييرات كبيرة مع انخفاض مستوى البروتين الدهني عالي الكثافة لدى مريضات السرطان. ومن الجدير بالذكر ان مريضات سرطان الثدي الثلاثي السلبي اظهرن مستويات سي-مايسين والهيموكلوبين السكري أعلى مقارنة بمريضات سرطان الثدي الثلاثي الايجابي.

الاستنتاج: ترتبط مستويات السي-مايسين المرتفعة بأعادة برمجة التمثيل الغذائي في سرطان الثدي وقد تعمل كهدف علاجي محتمل. يرتبط التعبير الاعلى عن السي-مايسين في سرطان الثدي الثلاثي السلبي بطبيعته الاكثر عدوانية, مما يشير الى دوره في تطور الورم.

الكلمات المفتاحية: سرطان الثدي, سي-مايسين, لاكتات الهيدروجين, بسرطان الثدي الثلاثي الايجابي, سرطان الثدي الثلاثي السلبي.

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Therapeutic Effect of Curcumin Oral Gel on Salivary Epidermal Growth Factor and Lactate Dehydrogenase Levels in Relation to Oral Mucositis in Head and Neck Cancer Patients Undergoing Concurrent Chemoradiation

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Abstract

Background: Curcumin is a traditional herbal medication, which has shown promise in a variety of pharmacologic applications. Epidermal growth factor an amino acid polypeptide found in saliva and other bodily fluids, which promotes cell proliferation and renewal. Lactate dehydrogenase is classified as altered protein markers present in saliva, and its levels showed significantly elevated in head and neck cancer. To study the effect of curcumin oral gel on levels of salivary epidermal growth factor and lactate dehydrogenase in head and neck cancer patients receiving concurrent chemoradiation induced oral mucositis.

Patients and Methods: Ninety head and neck cancer patients receiving concurrent chemoradiation, 45 patients in each group. Saliva levels of lactate dehydrogenase and epidermal growth factor measured by an enzyme linked immunosorbent assay. WHO scale used to assess oral mucositis.

Results: Highly significant increase in salivary epidermal growth factor and decrease in lactate dehydrogenase levels with less severe oral mucositis in group treated with curcumin. Significant differences were found between the two groups in the WHO scale at two weeks (P = 0.041) and six weeks (P=0.02).

Conclusion: The current study concludes that curcumin oral gel can increase epidermal growth factor and reduce lactate dehydrogenase levels in saliva, and it may be used as an alternate treatment for oral mucositis-induced by chemoradiation.

Keywords: Head and neck cancer, Curcumin, Oral mucositis.

Introduction

Curcumin goes under another name, The Zingiberaceae family includes turmeric. One to two percent curcuminoids and three to twelve percent volatile oil are the two main components of the root. A phenolic compound with possible health benefits, dimethylsulfoxide is also known as curcumin (1, 2). Numerous clinical investigations have shown the extensive variety of pharmacologic capabilities

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exhibited by Curcumin oral gel. These features include the ability to enhance wound healing, anti-inflammatory, antifungal, antibacterial, and anticarcinogenic actions (3). Curcumin improves epithelialization and wound healing by protecting and activating keratinocytes while acting as a reactive oxygen species scavenger and an antioxidant (4, 5). Curcumin may potentially increase the effectiveness of morphine by decreasing pain transmission channels and promoting the production of serotonin, dopamine, and noradrenaline at large dosages (6). Head and neck cancers (HNCs) encompass a wide range of malignancies that can develop in the oral cavity, nasal cavity, paranasal sinuses, pharynx, larynx, and salivary glands (7). Chemotherapy combined with radiotherapy is the first line of treatment when head and neck cancer has spread locally (8).

Cytotoxic concurrent chemoradiotherapy causes oral mucositis (OM), an inflammatory disorder of the mouth and throat that is a major problem in oncology (9). Confluent and deep ulcers are the most severe symptoms of oral mucositis. When a patient has pain due to mucositis, it may greatly compromise a patient's functional status and quality of life (10).

Epidermal growth factor (EGF) promotes cell renewal and proliferation (11). Evidence reveals a link between low-grade clinical oral mucositis and increased epidermal growth synthesis (12). Curcumin has a notable effect on numerous growth factors, including EGF (13).

Lactate dehydrogenase (LDH) is essential enzyme catalyzed the reversible conversion of pyruvate and lactate during glycolysis and gluconeogenesis (14). Under typical, healthy conditions, lactate dehydrogenase is located in the cytoplasm of cells. LDH is released into the extracellular environment when cells are exposed to cytotoxic substances (15). Salivary LDH levels may change in several oral diseases, including malignancy, periodontitis, and gingivitis. According to reports, it is theorized that severe mucositis cases would be associated with higher LDH levels due to the cytotoxic nature of chemoradiation (16).

To our knowledge, no previous studies have studied the effect of curcumin oral gel on salivary epidermal growth factor and salivary lactate dehydrogenase levels in HNC patients undergoing concurrent chemoradation-induced oral mucositis. Therefore, this study aimed to determine the effect of curcumin oral gel on levels of salivary epidermal growth factor and lactate dehydrogenase in head and neck cancer patients under concurrent chemoradiation-induced oral mucositis.

Patients and Methods

Study design: From March 2023 to June 2024, this study was carried out. There were 90 HNC patients that took part in this study. There were two groups created: the study group and the control group. For the trial, 45 patients were given oral gel containing curcumin, whereas 45 patients were given magic-solution as a control.

Inclusion criteria: Included patients between the ages of 30 and 70, diagnosed with head and neck cancer and scheduled for concurrent chemoradiotherapy. Patients were also required to wear a head and neck mask during radiation treatments, and their oral cavity mucosa had to be within the radiation range. Chemotherapy was cisplatin 40 mg/m2 administered weekly, and radiotherapy consisted of 33 fractions scheduled five days a week for six weeks with 50-70 Gray (Gy).

Exclusion criteria: included patients receiving palliative radiation or radiation treatment alone.

Assessment of oral mucositis clinical: On the 2nd week of chemoradiation and the last day of the chemoradiation treatments, patients were examined and scored on a scale from 0 to 4 developed by the World Health Organization. With a score of 0, no symptoms are present; with a score of 1, the oral mucosa is red and

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uncomfortable; and with a score of 2, the mouth is ulcerous and makes it hard to eat normally. When score reaches 3, the ulcer has already developed and the patient is limited to drink fluids; when it reaches 4, the patient is completely unable to consume any food or liquids (17).

Curcuma longa oral gel: The subjects in the curcumin group were given Curenext®, aTn product made by (Abbott Healthcare, India), which includes 10 milligrams of Curcuma longa root extract (rhizome) per gram of gel. From the beginning with the initial saliva sample collection until their chemoradiotherapy treatment was finished, patients were told to use a cotton swab or their fingers to apply the gel three times a day (18, 19). A standard mouthwash consisting of nystatin, dexamethasone, lidocaine, and tetracycline was administered to patients in the magic-solution group.

Saliva sample collection and storage: Each of the 90 patients had three complete saliva samples taken: once before chemoradiation, once after the second week of treatment, and again at the six-week chemoradiation. Patients spat into a plastic tube that was marked with their name, group, and visit date in order to collect their unstimulated saliva. The next step was to place it in an icebox and freeze it at -80°C until analysis.

Laboratory analysis: The levels of EGF and LDH in the saliva were determined using an enzyme-linked immunosorbent assay (ELISA). In accordance with the manufacturer's instructions, commercial quantitative sandwich (ELISA) kits from Cloud-Clone Corp (CCC, USA) were used. Salivary samples were taken to determine EGF and LDH levels using phosphate-buffered saline as a negative control and a manufacturer-supplied standard curve.

Statistical analysis

Data were handled in an Excel spreadsheet. Analysis was carried out using SPSS version 22, the Statistical Package for the Social Sciences. Tests used are the Wilcoxon Signed Ranks, Bonferroni, a paired t-test, an independent t-test, and chi-square ($\chi 2$) test. A P-value below 0.05 was defined significant.

Results

The results of this study showed that when comparing the two groups according to age and sex, no statistically significant differences were found.

Salivary epidermal growth factor (EGF): The comparison between the two studied groups with respect to the EGF marker, over different experimental periods, results show that mean values increase clearly over time, and at higher levels with respect to those treated with the curcumin group (Table 1 and Figure 1).

Table (1). Summary Statistics of EGF (pg/ml) marker along studied periods of the studied groups.

Periods	Groups	No.	Mean	Std. D.	Std. E.	95% C.I. for Mean		Min.	Max.
						L.b.	U.b.		
Initiation Period (P1)	Curcumin G1	45	122.6	10.64	1.6	119.4	125.8	100.8	152.4
	Magic Solution G2	45	118.6	9.86	1.5	115.6	121.5	93.6	136.9
After 2 weeks (P2)	Curcumin G1	45	250.0	56.3	8.4	233.1	266.9	129.1	404.5
	Magic Solution G2	45	200.1	42.1	6.3	187.5	212.8	122.7	280.9
After 6 weeks (P3)	Curcumin G1	45	407.5	61.7	9.2	389.0	426.1	304.2	545.8
	Magic Solution G2	45	257.6	50.2	7.5	242.6	272.7	170.4	349.8

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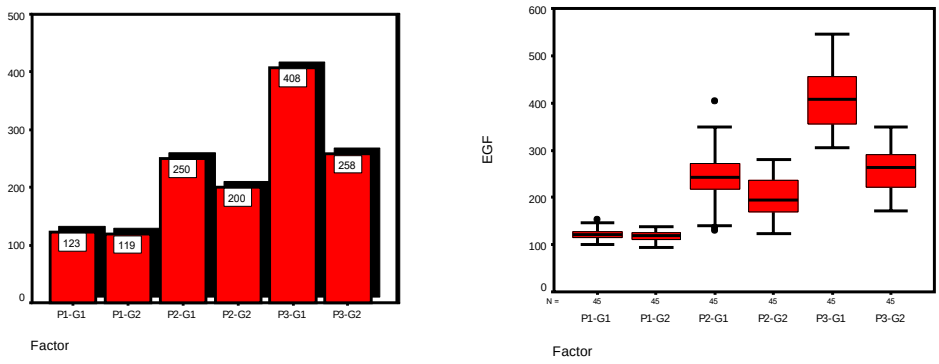

Figure 1. Stem-Leaf Plot with Bar Chart, and stem-leaf plot to explore the behavior of EGF marker readings' distribution along the study of sequential periods in each group.

Means of salivary EGF were highly significantly (P = 0.000) increased two and six weeks after chemoradiotherapy compared to that before chemoradiotherapy and six weeks after chemoradiotherapy compared to that at two weeks after chemoradiotherapy in both study groups. The increment in EGF two and six weeks after chemoradiotherapy was significantly greater in the group given curcumin than that treated with magic solution compared to that before chemoradiotherapy (Table 2).

Table 2. Significant levels for testing repeated measurements of EGF marker readings in each group independently over the sequential of studied periods.

Groups	Pairwise Comparisons		Mean Diff. (I-J)	Std. Error	Sig. Level	95% C. I. for Diff.	
	(I) EGF	(J) EGF				L.b.	U.b.
Curcumin	Initiation	After 2 w.	-127.5	10.25	0.000	-148.1	-106.8
		After 6 w.	-285.0	10.25	0.000	-307.6	-262.4
	After 2 w.	After 6 w.	-157.5	10.25	0.000	-187.2	-127.8
Magic Solution	Initiation	After 2 w.	-81.5	8.06	0.000	-97.1	-65.9
		After 6 w.	-139.1	8.06	0.000	-157.5	-120.6
	After 2 w.	After 6 w.	-57.5	8.06	0.000	-80.8	-34.2
(*) HS: Highly Significant at P<0.01; Testing are based on repeated measurers of several related groups, through using adjustment for multiple comparisons by "Bonferroni" test.							

Salivary lactate dehydrogenase (LDH): The comparison between the two studied groups with respect to the " LDH " marker, over the course of an experimental period. Results show that mean values are decreasing clearly over the time periods, and at a lower level with respect to those treated with curcumin (Table3 and Figure 2).

Table 3. Summary Statistics of LDH (ng/mL) marker along different periods of the studied groups.

