CME LIVE OPEN ACCESS

Journal of Clinical Immunology Research

Research Article

DNA Sequencing, Phylogenetic Tree and a New Recording Genes of Toll- like Receptors (2, 4 and 9) in Gene Bank – NCBI for Patient with Male Infertility in Basrah

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Received: 2023 June 15 **Accepted:** 2023 July 10 **Published:** 2023 July 30

Abstract

IInfertility remains a global health challenge with devastating psycho-social consequences in many communities, and the underlying long-term risk of couple separation is also a major clinical and social problem. Infertility is defined as the inability of a couple to conceive naturally after one year of intercourse.

The aim of the study was to determine the immune molecular characterization of TLR genes. Toll-like receptors (TLRs) are an important family of receptors that constitute the first line of defense against pathogens. They can recognize both invading pathogens and endogenous danger molecules released from dying cells and damaged tissues and play a key role in linking innate and adaptive immunity. TLRs are widely distributed in both immune and other body cells. A cross sectional case control study was carried out by ELISA technique, conventional PCR, and DNA sequencing among male infertility patients who attended to the Infertility and In Vitro Fertilization Center of Basrah Province on September 2021 to June 2022. The results from the sequencing of TLR2, TLR4 and TLR9 were shown as follows: TLR2 in the current study, the forward primer when compared with the sequence of NCBI by the basic local alignment search tool (BLAST) showed 96% identity with an expected value of 2e-46 and there were five mutations: -Gab (-) >G, gab (-) >G, G>C, T>C, T>C in various locations, resulting in a new amino acid or protein. The other hand, the TLR2 reverse primer showed three mutations: - G>Gab (-), C>T, T>C in different locations with identity 98% and 2e-44 expect value. The forward primer of TLR4 when compared with the sequence of NCBI showed three mutations: - G>T, C> Gab (-), Gab (-) >an in different locations with 97% identity and 6e-44 expect value. On the other hand, the reverse primer showed four mutations: - Gab (-) >G, G>A, G>A, A>T with 97% identities and a 5e-44 expect value. TLR9, the forward primer, when compared with the sequence of NCBI, showed six mutations: - C> Gab (-), Gab (-)> A, Gab (-)> A, C> G, Gab (-) > T, C> AA in different locations with 95% identity and 6e-50expect value. On the other hand, the reverse primer showed a single mutation: - A> Gab (-) in different locations with 99% identity and a 6e-50 expect value.

Keywords: Male Infertility, Toll like Receptors, DAMPs, PAMPs, Phylogenetic Tree, Gene.

1. Introduction

Infertility remains a major problem for couples throughout the globe. Clinically, it is referred to as the inability of a couple to conceive after one year of regular sex [1]. 13-18% of couples suffer infertility, with the male component accounting for up to 50% of all cases [2]. Primary infertility is defined by the World Health Organization (WHO) as a woman who has never conceived, while secondary infertility it's the inability to become pregnant after at least one successful pregnancy [3]. Primary infertility affects 67%-71% of patients, whereas secondary infertility affects 29%-33%. One in ten couple's experiences infertility for various reasons. Male infertility has several causes, More than 50% of infertile males have unknown (idiopathic) causes, which may be inherited or acquired [4].

Male infertility may be caused by medical (inherited or acquired), environmental (chemical substances, chemotherapeutic agents, radiation, pollution, and stress), and lifestyle variables (smoking, alcohol use, illegal recreational drug

use) [5]. To evaluate male infertility, the urologist collects an assessment of the patient's medical history and a physical assessment that involves a semen test [6]. An infertile male's sperm examination may reveal the following conditions: (a) Oli gozoospermia (low spermatozoa count), (b) Teratozoospermia (aberrant sperm), and (c) As the no zoos permia (low sperm motility). This disorder is known as Oli go as the not era to azoospermia syndrome when these anomalies are detected in sperm analysis [7].

The immune system, comprising adaptive and innate immunological processes, offers the first line of protection against external threats by recognizing and responding quickly to infections and other immunogens, and by inducing inflammation. Innate immunity is key to male reproductive system infection responses [8]. Recent studies demonstrate that the immune cells are indeed mounting an antitumor response and that tumors develop mechanisms to combat an immune response [9].

Pattern-recognition receptors, that identify certain motifs, or pathogen-associated molecular patterns (PAMPs), generated by bacteria, virus, fungi, and protozoan pathogens [10]. And damage-associated molecular patterns, are required for the trigger of the innate immune system (DAMPs) [11].

Toll-like receptors, often known as TLRs, are one of the primary categories of pattern recognition receptors. These receptors identify the molecular patterns of infections, which helps the body's innate immune system detect foreign pathogens [12]. Several TLRs react to distinct molecular patterns related to diseases, such as, lip open tides (TLR 1, 2, 6), lipopolysaccharide (TLR 4), double-strand RNA viruses (TLR 3, 7, 8) and Chg.-rich un methylated DNA (TLR 9), bacterial flagella (TLR 5) [13, 14]. As a mediator, TLR not only plays a pivotal function in the induction of innate immunity However, it also serves as a bridge between innate and adaptive immune systems. TLRs are found on immune cells and cells that are not part of the immune system. These cells include B lymphocytes, dendritic cells, macrophages, natural killer (NK) cells, endothelial cells, fibroblasts, and epithelial cells [15]. Furthermore, these receptors can dimerize on the cell membrane, in which case two identical proteins hem dimerize or two distinct TLRs het erodimerize. Specificity in these receptors has improved via het erodimerization [12]. On the surface of cells, TLR 1, 2, 4, 5, and 6 were shown to be connected with external microorganisms, whereas TLR 3, 7, 8, and 9 were found on the membranes of cytoplasmic organelles, such as endosomes, to sense pathogen-related nucleic acids [16].

TLR induction signaling pathways in the host as a defense against attackers and to heal injured tissue, causing the secretion of several inflammatory cytokines and immune mediator [17, 18]. As a result of excessive TLR activation, persistent production of chemokines and pro-inflammatory cytokines impairs the immunological balance and hence leads to numerous illnesses [19].

In the male reproductive system, TLRs are few, although they have been demonstrated to be expressed all across the male reproductive system, involving the testis, vas deferens, epididymis, and accessory glands of male reproductive tissues [20]. In men, TLRs seem to have a role in both normal and pathological testicular steroidogenesis and spermatogenesis [21]. Invasion of the testis or other regions of the reproductive organs by pathogens activates innate immune responses and TLRs [22]. TNF-α and NO, inflammatory mediators produced by activated testicular macrophages via TLRs, may limit Ley dig cell androgen synthesis and negatively impact sperm production if levels are elevated above normal [23].

2. Materials and Methods

2.1 Samples Sources: This case control study was conducted between 1 September 2021 and 1 June 2022 in the province of Basrah. A questionnaire paper was used to record special note including no. of file, age, family history, varicocele, duration of marriage, infertility type, other disease, drugs, smoking, in addition to seminal fluid analysis, regarding all these individuals. Samples of blood have been collected from the male patients at Infertility and IVF center in Basrah province. Ethical approval was attempted according to acceptance from Research and Development center- Ministry of health and the approval of head master of each hospital was obtained, the objective of the study was explained to each participant.

Exclusion Criteria

- All patients who have atopic diseases.
- All patients who have autoimmune diseases.
- Patients who have an infectious disease, varicocele and reproductive organ surgery.

Blood DNA Extraction

The DNA extraction was performed by using (Easy Pure® Blood Genomic DNA Kit), DNA was extracted from blood, according to the manufacturer

Table 1: Shows the reagents of Easy Pure® Blood Genomic DNA extraction Kit.

Component	EE121-01(50 rxns) EE121-11(50 rxns)
Binding Buffer 3 (BB3)	30ml
Clean Buffer 3 (CB3)	6ml
Wash Buffer 3 (WB3)	12ml
Elution Buffer (EB)	25ml
RNase A (20 mg/ml)	500 µl (EE121-01) 0 (EE121-11)
Proteinase K (20 mg/ml)	1ml
Genomi Spin Columns with Collection Tubes	50 each

Preparation of Agarose Gel

1% of agarose gel was Prepared by mixing 1 gram of agarose powder with 100ml of already prepared TBE buffer in Pyrex conical flask , then dissolved the mixture very well in microwave oven for about 4 min at medium temperature until it start boiling with no thread appearance throughout agarose liquid, allow the agarose to cool until 50 C° then ethidium bromide was added to the gel (5µl of the stain per 100ml of agarose gel), after that the gel poured into the mold and let it at room temperature to solidify and be ready to use.

Preparation of the PCR master mix reaction

Using (one taq quick-load) PCR Kit, a PCR master mix reaction was performed according to the manufacturer's instructions.

Notes: the reaction was thoroughly mixed. Then, if required, a rapid spin was used to collect all liquid
At the bottom of the tube. The PCR tubes were moved to a PCR machine and thermo-cycling was initiated.

Table 2: A Protocol for one Tag® Quick-Load 2X Master Mix with Standard Buffer

Component	25 µl reaction
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Template DNA	1.5 µl
OneTaq Quick-Load 2X Master Mix with Standard Buffer	12.5 µl
Nuclease-free water	10 µl
Total	25 µl

Table 3: Thermo-cycling conditions for a routine PCR

Genes	Temperature (°C) / Time					Cycles No.
	Initial denaturation	Cycling conditions			Final extension	
		denaturation	annealing	extension		
TLR2	94°C/30 sec.	94°C/30 sec.	59°C/60 sec.	68°C/1 min.	68°C/5 min.	30 Cycle
TLR4	94°C/30 sec. 94	94°C/30 sec.	65°C/60 sec.	68°C/1 min.	68°C/5 min.	30 Cycle
TLR9	94°C/30 sec.	94°C/30 sec.	59°C/60 sec.	68°C/1 min.	68°C/5 min.	30 Cycle

TLRs Primers

Table 4: Shown of TLRs primers sequences and product size.

Gene	Oligonucleotide Sequence (5'-3')	Amplicon Size, bp	Reference
TLR2			
Forward	CCAAGAGGAAGCCCAAGAAAG	**154**	(Che et al., 2017)
Reverse	AAGTCCCGCTTGTGGAGACAC		
TLR4			
Forward	TTGAGCAGGTCTAGGGTGATTGAAC	**143**	(Che et al., 2017)
Reverse	ATGCGGACACACACACTTTCAAAT		
TLR9			
Forward	AAGCTGGACCTCTACCACGA	**177**	(Wujcicka et al., 2015)
Reverse	TTGGCTGTGGATGTTGTT		

DNA Sequencing: The sequence of the nucleotide of TLRs genes was known in 3 samples, as 25 microliters of each sample of the PCR product with the Primers of each TLRs gene were sent to Macro-gene in the Korea and after obtaining the results, all the results were compared directly with the nucleotide of TLRs, Available in the internet (http: NCBI Reference Sequence) by computer program (Bio Edit Pro. version: 7.0.0). The results were registered in NCBI under accession numbers (LC712875, LC712876& LC712877).

