

Research Article,

Molecular Gene Expression of Toll-Like Receptors 4 & 10 in Cellular Subsets of Human Peripheral Blood among Patients with Prostatitis: Conventional, Real Time Pcr and DNA Sequencing Techniques

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Abstract:

Summary:

The Aim of this study was to determine Immunogenetic expression of Toll-like receptor gene clusters related to prostatitis, to give acknowledge about Role of TLR in prostatitis immunity in men from Basrah and Maysan provinces. A case-control study included 135 confirmed prostatitis patients And 50 persons as a control group. Data about age, marital status, working, infertility, family history and personal information like (Infection, Allergy, Steroid therapy, Residency, Smoking, Alcohol Drinking, Blood group, Body max index (BMI) and the clinical finding for all patients of Prostatitis were collected , The molecular expression study include extracting DNA from blood of Prostatitis patients , Prostitis patients and Control group by using specific primers for conventional PCR and Real Time PCR , the amplification of all extracted DNA from blood samples was preform and confirm by using electrophoresis with (100volt/30min). The amplification of all extracted DNA from blood samples was preform and confirm by using electrophoresis with (100volt/30min) , the result of this estimation revealed that the amplified DNA(PCR product) was 227bp for TLR4 on agarose gel (1%) , (50voltage for 1hour) with a presence 100% , (PCR product) was 279bp for TLR10 on agarose gel(1%) , (50volt/1hour) with a presence 80%. The results of the present study indicate that the Toll like receptor alleles associated with risk of prostatitis.

Key words: prostatitis, TLR4, TLR10, PCR

Introduction:

Toll like receptors a well-known group of pattern recognizing receptors in the innate immunity. Toll-like receptors are a group of transmembrane receptors act as a key role in the innate immunity. Tlrs block the invasion of the pathogens by recognizing the pathogen-associated molecular patterns (pamps), which are they highly preserved components derived from bacteria, viruses, fungi, and parasites. It can also recognize endogenous damage-associated molecular patterns (damps) in several disorders and diseases such as cancer. (Takeda and Akira. 2003). At present, there are 13

types of toll like receptors in the nature, 10 are present in human and other 3 in animals TLR1s, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, TLR3, TLR7, TLR8, and TLR9 are found exclusively inside endosomes. Various types of tlrs show specifically for ligand recognition like TLR2 recognizes bacterial lipoproteins, TLR3 recognizes double-stranded RNA/polyinosinic polycytidylic acid, TLR4 recognizes lipopolysaccharides (LPS), TLR5 recognizes flagellin, TLR7 recognizes single-stranded RNA, and TLR9 recognizes cpg-containing DNA (cpg-ODN). (Heil, *et al.*, 2004

and Poltoraka, *et al.*, 1998), TLR10 is so far an orphan receptor and highly expressed in the human spleen and B cells. (Hasan, *et al.*, 2005).

Role of tlrs in the defense against prostate infections is the most evolutionarily conserved role of tlrs in host defense is the regulation of antimicrobial responses by epithelial cells, the first line of defense at mucosal sites such as the respiratory, gastrointestinal and genitourinary tracts and the skin. Nevertheless, the widely accepted hypothesis is that non-sterile sites (i.e. Mouth, colon, or vagina) would require a response system different from that of sterile sites (bladder, kidney, prostate and testis). It is conceivable that the pattern of expression of tlrs would then differ at sterile versus non-sterile sites and that at non-sterile sites epithelial cells might be less efficient reactive than at sterile sites where even a low load of deleterious microorganisms should be rapidly detected and eliminated. (Quayle 2002).

Materials and methods:

Sampling

During collection process data about each patient were reported in the paper questionnaire for each one, which included age, marital status, infertility, family history, personal information and clinical finding of the diseases. Blood samples were collected from peoples that are symptomatic and asymptomatic patient in various hospitals of Basrah and Missan province. From a total number of (135) patients with prostatitis were taken from two provinces from the Basrah teaching hospital and Missan teaching hospital that included in the present study and the age of patients was between 40 - >70 years and (50) individuals regarded as a control group without any urological problems were also studied.

Molecular Study

The molecular expression includes extracting DNA from blood cells of prostatitis patients and the control group by using specific primers for Conventional PCR and Real Time PCR.

