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DETECTION OF ENOLASE AND PYRUVATE KINASE ENZYMES FROM FLUORIDE-SENSITIVE AND FLUORIDE RESISTANCE *STREPTOCOCCUS MUTANS* BY USING PCR.

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Abstract

Background: This study aimed to detect enolase and pyruvate kinase, key metabolic enzymes in glycolysis, in fluoride-resistant and fluoride-sensitive Streptococcus mutans strains isolated from diabetic dental caries. Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP), while pyruvate kinase converts PEP to pyruvate. **Methods:** This cross-sectional study involved 32 *Streptococcus mutans* strains isolated from dental caries in Khartoum, Sudan, between April and June 2022. The strains were identified using conventional methods, and their fluoride sensitivity was tested by culturing them in BHI agar under microaerophilic conditions with and without fluoride. The presence of enolase and pyruvate kinase was detected using conventional PCR, and the results were analyzed with SPSS software version 23.0.

Results: In this study, enolase and pyruvate kinase enzymes were detected in 32 *Streptococcus mutans* strains using PCR. Enolase was present in 59% of strains, while pyruvate kinase was found in 75%. The presence of pyruvate kinase was linked to fluoride resistance, with significant resistance observed at fluoride concentrations of 80, 125, and 250 ppm, showing p-values of 0.037, 0.002, and 0.009, respectively.

Conclusion: Among the isolated fluoride-resistant and fluoride-sensitive *Streptococcus mutans*, 53% harbored both enolase and pyruvate kinase. Only 6% carried the enolase gene, and 22% had the pyruvate kinase gene. The absence of pyruvate kinase in fluoride-resistant strains may contribute to higher fluoride resistance, with significant resistance observed at concentrations of 80, 125, and 250 ppm, where 87%, 75%, and 50% of strains resisted fluoride, respectively.

Keywords: Fluoride resistance, PCR, Streptococcus mutans, enolase, and pyruvate kinase.

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Introduction:

Fluoride is an inorganic, atomic anion with the chemical formula (F-). Its salts and minerals are crucial chemical reagents and industrial chemicals since it was discovered in 1931 as a preventing agent against dental caries. Fluoride has been used extensively to prevent caries in the teeth, it has markedly reduced caries. Fluoride prevents dental carried by inhibiting demineralization and increasing dental hard tissue remineralization. Additionally, it inhibits metabolic activity and affects bacterial growth by inhibiting *Streptococcus mutans* metabolic enzyme enolase and ATPase enzyme (1).

The excess fluoride exposure may be due to the natural source of freshwater containing high concentrations of fluoride in some geographical areas such as Africa, the Eastern Mediterranean, and southern Asia. The toothpaste or mouth rinse are fluoridated, as well as food sources and public water fluoridation (2). The emergence of Long-term fluoride usage increases the risk of bacterial resistance developing (Fluoride-resistant strains). Numerous studies have found strains that were resistant to fluoride. *Streptococcus mutans* can gain this resistance both phenotypically (unstable) and genotypically (stable), and it has been found less cariogenic than the wild-type *Streptococcus mutans* UA130 and *Streptococcus. Mutans* U159 (3).

Because at acidic pH, the resistant strains generate less acid at a slower pace than the wild type. Another feature is the ability of fluoride-resistant *Streptococcus mutans* to compete with the wild-type strains in binding to artificial tooth pellicle (3).

Enolase and pyruvate kinase are crucial glycolytic enzymes involved in the glycolysis pathway. The effect of fluoride on bacterial metabolism has been demonstrated in previous studies. It occurs in two ways, either directly by inhibiting the cellular enzyme or in combination with (metal or hydrogen) to form HF (hydrogen fluoride) that enhances the proton permeability of bacterial cell membrane (4).

Inhibition of fluoride by *streptococcus mutans* enolase enzyme has been demonstrated in several vitro studies, but it is still unclear if this inhibition would occur in whole bacterial cells since they used purified extract of the enolase gene only (5). A study on the Enzyme purification of *Streptococcus mutans* and suppression by fluoride shows that the inhibitory fluoride concentrations for the pyruvate kinase enzyme were around 10–100 times greater than those needed for enolase suppression, suggesting that the inhibition of fluoride on pyruvate kinase is improbable to occur in bacterial cells (4).

In vitro study on fluoride-resistant and fluoride resistive in *Streptococcus mutans:* a mini-review, they study the purified enolase and its permeabilized cell. They found no variation in the enzyme activity compared to the two strains (6).