Statistical Analysis: Statistical analysis was performed with SPSS (Standard Program for Social Science) statistical program version 23 and Microsoft Excel 2010. Numerical data were defined according to mean, standard deviation of mean. For comparison between different groups, logistic regression was used. The lowest accepted difference in statistical importance was 0.05 or less.

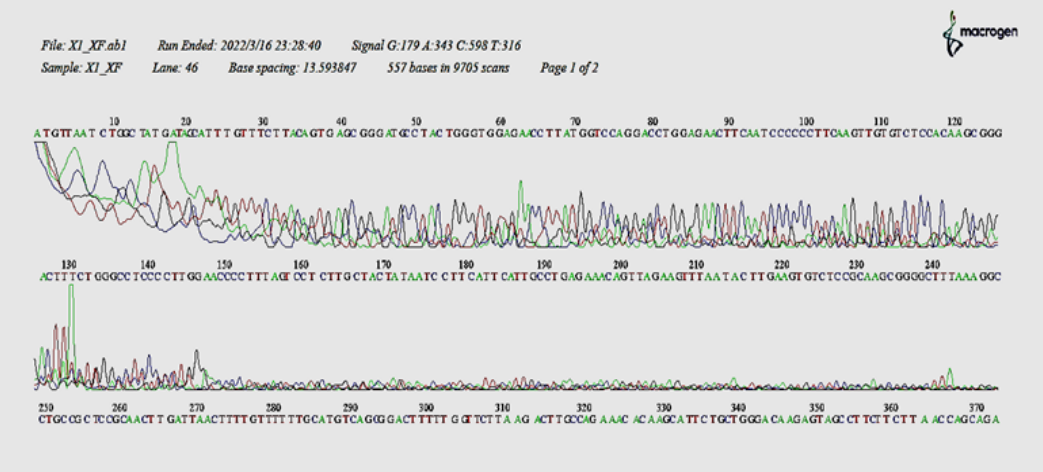
3. Results

DNA sequencing

DNA sequence analysis of (TLR2) gene: Two samples have sequenced through PCR-sequences by Macro Gen Company / Korea. Nucleotides substitutions have determined by comparing the data obtained from gene bank publish which is available at NCBI (https://www.ncbi.nlm.nih.gov) and the results were registered in NCBI under accession numbers (LC712877) which is available on this link (https://www.ncbi.nlm.nih.gov/nuccore/LC712877).

In Sample (1) presented 99 % identity of TLR2 gene compared to the genes with Sequence ID (NM_001318796.2) While Sample (2) presented % identity for TLR2 gene compared to the same genes of the (NM_001318796.2). In the Sample (1) and Sample (2) The results of gene sequence analysis TLR2 have shown that there were many polymorphisms in both forward and reverse primer amplification as shown in table (1) and table (2)?

Homo sapiens toll like receptor 2 (TLR2), transcript variant 8, mRNA



Score	Expect	Identities	Gaps	Strand
200 bits(108)	2e-46	119/124(96%)	2/124(1%)	Plus/Plus

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Sbjct  2084  ATCT-GCTATGAT-GCATTTGTTTCTTACAGTGAGCGGGATGCCTACTGGGTGGAGAACC  2141

Query  67    TTATGGTCCAGGACCTGGAGAACTTCAATCCCCCCTTCAAGTTGTGTCTCCACAAGCGGG  126
             |||||||||||||| ||||||||||||||||||||||||||||||||||  || |||||||
Sbjct  2142  TTATGGTCCAGGAGCTGGAGAACTTCAATCCCCCCTTCAAGTTGTGTCTTCATAAGCGGG  2201

Query  127   ACTT  130
              ||||
Sbjct  2202  ACTT  22
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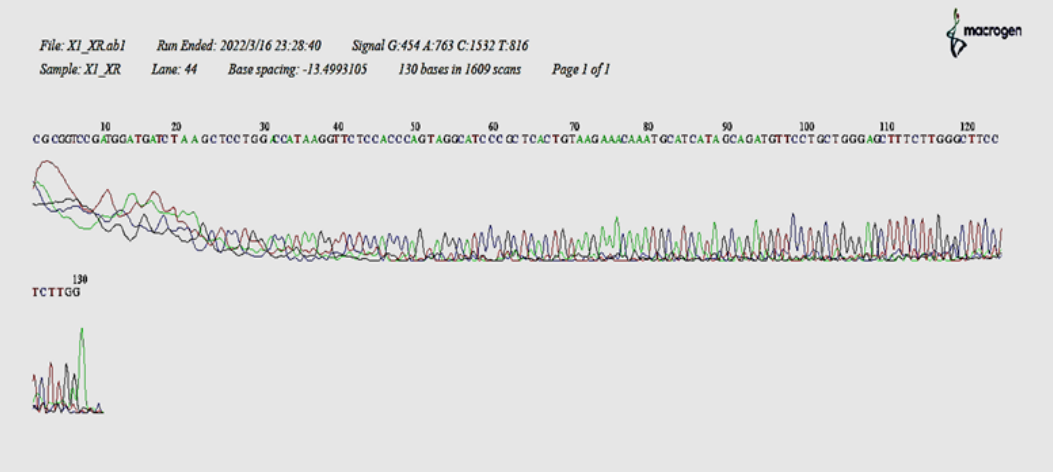
Figure 1: Alignment statistics for Sample (1) TLR2 Forward primer



Score	Expect	Identities	Gaps	Strand
191 bits(103)	2e-44	107/109(98%)	0/109(0%)	Plus/Minus

```
Query  22    AGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAACAAAT  81
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2157  AGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAACAAAT  2098

Query  82    GCATCATAGCAGATGTTCCTGCTGGGAGCTTTCTTGGGCTTCCTCTTGG  130
             |||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct  2097  GCATCATAGCAGATGTTCCTGCTGGGAGCTTTCCTGGGCTTCCTTTTGG  2049
```

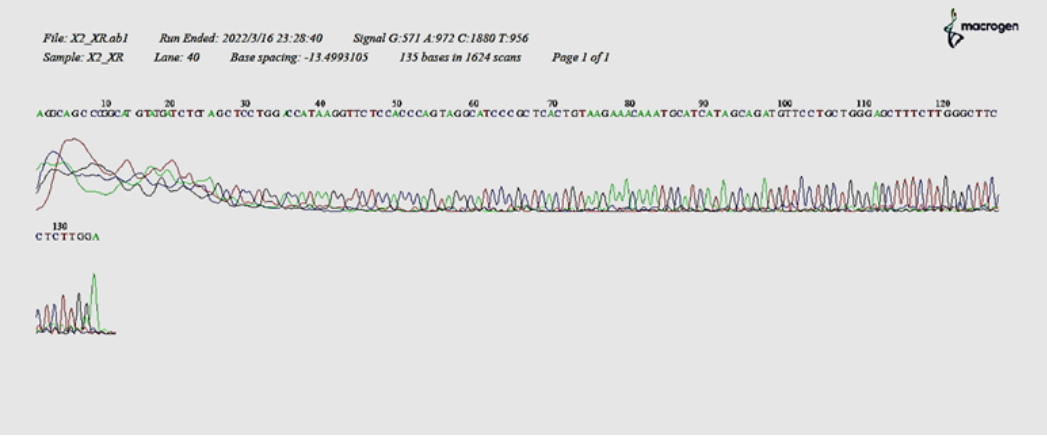
Figure 2: Alignment statistics for Sample (1) TLR 2 Reverse primer.

Table 5: Type polymorphism of in the TLR2 gene sequence (sample 1)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
	Insertion	2088	Gab(-)>G	CT-> CTG	No functional protein > Glutamine	The protein made by the gene may not function properly	2084 to 2205	NM_001318796.2	200 bits(108)	119/124(96%)	
	Insertion	2097	Gab(-)>G	AT-> ATA	No functional protein > Lysine	The protein made by the gene may not function properly	2084 to 2205	NM_001318796.2	200 bits(108)	119/124(96%)	

1 F	Tranversion	2155	G>C	GAG > GAC	Glutamine >Asparagine	The protein made by the gene may not function properly	2084 to 2205	NM_001318796.2	200 bits(108)	119/124(96%)	TLR 2
	Transition	2191	T>C	CTT >CTC	Glutamine >Histidine	The protein made by the gene may not function properly	2084 to 2205	NM_001318796.2	200 bits(108)	119/124(96%)	
	Transition	2195	T>C	CAT>CAC	Glutamine >Histidine	The protein made by the gene may not function properly	2084 to 2205	NM_001318796.2	200 bits(108	119/124(96%)	
1 R	Deletion	998	G>Gab(-)	GCG>GC-	Vilene > No functional protein	The protein made by the gene may not function properly	112 to 1002	MK878418.1	1607 bits(870)	885/891(99%)	TLR 2
	Transition	2034	C>T	TCC>TCT	Glutamine > No functional protein	The protein made by the gene may not function properly	2049 to 2157	NM_001318796.2	191 bits(103)	107/109(98%)	
	Transition	2045	T>C	CTT>CTC	Isolusine No functional protein	The protein made by the gene may not function properly	2049 to 2157	NM_001318796.2	191 bits(103)	107/109(98%)	

Homo sapiens toll like receptor 2 (TLR2), transcript variant 8,



Score	Expect	Identities	Gaps	Strand
195 bits(105)	1e-45	111/114(97%)	0/114(0%)	Plus/Minus

```
Query  21    TCTCTAGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAA  80
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2162  TCTCCAGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAA  2103

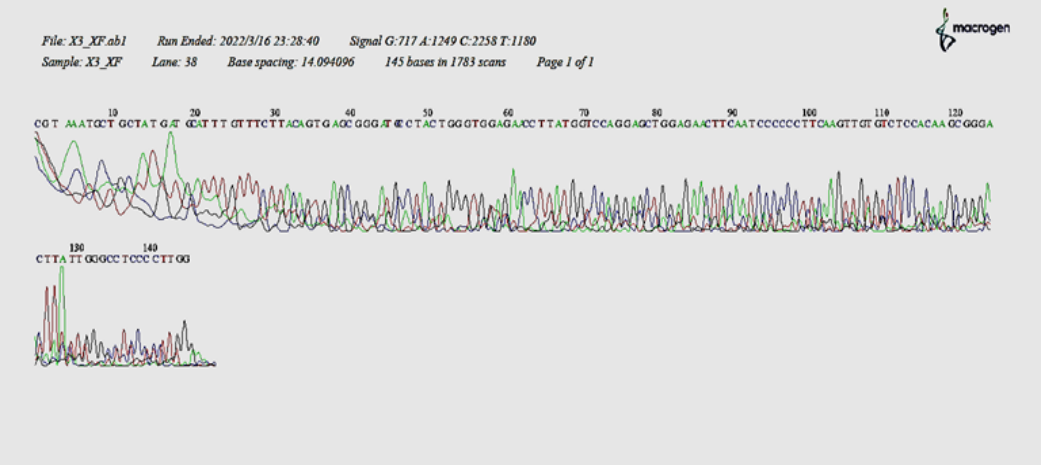
Query  81    CAAATGCATCATAGCAGATGTTCCTGCTGGGAGCTTTCTTGGGCTTCCTCTTGG  134
             |||||||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct  2102  CAAATGCATCATAGCAGATGTTCCTGCTGGGAGCTTTCCTGGGCTTCCTTTTGG  2049
```

Figure 3: Alignment statistics for Sample (2) TLR2 Reverse primer.

