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Biochemical and Genetic Study of Muramidase Enzyme in Serum of Patient with Crohn`S Disease with Crohn`S Disease

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Abstract
Abnormal immune reactions throughout the digestive tract are a hallmark of Crohn's disease, also known as chronic inflammatory bowel disease. This study looks on the relationship between Crohn's disease and biochemical markers in Mosul City. 140 individuals (70 Crohn's patients and 70 healthy controls) had their serum levels of lysozyme, interferon-gamma (IFN-γ), tumor necrosis factor-α, interleukin-12 (IL-12), interleukin-23 (IL-23) and malondialdehyde (MDA) measured.Additionally, ribonucleic acid (RNA) was extracted using the quantitative polymerase chain reaction (q-PCR) technique from 13 patients and 2 healthy individuals. The results are displayed. The levels of IFN-γ, TNF-α, IL-12, IL-23, MDA, and mur all significantly increased in Crohn's disease patients compared to healthy people. Lysozyme gene expression also increased.
Key word: *Crohn s Disease, Lysozyme, Interferon-gamma, Tumer nicrosis factor-alpha.*

1. INTRODUCTION

Crohn's disease is one type of chronic inflammation that affects the gastrointestinal tract. In the United States, it affects about 400,000 people and was first discovered in 1932. The disease usually affects the colon or small intestine, but it can appear anywhere in the digestive tract, from the mouth to the anus.Crohn's disease is characterized by an abnormal immune response to good gut bacteria that leads to the invasion of immune cells, such as T-lymphocytes, excessive cytokine release, and severe inflammation that may affect the layers of the gut wall (1) (Baumgart & Sandborn, 2012). Three primary criteria are used in the Montreal Classification System to classify Crohn's disease. First, according to diagnosis age: A1 is identified before the age of sixteen, A2 between the ages of seventeen and forty, and A3 after forty. Two, according to the location of inflammation in the gastrointestinal tract: Ileal inflammation, denoted as L1, only affects the terminal ileum (28%) experiencing diarrhea, abdominal cramps, and weight loss, as well as potential side effects such fistulas or abscesses. L2 indicates ileocolonic inflammation, which is the most prevalent type (35%) and manifests as diarrhea, weight loss, and pain in the middle or lower right abdomen. It affects both the end of the ileum and a portion of the colon. With symptoms like diarrhea, perirectal abscesses and ulcers, fistulas, joint pain, and rectal bleeding, L3 denotes colonic inflammation that is restricted to the colon and does not include the ileum (32%). Anorexia, weight loss, nausea, vomiting, and possibly intestinal obstruction are all possible outcomes of L4, which is a rare condition involving isolated upper gastrointestinal tract inflammation, also known as gastroduodenal Crohn's disease, which affects the duodenum, the first segment of the small intestine, and the stomach (Kusulas & Delint, 2016). Third, we have B1 (non-penetrating and non-stricturing inflammatory disease), B2 (stricturing, resulting in intestinal narrowing), and B3 (penetrating, characterized by the creation of fistulas and abscesses). According to Kusulas and Delint (2016), P also indicates the existence of perianal illness. Diarrhea, weight loss, abdominal pain, exhaustion, mucus in the stool, dry mucous membranes, anemia, and stunted growth in children are the most prevalent symptoms of Crohn's disease. (Higgins & Cushing, 2021). Following diagnosis, Crohn's disease is treated with either surgery or medicine, such as methotrexate and thiopurines (Higgins & Cushing, 2021) (Boyapati and others, 2015).

In the innate immune system, lysozyme is one of the most important antibacterial proteins and has been thoroughly investigated. Numerous immune cells, such as neutrophils and macrophages, secrete it, and it is present in a range of body fluids, such as tears and saliva. By dissolving the peptidoglycan structure, it primarily targets the bacterial cell wall, particularly in Gram-positive bacteria. Bacterial death results from the cell wall being weaker. Ragland and Criss (2017) and Lerner and Ganz (2002).