Genomic DNA Extraction

The quality and purity of extracting DNA from blood samples of prostatitis patients by using a Favogren DNA Extraction kit was high and every DNA for each sample was separately extracted and the results confirm throughout gel electrophoresis, DNA-based techniques have been further simplified benefiting from the introduction of PCR. Various strategies have been adopted in

attempting to use PCR for genome analysis and specification purposes. (Fairbrother *et al.*, 1998) as seen in the figure (1).

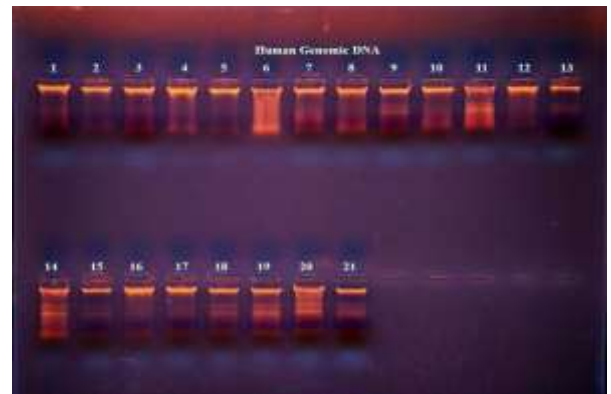


Figure (1) Show Agarose 1% gel electrophoresis image (100voltage for 30min). That show quality and purity of DNA products extracted from blood samples by commercial DNA extraction Kit.

Polymerase chain reaction technique

PCR is a very effective method to amplify a particular DNA as many copies of a specific DNA (Bartlett 2003), all samples were assayed for the presence of the *TLR1* and *TLR2* genes by PCR using previously described primers, for PCR used diluted forward and reverse primers and the primers working solution were prepared by diluting the stock solution with TE buffer to get final working solution (10 pmole/ µl) for each primer.

Table-1 PCR Master mix Volume:

PCR mix		Volume
Bio-Lab Master Mix		12. 5µL
DNA template		1. 5µL
Primer	Forward Primer	0. 5µL
	Reverse Primer	0. 5µL
Nuclease free water		10 µL
Total		25µL

Table (2) Oligonucleotide sequence and Amplicon Size for each gene used in this study

Gene	Oligonucleotide Sequence (5'-3')	Amplicon Size, bp	Reference
TLR4			
Forward	TGGGATCCCTC CCCTGTACCCTTC	227bp	Haidar, et al. 2015
Reverse	CTGGATCCGTT TCTGAGGAGGCTGGATG		
TLR10			
Forward	AGAAGAAAG GGAAGTGTATGAC	279bp	Huang, et al., 2012
Reverse	CCTGCCAGT AAATACCAAGT		

Table (3) the thermal cycler programs used in this study

Gene	Temperature (°C) / Time					Cycle No.
	Initial	Cycling condition			Final	
	Denaturation n	Denaturation n	Annealing	Extension	extension n	
TLR4	94/3 min	94/30 sec	73/60 sec	68/1 min	68/5min	30
TLR10	94/3 min	94/30 sec	55/60 sec	68/1 min	68/5min	30

Statistical analysis

Statistical analysis is performed with SAS JMP Pro statistical program version 13.2.1 and Microsoft Excel 2013. Numerical data were described as mean, standard deviation of the mean. Logistic regression was used for comparison between various groups. The lowest level of accepted statistical significant difference is below or equal to 0.0001.

Results:

The results of amplification of extracted DNA from blood samples was preform and confirm by using electrophoresis, in this analysis the resulted DNA bands that came from a successful binding between the extracted DNA and the target specific primers for each one of toll like receptors, and the bands will appear under UV light as a compact band by using ethidium bromide stain as indicator DNA stain, also electrophoresis allow to estimate the size of molecular DNA by using (100-1500bp DNA ladder) and (100- 1000bp DNA ladder) as DNA marker, the results will show the amplified DNA (PCR products) for each tlrs.