Table (3-13): Type polymorphism of in the TLR2 gene sequence (sample 2)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
2R	Transition	2166	C>T	TCC > TCT	Threonine > Threonine	Silent	2049 to 262	NM_001318796.2	195 bits(105)	111/114(97%)	TLR2
	Transition	2034	C>T	TCC > TCT	Threonine > Threonine	Silent	2049 to 2162	NM_001318796.2	195 bits(105)	111/114(97%)	
	Transition	2045	T>C	CTT > CTC	Glutamine > Histidine	The protein made by the gene may not	2049 to 2162	MK878418.1	195 bits(105)	111/114(97%)	

Homo sapiens toll like receptor 2 (TLR2), transcript variant 8, mRNA



Score	Expect	Identities	Gaps	Strand
211 bits(114)	2e-50	121/124(98%)	1/124(0%)	Plus/Plus

```
Query  9     CTGCTATGATGCATTTGTTTCTTACAGTGAGCGGGATGCCTACTGGGTGGAGAACCTTAT  68
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2086  CTGCTATGATGCATTTGTTTCTTACAGTGAGCGGGATGCCTACTGGGTGGAGAACCTTAT  2145

Query  69    GGTCCAGGAGCTGGAGAACTTCAATCCCCCCTTCAAGTTGTGTCTCCACAAGCGGGACTT  128
             |||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  2146  GGTCCAGGAGCTGGAGAACTTCAATCCCCCCTTCAAGTTGTGTCTTCATAAGCGGGACTT  2205

Query  129   -ATT  131
              |||
Sbjct  2206  CATT  2209
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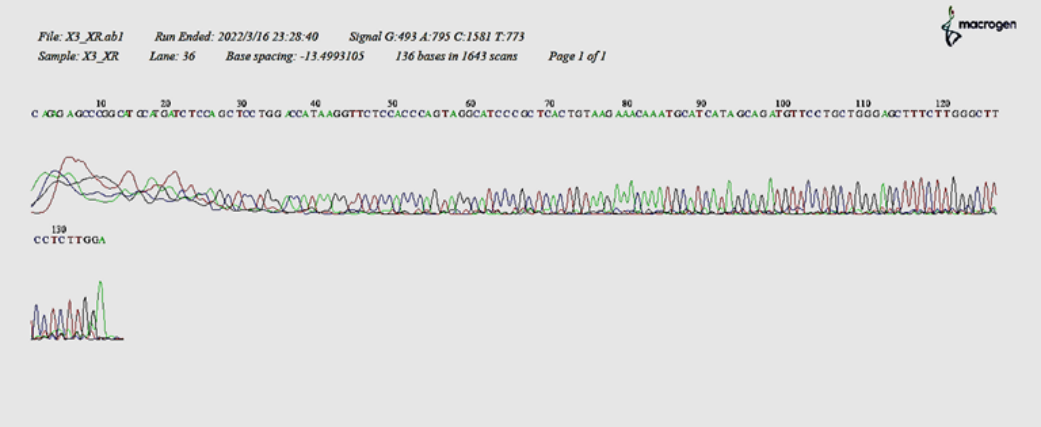
Figure 4: Alignment statistics for Sample (3) TLR2 Forward primer



Score	Expect	Identities	Gaps	Strand
200 bits(108)	3e-47	112/114(98%)	0/114(0%)	Plus/Minus

```
Query  22    TCTCCAGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAA  81
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2162  TCTCCAGCTCCTGGACCATAAGGTTCTCCACCCAGTAGGCATCCCGCTCACTGTAAGAAA  2103

Query  82    CAAATGCATCATAGCAGATGTTCCTGCTGGGAGCTTTCTTGGGCTTCCTCTTGG  135
             |||||||||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct  2102  CAAATGCATCATAGCAGATGTTCCTGCTGGGAGCTTTCCTGGGCTTCCTTTTGG  2049
```

Figure 5: Alignment statistics for Sample (3) TLR 2 Reverse primer

Table 7: Type polymorphism of in the TLR2 gene sequence (control).

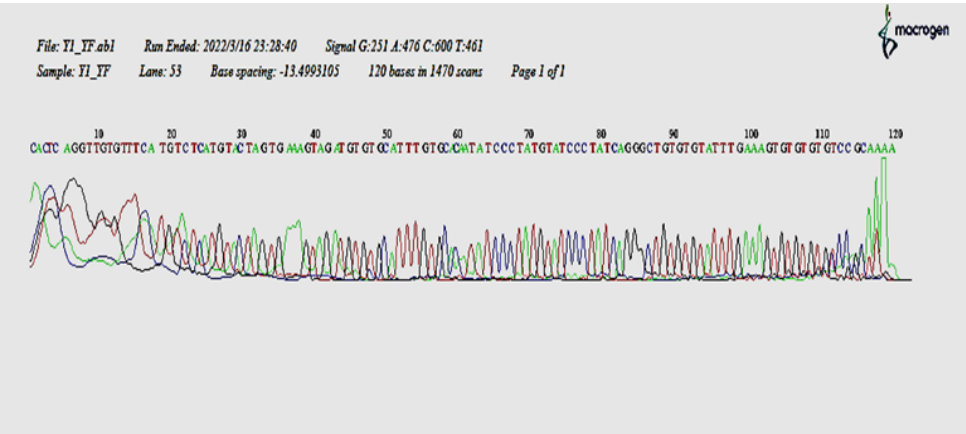
No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
3 F	Transition	2192	T>C	CTT > CTC	Glutamine > Histidine	The protein made by the gene may not function properly	2086 to 2209	NM_001318796.2	211 bits(114)	121/124(98%)	TLR2
	Transition	2194	T>C	CAT > CAC	Glutamine > Histidine	The protein made by the gene may not function properly	2086 to 2209	NM_001318796.2	211 bits(114)	121/124(98%)	
	Insertion	2206	C>Gab(-)	TTC > TT-	Asparagine > No functional protein	The protein made by the gene may not function properly	2086 to 2209	NM_001318796.2	211 bits(114)	121/124(98%)	
3 R	Deletion	2034	C>T	TCC >TCT	Therionine > Therionine	Silent	2049 to 2162	NM_001318796.2	200 bits(108)	112/114(98%)	
	Transition	2045	T>C	CTT >CTC	Glutamine > Histidine	The protein made by the gene may not function properly	2049 to 2162	NM_001318796.2	200 bits(108)	112/114(98%)	

DNA Sequence Analysis of (TLR4) Gene

Two samples have sequenced through PCR-sequences by Macro Gen Company / Korea. Nucleotides substitutions have determined by comparing the data obtained from gene bank publish which is available at NCBI (https://www.ncbi.nlm.nih.gov) and the results were registered in NCBI under accession numbers (LC712875) which is available on this link (https://www.ncbi.nlm.nih.gov/nuccore/LC712875) .

In Sample (1) presented 99 % identity of (TLR4) gene compared to the genes with Sequence ID (NM_003266.4) While Sample (2) presented 99 % identity for (TLR4) gene compared to the same genes of the (NM_003266.4). In the Sample (1) and Sample (2) The results of gene sequence analysis (TLR4) gene have shown that there were many polymorphisms in both forward and reverse primer amplification as shown in table (3) and table (4).

Homo sapiens toll like receptor 4 (TLR4), transcript variant 3, mRNA



Score	Expect	Identities	Gaps	Strand
189 bits(102)	6e-44	109/112(97%)	2/112(1%)	Plus/Plus

```
Query  7     GGTTGTGTTT-CATGTCTCATGTACTAGTGAAAGTAGATGTGTGCATTTGTGCACAATAT  65
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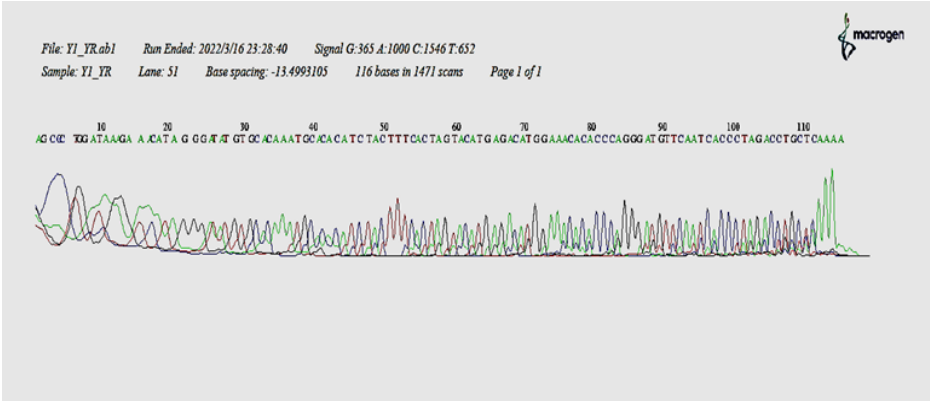
Sbjct  5079  GGGTGTGTTTCCATGTCTCATGTACTAGTGAAAGTAGATGTGTGCATTTGTGCAC-ATAT  5137