In order to destroy or impede bacterial development, the enzyme muramidase primarily breaks the bonds in the bacterial cell wall, specifically focusing on N-acetylglucosamine and N-acetylmuramic acid. As a result, the body becomes more resistant to infection and prevents the spread of bacteria. When bacterial

infections occur, the levels of the enzyme in the blood and other body secretions rise, making it an essential indicator of immunological activation (Nevalainen et al., 2021).
The structure of the enzyme Mur is composed of six tryptophan residues: Trp-28, Trp-62, Trp-63, Trp-108, Trp-111, and Trp-23.Three tyrosine residues (Tyr-53, Tyr-2, and Tyr-23) are also present. Three of the tryptophan residues are situated in the substrate's binding site, two are in the enzyme's hydrophobic area, and one is close to the enzyme molecule's edge (Galvez–Iriqui & Plascencia–Jatomea, 2020).

2. MATERIALS AND METHODS

2.1 Case Study

In Mosul City, the study included 70 healthy controls (30 men and 40 women, ages 18–50) and 70 Crohn's disease patients (48 men and 22 women, ages 12–56). Participants were selected from private hospitals and outpatient clinics between January 19 and February 25, 2025.

2.2 Blood Sample Collection

The sample was separated into three sections after five milliliters of venous blood were drowned: 5.0 0.5 milliliters of blood were placed in an EDTA tube for DNA extraction and methylation analysis, 0.25 milliliters of blood were placed in a tube containing TRIzol for RNA extraction, and a gel tube was used .for biochemical analysi

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2.3 Biochemical Analysis

Interleukin-12 (IL-12), interleukin-23 (IL-23), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) were tested using serum levels of muramidase (LYZ) and enzyme-linked immunosorbent assay (ELISA) technology from the Chinese company SUNLONG. MDA stands for malondialdehyde.

2.4 Gene Expression and Mutation Analysis

To measure the expression of the LYZ gene in comparison to a housekeeping gene, total RNA was extracted, reverse-transcribed, and then subjected to quantitative PCR (qPCR). Furthermore, LYZ gene mutations were found using DNA sequencing.

3.MATERAILS AND METHODS

3.1 RNA extraction

250 microliters of the blood sample and 750 microliters of trizol are properly mixed after the blood is drawn. Following the directions on the Transgenbiotech kit, the RNA is then extracted.
Procedure:
1. Thoroughly combine the trizol-containing sample, then centrifuge at 10,000 rpm for five minutes.
2. After removing 300 µl of the top layer of water, add 200 µl of trizol and 200 µl of chloroform, and thoroughly mix by vortexing. After five minutes of room temperature incubation, centrifuge for twelve minutes at 12,000 rpm.
3. Fill the new tube with 300 µl of the upper layer, then add 300 µl of the 100% ethanol. Gently swirl by hand.
4. Place a collecting tube in a filter column and centrifuge the mixture for one minute at 12,000 rpm.
5. Eliminate the contents of the collecting tube, then put 500 µl of the wash solution in a centrifuge set to 10,000 rpm for one minute. Finally, dispose of the filtrate.
6. Include 500 µl from wash 2, centrifuge at 12,000 for 2 minutes, and then discard the filtrate. Centrifuge at 12.000 rpm for 3 minutes.
7. After setting the filter column in the water bath at 60°C for two minutes, pour 75 µl of elusion into a fresh collecting tube. After that, store at -80°C.

Table (1): Components of the RNA extraction kit from Transgenbiotech

Component	ER501-01(100rxns)
TransZol UP	100ml
RNA extract agent	20ml
Clean buffer 9(CB9)	110ml
Wash buffer 9(WB9)	24ml
RNase-free water	40ml
RNase-free Tube (1.5ml)	100
RNA Spin Colum with Collection Tubes	100

transformation of the isolated mRNA molecules into cDNA. Following the mRNA extraction procedure, the reverse transcriptase enzyme's activity determines whether the mRNA is transformed into a cDNA molecule. This is done using the Transgenbiotech kit technique and the following table.

Table (2): kit components supply by Transgenbiotech

Components	PC5801	PC5802
5 ×RT PCR Master Mix	200 µl	400 µl
RNase free H2O	1 ml	2 ×1 ml

Procedure:

1. On ice, template RNA thawed. At room temperature, RNase-free water was thawed and promptly placed on ice.

2. Put the following ingredient in a PCR tube that is RNA-free.

Components	Volume
Total RNA/mRNA	10 µl
Reverse transcriptase - enzyme	4 µl
RNase free H2O	6 µl
Total volume	20 µl

Mix the solution gently (20 µl total) and let it sit at 42 °C for 15 minutes. Reverse transcriptase inactivation for five seconds at 85 °C
The cDNA products can be used directly for qPCR or stored at -20°C for short-term storage; for long-term storage, they should be stored at -70°C to prevent freeze-thaw cycles.