Periods	Groups	No.	Mean	Std. D.	Std. E.	95% C.I. for Mean		Min.	Max.
						L.b.	U.b.		
Initiation period	Curcumin	45	5.290	0.86	0.13	5.03	5.55	3.65	6.64
	Magic Solution	45	5.286	1.07	0.16	4.96	5.61	3.03	6.97
After 2 weeks	Curcumin	45	4.04	0.42	0.06	3.92	4.17	3.18	4.74
	Magic Solution	45	5.16	0.81	0.12	4.92	5.40	3.83	7.06
After 6 weeks	Curcumin	45	3.06	0.45	0.07	2.93	3.20	2.28	3.74
	Magic Solution	45	3.49	0.78	0.12	3.26	3.72	2.01	4.64

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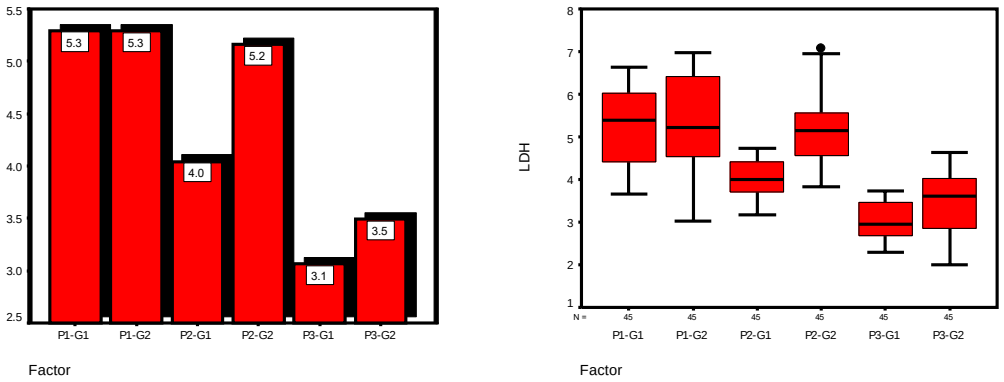

Figure 2. Bar Chart, and a stem-leaf plot for exploring the behavior of LDH marker readings' distribution along the study of sequential periods in each group.

The results in Table 4 showed that the mean salivary LDH was significantly decreased at two and six weeks after chemoradiotherapy compared to before chemoradiotherapy and at six weeks after chemoradiotherapy compared to two weeks after chemoradiotherapy in both study groups. The decrement in LDH two and six weeks after chemoradiotherapy in the patients treated with curcumin had a much greater decrease than those treated with the magic solution compared to that before chemoradiotherapy.

Table 4. Significant levels for testing the covariate of LDH (ng/mL) marker's readings in each group independently over the sequential periods.

Groups	Pairwise Comparisons		Mean Diff. (I-J)	Std. Error	Sig. Level	95% C. I. for Diff.	
	(I) LDH	(J) LDH				L.b.	U.b.
Curcumin	Initiation	After 2 w.	1.245	0.118	0.000	0.951	1.540
		After 6 w.	2.227	0.139	0.000	1.881	2.572
	After 2 w.	After 6 w.	0.981	0.062	0.000	0.826	1.136
Magic Solution	Initiation	After 2 w.	0.127	0.199	1.000	-0.368	0.621
		After 6 w.	1.797	0.192	0.000	1.318	2.275
	After 2 w.	After 6 w.	1.670	0.164	0.000	1.263	2.077
(*) HS: Highly Significant at P<0.01; NS: Non-Significant at P>0.05; Testing is based on repeated measures of several related groups, using adjustment for multiple comparisons by the "Bonferroni" test.							

Clinical evaluation of oral mucositis world health organization scale: The results in Table 5 and Figure 3 showed that during the 2-week and 6-week chemoradiation visits, the curcumin group had a lower mean WHO score than the magic-solution group with regard to oral mucositis.

Table 5. Summary Statistics of Grade of Mucositis WHO score along different periods of the studied groups.

Groups	Statistics	Periods		
		Initiation	After 2 weeks	After 6 weeks
Curcumin	Mean of Score	0.000	1.667	1.178
	Interquartile Range	0.000	1.000	1.000
	Minimum score	0.000	1.000	1.000
	Maximum score	0.000	3.000	2.000
Magic Solution	Mean of Score	0.000	1.689	1.378
	Interquartile Range	0.000	0.000	1.000
	Minimum score	0.000	1.000	1.000
	Maximum score	0.000	3.000	3.000

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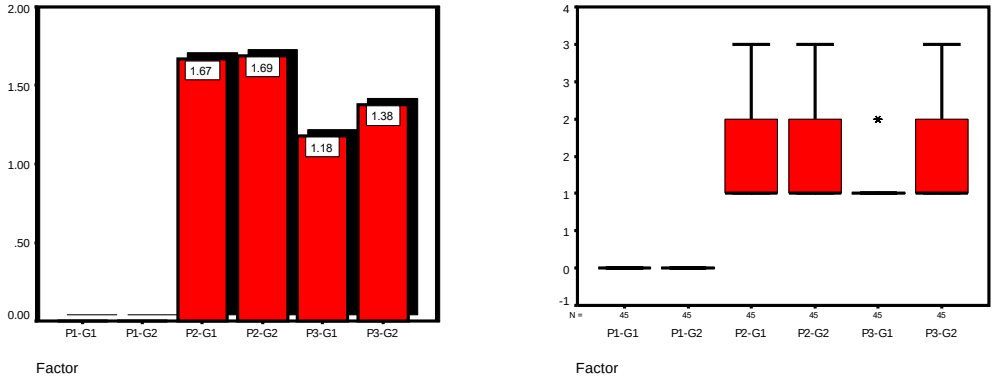

Figure 3. Bar Chart, and stem-leaf plot for exploring behavior of WHO Score reading's distribution along the studied of sequential periods in each group.

Results in Table 6 represent the WHO score's readings at a significance level of (P=0.000), there were highly significant differences with respect to all probable pairwise comparisons grade of mucositis GOM , either for curcumin or magic solution groups % independently.

Table 6. Significant levels for testing of the WHO score readings in each group independently over the sequential periods.

Groups	Pairwise Comparisons		Z-value	Sig. Level
	(I) GOM	(J) GOM		
Curcumin	Initiation	After 2 w.	-5.964	0.000
		After 6 w.	-6.283	0.000
	After 2 w.	After 6 w.	-4.491	0.000
Magic Solution	Initiation	After 2 w.	-5.970	0.000
		After 6 w.	-6.081	0.000
	After 2 w.	After 6 w.	-3.300	0.001
(*) HS: Highly Significant at P<0.01; Testing are based on the "Wilcoxon Signed Ranks" test.				

Grade of mucositis between study groups: The results in Table 7 showed the comparison in the grade of mucositis between the study group and the magic solution group after chemoradiotherapy. After two weeks, 66.7% of patients in the curcumin group were graded I compared to 42.3% in the magic solution group, with a p-value = 0.041 indicating statistical significance. After six weeks, 82.2% of patients in the curcumin group were graded I compared to 60% in the magic solution group, a statistically significant difference (P=0.02).

Table 7. Comparison between study groups by grade of mucositis.

Grade of mucositis (WHO)	Study group		X2 test	P - Value
	Curcumin (%) n= 45	Magic Solution (%) n= 45		
Two weeks after chemoradiotherapy				
1	30 (66.7)	19 (42.3)	6.351	0.041
2	11 (24.4)	15 (33.3)		
3	4 (8.9)	11 (24.4)		
Six weeks after chemoradiotherapy				
1	37 (82.2)	27 (60.0)	7.75	0.02
2	8 (17.8)	13 (28.9)		
3	0 (0)	5 (11.1)		
WHO: World Health Organization. $\chi 2$: chi-square test.				

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Discussion

Since it is simpler to apply, absorbs quickly, topical curcumin treatment, in the form of an oral gel, offers several benefits over systemic curcumin because it interacts with surrounding tissues, prolonging the contact period that increases its benefits, and because it has fewer evident bad effects. Patients with dysphagia or gastrointestinal issues may potentially benefit from oral gel formulations in reducing undesirable effects (17-20).

Concurrent chemoradiotherapy causes basal epithelial cell death, which may occur as a result of free radical production. These free radicals activate second messengers which carry messages from the cellular surface receptors to the inner cell environment, resulting in increased production of pro-inflammatory cytokines, tissue damage, and cell death (21).

In this study the majority of patients using curcumin group experienced only mild mucositis grade1 at the end of the chemoradiotherapy sessions; a few had grades 2 but none had severe mucositis (grades 3 and 4) whereas patients in magic solution group experienced grade 2 and grade 3 mucositis. The results agree with results of Alsalim et al., 2024, most patients treated with curcumin did not have any mucositis (grade 0) throughout their radiation treatments and small number of patients did experience mild mucositis (grades 1 and 2), but none of them had severe mucositis (grades 3 and 4) (22). A study done by Arun et al., 2020 revealed that the majority of patients in the curcumin group experienced only grade 1 mucositis after four weeks of treatment (23). Also, our findings are in

agreement with those of the Shah trial, which also indicated that, Grade 3 mucositis did not occur in the curcumin group, unlike the control group (1). Additionally, Patil's research showed that the two groups' WHO ratings were significantly different (1, 24). Oral mucositis caused by chemoradiation was less severe in HNC patients treated with curcuma long a gel compared to placebo gel (19), chlorhexidine gel (18).

Salivary EGF levels in HNC patients treated with chemoradiotherapy showed that lower EGF levels during treatment exacerbated OM severity, perhaps as a result of reduced cell proliferation and suppression of mucosal repair (25). In this study, when curcumin oral gel was applied a significant increase in salivary EGF after chemoradiation compared to before, as well as a reduction in the severity of OM, provide evidence that EGF may aid in speed up the healing process after chemoradiation damage to the mucosa and promote the oral mucosa's recovery (26). The enhanced salivary EGF and improved wound healing that occurred in the curcumin group may have been caused by curcumin's anti-inflammatory and combined antibacterial characteristics. Curcumin has many effects that promote healing and tissue remodeling, including promoting epithelization, restoring collagen architecture, and accelerating angiogenesis (27). These findings are in line with those of the research conducted by Alsalim et al. in 2024, which shown that use of curcumin oral gel significantly reduced the severity of OM and resulted in a significant increase in salivary EGF after radiation compared to before radiation (22).

Cancer cells often exhibit elevated levels of LDH activity, which facilitates promote uncontrolled cell proliferation and migration, especially those in hypoxic conditions (28). Patients with HNC had

significantly higher salivary LDH levels, according to previous studies (29, 30). Radiation exposure was linked to higher levels of salivary LDH, which in turn exacerbated the severity of oral mucositis, according to a study by Shivashankara et al. 2019 (31).

Also, in this study, when curcumin oral gel was applied, a significant decrease in salivary LDH after

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chemoradiation compared to before, and the severity of OM was reduced, suggesting that curcumin exhibits anti-oxidant properties and helps in the prevention of free radicals and toxic products resulting from oxidative stress, which contribute to cancer development. According to preclinical studies, curcumin inhibits reactive oxygen species (ROS) and free radicals, which protect DNA from damage by oxidative stress. This stress may be induced by ionizing radiation and other oxidative causes (32). The nuclear factor – kappaB NF κ-B plays an important role in the production of oxidative stress and nitric oxide synthase, which may cause cancer. Curcumin inhibits NF-kappaB production, which in turn suppresses the development of cancer growth (32). It has shown anti-cancer benefits under concurrent chemotherapy and radiotherapy .Studies have shown curcumin reduces reactive oxygen species (ROS) levels and decrease lactate dehydrogenase (LDH) release (33). According to Han et al., 2023, curcumin shows promise as a cancer treatment (34). Study by Chandrashekar et al., 2021 showed that patients with oral submucous fibrosis OSMF had significantly reduced LDH levels after treatment with curcumin oral gel (35). No previous studies have evaluated the therapeutic effect of curcumin oral gel on salivary LDH levels in HNC patients under concurrent chemoradiotherapy-induced oral mucositis.

Conclusions

Significant increase in the level of salivary EGF and significant reduction in salivary LDH level in HNC patients undergoing concurrent chemoradiotherapy after using topical curcumin oral gel compared to the magic solution, suggesting that it is effective and could be used as an alternative treatment in preventing and managing oral mucositis caused by concurrent chemoradiation. In addition, it's recommended for head and neck cancer patients to use curcumin oral gel as a preventive agent for chemoradiation-induced oral mucositis before concurrent chemoradiotherapy.

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Ethical clearance: This study was approved by a protocol number 934724 by the Research Ethics Committee of the University of Baghdad, College of Dentistry, Iraq.

Conflict of interest: None.