Query  66    CCCTATGTATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCA  117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5138  CCCTATGTATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCA  5189
```

Figure 6: Alignment statistics for Sample (1) TLR4 Forward primer.



Score	Expect	Identities	Gaps	Strand
189 bits(102)	5e-44	111/115(97%)	1/115(0%)	Plus/Minus

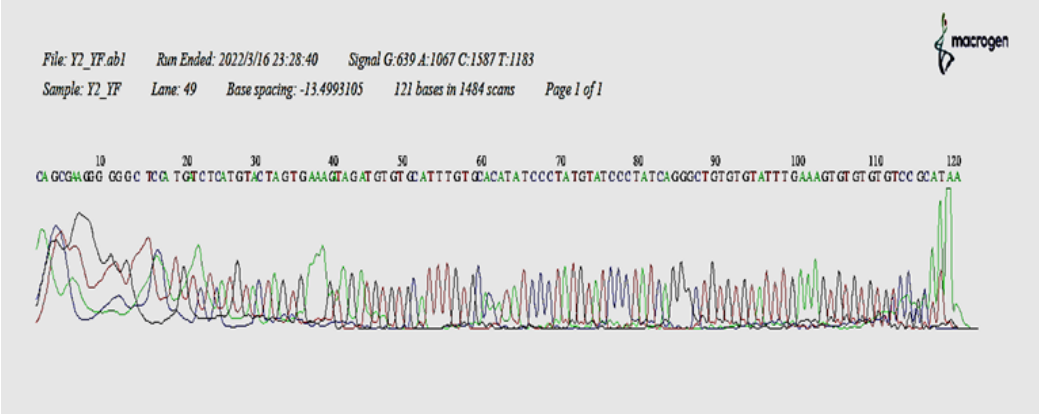
```
Query  1     AGCCCTGGATAAAGAAACATAGGGATATGTGCACAAATGCACACATCTACTTTCACTAGT  60
             |||||| ||||  || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5160  AGCCCT-GATAGGGATACATAGGGATATGTGCACAAATGCACACATCTACTTTCACTAGT  5102

Query  61    ACATGAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  115
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5101  ACATGAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  5047
```

Figure 7: Alignment statistics for Sample (1) (TLR4) reverse primer

Table 8: Type polymorphism in the TRL4 gene sequence (sample 1)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
1F	Transversion	5081	G>T	GGG> GGA	Glycine > Glycine	Silent	5079 to 5189	NM_003266.4	189 bits(102))	109/112(97%)	TLR3
	Deletion	5089	C> Gab (-)	TTC> TT-	Arginine > No functional protein	The protein made by the gene may not function properly	115 to 848	NG_050276.1	1279 bits(692)	720/734(98%)	
	Insertion	5133	Gab (-) >A	AC-> ACA	No functional protein > threonine	The protein made by the gene may not function properly	115 to 848	NG_050276.1	1279 bits(692)	720/734(98%)	
3 R	Insertion	5166	Gab (-) >G	CT- > CTG	No functional protein > Glutamine	The protein made by the gene may not function properly	5047 to 5160	NM_003266.4	189 bits(102)	111/115(97%)	
	Transition	5171	G>A	TAG > TAA	Lyscine > Lyscine	silent	115 to 848	NG_050276.1	1279 bits(692)	720/734(98%)	
	Transition	5172	G>A	TAG > AAA	Lyscine > Lyscine	silent	115 to 848	NG_050276.1	1279 bits(692)	720/734(98%)	
	Transversion	5175	A>T	AC-> ACA	No functional protein > threonine	The protein made by the gene may not function properly	115 to 848	NG_050276.1	1279 bits(692)	720/734(98%)	

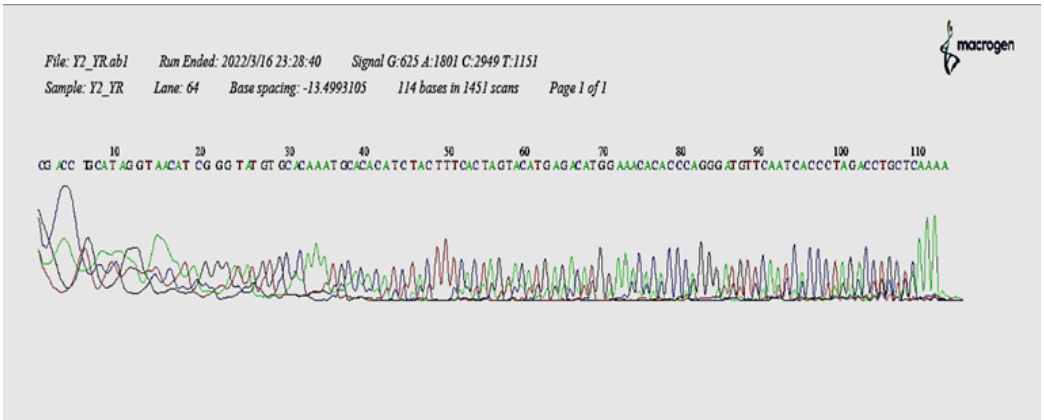


Score	Expect	Identities	Gaps	Strand
182 bits(98)	1e-41	103/105(98%)	2/105(1%)	Plus/Plus

```
Query  15    TCCATGATCTCATGTACTAGTGAAAGATAGATGTGTGCATTTGTGCACATATCCCTATGT  74
             ||||||  ||||||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  5088  TCCATG-TCTCATGTACTAGTGAAAG-TAGATGTGTGCATTTGTGCACATATCCCTATGT  5145

Query  75    ATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCAT  119
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5146  ATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCAT  5190
```

Figure 8: Alignment statistics for Sample (2) TLR4 Forward primer



Score	Expect	Identities	Gaps	Strand
176 bits(95)	4e-40	107/112(96%)	3/112(2%)	Plus/Minus

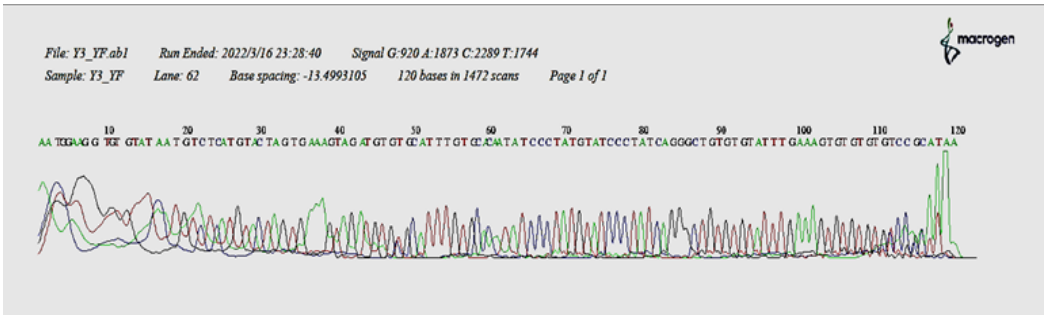
```
Query  4     CCTGCATAGGTA-ACATCGGG-TATGTGCACAAATGCACACATCTACTTTCACTAGTACA  61
             ||||  |||||  |  ||||  |||  ||||||||||||||||||||||||||||||||
Sbjct  5157  CCTG-ATAGGGATACATAGGGATATGTGCACAAATGCACACATCTACTTTCACTAGTACA  5099

Query  62    TGAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  113
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5098  TGAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  5047
```

Figure 9: Alignment statistics for Sample (2) TLR4 Reverse primer

Table 9: Type polymorphism of in the TLR4 gene sequence (sample 2)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
2F	Insertion	5094	Gab (-) >A	TG-> TGA	No functional protein > Arginine	The protein made by the gene may not function properly	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	TLR 4 gene
	Insertion	5112	Gab (-) >A	AG-> AGA	No functional protein > Arginine	The protein made by the gene may not function properly	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	
2R	Insertion	5161	Gab (-) >C	TG-> TGA	No functional protein > Arginine	The protein made by the gene may not function properly	5047 to 5157	NM_003266.4	176 bits(95)	107/112(96%)	
	Transversion	5167	G>T	GGG> GGT	Glysine > Glysine	Silent	5047 to 5157	NM_003266.4	176 bits(95)	107/112(96%)	
	Deletion	5169	T > Gab (-)	GAT > GA-	Glutamate > No functional protein	The protein made by the gene may not function properly	5047 to 5157	NM_003266.4	176 bits(95)	107/112(96%)	
	Transversion	5174	A>C	ATA > ATC	Glysine > Asparagine	The protein made by the gene may not function properly	5047 to 5157	NM_003266.4	176 bits(95)	107/112(96%)	
	Deletion	5178	A > Gab (-)	GGA > GG	Glysine > No functional protein	The protein made by the gene may not function properly	5047 to 5157	NM_003266.4	176 bits(95)	107/112(96%)	

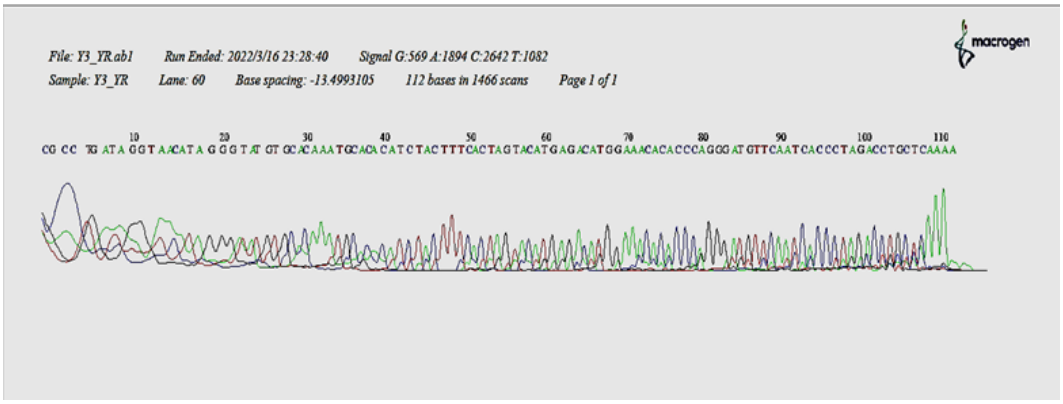


Score	Expect	Identities	Gaps	Strand
183 bits(99)	3e-42	108/112(96%)	2/112(1%)	Plus/Plus

```
Query  8     GGTGTGTAT-AATGTCTCATGTACTAGTGAAAGTAGATGTGTGCATTTGTGCACAATATC  66
             ||||||| |  ||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  5080  GGTGTGTTTCCATGTCTCATGTACTAGTGAAAGTAGATGTGTGCATTTGTGCAC-ATATC  5138

Query  67    CCTATGTATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCAT  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5139  CCTATGTATCCCTATCAGGGCTGTGTGTATTTGAAAGTGTGTGTGTCCGCAT  5190
```

Figure 10: Alignment statistics for Sample (3) TLR4 Forward primer



Score	Expect	Identities	Gaps	Strand
187 bits(101)	2e-43	108/111(97%)	2/111(1%)	Plus/Minus