Using the qPCR technique, determine the Lysozyme gene's expression levels
In addition to the housekeeping gene primers, particular primers for the Lysozyme gene were employed for a quantitative assessment of the degree of gene expression for apoptotic genes.

Table (3):the primer of the Lysozyme gene with the specific primer of the housekeeping gene

Primer	Sequence
LYZ F	GAGCAAAATATGGCCTTTCTTCTA
LYZ R	CGGAATGTACTGGAGATGTATTTG
H.K-F	GACCCAGATCATGTTTGAG

H.K-R	GACCCAGATCATGTTTGAG

Table (4) : The final reaction volume was 20 µl and as shown in the following table

Component	Volume
Ultra sybrgreen q-PCR master mix	10 µl
RT forward primer	0.5 µl
RT Reversed primer	0.5 µl
cDNA templet	4 µl
D.W	5 µl

➢ Table (5): the program use in RT- PCR reaction

Stage	Temperature	Time
Pre denaturation	95 °C	10 min
Denaturation	95 °C	15 sec
Annealing /Extension	60 °C	1 min
Melting curve analysis	95 °C 60 °C 95 °C 60 °C	15 sec 1 min 15 sec 15 sec

3.2 Calculate the rate of gene expression

The following formula was used to determine the rate of gene expression for the control and patient samples based on the CT value of the target gene with the housekeeping gene:(Haimes and others, 2013).

1. ΔCT (test) = CT (target, test) – CT (ref, test).

ΔCT (control)=CT (target, control) -CT (ref, control).

CT (target, test) refers to the number of mRNA cycles for the Bax and Bcl-2 genes in patient samples.

CT (ref, test) indicates the number of mRNA cycles for the housekeeping gene in patient samples.

CT (target, control) refers to the mRNA cycles of the Bax and Bcl-2 genes for control samples.

CT (ref, test) refers to the mRNA cycles of the housekeeping gene for control samples.

2. The equation of ΔCT for the treated sample relative to ΔCT for the

Table(6): kit components for DNA extraction

Item	Volume	Storage
Proteinase K solution	2ml	At(2-8°C)
Lysis buffer-2	35ml	At(15-25°C)
Washing buffer 1	55ml	At (15-25°C)
Washing buffer 2	55ml	At(15-25°C)
Elution buffer	20ml	At(15-25°C)
Collection tube and spin column	100 pcs	At(15-25°C)

Procedure:

1. Use a vortex to thoroughly mix the sample for 15–20 seconds.
2. Fill an Eppendorf tube that is 1.5 or 2 mL with 20 µL of Proteinase K solution.
3. Fill an Eppendorf tube that is 1.5 or 2 mL with 300 µL of Lysis buffer-2.
4. Add 200 µL of the liquid blood sample, mix by repeatedly inverting, and then vortex for ten to fifteen seconds.
5. The sample combination should be incubated at 60 oC for 15 minutes.
6. Flip the mixture every five minutes to mix it.
7. To get the drips to stick beneath the cover, quickly spin down. Let the tubes in.
8.To the lysate, add 200 µL of absolute ethanol, mix thoroughly by inverting, and vortex for 10 seconds.
9. To get the drips adhering beneath the cover, quickly spin down.
10. Insert a spin column into the tube used for collecting. Next, move all of the lysate (about 720 uL) to the column, screw on the cap, and spin at 11,000 rpm for 1 minute at room temperature.

11. Put the spin column in the same collecting tube after discarding the filtrate.
12. Include 500 µL of wash buffer-1 and centrifuge at 11.000 rpm for 1 minute.
13. Put the spin column in the same collecting tube after discarding the filtrate.
14. Centrifuge at 13,000 rpm for 1 minute after adding 500 µL of wash buffer-2.
15. Put the spin column in the same collecting tube (reuse) and discard the filtrate.
16. To get rid of the remaining ethanol, centrifuge again for three minutes at 13,000 rpm.