Toll like Receptor 4

The result of PCR amplification which was performed on the extracted DNA was confirmed by electrophoresis as the strands of the DNA,

which result from the successful binding, appear as a single band under U.V illuminator using ethidium bromide as a specific DNA stain. Only the bands with expected size 227bp (TLR4 - specific primer) were observed. As seen in figure (2). In real time polymerase chain reaction for toll like receptor 1 for 20 samples the starting time of amplification was begun after 20 cycles / min, and the quantitative account of the amplification line was between 700 - 1100ΔR. As seen in the figure (3).

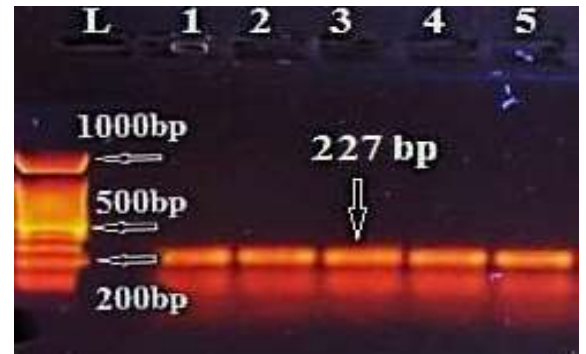


Figure (2) Positive and Negative results of PCR amplification; Lane (1) ladder marker; Lane (1,2,3,4,5) positive TLR4- specific gene (227bp); on 1% agarose, (50voltage for 1hour).

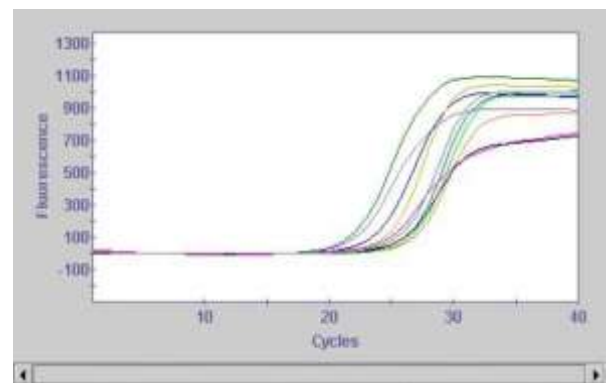


Figure (3) Real-Time PCR amplification plot of target gene for TLR4.

DNA Sequence of FTLR4 gene

Figure (4) showed a DNA sequence of FTLR4



Figure (4) DNA sequence of FTLR4

Alignment of FTLR4 gene

Homo sapiens toll like receptor 4 forward (F TLR4), transcript variant 3, mRNA Sequence ID: NM_003266.4 Length: 12797 Number of Matches: 1, Range 1: 3825 to 4014.

Score	Expect	Identities	Gaps	Strand
335 bits(181)	6e-88	187/190(98%)	0/190(0%)	Plus/Plus

```

Query 1
GCCGGGGCGGACTACGTGTGAAGGTATTCAAGGCAGGGAGTATACA
TTGCTGTTTCCTGTT 60
      |||                ||
      |||
      |||
Sbjct 3825
GCCAGGAGAACTACGTGTGAAGGTATTCAAGGCAGGGAGTATACA
TTGCTGTTTCCTGTT 3884
    
```

```

Query 61
GGGCAATGCTCCTTGACCACATTTTGGGAAGAGTGGATGTTATCA
TTGAGAAAACAATGT 120
      |||
      |||
      |||
Sbjct 3885
GGGCAATGCTCCTTGACCACATTTTGGGAAGAGTGGATGTTATCA
TTGAGAAAACAATGT 3944
    
```

```

Query 121
GTCTGGAATTAATGGGGTTCCTTATAAAGAAGGTTCCCAGAAAAGA
ATGTTTCATCCAGCCT 180
      |||
      |||
      |||
Sbjct 3945
GTCTGGAATTAATGGGGTTCCTTATAAAGAAGGTTCCCAGAAAAGA
ATGTTTCATCCAGCCT 4004
    
```

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Query 181 CCTCAGAAAC 190
      |||
Sbjct 4005 CCTCAGAAAC 4014
    