```
Query  3     CCTGATAGGTA-ACATAGGG-TATGTGCACAAATGCACACATCTACTTTCACTAGTACAT  60
             ||||||||| | |||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  5157  CCTGATAGGGATACATAGGGATATGTGCACAAATGCACACATCTACTTTCACTAGTACAT  5098

Query  61    GAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  111
             |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5097  GAGACATGGAAACACACCCAGGGATGTTCAATCACCCTAGACCTGCTCAAA  5047
```

Figure 11: Alignment statistics for Sample (3) TLR4 Reverse primer

Table 10: Type polymorphism of in the TLR4 gene sequence (Control)

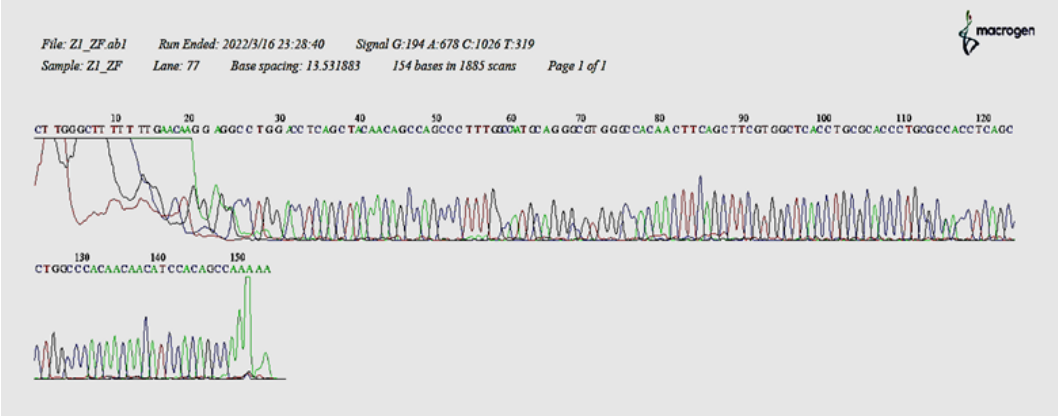
No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
3F	Transversion	5088	T>A	GTT > GTA	Glutamine > Glutamine	Silent	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	TLR 4 gene
	Insertion	5010	C > Gab (-)	TTC > TT-	Arginine> No functional protein	The protein made by the gene may not function properly	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	
	Transversion	5011	C>A	TCC> T-A	Therionine > No functional protein	The protein made by the gene may not function properly	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	
	Insertion	5133	Gab (-) >A	AC->ACA	Therionine > No functional protein	The protein made by the gene may not function properly	5088 to 5190	NM_003266.4	182 bits(98))	103/105(98%)	
3R	Transversion	5166	G>T	GGG > GGT	Glysine > Glysine	Silent	5047 to 5157	NM_003266.4	187 bits(101)	108/111(97%)	
	Deletion	5177	A > Gab (-)	GGA > GG-	Glysine > No functional protein	The protein made by the gene may not function properly	5047 to 5157	NM_003266.4	187 bits(101)	108/111(97%)	

DNA Sequence Analysis of (TLR9) gene

Two samples have sequenced through PCR-sequences by Macro Gen Company / Korea. Nucleotides substitutions have determined by comparing the data obtained from gene bank publish which is available at NCBI (https://www.ncbi.nlm.nih.gov) and the results were registered in NCBI under accession numbers (LC712876) which is available on this link (https://www.ncbi.nlm.nih.gov/nuccore/LC712876).

In Sample (1) presented 99 % identity of TLR9 gene compared to the genes with Sequence ID (NG_033933.1) While Sample (3) presented 99 % identity for TLR9 gene compared to the same genes of the (NG_033933.1). In the Sample (1) and Sample (2) The results of gene sequence analysis TLR9 gene have shown that there were many polymorphisms in both forward and reverse primer amplification as shown in table ( ) and table ( ).

Homo sapiens toll like receptor 9 (TLR9), RefSeqGene on chromosome 3



Score	Expect	Identities	Gaps	Strand
224 bits(121)	2e-54	129/132(98%)	3/132(2%)	Plus/Plus

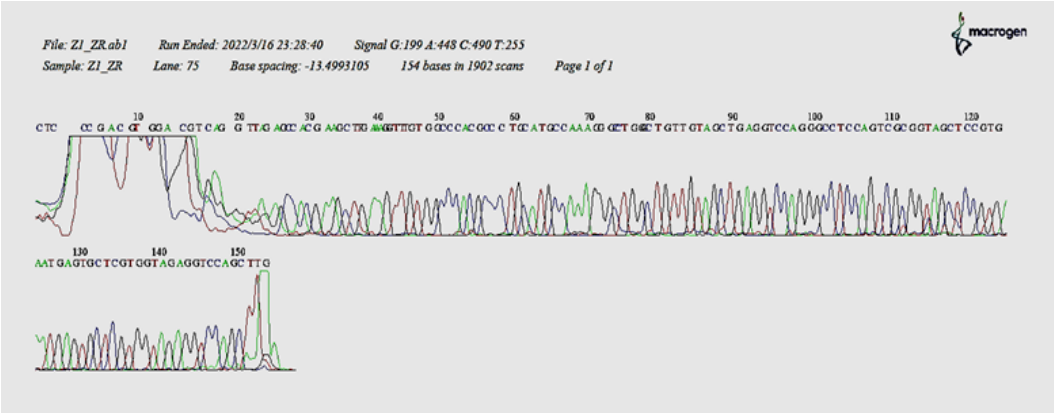
```
Query  21    GGAGG-CCTGGACCTCAGCTACAACAGCCAGCCCTTTGGCCAATGCAGGGCGTGGGCCAC  79
             |||||  ||||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  8489  GGAGGCCCTGGACCTCAGCTACAACAGCCAGCCCTTTGGC--ATGCAGGGCGTGGGCCAC  8546

Query  80    AACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCCACCTCAGCCTGGCCCACAACAAC  139
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8547  AACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCCACCTCAGCCTGGCCCACAACAAC  8606

Query  140   ATCCACAGCCAA  151
             ||||||||||||
Sbjct  8607  ATCCACAGCCAA  8618
```

Figure 12: Alignment statistics for Sample (1) TLR9 Forward primer

Homo sapiens voucher NARIPR04 toll-like receptor 9 precursor (TLR9) gene, partial cds.



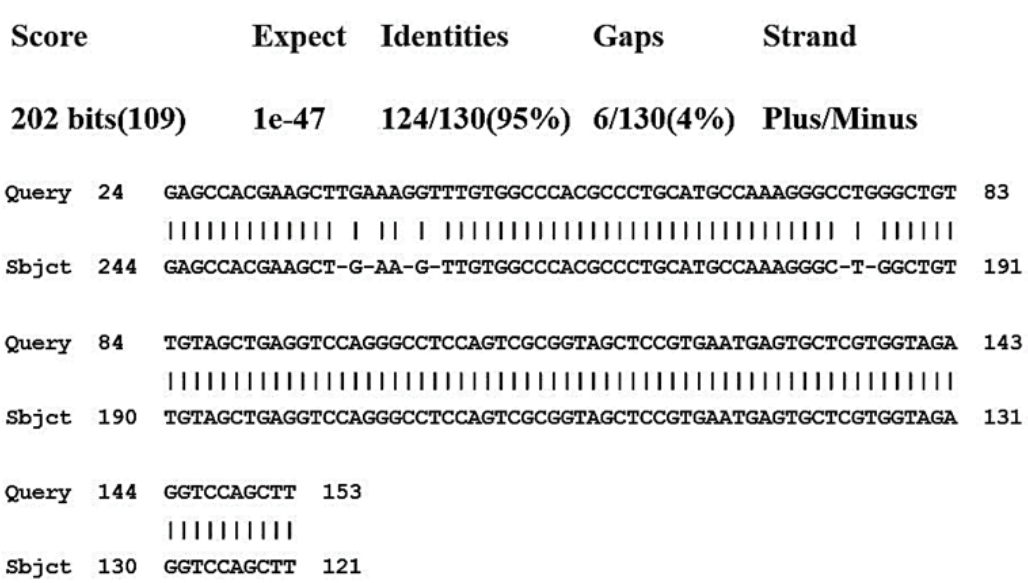


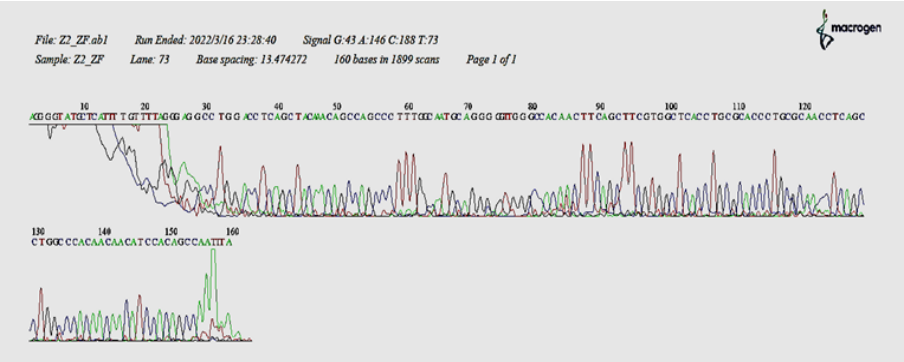
Figure 13: Alignment statistics for Sample (1) TLR9 reverse primer

Table 11: Type polymorphism of in TLR9 gene sequence (sample 1)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
1F	Deletion	8494	C>Gab (-)	GGC > GG-	Glycine > No functional protein	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	224 bits(121)	129/132(98%)	
	Insertion	8526	Gab (-)> C	GC- ->GCC	No functional protein > Glycine	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	224 bits(121)	129/132(98%)	
	Insertion	8527	Gab (-)> A	C-- >CCA	No functional protein > Proline	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	224 bits(121)	129/132(98%)	
1R	Insertion	257	Gab (-)> T	CT- >CTT	No functional protein > Glutamine	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	
	Insertion	259	Gab (-)> A	G- >TGA	No functional protein > Arginine	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	

	Insertion	262	Gab (-)> G	AA- >AAG	No functional protein > Lysine	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	
	Insertion	264	Gab (-)> T	-G- >GGT	No functional protein > Glysine	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	
	Insertion	183	Gab (-)> C	GC- >GCC	No functional protein > Alanine	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	
	Insertion	185	Gab (-)> G	-T- >CTG	No functional protein > Proline	The protein made by the gene may not function properly	121 to 244	MG322604.1	202 bits(109)	124/130(95%)	

Homo sapiens toll like receptor 9 (TLR9), RefSeq Gene on chromosome 3



Score	Expect	Identities	Gaps	Strand
209 bits(113)	6e-50	127/133(95%)	4/133(3%)	Plus/Plus

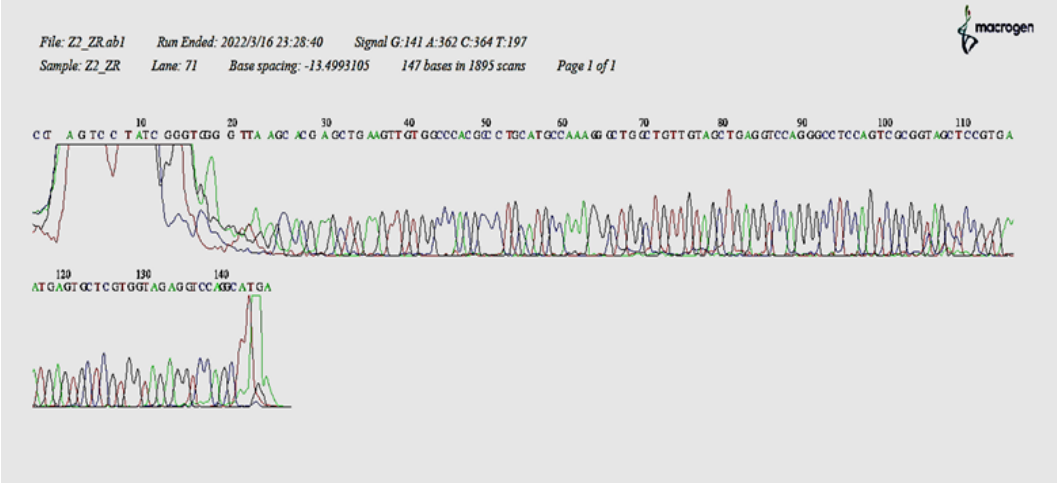
```
Query  25    GGAGG-CCTGGACCTCAGCTACAAACAGCCAGCCCTTTGGCAATGCAGGGGGTTGGGCCA  83
             |||||  |||||||||||||||| |||||||||||||||||||| ||||||| | |||||||
Sbjct  8489  GGAGGCCCTGGACCTCAGCTAC-AACAGCCAGCCCTTTGGCA-TGCAGGGCG-TGGGCCA  8545