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التأثير العلاجي لهلام الكركمين الفموي على مستويات عامل نمو البشرة اللعابي ونازعة هيدروجين اللاكتات وعلاقته بالتهاب الغشاء المخاطي الفموي لدى مرضى سرطان الرأس والرقبة الذين يخضعون للعلاج الكيميائي الإشعاعي المتزامن

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الملخص

خلفية الدراسة: الكركمين هو دواء عشبي تقليدي أظهر نتائج واعدة في مجموعة متنوعة من التطبيقات الدوائية. عامل نمو البشرة هو حمض أميني متعدد الببتيد موجود في اللعاب وغيره من سوائل الجسم، والذي يعزز تكاثر الخلايا وتجددها. نازعة هيدروجين اللاكتات يصنف على أنه علامات بروتينية متغيرة موجودة في اللعاب، وقد أظهرت مستوياته ارتفاعًا ملحوظًا في سرطان الرأس والرقبة.

اهداف الدراسة: دراسة تأثير هلام الكركمين الفموي على مستويات عامل نمو البشرة اللعابي و نازعة هيدروجين اللاكتات في مرضى سرطان الرأس والرقبة الذين يتلقون العلاج الكيميائي الإشعاعي المتزامن الناجم عنه التهاب الغشاء المخاطي الفموي.

المرضى والطرائق: تسعون مريضًا بسرطان الرأس والرقبة يتلقون علاجًا كيميائيًا إشعاعيًا متزامنًا، ٤٥ مريضًا في كل مجموعة. تم قياس مستويات اللعاب من نازعة هيدروجين اللاكتات وعامل نمو البشرة باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم .مقياس منظمة الصحة العالمية استخدم لتقييم التهاب الغشاء المخاطي الفموي.

النتائج: زيادة كبيرة في عامل نمو البشرة اللعابي وانخفاض في مستويات نازعة هيدروجين اللاكتات مع أقل حدة في التهاب الغشاء المخاطي الفموي في المجموعة المعالجة بالكركمين. تم العثور على اختلافات كبيرة بين المجموعتين في مقياس منظمة الصحة العالمية عند أسبوعين (**P = 0.041**) و ٦ أسابيع (**P = 0.02**).

الاستنتاجات: خلصت الدراسة الحالية إلى أن هلام الكركمين الفموي يمكن أن يزيد من عامل نمو البشرة ويقلل من مستويات نازعة هيدروجين اللاكتات في اللعاب ، ويمكن استخدامه كعلاج بديل لالتهاب الغشاء المخاطي الفموي الناجم عن العلاج الكيميائي والإشعاعي.

الكلمات المفتاحية: سرطان الرأس والرقبة، الكركمين، التهاب الغشاء المخاطي الفموي.

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Estimation of Sodium, Potassium, and Calcium Serum in Patients with Asphyxiated Neonates

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Abstract

Background: Birth asphyxia is a critical neonatal condition characterized by the failure to initiate and sustain breathing at birth, often due to impaired cerebral blood flow. Electrolyte imbalances may contribute significantly to its morbidity and mortality.

Objectives: To assess the serum sodium, potassium, and calcium levels in neonates with birth asphyxia of varying severities and healthy controls.

Patients and Methods: A hospital-based cross-sectional study was conducted at Al-Batool Teaching Hospital in Diyala, Iraq, from June to September 2023. A total of 200 term neonates were enrolled, 80 with birth asphyxia (defined by an Apgar score <7 at 5 minutes) and 120 healthy neonates as controls. Blood samples were collected within the first 24 hours of life, and serum levels of sodium, potassium, and calcium were measured using standard photometric methods.

Results: Hyponatremia (<130 mmol/L) was observed in 51.3% of asphyxiated neonates. Hyperkalemia (>5.2 mmol/L) was found in 50% and hypocalcemia (<2.2 mmol/L) in 55% of cases. Compared to controls, asphyxiated neonates had significantly higher potassium levels ($p < 0.05$) and lower calcium levels ($p < 0.05$); however, differences in sodium levels were not statistically significant ($p > 0.05$). Electrolyte abnormalities were more pronounced in neonates with altered tone, sucking, respiration, and heart rate.

Conclusion: Hyponatremia, hyperkalemia, and hypocalcemia are prevalent in neonates with birth asphyxia and correlate with clinical severity. Early identification and management of these disturbances are vital to improving outcomes and reducing neonatal morbidity and mortality.

Keywords: Birth asphyxia, Neonate, Hyponatremia, Hyperkalemia, Hypocalcemia.

Introduction

Asphyxia in a neonate is defined as failure to regulate breathing at birth. Many conditions can affect an asphyxiated baby's birth, but the underlying etiology is decreased blood flow to the brain (1). Birth asphyxia (BA) can cause a series of reactions resulting in changes in brain function known as hypoxic-ischemic encephalopathy. The likelihood of outcomes for surviving birth asphyxia varies widely, from normal outcomes to death, with a variety of disabilities in between, including long-term neurodevelopmental disabilities, cerebral palsy, neuromotor delays, and developmental delays. Treatment of hypoxic-ischemic encephalopathy centres on dampening or blocking biochemical pathways that cause nerve cell death (2,3). The brain has limited sources of stored energy and relies on adequate blood flow to extract the needed energy supplies for neuronal cells. With reduced blood flow, neuronal cells cannot extract enough glucose to convert to energy-storing adenosine triphosphate (ATP). This decrease in ATP stimulates a cascade of biochemical reactions that lead to

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early neuronal cell death via ischemia and necrosis (primary energy failure) or cell death via apoptosis (secondary energy failure). These reactions involve the destruction of cell membrane potentials as ATP levels decrease. Consequently, the control of ion movement across the cell membrane is impaired (4). Accumulations of intracellular calcium, sodium, chloride, and water reach toxic levels, and the level of the excitatory neurotransmitters is elevated at the synaptic junction. In a biological system, sodium, potassium, and calcium are the most important electrolytes, and any significant fluctuations in the blood concentrations can lead to metabolic derangements, causing convulsions and shock (5). Tight regulation and maintenance of normal blood concentrations of these electrolytes are essential for optimal functioning of the body. Assessment and management of electrolyte status in the newborn is a very crucial and challenging task. Water and electrolyte levels in the body can vary widely during the transition from fetal to newborn life. Before birth, the fetus receives the nutrients, including fluid and electrolytes, from the maternal blood, and their levels are predominantly controlled by the maternal regulatory system (4,5). The three main electrolytes in the human body are sodium, potassium, and calcium. Any change in these minerals' typical blood levels can result in convulsions, shock, and other metabolic disorders. Calcium is a crucial second messenger in the body that also aids in muscular contraction and serves as a cofactor for a number of enzyme processes (6). In addition, transitory alterations in the fluid and electrolyte levels can be expected, and even a minor change in the absolute concentrations of these electrolytes can suggest proportionately substantial variation for the newborn considering its relatively small size (7). Therefore, this study aimed to determine the serum sodium, potassium, and calcium levels in neonates with birth asphyxia.

Patients and Methods

Study design and sample collection: This was a hospital-based cross-sectional study conducted from the first of June to the 30th of September 2023, in the department of Pediatrics at Al-batool Teaching Hospital (neonatal Care unite and operating room) serum sodium (Na) and potassium (K) were measured in 80 term asphyxiated newborn (low Apgar score at 5 minute) and 120 healthy Newborn babies immediately after birth. A detailed antenatal and postnatal history was taken, and the findings were recorded using a well-prepared questionnaire. Electrolyte estimation (serum sodium, potassium, and calcium) was analyzed using a selective electrode by an automated machine. The cases were collected according to early signs of birth asphyxia (Tone, LOC, Moro Reflex, Sucking Reflex, HR, and RR) and applying inclusion and exclusion criteria.

Inclusion criteria: (neonate born admitted with appropriate gestational age and birth weight of 2.5-4 kg) who has birth asphyxia according to WHO, defined as failure to initiate and sustain breathing at birth, and also based on an APGAR score of less than 7 at 5 minutes of life, even after resuscitation according to NRP guidelines.

Exclusion criteria: Individuals with intrauterine growth restriction (IUGR) and those with gross congenital malformations were excluded from the study.

Determination of sodium: Sodium is estimated by the colorimetric method based on the modified Maruna and Trinder method. Sodium and proteins are precipitated together by magnesium uranyl acetate as uranyl magnesium sodium acetate salt. The intensity of the colour is measured photo metrically at 530 nm (500-546 nm). Precipitation shake vigorously and incubate at room temperature for 5 min, then centrifuge at 2000-3000 rpm for 2 min to obtain a clear supernatant, and then transfer the supernatant immediately after centrifugation for standard and test. Sodium

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RI which is consist of precipitating REA standard (1 ml) and test (1 ml) while from the sodium standard, it used 10 ml of standard and 10 ml from the serum of the test. Sodium estimation was done by sodium RI precipitating in blank (1 ml) and in standard (1 ml) and test (1 ml), sodium standard in blank and standard (20 ml) and in test (20 ml) and in serum (20 ml), then they mixed well and allow to stand at room temperature for 5 min, then it was measured the absorbance of standard and sample against reagent blank calculation; sodium concentration (mmol)= Abs of test – Abs of blank/ Abs of standard – Abs of blank*standard concentration (8).

Determination of potassium: Potassium is estimated using the turbidimetric method (9). The extent of turbidity is proportional to the potassium concentration. It is measured photometrically at 578 nm (570-620 nm). Potassium RI (precipitate Rea) standard (1000 ml) and test (1000 ml) and standard (10 ml) and in sample and 25 ml in test, mixed well and allow it to stand at room temperature for 5 min and measure the absorbance of standard and sample against distal water within 10 min, calculation; potassium concentration (mmol) =Abs of sample \Abs of standard *5.

Determination of calcium: Calcium OCPC procedure is based on the reaction of calcium ions (Ca+) with O-cresolphthalein complex in an alkaline solution to form an intense viol coloured complex which shows maximum absorbance at 578 nm. Reagent in blank (1000 µL), standard (1000 µL), and in test (1000 µL), and ca standard in blank and 10 mL in standard and test and sample in blank and in standard and 10 mL in test. The calculation for Calcium concentration (mmol) = Abs of sample/ Abs of standard *10 (10).

Statistical analysis

The statistical analysis was performed using XLSTAT version 2019. Student's t-test determined the normality of distribution.

Results

Percentage of electrolyte concentrations in asphyxiated neonates: Patients divided into subgroups according to: gender, Tone, LOC, Moro Reflex, Sucking, Respiration and Heart Rate.

The baseline characteristics of the cases showed that: 51.3% had sodium concentration <130mmol, 31.3% had sodium concentration 130_146, and 17.4% had > 146, 7.5%had potassium concentration <3.5mmol, 41.3% had potassium concentration 3.3-5.5mmol, 50% had potassium concentration > 5.2, 55% had a calcium concentration <2.2 mmol, and 45% had a calcium concentration of 2.2-2.7 (Table 1).

Table 1. Percentage of electrolyte concentrations in asphyxiated newborns.

Parameter	Conc.	n	%
Sodium mmol/L	<130	41	51.3
	130-146	25	31.3
	> 146	14	17.4
Potassium mmol/L	<3.3	7	7.5
	3.3-5.2	33	41.3
	>5.2	40	50.0
Calcium mmol/L	<2.2	44	55.0
	2.2-2.7	36	45.0

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Total electrolyte concentrations in the studied groups: The mean (±SD) of total electrolyte concentrations in the serum of the control group (healthy individuals) and the patients is illustrated in Table 2.

Table 2. Total electrolytes concentrations in studied groups, which demonstrated as mean ±SD.

Groups	Sodium mmol/L	Potassium mmol/L	Calcium mmol/L
Total Patients n=80	131.26±11.12	3.24±0.38	8.19±0.80
Total Control n=120	132.2±10.18	2.70±0.40	8.75±0.75
P value	>0.05	<0.05	<0.05

Effect of gender on electrolytes concentrations: The mean (±SD) of electrolytes concentrations in the serum of patients and control groups according to gender are illustrated in Table 3.

Table 3. Mean ±SD of electrolyte concentrations according to sex.

According to Sex					
Groups		%	Sodium mmol/L	Potassium mmol/L	Calcium mmol/L
Patients n=80	Male n=50	(62.5%)	11.79±8.9	2.77±0.36	8.41±0.74
	Female n=30	(37.5%)	128.3±10.9	2.68±0.41	8.96±0.64
control n=120	Male n=78	(65%)	130.0±11.2	3.60±0.39	8.19±0.73
	Female n=42	(35%)	133.9±9.89	2.80±0.37	0.803±0.79
P value					
Parameters		**Male/Female Control**	**Male/Female Patients**	**Male Control / MalePatients**	**Female Control / FemalePatients**
Sodium		>0.05	>0.05	>0.05	>0.05
Potassium		>0.05	<0.05	>0.05	>0.05
Calcium		>0.05	>0.05	>0.05	>0.05

Electrolytes concentrations according to Tone and LOC: The study included mean (±SD) of electrolytes concentrations in serum of patients groups according to early clinical sign. The mean (±SD) of electrolytes concentrations in serum of patients groups according to Tone and LOC signs are illustrated in Table (4).