```

Figure (5) shows Alignment of FTLR4 gene

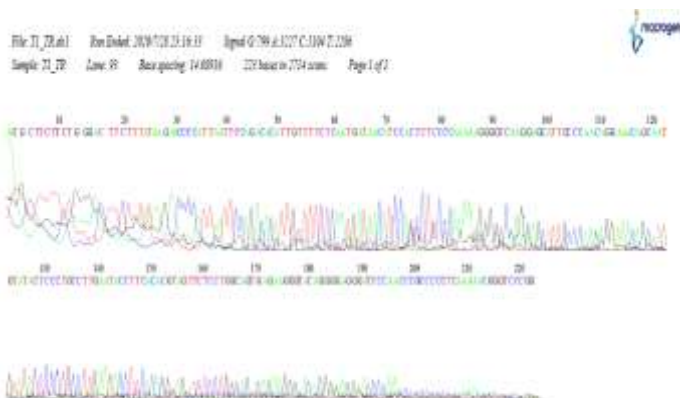


Figure (6) showed a DNA sequence of RTLR4

Alignment of RTLR4 gene

Homo sapiens toll like receptor 4 reverse (R TLR4), transcript variant 3, mRNA Sequence ID: NM_003266.4, Length: 12797 Number of Matches: 1, Range 1: 3793 to 3988

Score	Expect	Identities	Gaps	Strand
322 bits (174)	5e-84	190/197(96%)	3/197(1%)	Plus/Minus

```

Query 5 CTTCTTCTGGG-A-
CTTCTTTATAAGAACCCCATTAATTCCAGACACATTGTTTTCTCA
A 62
      ||| ||||| |
      |||
      |||
Sbjct 3988 CTT-
TTCTGGGAACCTTCTTTATAAGAACCCCATTAATTCCAGACACAT
TGTTTTCTCAA 3930
    
```

```

Query 63
TGATAACATCCACTCTCCCCAAAAGGGTCAAGGAGCATTGCC
AACAGGAAACAGCAA 122
      |||
      |||
      |||
Sbjct 3929
TGATAACATCCACTCTTCCCCAAAATGTGGTCAAGGAGCATTGCC
AACAGGAAACAGCAA 3870
    
```

```

Query 123
TGTATACTCCCTGCCTTGAATACCTTCACACGTAGTTCTCCTGGC
AGTGAGAAGGGTACA 182
      |||
      |||
      |||
Sbjct 3869
TGTATACTCCCTGCCTTGAATACCTTCACACGTAGTTCTCCTGGC
AGTGAGAAGGGTACA 3810
    