Query  84    CAACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCAACCTCAGCCTGGCCCACAACAA  143
             ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  8546  CAACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCCACCTCAGCCTGGCCCACAACAA  8605

Query  144   CATCCACAGCCAA  156
             |||||||||||||
Sbjct  8606  CATCCACAGCCAA  8618
```

Figure 14: Alignment statistics for Sample (2) TLR9 Forward primer

Homo sapiens voucher NARIPR04 toll-like receptor 9 precursor (TLR9) gene, partial cds



Score	Expect	Identities	Gaps	Strand
209 bits(113)	6e-50	116/117(99%)	1/117(0%)	Plus/Minus

```
Query  26   CACG-AGCTGAAGTTGTGGCCCACGCCCTGCATGCCAAAGGGCTGGCTGTTGTAGCTGAG  84
           ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  CACGAAGCTGAAGTTGTGGCCCACGCCCTGCATGCCAAAGGGCTGGCTGTTGTAGCTGAG  181

Query  85   GTCCAGGGCCTCCAGTCGCGGTAGCTCCGTGAATGAGTGCTCGTGGTAGAGGTCCAG  141
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  GTCCAGGGCCTCCAGTCGCGGTAGCTCCGTGAATGAGTGCTCGTGGTAGAGGTCCAG  124
```

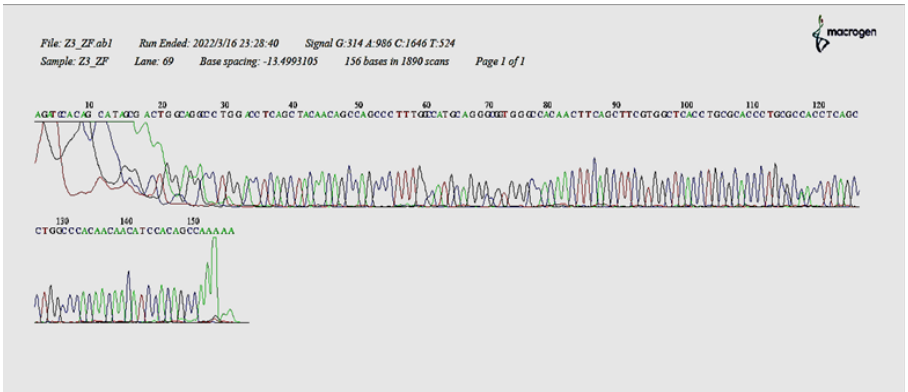
Figure 15: Alignment statistics for Sample (2) TLR9 reverse primer

Table 12: Type polymorphism of in TLR9 gene sequence (sample 2)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
2F	Deletion	8494	C>Gab (-)	GGC > GG-	Glycine > No functional protein	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	TLR9 gene
	Insertion	8511	Gab (-)> A	AC- > ACA	No functional protein > Threonine	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	
	Insertion	8531	Gab (-)> A	CA- > CAA	No functional protein > Glutamate	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	

2F	Trans-version	8535	C> G	GGC > GGG	Glycine > Glycine	Silent	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	
	Insertion	8537	Gab (-)> T	CG- >CGT	No func-tional protein > Arginine	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	
	Trans-version	8583	C> AA	CTC>CTA	Histidine > Gluta-mine	The protein made by the gene may not function properly	8489 to 8618	NG_033933.1	209 bits(113)	127/133(95%)	
2R	Deletion	244	A> Gab (-)	CGA > CG-	Arginine > No func-tional protein	The protein made by the gene may not function properly	124 to 240	MG322604.1	209 bits(113)	116/117(99%)	

Homo sapiens toll like receptor 9 (TLR9), RefSeqGene on chromosome 3



Score	Expect	Identities	Gaps	Strand
237 bits(128)	3e-58	136/139(98%)	3/139(2%)	Plus/Plus

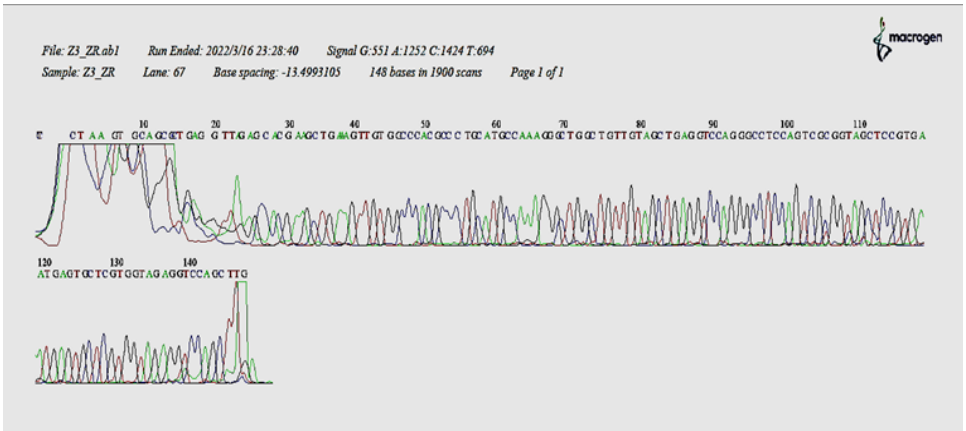
```
Query  15    GCGACTGGCAGGCCCTGGACCTCAGCTACAACAGCCAGCCCTTTGGCCATGCAGGGCGGT  74
             ||||||||  ||||||||||||||||||||||||||||||||||||  |||||||||  ||
Sbjct  8483  GCGACTGG-AGGCCCTGGACCTCAGCTACAACAGCCAGCCCTTTGGC-ATGCAGGGC-GT  8539

Query  75    GGGCCACAACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCCACCTCAGCCTGGCCCA  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8540  GGGCCACAACTTCAGCTTCGTGGCTCACCTGCGCACCCTGCGCCACCTCAGCCTGGCCCA  8599

Query  135   CAACAACATCCACAGCCAA  153
             ||||||||||||||||||
Sbjct  8600  CAACAACATCCACAGCCAA  8618
```

Figure 16: Alignment statistics for Sample (3) TLR9 forward primer

Homo sapiens voucher NARIPR04 toll-like receptor 9 precursor (TLR9) gene, partial cds



Score	Expect	Identities	Gaps	Strand
219 bits(118)	1e-52	123/125(98%)	2/125(1%)	Plus/Minus

```
Query  24   GAG-CACGAAGCTGAAAGTTGTGGCCCACGCCCTGCATGCCAAAGGGCTGGCTGTTGTAG  82
            ||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  GAGCCACGAAGCTG-AAGTTGTGGCCCACGCCCTGCATGCCAAAGGGCTGGCTGTTGTAG  186

Query  83   CTGAGGTCCAGGGCCTCCAGTCGCGGTAGCTCCGTGAATGAGTGCTCGTGGTAGAGGTCC  142
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  CTGAGGTCCAGGGCCTCCAGTCGCGGTAGCTCCGTGAATGAGTGCTCGTGGTAGAGGTCC  126