17. Insert the spin column into a fresh, unsupplied 1.5 ml tube.
18. Fill the column with 100–150 µL of preheated (60°C) elution buffer, then incubate for 5 minutes at room temperature.
19. Centrifuge at ambient temperature for two minutes at 11,000 rpm.
20. Throw away the column; it can be used right away or kept for analysis at 4 °C. DNA should be kept at -20 °C for extended periods of storage.

3.3 Estimating the concentration and purity of the extracted DNA

The concentration and purity of the extracted DNA samples were measured using the biodrop.

3.4 DNA sequence

The sequences of the nitrogenous bases of the SLC gene were determined for the study samples to ensure the validity of the designed primer used in the PCR technique and to detect the presence of additional variations in the target genes.

The products of the PCR reaction for the Bcl-2 and Bax genes were sent with the primers. The sequence was then read at the Psomagen Center in the United States of America. After that, the gene sequences were matched with the gene sequences documented at the National Center for Biotechnology Information (NCBI), and the results were analyzed using the BLAST program.

Table (7): Include the primer that have been used in DNA sequence

Primer	Sequence
SLYZ F	TATGTGACTCCAGTTATTTCCCATT
SLYZ R	AACATCACTGTTAAAAATCCCATGT

Table (8): Show the PCR program for LYZ gene.

Stage	Temperature	Time	Number of cycle
Initial denaturation	**94 °C**	**5 min**	**1**
Denaturation	**94°C**	**45min**	**35**
Annealing	**56°C**	**1min**	**1**
Extension	**72**	**1min**	**1**
Final extension	**72**	**7min**	**1**
Stop reaction	**4**	**4min**	**1**

4. RESULT AND DISCUSSION

4.1 Biochemical Analysis

The result in the table (9) shown an increase in the level of each of Muramidase,Interferon-γ,Interleukin-12, Interleukin-23 and MDA

Table (9) the level of biochemical parameters in patient with crohn s disease and healthy people

Biochemical parameters		Mean	Std.Deviation	P-value
Muramidase Pg/ml	control patient	0.83 8.99	0.49 1.99	0.001
Interferon-gamma Pg/ml	control patient	109.4 132.7	20.36 33.59	0.001
Tumer nicrosis factor-alpha pg/ml	Control patient	150.2 174..2	26.25 52.67	0.008
Interelukin-12 Pg/ml	control patient	9.12 12.79	1.99 6.67	0.001
Interleukin-23 Pg/ml	control patient	13.39 17.37	2.18 8.12	0.002
Malondialdehyde µmol/L	Control patient	0.27 0.40	0.20 0.24	0.004

P is Significant at the level p≤0.05

The complex interaction of microbial, environmental, and genetic factors results in increased immunological activity that enhances intestinal inflammation, as seen by elevated muramidase levels in Crohn's disease patients. In 1977, Helman et al.
An key sign that the cellular immune response, particularly the Th1 type, is vital to the development of disease is the rise in interferon-dz levels. Natural killer (NK) cells and type 1 helper T cells both release

the cytokine interferon-gamma. Additionally, it directly triggers the intestinal chronic inflammatory response (Grabarek et al., 2017).

In patients with Crohn's disease, tumor necrosis factor-alpha (TNF-α) levels are considerably higher than in healthy individuals (p=0.008), as indicated by the results from Table 9. Jankeer & Al-Taii, 2025 This rise is the consequence of a complicated interaction between environmental, immunological, and genetic factors that causes an inflated and disjointed immune response. These genetic changes, such as those affecting the NOD2 gene, have been shown to decrease the ability of phagocytic cells to efficiently eradicate specific bacteria. The inflammatory processes found in Crohn's disease are exacerbated by the bacterial buildup in tissues, which encourages the overproduction of TNF-α. (Cho & Abraham 2009).