```

```

Query 183 GGGGAGGGATCCCAACC 199
      |||
Sbjct 3809 GGGGAGGGATCCAGCC 3793
    
```

Figure (7) shows Alignment of RTLR4 gene

Phylogenetic tree analysis for TLR4

Phylogenetic analysis of the TLR4 isolates were analyzed by macrogen and compared with sequences of different TLR4 available in a Gen Bank database, show a clear convergence between our TLR4isolates and that of the Gen Bank database. As seen in the figure (8).

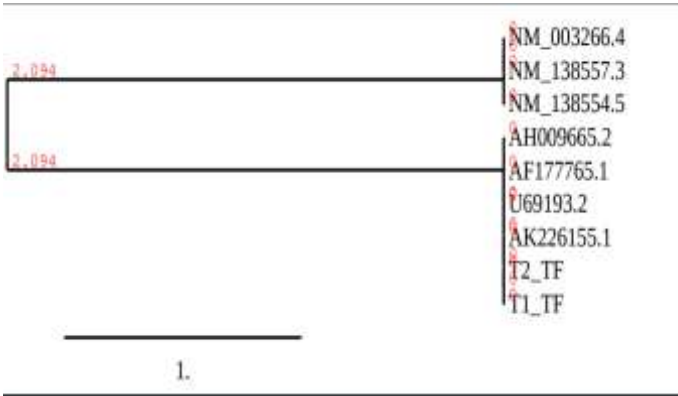


Figure (8) Phylogenetic tree analysis for TLR4

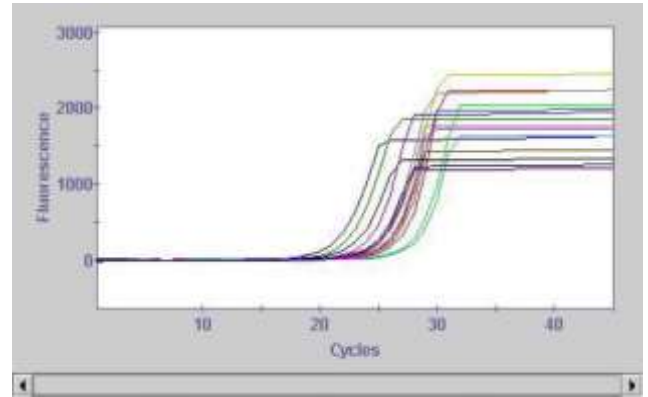


Figure (10) Real-Time PCR amplification plot of target gene for TLR10.

Toll like Receptor 10

The result of PCR amplification which was performed on the extracted DNA was confirmed by electrophoresis as the strands of the DNA, which result from the successful binding appear as a single band under U.V illuminator using ethidium bromide as a specific DNA stain. Only the bands with expected size 279bp (TLR10 - specific primer) were observed. Figure (9).

DNA sequencing for (FTLR10)

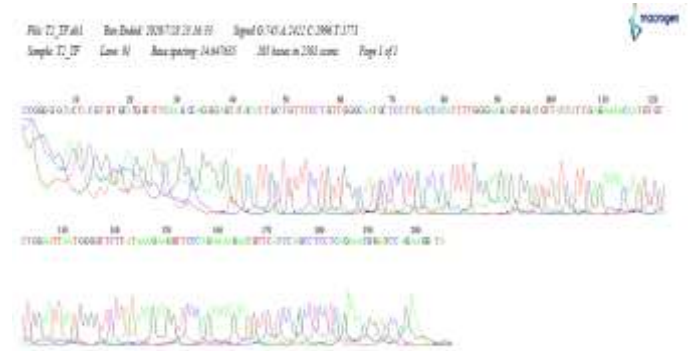


Figure (11) DNA sequence of FTLR10

Figure (9) Positive and Negative results of PCR amplification; Lane (1) ladder marker; Lane (1,2,3,4,6,7,9,10,11,12,13,14,15,16,17,18,20) positive TLR10- specific gene (279bp); Lane (5,8,19) Negative TLR7- specific primer on 1% agarose, (50voltage for 1hour).

Alignment of FTLR10 gene

Homo sapiens toll like receptor 10 forward (F TLR10), mRNA, Sequence ID: NM_001195108.2, Length: 3705Number of Matches: 1, Range 1: 473 to 705

Score	Expect	Identities	Gaps	Strand
431 bits (233)	9e-117	233/233(100%)	0/233(0%)	Plus/Plus

Query 16
AAGGTTCCCGCAGACTTGACCCAGCCACAACGACTGGATT
TCCTATAACCTCCTT 75

|||||
Sbjct 473
AAGGTTCCCGCAGACTTGACCCAGCCACAACGACTGGATT
TCCTATAACCTCCTT 532

Query 76
TTTCAACTCCAGATTTCAGATTTTCATTCTGTCTCCAACTGAGA

In real time polymerase chain reaction for toll like receptor 1 for 20 samples the starting time of amplification was begun after 20 cycles / min, and the quantitative account of the amplification line was between 1200 - 2500ΔR. As seen in the figure (10).

```
GTTTTGATTCTATGC 135
|||||
|||||
Sbjct 533
TTTCAACTCCAGAGTTCAGATTTTCATCTGTCTCCAACTGAGA
GTTTTGATTCTATGC 592
Query 136
CATAACAGAATTCAACAGCTGGATCTCAAAACCTTTGAATTCAAC
AAGGAGTTAAGATAT 195
|||||
|||||
Sbjct 593
CATAACAGAATTCAACAGCTGGATCTCAAAACCTTTGAATTCAAC
AAGGAGTTAAGATAT 652
Query 196
TTAGATTTGTCTAATAACAGACTGAAGAGTGTAACCTGGTATTTA
CTGGCAGG 248
|||||
|||||
Sbjct 653
TTAGATTTGTCTAATAACAGACTGAAGAGTGTAACCTGGTATTTA
CTGGCAGG 705
```