Query  143  AGCTT  147
            |||||
Sbjct  125  AGCTT  121
```

Figure 17: Alignment statistics for Sample (3) TLR9 reverse primer

Table 13: Type polymorphism of in TLR9 gene sequence (control)

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
3F	Insertion	8491	Gab (-)> C	GG-> GGA	No functional protein > Arginine	The protein made by the gene may not function properly	8483 to 8618	NG_033933.1	237 bits(128)	136/139(98%)	TLR9 gene
	Insertion	8539	Gab (-)> C	GC->CGC	No functional protein > Arginine	The protein made by the gene may not function properly	8483 to 8618	NG_033933.1	209 bits(113)	136/139(98%)	
	Insertion	8526	Gab (-)> G	GC->GCG	No functional protein > Arginine	The protein made by the gene may not function properly	8483 to 8618	NG_033933.1	209 bits(113)	136/139(98%)	

3R	Deletion	247	A> Gab (-)	AGC > AG-	Serine > No functional protein	The protein made by the gene may not function properly	121 to 244	MG322604.1	219 bits(118))	123/125(98%)	
	Insertion	8554	Gab (-)> A	TG- >TGA	No functional protein > Arginine	The protein made by the gene may not function properly	121 to 244	MG322604.1	219 bits(118))	123/125(98%)	

Phylogenic tree of TLR2, 4 and 9

A phylogenic tree of based in the (TLR2, 4 and 9) genes Molecular phylogenetic is the branch of phylogeny that analyses hereditary molecular differences, mainly in DNA sequences, to gain information on an organism's evolutionary relationships. However, phylogeny estimated from a single gene should be treated with caution. The phylogenetic tree derived from ((TLR2, 4 and 9) genes respectively sequences 3 sample s with different sequences available at NCBI showed in ( ) which revel ( ) lies in the same branch of the phylogenetic tree with ( ). As mentioned in figure (34, 35, 36) respectively.

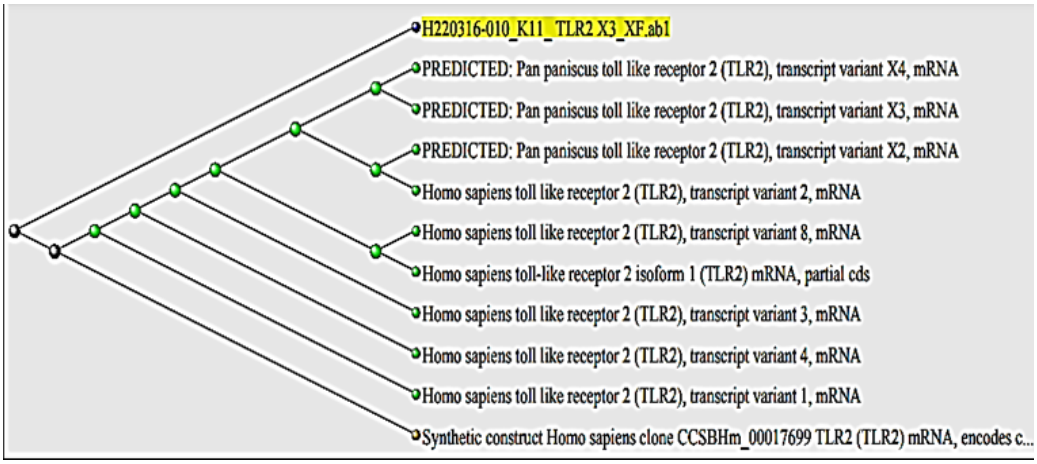


Figure 18: Phylogenetic tree of TLR2 gene sequence analysis.

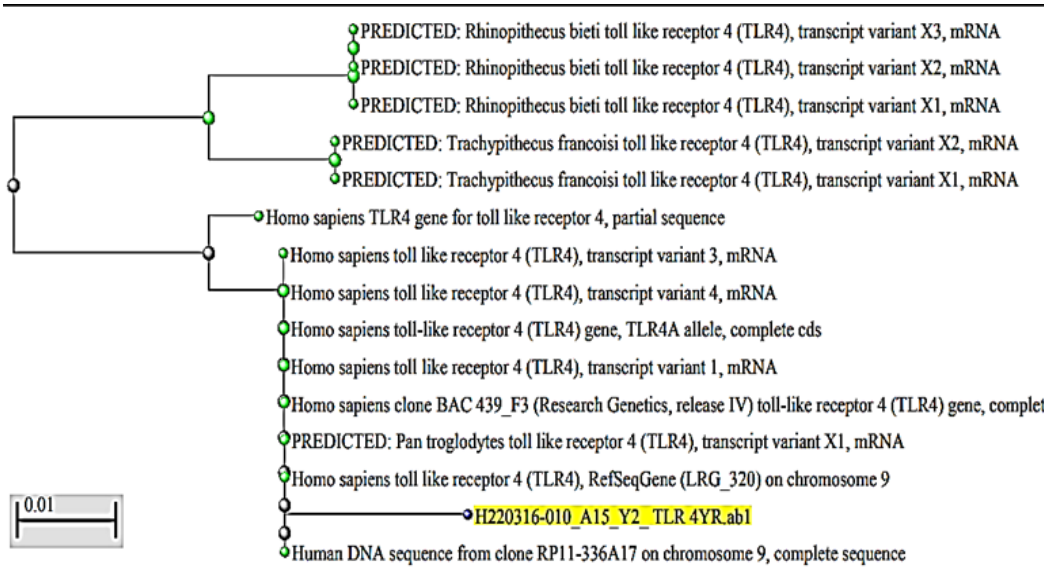


Figure 19: Phylogenetic tree based on TLR4 gene sequence analysis.

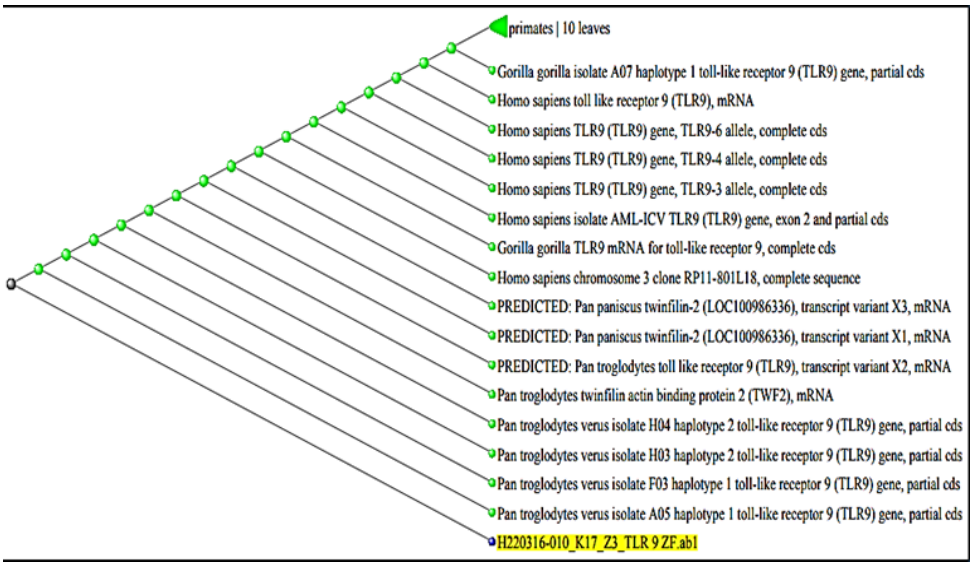


Figure 20: Phylogenetic tree based on TLR9 gene sequence analysis.

Gene recording of Toll Like Receptors: -

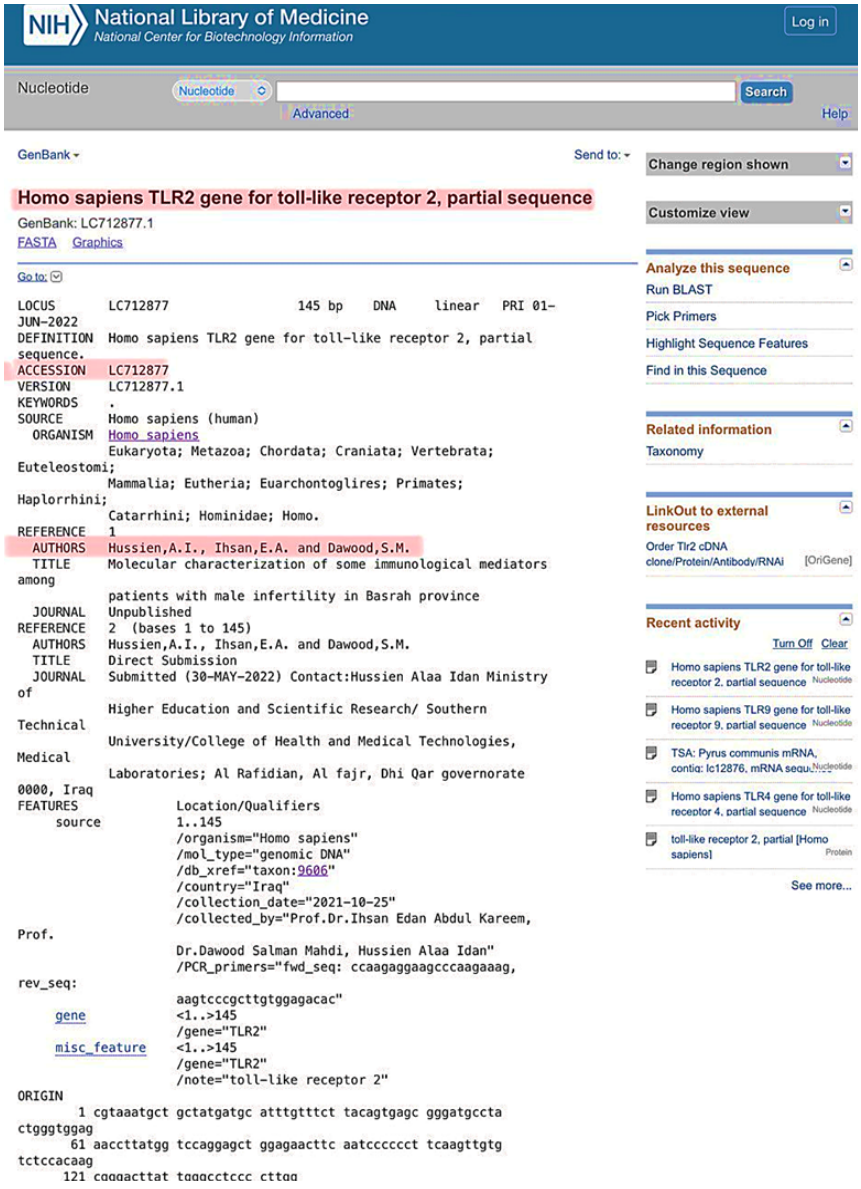


Figure 21: Recording of TLR2 gene in the Gene Bank

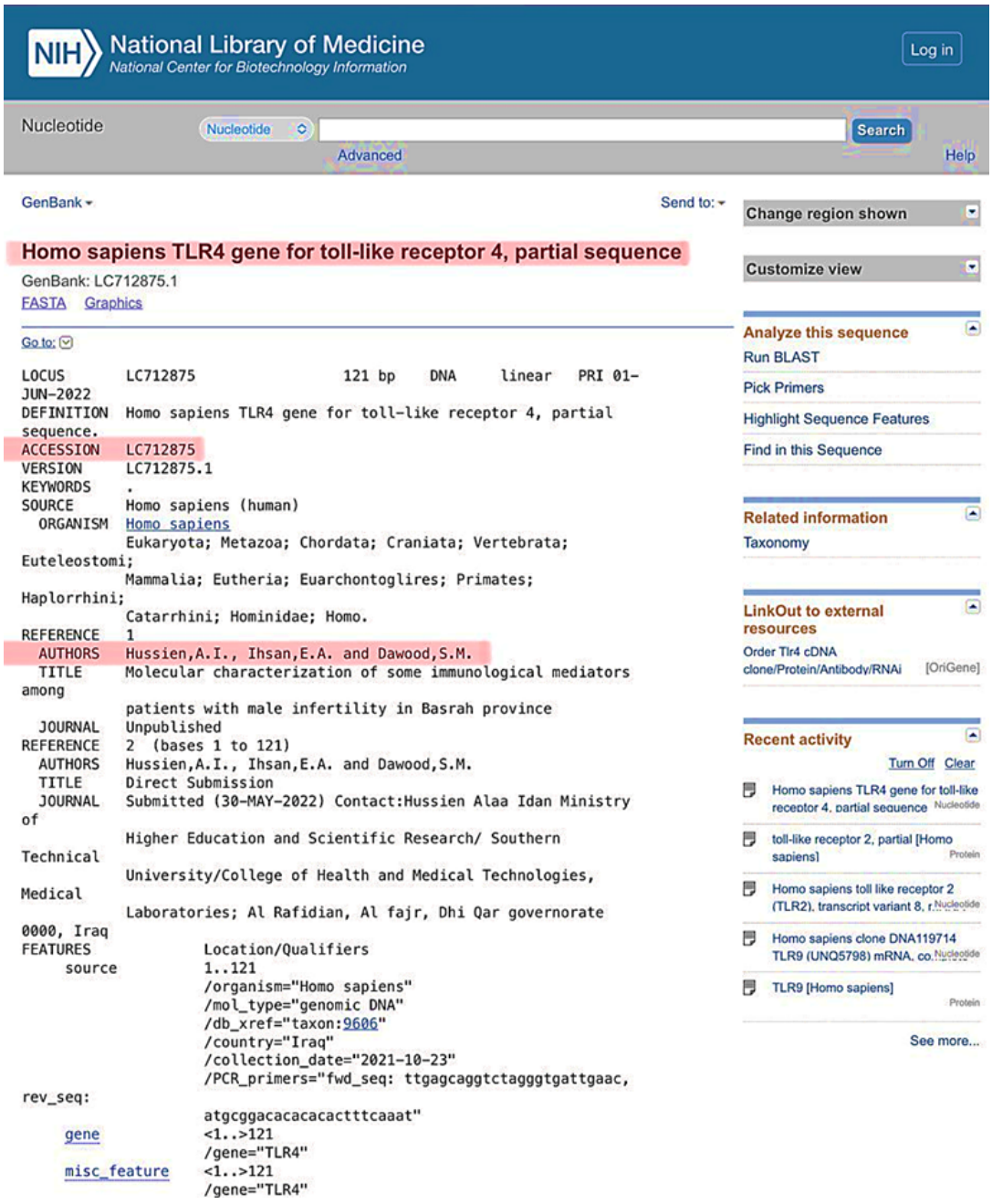


Figure 22: Recording of TLR4 gene in the Gene Bank.

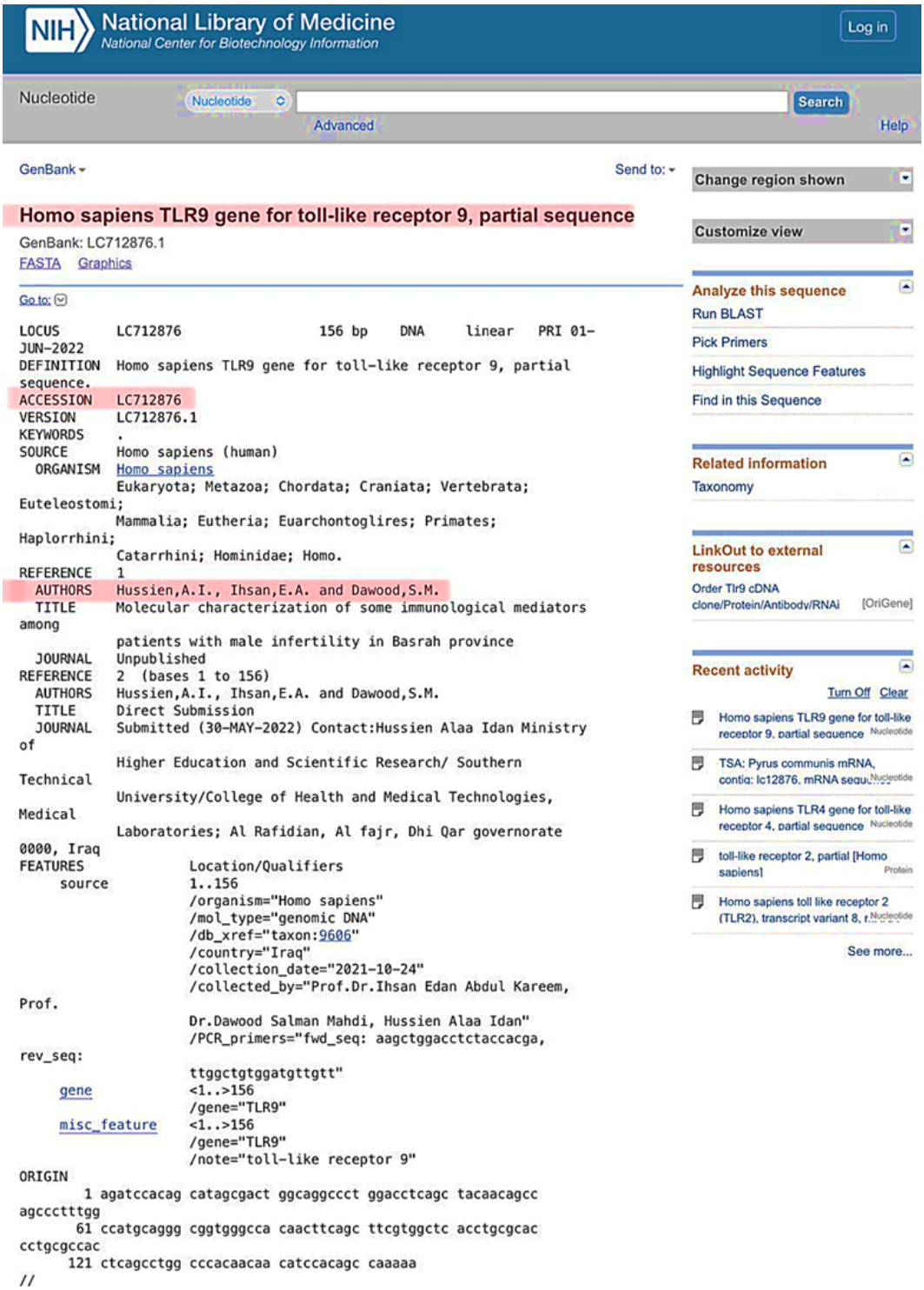


Figure 23: Recording of TLR9 gene in the Gene Bank.

4. Discussion

TLR2 in the current study, the forward primer when compared with sequence of NCBI by basic local alignment search tool (BLAST) showed 96% identities with expected value 2e-46 and there were five mutations: - Gab (-) >G, gab (-) >G, G>C, T>C, T>C in different location which give new amino acid or protein as shown in table on the other hand, TLR2 reverse primer showed three mutations: - G>Gab (-), C>T, T>C in different location with identity 98% and 2e-44 expect value.

TLR4, the forward primer when compared with sequence of NCBI showed three mutations: - G>T, C> Gab (-), Gab (-) >an in different location with 97% identities and 6e-44 expect value. On the other hand, the reverse primer showed four mutations: - Gab (-) >G, G>A, G>A, A>T with 97% identities and 5e-44 expect value.