Table 4. Mean ±SD of electrolytes concentrations according to Tone and LOC signs.

Study	Groups	Sodium mmol/L	Potassium mmol/L	Calcium mmol/L
Tone n=80	Flaccid (n=20) (25%)	129.7±10.73	3.17±0.46	8.07±0.65
	Hypotonic (n=60) (75%)	133.2±14.25	3.30±0.31	8.12±0.84

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P value		>0.05	>0.05	>0.05
LOC n=80	Alert or irritable n=15 (18.75%)	134.52±10.7	3.31±0.33	7.90±0.82
	Comatose (n=20) (25%)	131.33±7.51	3.23±0.32	8.12±0.66
	Lethargy (n=45) (56.25%)	132.81±13.5	3.30±0.31	8.38±0.89
P value	**Parameters**	**Alert or irritable/Lethargy**	**Comatose/ Lethargy**	**Alert or irritable/Comatose**
	Sodium	>0.05	>0.05	>0.05
	Potassium	>0.05	>0.05	>0.05
	Calcium	<0.05	>0.05	>0.05

Electrolytes concentrations according to Moro reflex and sucking signs: The study included mean (±SD) of electrolytes concentrations in serum of patients groups according to Moro reflex and sucking signs are illustrated in Table 5.

Table 5. Mean ±SD of electrolytes concentrations according to Moro reflex and sucking signs.

Study	**Groups**	**Sodium mmol/L**	**Potassium mmol/L**	**Calcium mmol/L**
Moro Reflex n=80	Absent (n=15) (18.75%)	133.5±7.70	3.19±0.31	8.12±0.75
	Weak (n=65) (81.25%)	131.65±11.32	3.23±0.35	0.90
P value		>0.05	>0.05	>0.05
Sucking n=80	Absent (n=21) (26.25%)	128.5±11.18	5.8±0.33	8.10±0.78
	Unable to suck (n=30) (27.5%)	131.0±6.19	3.11±0.39	8.14±0.72
	Weak (n=29) (36.25%)	138.5±13.18	3.31±0.38	8.21±0.73
P value	**Parameters**	**Absent/ Unable to suck**	**Absent/ Weak**	**Unable to suck/ weak**
	Sodium	>0.05	>0.05	>0.05
	Potassium	>0.05	<0.05	>0.05
	Calcium	>0.05	>0.05	>0.05

Electrolyte concentrations according to respiration and heart rate: The study included the mean (±SD) of electrolyte concentrations in the serum of patient groups according to respiration and heart rate signs, which were illustrated in Table 6.

Table 6. Mean ±SD of electrolytes concentrations according to respiration and heart rate signs.

Study	**Groups**	**Sodium mmol/L**	**Potassium mmol/L**	**Calcium mmol/L**
Respiration n=80	Apneic (n=37) (46.25%)	128.9±12.0	5.4±0.76	8.10±0.89
	Periodic (n=43) (53.75%)	134.2±14.45	3.55±0.78	8.21±1.33
P value		>0.05	>0.05	>0.05

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Heart Rate n=80	Bradycardia and Tachycardia n=28 (35%)	129.1±11.23	5.6±0.44	8.09±0.64
	Normal n=52(65%)	134.7±13.48	3.6±0.41	8.21±0.86
P value		>0.05	<0.05	>0.05

Correlations study: Correlation Coefficient (r) is measure the association between two variables to same sample. The values of r above 0.38 have stronger correlation (Table 7).

Table 7. Correlation Coefficient between parameters.

Correlation coefficient (r)			
Groups	**Parameters**	**Sodium mmol/L**	**Potassium mmol/L**
Patients	Potassium	0.20	-
	Calcium	0.313	0.485
Control	Potassium	0.023	-
	Calcium	0.234	0.342
Male Control	Potassium	-0.004	-
	Calcium	0.21	0.35
Female Control	Potassium	0.086	-
	Calcium	0.26	0.32
Male Patients	Potassium	0.17	-
	Calcium	0.34	0.48
Female Patients	Potassium	0.31	-
	Calcium	0.29	0.49

Discussion

There was no significant difference in the serum sodium levels between the control and patient groups. At the same time, there was a substantial increase in the serum levels of potassium, and a significant decrease in the serum levels of calcium in the patient groups when compared with the control group. These results are different from other study that found, hyponatremia in 34% of neonates, hypernatremia in 0% of asphyxiated neonates, hyperkalaemia in 0% and found hypocalcaemia observed in 4 (8%) asphyxiated neonates, compared to 2 (4%) cases in comparison group, which is statistically not significant, but proportionally, it is comparable to other studies in this issue in newborns (11). It was shown that specific symptoms of electrolyte abnormalities commonly coexist with indicators of underlying hypoxic ischemic encephalopathy, or HIE, and the use of fluid and electrolytes in such cases increases morbidity and death. The results disagreed with those of Lackmann et al. (12), who measured potassium levels in 98 asphyxiated newborns, and none of them showed significant hyperkalaemia in the initial 144 h of life. *Basu et al.* (13) concluded that decreased calcium levels are associated with increased severity of birth asphyxia. In case–control study by *Jajoo et al.* (14), and *Rai et al.* (15) they established lower serum calcium level in asphyxiated newborns compared to their controls, and in disagreement with a case control study by *Varma V et al.* (16) among asphyxiated newborns, mean values of electrolytes

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showed no significant difference among cases and controls. Hyperkalaemia can be explained by the fact that asphyxia is associated with acidosis, and in metabolic acidosis, more than half of the excess hydrogen ions are buffered in the cells. In this setting, electro-neutrality is maintained partially by the movement of intracellular potassium into the extracellular fluid. It can also be due to acute renal failure secondary to birth asphyxia, which leads to reduced excretion of potassium and hence hyperkalaemia (17). The modification of potassium from the intracellular to extracellular space in early neonatal period may lead to hyperkalaemia and depends on the degree of immaturity; further premature babies are more possibly to have hyperkalaemia. Acute renal failure secondary to asphyxia causes hyperkalaemia by declining the elimination of potassium (18). Normally, gestational age is directly proportional to cord plasma total calcium concentration. At the time of delivery, unexpected cessation of calcium transport through the placenta decreases the serum calcium levels, which in turn leads to augmented secretion of serum parathyroid hormone [PTH] (19).

In this study, it was shown that there were no significant differences in the serum level of sodium in age groups, except a significant decrease in the serum levels of potassium in female patients group compared with male patients group. In addition, in the current study it was illustrated that among enrolled patients, males predominated (65%) while female (35%). These results were agreement with the other studies by Ahmed N et al (20), who reported that the percentage of males was 64%, and Bahatkar and Aundhakar were found 72% of patients were males (21). Furthermore, many studies were agreed with our study that showed predominated male (54.3%) (13, 22). The difference in the percentage between male and female could be due to differences in time, area of the study, and registration of neonatal data. In addition, other reason for male babies being more affected is due to the death of respiratory control neurons in brainstem which mediates the function of emergency resuscitation in male gender (20).

Conclusions

According to the results of study was conducted that included hyponatremia, hypocalcaemia and hyperkalaemia occur in neonates with birth asphyxia which may cause increased morbidity and mortality, and its percentage in male more than in female. In addition, serum sodium levels in the asphyxiated newborns were in the hyponatremic range and in proportion to the severity of asphyxia. As serum sodium levels are low in birth asphyxia, fluids must be managed judiciously in asphyxiated newborns. The study findings revealed that birth asphyxia was more common in irregular or no neonatal care cases. It was recommended to follow up on optimal electrolyte disturbances, which are essential to improve outcomes and prevent life-threatening events.

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Ethical clearance: Ethical approval for this study was obtained from the Research Ethical Committee of the College of Medicine/ University of Diyala (No:2023DTA798)

Conflict of interest: None.

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تقدير مصل الصوديوم والبوتاسيوم والكالسيوم لدى حديثي الولادة المختنقين

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الملخص

الخلفية: يعرف الاختناق عند الأطفال حديثي الولادة بأنه الفشل في تنظيم التنفس عند الولادة. يمكن أن تؤثر العديد من الحالات على ولادة طفل مختنق، ولكن المسببات الأساسية هي انخفاض تدفق الدم إلى الدماغ. يمكن أن يسبب الاختناق الولادي (**BA**) سلسلة من التفاعلات تؤدي إلى تغيرات في وظائف المخ المعروفة باسم اعتلال الدماغ بنقص الاوكسجين.

الأهداف: قياس مستوى الصوديوم والبوتاسيوم والكالسيوم في الدم عند حديثي الولادة المختنقين بدرجات مختلفة في فترة مابعد الولاده مع مجموعة المولودين حديثين الولادة.

المرضى والطرق: هذه الدراسة مقطعية وتم إجراؤها في. مستشفى البتول التعليمي في محافظة ديالى من ١ يونيو إلى ٣٠ سبتمبر ٢٠٢٣ , توخذ عينات من الدم من ٢٠٠ طفل حديثي الولادة في وحدة رعاية الأطفال حديثي الولادة وغرفة العمليات لقياس مستوى المصل بالكهرباء. تم تضمين مجموعه ٨٠ من حديثي الولادة المختنقين و١٢٠ عينة من الأطفال حديثي الولادة الاصحاء ، وتم سحب دم من الأوردة ٥ مل. من المريض في أنبوب هلام لاختبار الكيمياء الحيوية تتمثل النتائج التي تم قياسها بتأثيرمجموعة من العوامل حسب الجنس ، الموضع، منعكس مورو. المص، التنفس، معدل ضربات القلب.

النتائج: ظهرت الدراسة للحالات ١,٣٪ من الأشخاص المصابين بالمرض كان تركيز الصوديوم أقل من ١٣٠ أقل من ٣١,٣٪. كان تركيز الصوديوم ١٣٠-١٤٦ و ١٧,٤٪ كان > ١٤٦ بينما ٥٠٪ من حالات حديثي الولادة كان تركيز البوتاسيوم أقل من ٣,٥ ملم، ٤١,٣٪. كان تركيز البوتاسيوم ٣,٣-٥,٥ ملم و ٧,٥٪ مل مول و ٦ ملم ٥٥٪. من الحالات المختنقة كان تركيز الكالسيوم أقل من ٢,٢ مل مول، ٤٥٪ منها كان تركيز الكالسيوم فيها ٢,٢,٢,٧.

الاستنتاج: نقص صوديوم الدم، نقص كالسيوم الدم وفرط بوتاسيوم الدم يحدث عند حديثي الولادة المصابين بالاختناق عند الولادة مما قد يسبب زيادة في معدلات المرضى والوفيات. كانت مستويات الصوديوم في الدم عند الأطفال حديثي الولادة المختنقين في نطاق نقص صوديوم الدم ومتناسبة مع شدة الاختناق. بما أن مستويات الصوديوم في الدم تكون منخفضة في حالة الاختناق الولادي، فيجب إدارة السوائل بحكمة عند الأطفال حديثي الولادة المختنقين. وكشفت نتائج الدراسة أن حالات الاختناق الولادي كانت أكثر شيوعاً في حالات رعاية الأطفال حديثي الولادة غير المنتظمة أو التي لم توجد على الإطلاق.

الكلمات المفتاحية: اختناق الولادة، حديثي الولادة، نقص صوديوم الدم، فرط بوتاسيوم الدم، نقص كالسيوم الدم.

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Type 3 Secretion System Virulotypes in Clinical Isolates of Multidrug Resistant *Pseudomonas aeruginosa*

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Abstract

Background: Multidrug-resistant *Pseudomonas aeruginosa* has epidemiological impact on human health. It poses a threat to the health systems of the world including Iraq. Type 3 Secretion System effectors are among the several virulence factors that this bacterium possess. Determining the virulence profile is essential in prevention of infection. This study investigates the frequency of the four classical Type 3 Secretion System effectors in multidrug resistant *Pseudomonas aeruginosa*.

Patients and Methods: This study initially included 120 bacterial isolates from different clinical samples which were preliminary identified as *P. aeruginosa.* Out of those, 80 isolates were confirmed to be *P. aeruginosa*. Antibiotic susceptibility profile was studied and the existence of *exoY, exoT, exoS and exoU* was investigated by PCR.