A report on the same study finds that the enolase and ATPase activity of Fluoride-sensitive and fluoride-resistant *Streptococcus mutans* strains were affected by different fluoride types. The enolase from the fluoride-resistant strain was found to be less sensitive to fluoride, but the variation was not significant enough to be considered the reason behind the resistant metabolism mechanism.

In a study of identification and functional evaluation of genome mutation in a fluoride-resistant *Streptococcus mutans* strain (2015) (7). They identified a change in the fluoride resistance of *Streptococcus mutans* strain (C180-2FR) using a whole genome shotgun (WGC), and they compared the gene expression of fluoride-sensitive (C180-2In the genome of S.mutans C180-2FR, they discovered eight single nucleotide polymorphisms (SNPs), which Sanger sequencing verified (7).

Another study was done by Ying et al., (2018) (4), on genetic loci associated with fluoride resistance in *Streptococcus mutants*. The researcher used wild type of *Streptococcus mutans* UA159, C180-2, and their derived fluoride-resistant strains UA159-FR

and C180-2FR. Both fluoride-resistant strains were produced by culturing the wild type on a plate containing a high amount of NaF (not more than 52.6mM/L). The colonies were able to flourish on high concentrations of fluoride. The genomic sequence of *Streptococcus mutans* UA159 (NCBI accession: NC_004350.2), was compared with the genome sequence of *S.mtans* UA159-FR(.NCBI accession: NZ_CP007016.1), to identify a single nucleotide polymorphism (SNP). Streptococcus mutans. UA159-FR's identified non-synonymous and intragenic SNPs were compared to Streptococcus mutants C180-2FR's published SNPs according to Liao et al., (2015) (7). The activity of enzyme enolase (eno) and pyruvate kinase (pyk) (glycolytic enzyme), were elevated in both fluoride-resistant strains *S.mutans* C180-2FR and *S.mutans* UA159-FR. *Streptococcus mutans* UA159-FR demonstrated greater resistance to fluoride during development in comparison to S. mutans UA159. 24 SNPs were found in the S. mutans UA159-FR genome by bioinformatics analysis. These SNPs were compared to those discovered in the *Streptococcus mutans* C180-2FR genome. They were able to identify three shared loci with mutations in both fluoride-resistant strains. Analysis of these shared loci revealed modifications in the expression of genes or protein function changes (7).

The objective of this study was to detect the enzymes pyruvate kinase and enolase in S. mutans using uniplex conventional PCR. And the effect of these enzymes on the sensitivity of bacteria to fluorine salt.

Material and methods:

That research was a descriptive, cross-sectional study that involved 32 *Streptococcus mutans* strains isolated from dental caries and identified by using conventional methods. Fluoride- sensitivity was characterized by examining growth in BHI (brain heart infusion agar) under microaerophilic condition (5% CO2) at 37ºC with and without fluoride being added. Growth in fluoride-containing plates indicates the resistance strain. This study was conducted in Khartoum Sudan from the period 2022 April -June 2022.

Genomic DNA Extraction:

When the fluoride sensitivity and fluoride resistance of *S. mutans* strains were verified, The FAVOPrepTM Tissue Genomic DNA extraction mini kit (FAVRGEN) was used to isolate the genomic DNA from the S. mutans culture.

Preparation of primer sets:

Primer sets used were obtained from Germany (Metabion Company). Each primer of the two enzymes (Enolase eno coding gene and pyruvate kinase pyk coding gene) was diluted from 100µg\lµl stock solution and stored at -20C by adding nuclease-free PCR water. The dilution ratio was 1:10 with nuclease-free PCR water following the instructions of the manufacturer. The working solution of the PCR master mix was prepared by adding 0.5 µl of primers (each forward and reverse) to 4µl of master mix ready to load and 5µl from the template DNA finally the volume was completed by adding 10 µl of water injection. The master mix was used in a concentration suitable to give the best bands on gel electrophoresis.

PCR amplification conditions:

Amplification was done by the following thermal cycler (Aeris machine Peltier affect thermal cycler technology) the initial duration at 94ºC for 5 minutes and denaturation at 94ºC for 25 sec, annealing at 57ºC at 30 sec, and extension at 72ºC at 1 mint, with 5 min at 72ºC for final extension followed by 36 cycles. The PCR cycle takes from 2-3 hours. The PCR annealing temperature was considered according to the equation 4* (C+G)+ 2 (A+T) – 5.[44]

Loading and electrophoresis of amplified PCR product:

2.5 µl of ethidium bromide was added. The agarose TAE solution was poured onto a casting tray. The gel solution was cooled down and solidified. A gel slab was created with a row of wells at the top. Each

well was loaded from 5µl of PCR product, with molecular marker 50 bp DNA ladder (APSLABS, India) used as an external reference for size comparisons.