Patients with Crohn's disease have higher levels of interleukin-12 and interleukin-23 because their immune systems overreact to microbial antigens. the intestine, especially in those who are genetically predisposed. This leads to overactivation of antigen-presenting cells such as macrophages and dendritic cells.
High quantities of IL-12 and IL-23 are produced by these cells in response to continuous microbial exposure caused by dysbiosis of the gut microbiota. subsequently, naive T cells are stimulated to differentiate into Th1 cells by IL-23 and IL-12. These Th1 cells subsequently release interferon-gamma (IFN-γ), which causes inflammation. Genetic variables that affect the regulation of this inflammatory response and increase the production of IL-12 and IL-23 include mutations in the NOD2 and IL23R genes. This series of interactions eventually results in intestinal wall inflammation, which explains why patients with Crohn's disease have significantly elevated levels of IL-12 and IL-23.(Neurath 2014).

Malondialdehyde (MDA) levels in the serum of Crohn's disease patients are significantly higher than those of healthy controls, with a probability threshold of (p=0.004), indicating increased oxidative damage in their cells. Shihab, Yaseen, and Al-Abachi, 2022).
Crohn's disease patients' higher blood MDA levels are a reflection of the increased harm that oxidative stress and chronic inflammation have done to their bodies. The production of free radicals by immune cells in response to intestinal inflammation causes lipids in body cells to oxidize, causing cellular damage and the buildup of malondialdehyde, a sign of oxidative injury. Accordingly, patients with Crohn's disease have greater MDA levels than healthy individuals who do not suffer from this kind of inflammation or oxidative damage (Rezaie et al., 2007).

4.2 Gene Expression Analysis

Lyz gene expression levels were assessed by q-PCR and compared to a housekeeping gene (Attash & Hamed, 2023). Compared to healthy controls, patients with Crohn's disease exhibit significantly higher levels of Lyz expression, as indicated by the results displayed in Table (10) and Figure (2). Higher enzyme levels in tissues and physiological fluids may result from the production of the antimicrobial enzyme muramidase by immune cells, monocytes, and Paneth cells in response to chronic immunological activation in Crohn's disease (Peeter & Geboes, 1975), (Hamed, 2021).

Table (10) Ct valueans,LYZ gene expression levels ,and housekeeping gene expression levels for patient compared to those of healthy people

Lys. gene						
Sample	**CT target gene**	**CT housekeeping gene**	**Δ CT target gene**	**Δ CT control**	**Δ Δ CT**	**Gene Expression folding**
C	32.5	30.66	1.84	1.84	0	1
1	33.75	33.07	0.68	1.84	-1.16	2.23
3	31.33	30.21	1.12	1.84	-0.72	1.64
4	32.37	31.01	1.36	1.84	-0.48	1.4
5	32.15	31.05	1.1	1.84	-0.74	1.67
6	26.51	28.37	-1.86	1.84	-3.7	12.99
8	29.71	29.41	0.3	1.84	-1.54	2.9
25.70	31.36	-5.66	1.84	-7.5	100	4.4

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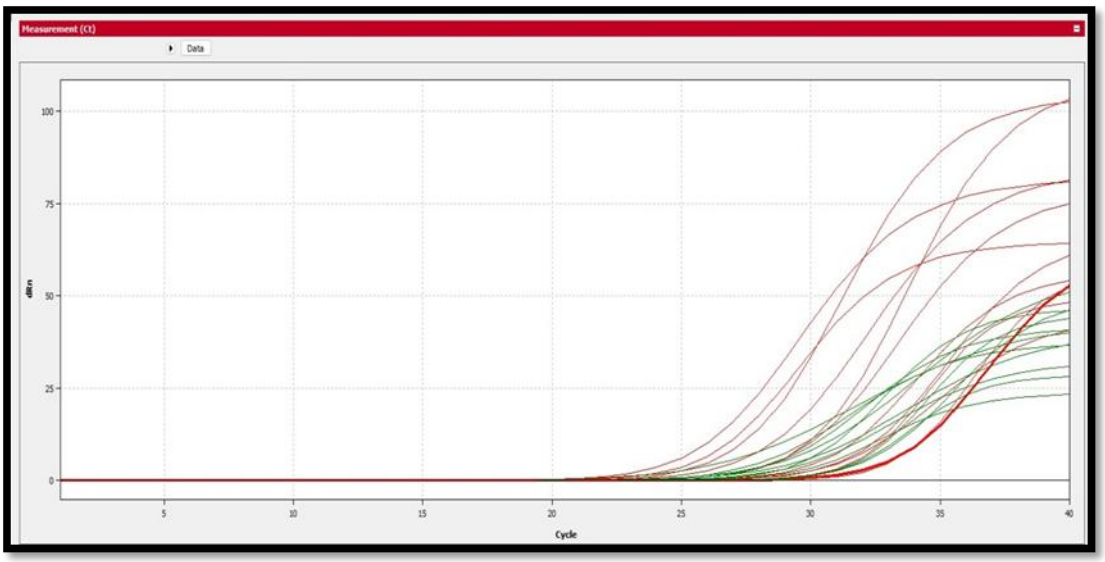

Figure(1): Gene expression level of the Lys gene measured by qPCR.