Results: 95%, 3.75%, and 1.25% of the isolates were classified as multidrug resistant, extensive drug resistant, and pan drug resistant, respectively. Among the selected isolates, *exoT* was found in 86.7%, *exoY* in 76.7%, *exoS* in 50% and, *exoU* in 30%.

Conclusion: This study highlights an increase in the emergence of multidrug resistant profiles in clinical isolates of *P. aeruginosa*, besides the co-existence of the four classical Type 3 Secretion System effectors in variable frequencies (86.7%, 76.7%, 50%, and 30%, respectively).

Keywords: *P. aeruginosa*, Clinical isolates, T3SS, MDR.

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic bacterium known for its ability to overcome the host immune response and thereby cause severe cellular damage. *P. aeruginosa* is extremely challenging to treat due to the frequent occurrence of antibiotic resistance and persistent colonisation on humid surfaces. *P. aeruginosa* employs a range of intrinsic and acquired resistance mechanisms, including antibiotic inactivation, drug target modification, attenuation of membrane permeability, expression of efflux systems, biofilm formation, and quorum sensing, to achieve a remarkably high level of antibiotic resistance (1). Infections caused by *P. aeruginosa* include pneumonia, burns, urinary tract infection, sepsis, nephrotic syndrome and wound infection (2-8). Nine different secretion systems (T1SS to T9SS) have been identified in bacteria, most of which are distributed in Gram-negative bacteria (9). These systems can either inject molecules into bacterial

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cells or transport molecules from inside bacteria to the extracellular environment. These systems play a pivotal role in bacterial survival in harsh environments and contribute significantly to evasion of the host's immune system (10). The type III secretion system (T3SS), one of *P. aeruginosa's* several secreted virulence factors (toxins, siderophores, proteases, and polysaccharides) (11), contributes to host cell damage. The T3SS's potential to attack the host immune response is demonstrated by its use of a needle-like structure to identify eukaryotic cells and inject toxins directly into their cytoplasm (12). Although several of the nine secretion systems have been identified in this microbe, the T3SS is the most characterized one in human infections. The T3SS plays a prominent role in bacterial pathogenesis by injecting several products known as effectors, which alter the host's signal transduction and actin cytoskeletal pathways, thereby contributing to colonization and replication in host cells (13). T3SS constitute of five functional parts; needle structure (which connects the bacterium with hosts cells), a structure responsible for the injection of effectors into host cells known as the translocation apparatus; 25 genes involved regulation known as the regulatory system; a group of small proteins (chaperones) whose function is to interact with secretions of the needle structure and prevent premature aggregation with bacterial cytoplasm and effectors. Four effectors (ExoS, ExoT, ExoU, and ExoY) have been described in *P. aeruginosa* (14). However, Burstein et al. (2015) found two additional effector proteins (PemA and PemB) in *P. aeruginosa* (15). Nevertheless, the exact role of these two newly described T3SS effectors has yet to be fully elucidated. Nolasco-Romero et al. (2024) identified 11 virulotypes in *P. aeruginosa* based on the presence or absence of T3SS, suggesting that these virulotypes can be linked to the sample type, in addition to playing a role in predicting patients' prognosis (16).

Due to the relevance of T3SS in *P. aeruginosa* clinical infections, this study aimed to investigate the prevalence and virulotypes of the four classical T3SS effectors in *P. aeruginosa* isolated from various clinical samples. In addition, this is the first study to investigate all four classical T3SS effectors in Thi-qar city, Iraq.

Patients and Methods

Study design: A total of 120 bacterial isolates whose preliminary diagnosis referred to *Pseudomonas* species were obtained from different clinical samples (sputum, wound swabs, ear swabs, burn swabs, and bronchoalveolar lavage (BAL)) in Al-Nasiriyah Teaching Hospital, Al-Haboubi Teaching Hospital, and Mohammed Al-Moussawi Children's Hospital, during the period from January 2023 to June 2023. Inclusion criteria included patients whose preliminary cultural results referred to the identification of Pseudomonas species and had not yet been administered antibiotics, while exclusion criteria included patients who had already been administered antibiotics.

Identification of bacterial isolates: Colony morphology, fruity odor, gram staining, inability to ferment lactose, catalase test (positive), oxidase test (positive), indole, methyl red, Voges-Proskauer, citrate utilization, and the ability to grow at 42°C were used for primary diagnosis. Subsequently, the isolates were sub-cultured on a selective medium (cetrimide agar, HiMedia) to confirm the diagnosis of the target microbe.

Antibiotic sensitivity tests: To classify the isolates included in the study according to the type of drug resistance, *P. aeruginosa* isolates were tested on Muller-Hinton agar for their sensitivity to 10 commonly used antibiotics by HiMedia-India; Amikacin (10µg), Gentamicin (10µg), Meropenem (10µg), Imipenem (10µg), Ceftazidime (30µg), Cefepime (10µg), Ciprofloxacin (5µg), Piperacillin (100 µg),

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Aztreonem (30 µg) and Colistin Sulphate (10µg) using the Kirby bauer method. The results were recorded by measuring the inhibition zone (in millimeters) and interpreted in accordance with the Clinical and Laboratory Standards Institute document (13). This bacterium was considered multidrug resistant (MDR), extensively drug resistant (XDR), and pan-drug resistant (PDR) based on the criteria previously described (14).

Genomic DNA extraction and polymerase chain reaction: The genomic DNA of *P. aeruginosa* was extracted from the bacterial growth of thirty randomly selected MDR isolates according to the protocol of FavorPrep Total DNA Mini Kit (FAVORGEN / Korea). Then, the presence of the four classical T3SS genes was investigated using conventional PCR with the Applied Biosystems ProFlex PCR System (Fisher Scientific, USA) and a set of previously published primers (15) listed in Table 1, along with the GoTaq Green Master Mix (Promega, USA).

Table 1. The primers used in this study.

Primer	Primer sequence 5'- - 3'		Size of Product (bp)
exoT	F	AATCGCCGTCCAACTGCATGCG	152
	R	TGTTCGCCGAGGTACTGCTC	
exoY	F	CGGATTCTATGGCAGGGAGG	289
	R	GCCCTTGATGCACTCGACCA	
exoU	F	CCGTTGTGGTGCCGTTGAAG	134
	R	CCAGATGTTCACCGACTCGC	
exoS	F	GCGAGGTCAGCAGAGTATCG	118
	R	TTCGGCGTCACTGTGGATGC	

The cycling conditions used for *exoT* and *exoY* were: 1 cycle of Initial denaturation at 95 °C, then 35 cycles of denaturation at 95 °C (30 seconds), annealing at 55 °C (30 seconds), and extension at 72°C (1 minute). Finally, seven minutes of final extension at 72°C. While the cycling conditions for *exoS* were one cycle of Initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C (45 seconds), annealing at 60 °C (45 seconds), and extension at 72°C (1 minute), finally, 7 minutes of final extension at 72°C. The cycling conditions of *exoU* were the same as those for *exoT* and *exoY*, except that the annealing was at 58°C. Confirmation of the presence of the PCR product was by running an agarose gel electrophoresis (Clarivate /UK) at 80V, 65 Amp for 1 hour. The DNA was visualized under a UV transilluminator (Vilber Lourmat Sté, France).

Statistical analysis

The statistical software IBM SPSS-29 (IBM Statistical Packages for Social Sciences, version 29, Chicago, IL, USA), was used to analyse the data. Simple frequency and percentage measures were used to display the data. The Pearson Chi-square test (x2-test) or Fisher Exact test, as appropriate, were used to assess the significance of differences in various percentages (qualitative data). The results were considered non-statistically significant when the p-value was greater than 0.05, while those with a p-value less than 0.05 were regarded as statistically significant, and those with a p-value less than 0.01 were considered highly significant.

Results

Demographic data and samples: This study initially involved 120 bacterial isolates, which were preliminarily identified as *P. aeruginosa* based on cultural characteristics, colony morphology, and conventional microbiological methods; subsequently, 80 were confirmed to be

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P. aeruginosa.

The demographic and sample characteristics of isolates included in the study are listed in Table 2. This table shows that males were more than females (76.25 vs 23.75%). The patient's age ranged from 17 to 70 years, with a mean age of 33.42±11.73 years. Out of the 80 *P. aeruginosa* isolates, 32 (40%) were isolated from wounds, 27(33.75%) from burns, 11(13.75%) from sputum, 7(8.75%) from ear swabs and 3 (3.75%) from BAL.

Distribution of *P. aeruginosa isolates* according to sex, with the source of clinical sample, showed that out of the 11 sputum samples included in the study, nine were isolated from males and two from females. Twenty-two isolates out of the 32 wound swabs included in the study were isolated from males and 10 from females. Out of the seven ear swabs, six isolates were collected from males and one from females. Regarding burn patients from whom *P. aeruginosa* were isolated, 22 were males and 5 were females.

Table 2. Demographic and sample characteristics of the patients included in the study.

Character		N	%
Sex N (%)	Male	61	76.25
	Female	19	23.75
Age (years)	Mean	33.42±11.73	
	Range	12-70	
Sample N (%)	Sputum	11	13.75
	wound swab	32	40
	Ear swab	7	8.75
	burn swab	27	33.75
	Bronchoalveolar lavage	3	3.75

Finally, out of the three of *P.aeruginosa* isolates identified in BAL samples, two were males and only one was a female. Statistical analysis revealed no significant association (p = 0.71) between sex and the type of clinical sample, as illustrated in Table 3.

Table 3. Distribution of *P.aeruginosa* isolates according to sex with the type of clinical samples. N.S. =Non- statistically significant (Chi-square).

Sex		Clinical Sample type					Total	P-value
		Sputum	wound swab	Ear swab	burn swab	BAL		
Male	N	9	22	6	22	2	61	0.71 (N.S)
	%	11.3%	27.5%	7.5%	27.5%	2.5%	76.3%	
Female	N	2	10	1	5	1	19	
	%	2.5%	12.5%	1.3%	6.3%	1.3%	23.8%	
Total	N	11	32	7	27	3	80	
	%	13.8%	40.0%	8.8%	33.8%	3.8%	100.0%	

Antibiotic resistance profile: Classifying the type of drug resistance in the 80 *P. aeruginosa* isolates showed that 76(95%), 3(3.75%), and 1 (1.25%) isolates were MDR, XDR, and PDR, respectively (Figure 1).

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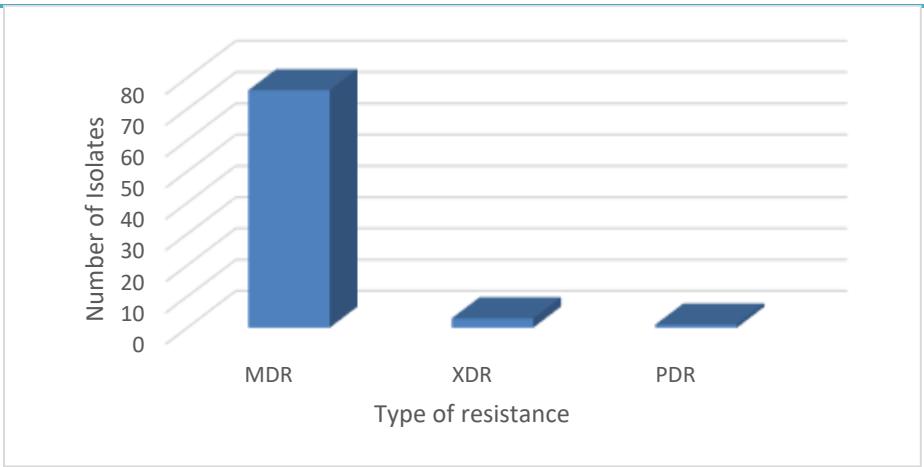

Figure 1. Type of drug resistance in *P. aeruginosa* isolates included in the study. MDR=Multidrug resistant, XDR=extensive drug resistant, PDR=Pan Drug Resistant.

Prevalence of *exoT, exoY, exoS* and *exoU* in *P.aeruginosa*: Figures 2, 3, 4, and 5 show images of PCR products for exoT, exoY, exoS, and exoU of *P. aeruginosa*, fractionated by 1.5% agarose gel and visualized under UV light after staining with a red dye.

