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, Prostatitis 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 perform and confirm by using electrophoresis with (100volt/30min). The amplification of all extracted DNA from blood samples was perform 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 tlr5 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 tlr in the defense against prostate infections is the most evolutionarily conserved role of tlr 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 tlr 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:

<table>
<thead>
<tr>
<th>PCR mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Lab Master Mix</td>
<td>12.5µL</td>
</tr>
<tr>
<td>DNA template</td>
<td>1.5µL</td>
</tr>
<tr>
<td>Primer</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.5µL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>10 µL</td>
</tr>
<tr>
<td>Total</td>
<td>25µL</td>
</tr>
</tbody>
</table>
Table (2) Oligonucleotide sequence and Amplicon Size for each gene used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide Sequence (5’-3’)</th>
<th>Amplicon Size, bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>TGGAATCCCTC CCGTGACCTCC</td>
<td>227bp</td>
<td>Haidar, et al. 2015</td>
</tr>
<tr>
<td>TLR10</td>
<td>AGAAGAAAG GGAATGTGAC</td>
<td>279bp</td>
<td>Huang, et al., 2012</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Initial Denaturation (°C)</th>
<th>Denaturation (°C)</th>
<th>Annealing (°C)</th>
<th>Extension</th>
<th>Final extension</th>
<th>Cycle No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>94.5 min</td>
<td>94.90 sec</td>
<td>76/60 sec</td>
<td>68.1 min</td>
<td>68.5/6 min</td>
<td>30</td>
</tr>
<tr>
<td>TLR10</td>
<td>94.5 min</td>
<td>94.90 sec</td>
<td>55/60 sec</td>
<td>68.1 min</td>
<td>68.5/6 min</td>
<td>30</td>
</tr>
</tbody>
</table>

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 come 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 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 - 1100AR. 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 (FTLR4), transcript variant 3, mRNA Sequence ID: NM_003266.4 Length: 12797 Number of Matches: 1, Range 1: 3825 to 4014.

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
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<td>335 bits (181)</td>
<td>6e-88</td>
<td>187/190 (98%)</td>
<td>0/190 (0%)</td>
<td>Plus/Plus</td>
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</tbody>
</table>

Query
GCCGGCGGACTACGTGTGAAGTTATCTCAAGGGGAGGATATACA
TTGGCTGTTCCTCGTT 60

Sbjct 3825
GCCAGGAAGACTAGGTGCAAGGCTATTCAGGCGAGGATATACATGTTGTTCCTGTT 3884

Query 61
GGGCAATGCTCTTTTGAGACTTTTGTGGGAAGGATGTTATATCA
TTGGGAAACAAATGT 120

Sbjct 3885
GGGCAATGCTCTTTTGAGACTTTTGTGGGAAGGATGTTATATCA
TTGGGAAACAAATGT 3944

Query 121
GTCTGGAATTAAAGGGGTTCTTTATATAAGAAGGGGCTACCTCAGAAAAGAAGGATGTTATATCA
TTGGGAAACAAATGT 180

Sbjct 3945
GTCTGGAATTAAAGGGGTTCTTTATATAAGAAGGGGCTACCTCAGAAAAGAAGGATGTTATATCA
TTGGGAAACAAATGT 3900

Query 181
CCTCAAGAAC 190

Sbjct 4005
CCTCAAGAAC 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 (RTLR4), transcript variant 3, mRNA Sequence ID: NM_003266.4 Length: 12797 Number of Matches: 1, Range 1: 3793 to 3988

<table>
<thead>
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<th>Gaps</th>
<th>Strand</th>
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<td>322 bits (174)</td>
<td>5e-84</td>
<td>190/197 (96%)</td>
<td>3/197 (1%)</td>
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</tbody>
</table>

Query 5
CTCTTTCTGGG-GGTTTTATAAGGCGATCTCCCGACACTATGTTTCTCA
A 62

Sbjct 3988
CTTTTCTGGGACCTTCTTTATATAAGGCGATCTCCCGACACTATGTTTCTCA
TTGGGAAACAAATGT 3930

Query 63
TGATAAACATCCACTCTCCTCCCAAAAGGGGTGAGGAGATCGAGCTTGC
AACAGGAAAACAGA 122

Sbjct 3929
TGATAAACATCCACTCTCCTCCCAAAAGGGGTGAGGAGATCGAGCTTGC
AACAGGAAAACAGA 3870

Query 123
TGATATCTCCCTCGCTGGAATACTCTCTCACACGTAGTCTCTGCC
AGTGAAGAGGTACA 182

Sbjct 3869
TGATATCTCCCTCGCTGGAATACTCTCTCACACGTAGTCTCTGCC
AGTGAAGAGGTACA 3810

Query 183
GGGGAGGGATCCCAACC 199

Sbjct 3809
GGGGAGGGATCCCAACC 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 TLR4 isolates and that of the Gen Bank database. As seen in the figure (8).
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).

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).

DNA sequencing for (FTLR10)

Alignment of FTLR10 gene

Homo sapiens toll like receptor 10 forward (FTLR10), mRNA, Sequence ID: NM_001195108.2, Length: 3705Number of Matches: 1, Range 1: 473 to 705

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<thead>
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<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
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<td>9e-117</td>
<td>233/233(100%)</td>
<td>0/233(0%)</td>
<td>Plus/Plus</td>
</tr>
</tbody>
</table>

Query 16
AAGGTTCGGCGAGCCTTGAACCCACGACACTGATTTA
TCCTAAACCTCTTT 75

Subject 473
AAGGTTCGGCGAGCCTTGAACCCACGACACTGATTTA
TCCTAAACCTCTTT 532

Query 76
TTTCAACTCCAGAGGTTCTCATTCTGCTCTCAGACTGAGA
GTTTGGATTCTATG 135
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Figure (12) shows Alignment of FTLR4 gene

DNA sequencing for (R TLR10)

Figure (13) DNA sequence of RTLR10

Alignment of RTLR4 gene
Homo sapiens toll like receptor 10 (TLR10), transcript variant 1, mRNA, Sequence ID: NM_030956.4, Length: 3991 Number of Matches: 1
Range 1: 712 to 944.

<table>
<thead>
<tr>
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<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
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<tbody>
<tr>
<td>425</td>
<td>4e-115</td>
<td>233/234(99%)</td>
<td>1/234(0%)</td>
<td>Plus/Minus</td>
</tr>
</tbody>
</table>

Figure (15) Phylogenetic tree analysis for TLR10.

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).
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%) ,(50voltage/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.

References:
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