According to Figure (2), those with Crohn's disease had significantly higher Lys gene expression (3.89), compared to controls (1.0). This rise is a result of increased mRNA synthesis, which produces more muramidase, an enzyme vital to the development of the illness. The increase in expression is associated with intestinal cell inflammatory pathway activation and immunological responses against persistent bacterial infection (Stamp et al., 1992). (Yunos & colleagues, 2023).

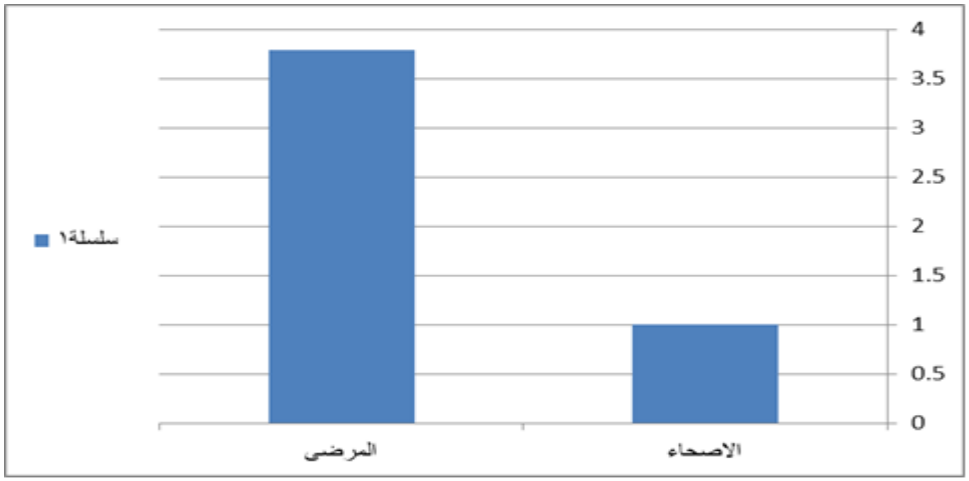

Figure (2)

The PCR results showed an association between the patients and genetic mutations in the Lys gene, as illustrated in the figure (3)