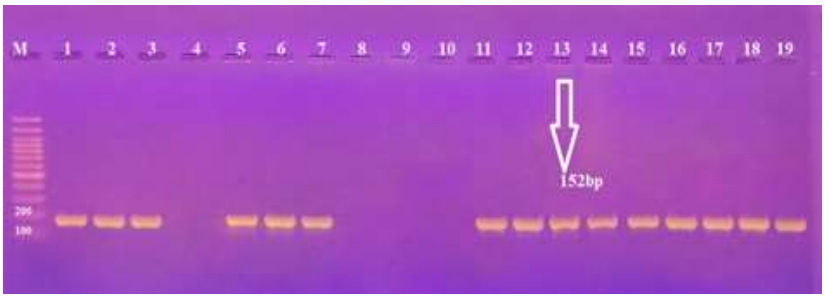

Figure 2. Gel electrophoresis (1.5%) of amplified exoT in P.aeruginosa. Agarose gel electrophoresis analysis shows the amplified exoT of P.aeruginosa. M: (100 ng/5 µl) of 100bp plus DNA ladder (Transgen/China). Lanes 1-3, 5-7 and 11-19: P.aeruginosa harboring exoT (152bp).

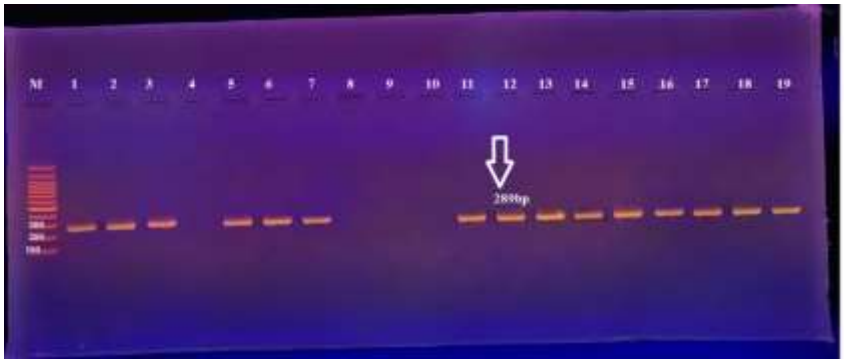

Figure 3. Gel electrophoresis (1.5%) of amplified exoY in P.aeruginosa. Agarose gel electrophoresis analysis shows the amplified exoY of P.aeruginosa. M: (100 ng/5 µl) of 100bp plus DNA ladder, (Transgen/China). Lanes 1-3, 5-7, 11-19: P.aeruginosa isolates harboring exoY (289bp).

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Figure 4. Gel electrophoresis (1.5%) of amplified exoS in P.aeruginosa. Agarose gel electrophoresis analysis shows the amplified exoS of P.aeruginosa. M: (100 ng/5 µl) of 100bp plus DNA ladder, (Transgen/China). Lanes 1-2, 5, 7, 16-19: P.aeruginosa isolates harboring exoS (118bp).

Figure 5. Gel electrophoresis (1.5%) of amplified exoU in P.aeruginosa. Agarose gel electrophoresis analysis shows the amplified exoU of P.aeruginosa M: (100 ng/5 µl) of 100bp plus DNA ladder, (Transgen/China). Lanes 1, 2, 5-7: P.aeruginosa isolates harboring exoU (1348bp).

The distribution of T3SS genes among the studied *P. aeruginosa* isolates revealed that the gene with the highest prevalence (86.7%) was *exoT*, followed by *exoY* (76.7%) and *exoS* (50%). The lowest prevalence was for exoU (30%), as illustrated in Table 4.

Table 4. Distribution of T3SS genes among studied *P. aeruginosa* isolates.

***T3SS* gene**	***Pseudomonas aeruginosa* Isolates N (%)**
exoT	26 (86.7%)
exoY	23 (76.7%)
exoS	15 (50%)
exoU	9 (30%)

The distribution of T3SS effector genes according to the origin of the sample (Table 5) revealed no significant association between the origin of the sample and the prevalence of exoS and exoU. On the other hand, *exoT* and *exoY* were mainly associated with burn patients (p = 0.04 and 0.007).

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Table 5. Distribution *of T3SS* genes in *P. aeruginosa* according to origin of sample**.**

Name of gene	Status	Type of samples				Total	P-value
		Sputum	Wound	Burn	BAL		
exoS	+Ve	3 (10.0%)	4 (13.3%)	8 (26.7%)	0 (0.0%)	15 (50.0%)	0.06
	-Ve	0 (0.0%)	8 (26.7%)	6 (20.0%)	1 (3.3%)	15 (50.0%)	
Total		3 (10.0%)	12 (40.0%)	14 (46.7%)	1 (3.3%)	30 (100.0%)	
exoT	+Ve	3 (10.0%)	8 (26.7%)	14 (46.7%)	1 (3.3%)	26 (86.7%)	0.04
	-Ve	0 (0.0%)	4 (13.3%)	0 (0.0%)	0 (0.0%)	4 (13.3%)	
Total		3 (10.0%)	12 (40.0%)	14 (46.7%)	1 (3.3%)	30 (100.0%)	
exoY	+Ve	2 (6.7%)	6 (20.0%)	14 (46.7%)	1 (3.3%)	23 (76.7%)	0.007
	-Ve	1 (3.3%)	6 (20.0%)	0 (0.0%)	0 (0.0%)	7 (23.3%)	
Total		3 (10.0%)	12 (40.0%)	14 (46.7%)	1 (3.3%)	30 (100.0%)	
exoU	+Ve	0 (0.0%)	5 (16.7%)	3 (10.0%)	1 (3.3%)	9 (30.0%)	0.12
	-Ve	3 (10.0%)	7 (23.3%)	11 (36.7%)	0 (0.0%)	21 (70.0%)	
Total		3 (10.0%)	12 (40.0%)	14 (46.7%)	1 (3.3%)	30 (100.0%)	

***T3SS* virulotypes:** The presence of different combinations of Exotoxins was analyzed according to the combinations published by Nolasco-Romero and co-workers (16) as illustrated in Table 6. The most abundant virulotypes were V3, V5, and V9 (23.3% each) followed by V6 (13.3%), V1 (6.7%), V4 (6.7%), and V7 (3.3%). Whereas, virulotypes V2, V10, and V11 did not exist in any of the *P. aeruginosa* clinical isolates included in the study.

Table 6. T3SS virulotypes in P. aeruginosa clinical isolates included in the study. (+ indicates presence of gene, - Indicates absence of gene).

Virulotype		N	%
V1	exoU+/exoS−/exoT+/exoY+	2	6.7
V2	exoU+/exoS−/exoT+/exoY−	0	0
V3	exoU−/exoS+/exoT+/exoY+	7	23.3
V4	exoU−/exoS−/exoT+/exoY−	2	6.7
V5	exoU−/exoS−/exoT+/exoY+	7	23.3
V6	exoU−/exoS−/exoT−/exoY−	4	13.3
V7	exoU−/exoS+/exoT+/exoY−	1	3.3
V8	exoU−/exoS−/exoT−/exoY+	0	0
V9	exoU+/exoS+/exoT+/exoY+	7	23.3
V10	exoU−/exoS+/exoT−/exoY+	0	0
V11	exoU+/exoS+/exoT+/exoY−	0	0

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Discussion

The WHO has listed antibiotic resistant *P. aeruginosa* among the "critical" group of pathogens necessitating urgent novel antibiotics (17).

Type III secretion system is among the many *P. aeruginosa* virulence factors that have been associated with host cell pathogenicity via activating the immune response and promoting the development of *P. aeruginosa* infections (12) posing as an additional risk factor in hospitals particularly in immune compromised patients (13).

In line with the current findings, the predominance of male patients in infections caused by *P. aeruginosa* has been previously reported (18, 19). The average age of patients included in the study was quite similar to what has been reported in a cross-sectional study isolating *P. aeruginosa* from infectious hospital departments (20). Regarding the source of isolates, most were from wound and burn swabs, coinciding with previous studies in Iraq (21), Saudi Arabia (22), and Pakistan (23). On the other hand, this isolation rate in wounds and burns was considerably higher than those reported in other studies (24, 25) which may be due to different inclusion criteria and different sampling protocols. The reported isolation rate of *P. aeruginosa* from sputum and BAL is 0% to 23%, which is in line with the current study (26). Despite the male predominance, no significant association was noted between sex and the type of clinical sample from which *P. aeruginosa* was isolated.

The current study showed a high prevalence of multidrug-resistance in clinical isolates of *P. aeruginosa* which is consistent with other studies in Iraq (27, 28) and abroad (29, 30).

The increase in the emergence of MDR *P. aeruginosa* is a global problem affecting many countries. The prevalence of MDR *P. aeruginosa* was higher (95 vs 72.63% and 85.49%) than the percentage previously reported in the cities of Basrah (27) and Babylon (28) in Iraq. In contrast to the 95% of MDR reported in the current study, an Iraqi study previously published in 2020 (31) reported that only 42% of *P. aeruginosa* isolates included in their study were MDR highlighting a sharp increase in the emergence of multidrug resistance in *P. aeruginosa* in Iraq. The increased prevalence of multidrug resistance in *P. aeruginosa* may be due to the selection of resistant strains which have emerged due to high consumption of antibiotics used to treat Covid-19 associated secondary bacterial infections (18).

Among the four classical T3SS effector genes, *exoT* was the most prevalent one which agrees with what has been previously published (32). The current result disagrees with the study of Waham and Naser in the city of Misan Iraq who found that the most prevalent exoenzyme was *exoY*. This difference may be attributed to the source of isolate as all of their isolates were from ear swabs (33). Both genes are part of the core genome of the bacterium (34). Statistical analysis has linked the existence of *exoY* and *exoT* with burn patients. Elnagar *et al.* (2022) reported that all *P. aeruginosa* strains isolated from burn sites harbored *exoY and exoT* (35). The second most prevalent gene in the current study was *exoY* followed by *exoS* and *exoU*. This comes in line with previous studies which have documented that *exoS* is more prevalent compared to *exoU* (35, 36). A previous study in the city of Wasit in Iraq has reported a frequency rate of the *exoU* and *exoS* genes of 60.31%, 90.47% in *P. aeruginosa* (37). It has been reported that exoU and exoS were found in 42.22% and 62.22% of *P. aeruginosa* clinical isolates, respectively (35). It is thought that *exoS+/exoU+ P. aeruginosa* strains have increased pathogenicity (38). Published data have reported that the potent A2-family phospholipase encoding *exoU* gene is the most virulent among the T3SS and can result in undesirable outcome such as multidrug

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resistance and death when over-regulated (39).

An *in vivo* study has shown that deletion of *exoU* in *P. aeruginosa* resulted in significant reduction in cytotoxicity and virulence highlighting its major role in pathogenesis (40). *ExoU* is the only *P. aeruginosa* T3SS effector encoded within a Genomic Island environment and Jaun and co-workers have linked its presence with an invasive phenotype (41). On the other hand, *exoS* has a cytotoxic phenotype (42-45). The most frequent virulotypes in the current study were V3, V5 and V9 in the current study. Similarly, a previous study has identified V3 as the most abundant virulotype followed by V1 and V7. The coexistence of the four T3SS genes (V9) was identified in 23.3% of the isolates included in the current study which is more than what has been previously reported (16).

Conclusions

This study highlights an increase in the emergence of multidrug-resistant *P. aeruginosa* in various clinical samples, as well as the presence of the four classical Type 3 Secretion System effectors at variable frequencies, underscoring their significant role in pathogenicity. *exoT* was the most prevalent among the other three Type 3 Secretion System effectors, suggesting that it plays a vital role in the virulence and survival of this pathogen. Furthermore, it was recommended to determine the profile of T3SS effector cases and precisely detect antibiotic susceptibility patterns, which are strongly essential for creating effective measures to prevent *P. aeruginosa* infections. Future studies with a larger sample size that focus on a specific infection site are required to reach a comprehensive conclusion.

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نمط الضراوة لنظام الإفراز من النوع الثالث في العزلات السريرية لبكتيريا الزائفة الزنجارية المقاومة للأدوية المتعددة

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الملخص

الخلفية: الخلفية: لبكتيريا الزائفة الزنجارية المقاومة للأدوية المتعددة تأثير وبائي على صحة الإنسان. فهي تُشكل تهديدًا للأنظمة الصحية في العالم، بما في ذلك العراق. تُعد مُفعِّلات نظام الإفراز من النوع الثالث من بين عوامل الضراوة العديدة التي تمتلكها هذه البكتيريا. يُعد تحديد نمط الضراوة أمرًا أساسيًا للوقاية من العدوى.