TLR9 the forward primer when compared with the sequence of NCBI showed six mutations: - C> Gab (-), Gab (-)> A, Gab (-)> A, C> G, Gab (-)> T, C> AA in different location with 95% identities and 6e-50expect value. On the other hand, the reverse primer showed single mutation: A> Gab (-) in different location with 99% identities and 6e-50 expect value. There were no studies interested in the relationship of TLRs with male infertility, so we will compare our findings with researches on other diseases. In a study performed on prostatitis patients in Basrah province, there was a clear resemblance between TLR4 extracts and those database of the Gen Bank. Where a single mutation showed in forward TLR4 as A to G 317 1, 2. And for reverse TLR4 showed five mutations when compared with a database of Gen Bank as T to C 822 1, 2. T to A 296 1, 2. T to G 298 1, 2. A to C 301 2. G to A 406 1, 2. And there was no resemblance between TLR10 extracts and those database of the Gen Bank [24]. Other research on TLR2 and TLR4 polymorphisms in colorectal cancer development discovered that TLR2+597T>C and TLR4 Asp299Gly SNPs substantially increase the risk of CRC development, suggesting that slight alterations in the normal function of these receptors owing to functional SNPs may lead to an imbalanced cytokine and pro-oncogenic cellular microenvironment, hence increasing the risk of tumor progression [25]. Genotypes and alleles were investigated for the existence of polymorphisms in the TLR2 gene (Arg677Trp, Arg753Gln) and the TLR4 gene (As- p299Gly, Thr399Ile) in control people and TB patients in a southeastern Chinese population. The polymorphisms were identified by PCR followed by direct sequencing. The TLR2 Arg753Gln polymorphism was found at a relatively low frequency (P = 0.094) in TB patients. Neither group showed evidence of the TLR2 Arg677Trp polymorphism. No Asp299Gly or Thr399Ile SNPs were identified in TLR4 from either the TB or normal control groups [26]. Other research focused on determining the significance of TLR 3 (c.1377C/T) [rs3775290] and TLR 9 (G2848A) [rs352140] gene polymorphisms in the development of cervical cancer in North India. The genotypic and allelic frequency distributions of TLR 3 and 9 between cases and healthy controls were equal, and there was no significant connection with the development of cervical cancer [27].

In addition, Phylogenetic tree of the TLR2 and TLR9 sequences in the present study showed new speciation event which give rise to new lineage as shown in figures which had lower level of convergence with the other lineage or taxon so it was had different derived trait but shared the common ancestor. On the other hand, TLR4 sequence was undergo with more than speciation event to give the new lineage and had more than one common ancestors.

5. Conclusions

DNA sequencing of TLRs showed different mutation in different locations in forward and reverse primer when compared with NCBI. Phylogenetic tree of the TLRs sequences in the present study showed new speciation event which give rise to new lineage or taxa (new gene recording).

Acknowledgments: We would like to thank all participants in this study.

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