```
Query 17
ATCTAAATATCTTAACTCCTTGTGAATTCAAAGGTTTTGAGATC
CAGCTGTTGAATTCT 76
|||||
|||||
Sbjct 944
ATCTAAATATCTTAACTCCTTGTGAATTCAAAGGTTTTGAGATC
CAGCTGTTGAATTCT 885
Query 77
GTTATGGCATAGAATCAAACTCTCAGTTTGGAGACAGAATGAAA
ATCTGAACTCTGGAG 136
|||||
|||||
Sbjct 884
GTTATGGCATAGAATCAAACTCTCAGTTTGGAGACAGAATGAAA
ATCTGAACTCTGGAG 825
Query 137
TTGAAAAGGAGGTTATAGGATAAATCCAGTGTCTGTTGTGGCTGG
GGTCAAGTCTGCGGG 196
|||||
|||||
Sbjct 824
TTGAAAAGGAGGTTATAGGATAAATCCAGTGTCTGTTGTGGCTGG
GGTCAAGTCTGCGGG 765
Query 197
AACCTTTCTTAGAGACATGTTGGAGCAGTTGGTCATCAGTCCCC
TTTCTTCTG 250
|||||
|||||
Sbjct 764
AACCTTTCTTAGAGACATGTTGGAGCAGTTGGTCATCAGTCC-
TTTCTTCTG 712
```

Figure (12) shows Alignment of FTLR4 gene

DNA sequencing for (R TLR10)

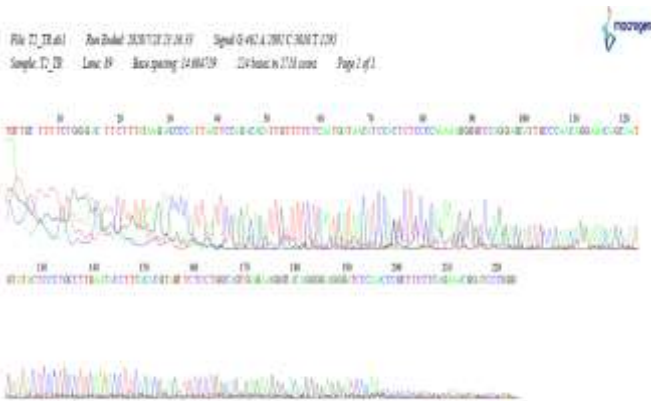


Figure (13) DNA sequence of R TLR10

Alignment of R TLR4 gene

Homo sapiens toll like receptor 10 (TLR10), transcript variant 1, mRNA, Sequence ID: NM_030956.4, Length: 3991Number of Matches: 1
Range 1: 712 to 944.

Score	Expect	Identities	Gaps	Strand
425 bits(230)	4e-115	233/234(99%)	1/234(0%)	Plus/Minus