الأهداف: تبحث هذه الدراسة في تكرار مُفعِّلات نظام الإفراز من النوع الثالث الأربعة الكلاسيكية في بكتيريا الزائفة الزنجارية المقاومة للأدوية المتعددة.

المرضى والطرق: شملت هذه الدراسة في البداية ١٢٠ عزلة بكتيرية من عينات سريرية مختلفة، والتي تم تحديدها مبدئيًا على أنها الزائفة الزنجارية. من بين هذه العزلات، تم تأكيد أن ٨٠ عزلة هي الزائفة الزنجارية. تمت دراسة نمط حساسية المضادات الحيوية، وتم التحقق من وجود exoY وexoT وexoS وexoU بواسطة تفاعل البوليميراز المتسلسل (PCR).

النتائج: صُنفت ٩٥٪ و٣,٧٥٪ و١,٢٥٪ من العزلات على أنها مقاومة للأدوية المتعددة، ومقاومة واسعة للأدوية، ومقاومة لجميع الأدوية على التوالي. من بين العزلات المختارة، وُجد exoT في ٨٦,٧٪، وexoY في ٧٦,٧٪، وexoS في ٥٠٪، وexoU في ٣٠٪ على التوالي.P=0.0001.

الاستنتاج: تُبرز هذه الدراسة زيادة في ظهور أنماط مقاومة الأدوية المتعددة في العزلات السريرية لبكتيريا الزائفة الزنجارية، بالإضافة إلى تواجد مُفعِّلات نظام الإفراز من النوع الثالث بنسب متفاوتة (٨٦,٧٪، و٧٦,٧٪، و٥٠٪، و٣٠٪ على التوالي).

الكلمات المفتاحية: الزائفة الزنجارية, المقاومة للأدوية المتعددة, نظام الإفراز من النوع الثالث.

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Enhancing the Mechanical and Physical Properties of Zirconium Dental Filling Material by Chemical Mixing with Polyvinyl Alcohol Polymer in Different Cure Times

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Abstract

Background: Zirconium is mainly composed of zirconia/silica particles. Zircon filling material is a light-curing, radiopaque nanohybrid composite resin designed for anterior and posterior tooth restorations with a high inorganic filler component. One synthetic polymer that dissolves in water is polyvinyl alcohol. Polyvinyl Alcohol's low toxicity, low propensity for protein adhesion, and biocompatibility make it useful in a range of medical applications. Contact lenses, eye drops, and cartilage substitutes are among the specific applications.

Objectives: evaluate the effect of chemical mixing polyvinyl alcohol polymer on the hardness, strength, water solubility, and water sorption properties of zircon dental filling material.

Patients and Methods: The biocomposite was prepared by dissolving 5g of polyvinyl alcohol in distilled water and stirring the mixture continuously for 30 minutes at 80°C. The mixture was stirred until a gel formed. Then, the mixed Universal Restorative material with zircon fillings was exposed to visible violet curing light for hardening, with different curing times (1, 2, 5, 10, 15, and 20 seconds). Then, we measured the strength, hardness, sorption, and solubility of the new mixed filling.

Results: Showed an increase in the hardness and the strength of the zircon filling materials after chemical mixing with Polyvinyl Alcohol polymer, compared with traditional zircon material that cured without Polyvinyl Alcohol polymer, and a decrease in the solubility and sorption of the filling material after chemical mixing.

Conclusion: An addition of polyvinyl Alcohol polymer particles to impact light-cure zircon dental filling material improves the impact physical and mechanical properties of the new mixed filling material.

Keywords: Zirconium, Nanohybrid, Polyvinyl alcohol polymer, Strength, Hardness, Sorption, Solubility.

Introduction

Zirconium, obscure before the late 1940s, became a significant engineering material for nuclear energy. Dental zircon has many advantageous properties, including biocompatibility and the ability to mimic the optical properties of real teeth (1, 2). A polymer nanocomposite is a blend of materials in which the filler is a nanomaterial and the matrix polymer. Adding nanofillers to the polymer matrix typically modifies the characteristics of the polymer (3). A restoration with full contour would exhibit

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excellent occlusal detail and a final shape, and a high translucency would enable the material to blend naturally with surrounding teeth (4). Due to their exceptional strength, monolithic zirconia crowns offer sufficient fracture resistance for dental crown restorations. Stress-induced transition toughening in stabilized zirconia is responsible for this resistance (5). Since zirconium does not absorb neutrons, it might be used as a perfect material in nuclear power plants (6). Zirconium metal is protected by a thin oxide layer, making it exceptionally resistant to corrosion by acids, alkalis, and seawater. For this reason, it is extensively used by the chemical industry. (7) ZrO2, or zirconia, is a wide bandgap metal oxide with potential applications in many scientific and technological domains. It exhibits admirable mechanical, optical, electrical, and thermal properties, including outstanding corrosion resistance, high fracture toughness, high hardness, a high refractive index, optical transparency, low thermal conductivity, and polymorphism features (8). One of the many polymer options is polyvinyl alcohol (PVA), a water-soluble polyhydroxy polymer characterized by its chemical structure, which features CH, CH2, and OH side groups. Due to its exceptional mechanical strength, biocompatibility, and non-toxicity, PVA has been widely investigated as an implant material in various biomedical applications, including drug delivery systems, dialysis membranes, wound dressings, artificial skin, cardiovascular devices, orthopedic implants, and maxillofacial surgeries when combined with ceramic materials (9, 10). Since poly PVA has great mechanical qualities, it is one of the more often used polymers. It is also biodegradable under the right circumstances (11). Handheld light-emitting devices, known as dental light curing units (LCUs), are used to cure photo-activated polymer-based restorative materials (PBRMs) (12). Using visible light energy, a photo initiator system is triggered in photo polymerization. This light-activated reaction produces reactive species (free radicals) that start the polymerization process by absorbing light photons (13, 14). To create a high-impact PVA/Zirconium nanocomposite, zirconium nanoparticles treated with polymers, specifically PVA, and cured for hardening filling material, were used in this study. The effects of this addition on various mechanical and physical properties over different curing times were also investigated. The objective of this study was to evaluate the impact of chemical mixing of PVA polymer on the hardness properties of zircon dental fillings.

Patients and Methods

Study design: The experiment was conducted at the University of Diyala, College of Science, from October 1, 2023, to March 1, 2024. The biocomposite was prepared by dissolving 5 g of PVA in distilled water, with continuous stirring for 30 minutes at 80°C. The mixture was then stirred continuously until gel formation occurred. We mix it with scope zircon fillings (3 M ESPER Filtek Z350 XT), approximately 5 g of universal restorative material. After mixing, the resulting materials were hardened using an O-Light device (Woodpecker O-Light unit, a product from DTE Woodpecker) with varying curing times (1, 2, 5, 10, 15, and 20 seconds) and a wavelength of light ranging from 420 nm to 490 nm. Additionally, the zirconium is hardened in two groups: a study group consisting of zirconium filling material mixed with PVA polymer, and a control group consisting of hardened zirconium filling material only. Then, the Shore-D device (Shore Durometer) was used to measure the hardness of the mixed and hardened materials. Fourier Transform Infrared Spectroscopy test (FTIR) was done to zirconium filling after mixing with the PVA polymer to evaluate the active groups. The PVA polymer imparted a strong rubber-like plasticity to the zirconium, and it hardened when exposed to an

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O-light device with a wavelength of light ranging from 420 nm to 490 nm, at varying time intervals. The test method includes placing the device perpendicular to the desired sample. Measure its hardness so that it is in contact with the surface of the sample whose hardness is to be measured, to insert the needle into the surface of the material, and for a waiting period of about three seconds, after which the hardness value is taken from the device. The applied pressure is in accordance with the specifications (DIN 53505) and equals 50 N, which is equivalent to 5 kPa. For Shore, several readings were taken at different places on the surface of the sample.

Strength was measured using a universal Instron testing machine. Each specimen was positioned on the bending fixture, and the load was applied with a crosshead speed of 1 mm/min by a rod placed centrally between the supports. Deflection was allowed to occur until fracture, and the scale of strength was recorded (15). Three square-shaped specimens, measuring 1.5 mm in thickness and 2 cm in size, were used to assess the solubility and water sorption of pure zirconium and a mixture of PVA and zirconium-based filling material. The specimens had one polished surface that was softening. For thirty minutes, the specimens were stored at room temperature. This weight figure was regarded as the specimen's starting weight (M1) Every day until a consistent weight (M2) was reached, all specimens were weighed using an analytical scale "Model JK-180; Chyo, Tokyo, Japan with an accuracy of 0.0001 g, in a water bath maintained at 37°C". The samples were weighed once again after being dried at 37°C in a vacuum oven to maintain their weight (M3). For every specimen, the values of water sorption (Wsp) and solubility (Wsl), expressed in µg/mm3, were computed using: $M2 - M3/V$ and $Wsl = M1 - M3/V$. The symbol V represents the volume of the specimen, in mm^3 (16). We followed the same steps as for the zirconium filling material, without mixing it with PVA, and measured it using all the tests applied to the zirconium with PVA. The results were recorded as a control group.

Statistical analysis

The data analysis was conducted using IBM SPSS 29 (IBM Statistical Package for the Social Sciences, version 29, Chicago, IL, USA). Descriptive statistics, including simple frequency and percentage calculations, were employed to present the data. Results were deemed non-statistically significant if the p-value exceeded 0.05. Conversely, p-values below this threshold were considered statistically significant, with those less than 0.01 classified as highly significant.

Results

Fourier transform infrared spectroscopy test (FTIR): The structures of the PVA polymer and zirconium were confirmed by Fourier Transform Infrared Spectroscopy test (FTIR). The FTIR spectrum showed absorption bands at 3309 cm^{-1} (OH) stretching (broad) of shellac, 2910.68 cm^{-1} (CH2) of aliphatic, 1730 cm^{-1} (C=O) of Ester, 1095 cm^{-1} (CO) Extended Ester, 1658 cm^{-1}(C=C) Shellac 1018 cm^{-1} (CO) (Extended Shellac) (Figure 1).

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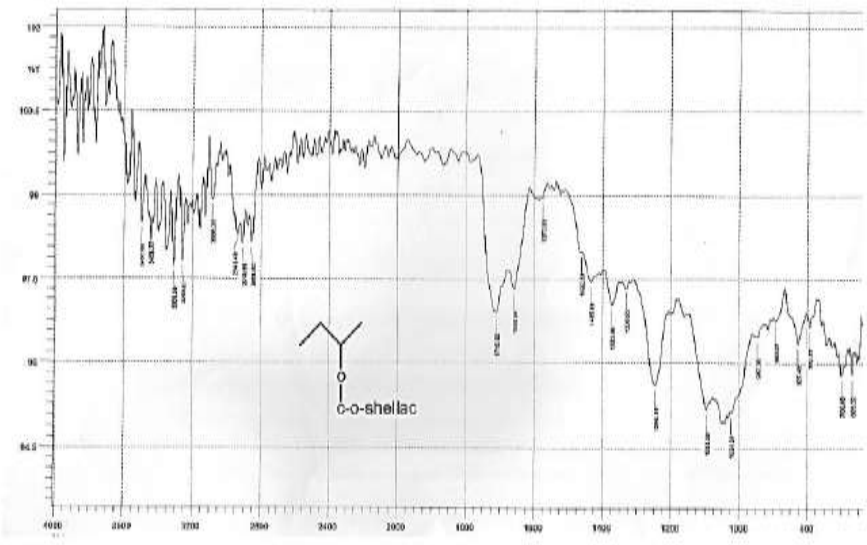

Figure 1. The FTIR test for zirconium filling material and PVA polymer.

The hardness: The results of the hardness show a high value for the study group compared to the control group. The groups' difference in hardness shows a highly significant difference in the study group at 10-15-20 sec curing time compared to the control group, which showed a non-significant difference at the same curing time. While the study showed a significant difference in the study group at 1-2-5 sec hardening or curing time compared to the control group, which showed a non-significant difference (Table 1).