Bands visualization on agarose gel:

To confirm the PCR products, the amplified products were visualized with a transilluminator (Barometers, an analytical Jena Company).

Table (1): Primers set for amplification of enolase and pyk genes:

PCR	Primers	Sequence (5´-3´)	Size pb
Uniplex	Enolase F	GGTGAAGATGGCTATTGG	85
	Enolase R	CATTGGCTCCTTCTGTAAT	100
Uniplex	PYK F	GCCACATATGAATAAACGCGTAAAAATTGT	100
	PYK R	CATGCCGCGGTTATTGAACGGTACGAACAC	

Statistical Analysis:

We analyzed the data using Statistical Package for Social Sciences software, version 23.0 (IBM SPSSInc., Chicago, IL, SPSS (RRID: SCR_002865).

Results and discussion:

For the detection and characterization of two enzymes, with considered PCR technique is a gold standard. Among 32 *Streptococcus mutans*, strains were positively detected for enolase enzyme which presents in 19 strains of *Streptococcus mutans* (59%) and not detected in 13 strains (41%) of the isolates. Regarding pyruvate kinase enzyme, it was detected in 24 strains of *Streptococcus mutans* (75%) and not detected in 8 stains (25%).

Table (2): The Prevalence of Enolase and Pyruvate Kinase Among Isolated *Streptococcus mutans*;

Enzymes	Present	Absent
Enolase	19(59%)	13(41%)
Pyruvate kinase	24(75%)	8(25%)

n=32

Distribution of enolase and pyruvate kinase enzymes. The strains carrying only one gene of enolase (Eno) were found to be 6% of the total isolates, while strains carrying only the pyruvate kinase gene (pyk) were found to be 22% of the total isolates.

The strains carrying the combination of two genes were 53% of the isolates. It is represented in Figure (1).

Comparing the results of PCR for enzymes and their presence or absence in the isolated bacteria and their sensitivity to fluoride, it was found that the presence of the enzyme pyruvate kinase proved its important correlation which was statistically significant (p-value 0.037). The isolates that harbor two enzymes were statistically significant (p-value 0.051) when the isolates were sensitive to sodium fluoride at a concentration of 80Ppm. The result is represented in Table (3).

Detection of enolase and pyruvate kinase enzymes and both of them (pyruvate kinase and enolase) enzymes, was found to be statistically significant (p-value0.015, 0.002, and 0.001) respectively when the isolates were sensitive to sodium fluoride at concentration 125 Ppm. Results are represented in Table (4).

When the isolates harbored pyruvate kinase enzyme only or harbored both of them, it was shown to be statistically significant (p-value 0.009 and 0.006) respectively, when the isolates were sensitive to sodium fluoride at a concentration of 250 Ppm. Results are represented in table (5).

There was no significant difference when the isolates harbored only enolase enzyme, and pyruvate kinase enzyme and harbored both, at a concentration of 500Ppm sodium fluoride. The results are represented in table (6).