```
Query 197
AACCTTTCTTAGAGACATGTTGGAGCAGTTGGTCATCAGTCCCC
TTTCTTCTG 250
|||||
|||||
Sbjct 764
AACCTTTCTTAGAGACATGTTGGAGCAGTTGGTCATCAGTCC-
TTTCTTCTG 712
```

Figure (14) shows Alignment of R TLR4 gene

Phylogenetic tree analysis for TLR10

Phylogenetic analysis of the TLR10 isolates were analyzed by macrogen and compared with sequences of different TLR10 available in a Gen Bank database, there is no convergence between our TLR10 isolates and that of the Gen Bank database. As seen in figure (15).

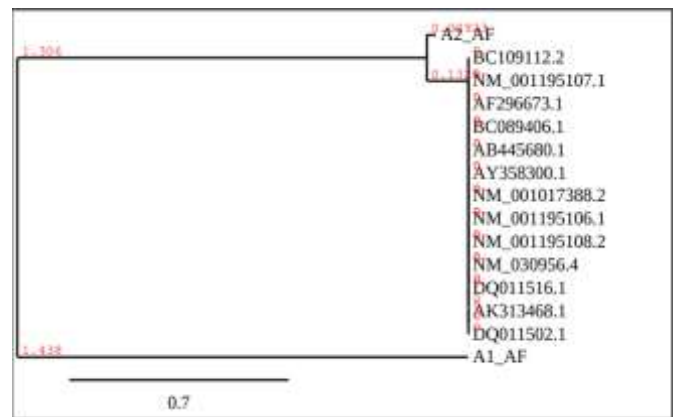


Figure (15) Phylogenetic tree analysis for TLR10.

Discussion:

Findings obtained by this study showed that after the amplification of all extracted DNA from blood samples was preform and confirm by using electrophoresis with (100volt/30min) , the result of this estimation revealed that the amplified DNA(PCR product) was 135bp for TLR1(50voltage for 1hour) and the presence of TLR1 was 75% from total samples , (PCR product) was 125bp for TLR2 on agarose gel(1%) ,(50volt/1hour) with a presence of 95% , there are few reports concerning TLR expression by the prostate (Konig , *et al.*,2004) , which is an organ usually neglected by immunologists, in spite of the many pathologies that affect it and the tremendous health impact that.

They have on human population. Although only a small percentage of men suffer of infectious chronic or acute prostatitis [National Institutes of Health (NIH) Categories I and II], chronic, noninfectious prostatitis (NIH Category III) is a highly prevalent disease among young adults. Chronic, nonbacterial prostatitis is an inflammatory state of the prostate with direct impact on the quality of life of the patients. Yet, its etiology is unknown. Furthermore, prostate cancer is one of the major causes of death in Western population, being the most commonly diagnosed cancer in men in industrialized countries. There is an expanding body of literature suggesting a link between chronic inflammation and cancer (Gatti , *et al.*,2006) , (Fan , *et al.*,2019) agree with our results in expression of TLR2and TLR10 his study found that prostate tissues expressed TLR2 and TLR10 in both epithelial and stromal cells and that TLR2 and TLR10 were co expressed on plasma membrane and in cytosol of prostate epithelial cells (RWPE-1). (AlSaimary, 2014). In addition, TLR10 expressed in the RWPE-1 cells consisted predominantly of its isoform type.

In the research of (Nagashima, *et al.*,2015) microarray analysis of gastric biopsy specimens from *Helicobacter pylori* (*H. Pylori*) -positive and uninfected subjects showed that TLR2, TLR4, and TLR6–10 were upregulated >2-fold in infected subjects. They then used *H. Pylori* to infect NCI-87 gastric epithelial cells for 24 h and found increases in TLR1, TLR2, TLR6, and TLR10 mrna levels.(Amer, *et al.*,2014) As far as their studies and our findings are concerned, the expression of all tlrs should undoubtedly increase with inflammatory grades.(Tajalli , *et al.*,2019) their review focused on aspects of TLR signaling during

acute neuropathological challenges in the brain and highlighted the potential neuroprotective capacity of estrogen, progesterone and vitamin D3 and their putative interactions with TLR signaling pathways after cerebral ischemia and TBI. In particular, TLR2 and TLR4 signaling appeared to be pivotal for controlling pathogenic immune responses following stroke. All three hormones were able to modulate TLR2 and TLR4 signal transduction. Thus, these steroids and the vitamin can be considered as therapeutic options for stroke therapy. The results of our study of sequencing of TLR4 and TLR10 were shown as following there are clear convergence between our TLR4 isolates and that of the Gen Bank database. Where there were single mutation appeared in forward TLR4 as A to G 317 1,2. And for reverse TLR4 showed five mutations when we compared with a Gen Bank database as T to C 288 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 is no convergence between our TLR10 isolates and that of the Gen Bank database.

There are no studies interested with sequencing of tlrs with the relation of prostatitis so we will compare our results with studies on other diseases. (Kim, *et al.*, 2012) suggested in their study that polymorphisms of the TLR4 gene might be associated with the risk of prostate cancer in Korean men because their results showed a significant association with the risk of prostate cancer (P (Corr) = 0.005, OR = 1.81). One common haplotype (ht2) was also significantly associated with the risk of prostate cancer (P (Corr) = 0.009, OR = 1.77). Also (Sun, *et al.*, 2005) said The TLR6-TLR1-TLR10 gene cluster may play a role in prostate cancer risk, although further functional studies are needed to pinpoint the disease-associated variants in this gene cluster. (Zhou, *et al.*, 2016) found there were no association between TLR4 and coronary artery disease.

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