Table 1. Descriptive statistics and groups' difference for the Hardness.

group	Time in sec	Descriptive statistics						Groups' difference		
		N	Mean	S.D	S.E	Min	Max	Mean difference	t-test	p-value
Control group	1	5	5.4	.60	1.34	4.000	7.000	6.013	-6.987	.076
	2	5	5.4	.67	1.51	3.000	7.000	6.544	-5.678	0.78
	5	5	6.8	.77	2.03	4.000	8.000	6.788	4.645	0.9
	10	5	7.4	1.12	2.50	5.000	10.000	8.800	-8.586	0.56
	15	5	8.8	2.02	2.70	6.000	11.000	8.999	-6.987	0.65
	20	5	9	3	3.3	8.000	13.000	9.987	-9.867	0.66
Study group	1	5	16.0	1.14	2.54	14.000	20.000	6.016	-11.345	.015*
	2	5	17.0	2.00	4.47	12.000	23.000	7.669	-12.45	.032*
	5	5	17.6	1.66	3.71	15.000	24.000	5.678	-13.456	.041*
	10	5	17.0	2.09	4.69	11.000	21.000	9.755	-12.456	.003**
	15	5	18.2	1.49	3.34	13.000	21.000	11.044	-13.542	.002**
	20	5	23.4	3.41	7.63	14.000	34.000	13.466	-14.453	.001**

The strength: The results of strength represented a high value in the study group that mixed zirconium with PVA, compared to the control group that used pure zirconium (Table 2). It showed a highly significant difference in the study group at 1-5-10-20 sec curing time, and showed a significant difference in the study group at 2-5 sec curing time, and showed a non-significant difference in the control group at (1-2-5-10-15-20) sec curing time.

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Table 2. Descriptive statistics and groups' differences for the strength (N/mm2).

group	Time in sec	Descriptive statistics						Groups' difference		
		N	Mean	S.D	S.E	Min	Max	Mean difference	t-test	p-value
Control group	1	5	99.654	4.64	0.34	79.000	104.000	8.099	-6.654	.096
	2	5	97.875	4.66	1.58	63.000	102.000	7.146	-4.118	0.38
	5	5	93.875	2.87	2.93	94.000	101.000	6.987	-6.633	0.66
	10	5	121.789	11. 2	1.50	85.000	140.000	8.895	-7.006	0.50
	15	5	88.974	12. 2	2.79	66.000	151.000	8.326	-8.337	0.25
	20	5	101.432	3.87	2.3	84.000	133.000	9.976	-9.212	0.16
Study group	1	5	133.453	3.14	3.53	104.000	240.000	9.916	-11.345	.005**
	2	5	144.432	5.05	4.44	120.000	293.000	10.459	-12.45	.002*
	5	5	157.624	7.60	2.72	105.000	254.000	9.078	-13.456	.031*
	10	5	187.011	4.09	3.99	131.000	210.000	11.795	-12.456	.000**
	15	5	188.232	3.99	4.39	163.000	210.000	13.004	-13.542	.002**
	20	5	203.411	7.41	6.63	188.000	304.000	13.065	-14.453	.001**

Water sorption: The results of the Water sorption of the zirconium mixed with PVA decreased in the study group compared to the control group, as shown in Table 3. It was demonstrated that water sorption results show a significant difference in the study group at all curing times (1-2-5-10-15-20 sec), while showing a non-significant difference in the control group at all curing time intervals.

Table 3. Descriptive statistics and groups' difference for the Water sorption (mg/cm2).

group	Time in sec	Descriptive statistics						Groups' difference		
		N	Mean	S.D	S.E	Min	Max	Mean difference	t-test	p-value
Control group	1	5	0.468	0.019	0.006	0.433	0.502	0.280	32.155	.196
	2	5	0.485	0.021	0.004	0.455	0.500	0.342	33.456	0.33
	5	5	0.534	0.022	0.003	0.590	0.585	0.345	23.456	0.66
	10	5	0.543	0.31	0.004	0.499	0.560	0.432	32.433	0.54
	15	5	0.541	0.30	0.004	0.489	0.555	0.234	30.554	0.55
	20	5	0.431	0.231	0.005	0.389	0.470	0.295	23.456	0.56
Study group	1	5	0.355	0.366	0.006	0.302	0.387	0.294	34.567	.035*
	2	5	0.432	0.905	0.006	0.402	0.460	10.459	12.45	.052*
	5	5	0.324	0.460	0.005	0.312	0.356	9.078	13.456	.038*
	10	5	0.311	0.309	0.007	0.288	0.345	11.795	12.456	.050*
	15	5	0.232	0.299	0.005	0.202	0.260	13.004	13.542	.042*
	20	5	0.311	0.341	0.006	0.279	0.389	13.065	14.453	.021*

Water solubility: The results show that the Water solubility of zirconium mixed with PVA decreases in the study group compared to the control group at the same curing time, as shown in Table 4. The results represent water sorption data that show a highly significant difference in the study group at all curing times (1-2-5-10-15-20 sec), while showing a non-significant difference in the control group at all time intervals.

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Table 4. Descriptive statistics and groups' difference for the Water solubility (mg/cm2).

group	Time in sec	Descriptive statistics						Groups' difference		
		N	Mean	S.D	S.E	Min	Max	Mean difference	t-test	p-value
Control group	1	5	0.079	0.008	0.006	0.043	0.072	0.068	7.055	.196
	2	5	0.060	0.008	0.006	0.045	0.070	0.054	7.956	0.33
	5	5	0.086	0.006	0.005	0.050	0.0 85	0.064	8.456	0.66
	10	5	0.071	0.007	0.006	0.049	0.060	0.061	9.003	0.54
	15	5	0.096	0.006	0.006	0.069	0.105	0.075	6.054	0.55
	20	5	0.071	0.007	0.005	0.039	0.060	0.064	7.956	0.56
Study group	1	5	0.021	0.003	0.005	0.012	0.037	0.021	4.067	.000**
	2	5	0.030	0.004	0.004	0.022	0.040	0.023	4.045	.000**
	5	5	0.031	0.002	0.005	0.022	0.050	0.021	5.006	.000**
	10	5	0.029	0.004	0.003	0.018	0.035	0.025	3.406	.000**
	15	5	0.029	0.003	0.004	0.022	0.032	0.021	3.042	.000**
	20	5	0.030	0.002	0.004	0.021	0.039	0.022	3.153	.000**

Discussion

The present study showed an increase in the hardness and strength of the zirconium filling material after mixing with PVA in most curing times, compared to pure zirconium. Conversely, it also showed a decrease in water sorption and solubility of the zirconium filling material after mixing with PVA in most curing times, compared to pure zirconium. The results of the present study indicate a significant increase in the hardness of materials due to the addition of PVA to ZrO2 nanoparticles, which have a more uniform surface and a higher accumulation of nanoparticle material, as noted by Hudson et al. (15), resulting in a significantly larger filler content. The current study's findings indicate that adding zirconium oxide to high-impact PVA significantly increases impact hardness, particularly with curing times of 1-2–5 seconds, and exhibit highly significant differences at 10-15–20 seconds. When Al-Hiloh et al. (16) used zerconia nanoparticles with traditional heat-cured denture base resin, he obtained similar results. Safi (18) also agree with the hardness result when using heat-cured acrylic denture bases and the addition of silanized nano-ZrO2 fillers, which showed an increase in the hardness of the zirconium filling material. The results of the strength tests of the zirconium show high significant difference at 1-10-15-20 sec than the 2-5 sec that show significant difference, Due to the formation of supra-molecular bonds or cross-links that envelop or shield the nano-fillers and impede the spread of fractures, the interfacial shear strength between the zirconium filler and PVA is high, contributing to the increase in strength of the zirconium dental filling. Additionally, a strong link between the dental filling material (zirconium and PVA) can alter the development of cracks. This finding agrees with Al-Hiloh A.,and Sun L., et al. (16, 19).

The diffusion property of water molecules through the mixture of zirconium dental material and PVA is significantly lower at all interval curing time than that control group of pure zirconium dental material without PVA mixing, the reduction in water sorption may be caused by decrease surface roughness of

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zirconium dental filling material by PVA partials due to the PVA particles have very small size and well dispersion, and will be improve the surface of the specimen and also replacing the hydrophilic resin of the zirconium dental material particles. Mohammed (20) concurred with the study's findings, which claimed that a rise in the proportion of ZrO2 nanofillers reduced water sorption. The absorbed polymer's total volume may be decreased by the ZrO2 nanoparticles, which are insoluble in water, as Ferracane J (21) and Panyayong, W., et al. (22).

The result of water solubility showed a significant decrease in the study group compared to the control group. The reason for the decrease in water solubility might be attributed to the insoluble nature of the zirconium filler material. Consequently, the inclusion of PVA as an additive in the specimen mass would lower the overall solubility of the mixture. The findings of this investigation agreed with those of Gurbuz O and Pinarkursoglu F. (23), who discovered that the solubility of acrylic resin decreased upon the addition of milled glass fiber fillers. The findings also supported and agreed with Mohammed's (20) claim that water solubility reduced as the percentage of ZrO2 nanofillers increased. Furthermore, the current results agree with those of Al-Hiloh A. (16), which indicate that the addition of ZrO2 to the acrylic matrix improves and decreases the water solubility of ZrO2. Recently, the use of nanomaterials has increased in the fields of physics and microbiology due to the strength and effectiveness of these materials and the spread of their uses in most fields, as seen in (24), which uses TiO2 in microbiological applications. In addition, the uses of nanomaterials were not limited to increasing physical properties, such as hardness; chemicals were also used to enhance the hardness of living tissues, including bones, by using bisphosphonates to heal and increase bone strength (25).

Conclusions

Numerous physical features of ZrO2 Nanoparticles, which are represented by zirconium dental materials, are improved by the addition of polyvinyl alcohol, or PVA. When zirconium and PVA are mixed, the new mixing filling material's strength and impact hardness of modified zirconium rise. At the same time, the solubility and water sorption of the mixing filling material decrease. PVA material is used to enhance the properties of the zirconium dental material. Therefore, it can be used with other filling materials to produce new mixtures with new physical and chemical properties, or with the same filling material at different concentrations to achieve other desirable results.

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Ethical clearance: This study was approved by the Research Ethical Committee of College of Medicine/ University of Diyala (No:2024MAH873).

Conflict of interest: None.

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تحسين الخواص الميكانيكية والفيزيائية لمادة حشو الأسنان الزركونيوم عن طريق الخلط الكيميائي مع بوليمر كحول البولي فينيل في أوقات معالجة مختلفة

منار عبد الرزاق حسن [١]

الملخص

الخلفية: يتكون الزركونيوم بشكل رئيسي من جزيئات الزركونيا/السيليكا. مادة حشو الزركون هي راتنج مركب نانوي هجين، مقاوم للأشعة فوق البنفسجية، مقاوم للتصلب بالضوء، مصمم لترميم الأسنان الأمامية والخلفية، ويحتوي على نسبة عالية من الحشو غير العضوي. أحد البوليمرات الاصطناعية التي تذوب في الماء هو كحول البولي فينيل. سمية كحول البولي فينيل المنخفضة، وقابليته المنخفضة للالتصاق بالبروتين، وتوافقه الحيوي، تجعله مفيدًا في مجموعة من التطبيقات الطبية. من بين التطبيقات المحددة العدسات اللاصقة، وقطرات العين، وبدائل الغضاريف.

الأهداف: تقييم تأثير الخلط الكيميائي لبوليمر كحول البولي فينيل على صلابة مادة حشو الأسنان الزركونية، ومتانتها، وقابليتها للذوبان في الماء، وخصائص امتصاص الماء.

المرضى والطرق: تم تحضير المركب الحيوي عن طريق إذابة ٥ غرامات من كحول البولي فينيل في الماء المقطر وتحريك الخليط باستمرار لمدة ٣٠ دقيقة عند درجة حرارة ٨٠ درجة مئوية. تم تحريك الخليط حتى تشكل هلام. بعد ذلك، عُرضت مادة الترميم العالمية المختلطة مع حشوات الزركون لضوء معالجة بنفسجي مرئي للتصلب، بأزمنة معالجة مختلفة (١، ٢، ٥، ١٠، ١٥، و٢٠ ثانية). بعد ذلك، قيست قوة وصلابة وامتصاص وذوبان الحشوة المختلطة الجديدة.

النتائج: أظهرت زيادة في صلابة ومتانة مواد حشو الزركون بعد الخلط الكيميائي مع بوليمر كحول البولي فينيل، مقارنةً بمادة الزركون التقليدية التي تصلب بدون بوليمر كحول البولي فينيل، وانخفاضًا في ذوبان وامتصاص مادة الحشوة بعد الخلط الكيميائي.

الاستنتاج: إضافة جزيئات بوليمر كحول البولي فينيل إلى مادة حشو الأسنان الزركونية المعالجة بالضوء تُحسّن الخصائص الفيزيائية والميكانيكية للحشوة المختلطة الجديدة.

الكلمات المفتاحية: الزركونيوم، النانو هجين، بوليمر كحول البولي فينيل، القوة، الصلابة، الامتصاص، والذوبان.

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