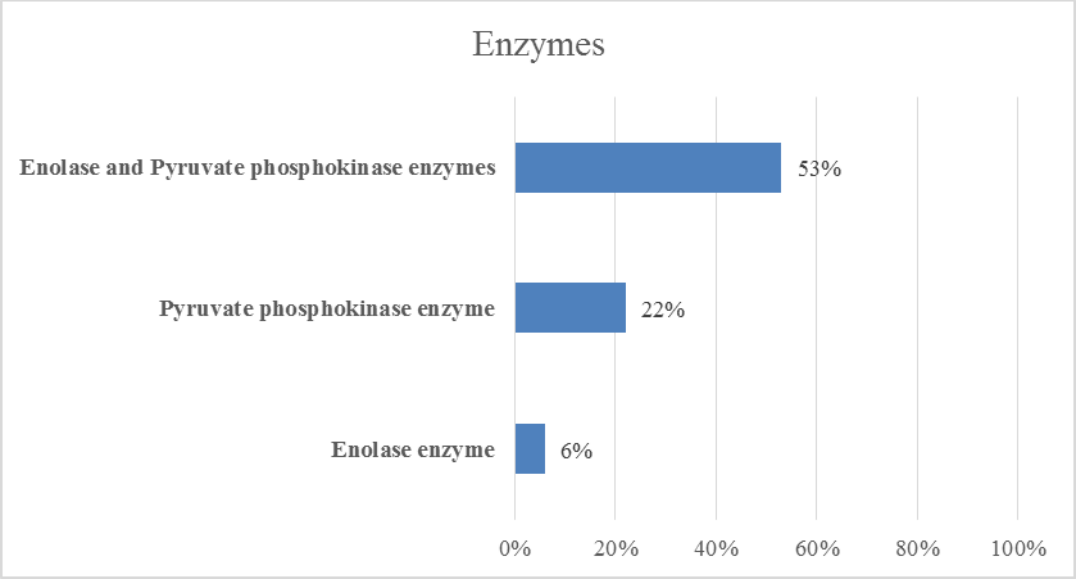

Figure 1: The Distribution of Enolase and Pyruvate Kinase Enzymes among Isolated *Streptococcus mutans*.

Table (3): Correlation Detection of Enzymes Enolase and Pyruvate Kinase on Streptococcus mutans and Antimicrobial Susceptibility using 80Ppm Fluoride:

Variables		Fluoride 80Ppm		Fisher's Exact Test P value
		Sensitive	Resistant	
		12	7	
	Present	63.2%	36.8%	
		4	9	
Enolase enzyme	Absent	30.8%	69.2%	0.149*
		15	9	
	Present	62.5%	37.5%	
		1	7	
Pyruvate kinase enzyme	Absent	12.5%	87.5%	0.037**
		1	5	
	Present	16.7%	83.3%	
		0	2	
	Enolase	0.00%	100.0%	
		3	4	
	Pyruvate kinase	42.9%	57.1%	
		12	5	
Enzymes	Enolase and Pyruvate kinase	70.6%	29.4%	0.051**

Table (4): Correlation Detection of Enzymes Enolase and Pyruvate Kinase on *Streptococcus mutans* Antimicrobial Susceptibility using 125 Ppm Fluoride.

Variables		Fluoride 125Ppm		Fisher's Exact Test P value
		Sensitive	Resistant	
		17	2	
	Present	89.50%	10.50%	
		6	7	
Enolase enzyme	Absent	46.20%	53.80%	0.015*
		21	3	
	Present	87.50%	12.50%	
		2	6	
Pyruvate kinase enzyme	Absent	25.00%	75.00%	0.002**
		1	5	
	Absent	16.70%	83.30%	
		1	1	
	Enolase	50.00%	50.00%	
		5	2	
	Pyruvate phosphokinase	71.40%	28.60%	
	Enolase and Pyruvate	16	1	
Enzymes	phosphokinase	94.10%	5.90%	0.001**

Table (5): Correlation Detection of enzymes enolase and pyruvate kinase on *streptococcus mutans* using 250 Ppm fluoride:

Variables		Fluoride 250Ppm		Fisher's Exact Test P value
		Sensitive	Resistant	
Enolase enzyme	Present	18 94.70%	1 5.30%	0.132*
	Absent	9 69.20%	4 30.80%	
Pyruvate phosphokinase enzyme	Present	23 95.80%	1 4.20%	0.009**
	Absent	4 50.00%	4 50.00%	
Enzymes	Absent	2 33.3%	4 66.7%	0.006**
	Enolase	2 100.00%	0 0.00%	
	Pyruvate phosphokinase	7 100.00%	0 0.00%	
	Enolase and Pyruvate phosphokinase	16 94.10%	1 5.90%	

Table (6): Correlation Detection of Enzymes Enolase and Pyruvate Kinase on *Streptococcus mutans* and Antimicrobial Susceptibility using 500 Ppm Fluoride

Variables		Fluoride 500Ppm		Fisher's Exact Test P value
		Sensitive	Resistant	
Enolase enzyme	Present	19 100.00%	0 0.00%	0.406*
	Absent	12 92.30%	1 7.70%	
Pyruvate kinase enzyme	Present	24 100.00%	0 0.00%	0.250*
	Absent	7 87.50%	1 12.50%	
Enzymes	Absent	5 83.30%	1 16.70%	0.245*
	Enolase	2 100.00%	0 0.00%	
	Pyruvate phosphokinase	7 100.00%	0 0.00%	
	Enolase and Pyruvate phosphokinase	17 100.00%	0 0.00%	

DISCUSSION:

Enolase and Pyruvate kinase are two significant fluoride-sensitive enzymes. They were supposed to play an important role in fluoride resistance mechanisms because they were the most probable sites among shared fluoride resistance strains. Pyruvate kinase plays an important central role in carbohydrates' fermentation and breakdown by linking glycolysis and sugar uptake. An alteration in its activity or configuration can lead to a change in sugar metabolism. Additionally, pyruvate kinase is located downstream of enolase in mechanisms for the antimicrobial effects of fluoride cascade (7).