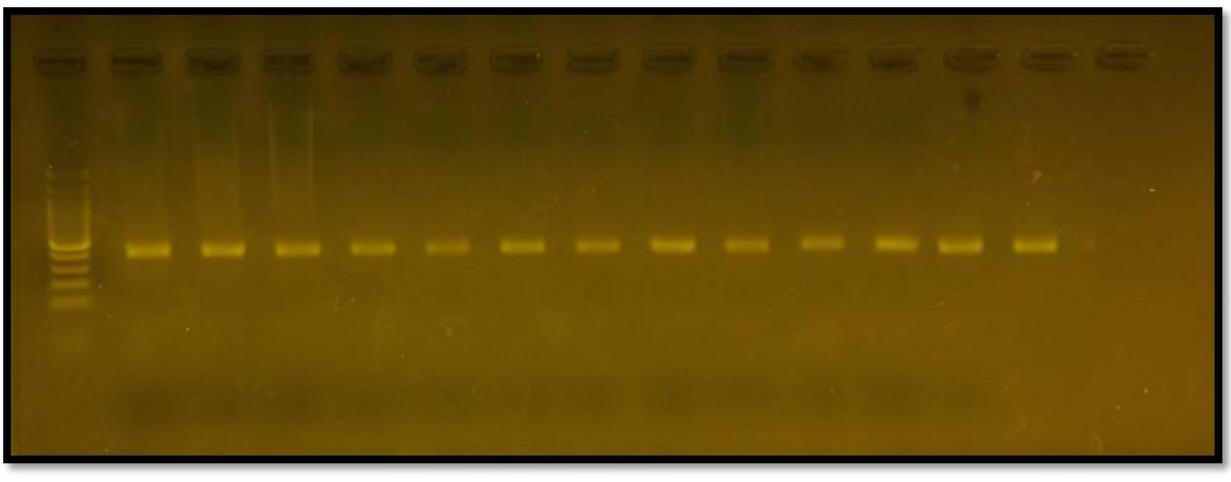

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Figure 3: Lys gene PCR findings, separated on a 2% agarose gel. The results revealed a range of genetic alterations, most of which were transversion mutations (A ↔ T), (A ↔ C), (T ↔ G), and (C ↔ G), as well as transition mutations (C → T), and (A ↔ G). Transition mutations may cause nonsense mutations or silent mutations, depending on the codon in issue. The structure and function of the protein may be changed by these mutations, which would significantly impact the protein's overall efficacy and biochemical interactions. Fernandez and Puglisi, 2022).

Figure(4) demonstrates how the LYZ gene in blood samples from Crohn's disease patients aligns with the NCBI database's reference gene sequence.

Figure (4) Sample (1)

Figure(5) demonstrates how the reference gene sequence in the NCBI database and the LYZ gene in blood samples from Crohn's disease patients align.

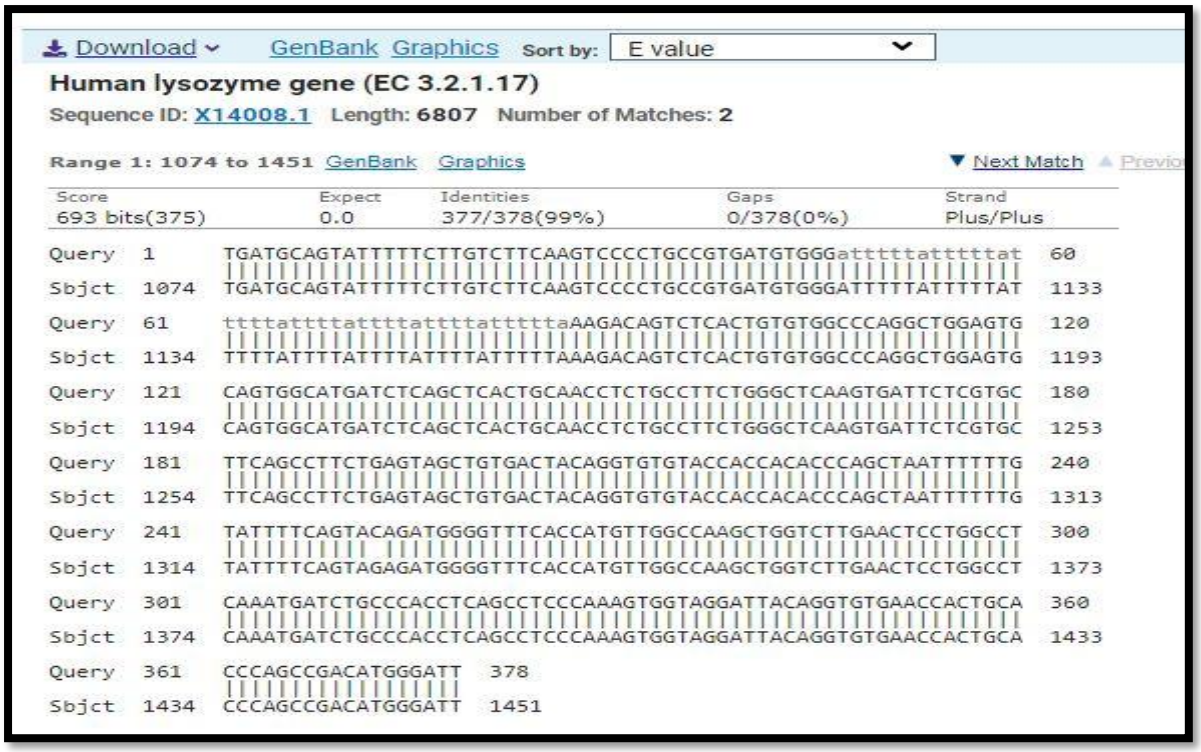

Figure (5) Sample (2)

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