Our studies disagree with the in vitro study on fluoride resistance *Streptococcus mutans*: A mini review by Ying Liao et al, (2017) compared the enzyme (enolase), and activity between fluoride resistant and fluoride sensitive *streptococcus mutans* strains. They observed a difference in activity when comparing the two strains, and that the fluoride suppression of pyruvate kinase was considered improbable, as the fluoride concentrations required to inhibit pyruvate kinase enzyme were around 10 - 100 times greater than those necessary to inhibit enolase inhibition (6). However, in that recent report, we were able to identify significant differences when using 125 Ppm fluorides. When the enzyme enolase was detected, the sensitivity to fluoride isolates was found to be 46.2% and the resistance was 53.8% with a significant (p-value 0.015).

Our results agreed with the finding of Van Loveren et al, (2008) who investigated the impact of various kinds of fluorides on enolase and ATPase activity of fluoride-resistant and fluoride-sensitive *Streptococcus mutans* strain (8). They find that the enzyme enolase from fluoride-resistant strain is less sensitive to fluoride, which is similar to our finding, the significant difference only when using sodium fluoride at a concentration of 125Ppm.

Our recent study confirmed that the pyruvate kinase enzyme plays a vital role in fluoride resistance. The presence or absence of this enzyme significantly influences the resistance of *Streptococcus mutans* isolates to fluoride when exposed to a concentration of 80,125 and 250Ppm.with p-values 0.037, 0.002, and 0.009. However, we didn't find a difference when using 500Ppm fluoride concentration.

Conclusion:

It can be concluded that, among the total isolates of fluoride resistance and fluoride sensitive *streptococcus mutans*, these isolates were found to harbor both enzymes (53%), while the detection of enolase enzyme was on (59%) and pyruvate kinase on (75%), regarding the distribution of the enzymes. The strain found to harbor only enolase coding gene was (6%), while strains that carry pyruvate kinase coding gene were (22%).

Our research indicates that the absence of pyruvate kinase enzyme in fluoride-resistant *streptococcus mutans* may enhance their ability to withstand fluoride with significant resistance when using 80,125and 250Ppm 87.0%, 75%, and 50% were found to be resistant to fluoride respectively. Moreover, when the two enzymes were not detected 83.3% were resistant to fluoride concentrations 80 and 125 Ppm, and 66.7% resisted 250 Ppm of fluoride. It was discovered that 16.7% were resistant to 500 Ppm fluoride concentrations. Our findings indicated a possible contribution of pyruvate kinase in the fluoride resistance of streptococcus. The last point shows us that other factors play an important role in the resistance to fluoride as we mentioned in the introduction.

More research is needed to study the fluoride's distinctive inhibition of bacterial glycolysis on those enolase and pyruvate kinase enzymes, the vital role of enzymes in glycolysis by studying their genomic sequence, and bacterial fluoride resistance merits further research.

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Ethical Approval:

The ethical approval serial number: KMOH-REC-018.2-2022, was obtained from the Ministry of Health, Khartoum state, general administration of strategic and information innovation, developments, and scientific research.

Consent:

Written informed consent for the publication of these patients' data was obtained from the participants

Conflict of interest: NIL

Data availability:

DRYAD (Isolation and Identification of Fluoride Sensitive and Fluoride Resistant *streptococcus mutans Strains from Diabetics Dental Carries raw data]*.

https://doi.org/10.5061/dryad.g1jwstqtq

The project contains the following underlying data:

- *[README_datadrnuha.sav] (sav, raw data).*
- *[the antimicrobial effect of fluoride against streptococcus mutan](figure.docx)*
- *[README_raw_data.xlsx] (excel sheet, raw data)*

Reporting guidelines:

Dryad: [STROBE checklist for 'Isolation and Identification of Fluoride Sensitive and Fluoride Resistant Streptococcus mutans Strain from Diabetics Dental Carries raw data]'. https://doi.org/10.5061/dryad.g1jwstqtq

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