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Research Article

Molecular Detection of Toll like Receptors (2, 4, 9) Among Patient with Male Infertility in Basrah Province

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Abstract

Infertility 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 1st, 2021, and June 1st, 2022. A questionnaire paper was used to record special notes. Samples were collected from (176 patients and controls divided as 88 patients of male samples and 88 samples of the control group) including seminal fluid and blood. The molecular study in conventional polymerase chain reaction showed a clear and well-focused DNA band, and this indicates the presence of TLR genes in a high concentration, and the percentage of the detection was 100% for patients and control groups.

Keywords: Infertility, Molecular Detection, Immune Molecular, PCR Kit, TLRs Primers

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) oligo azoospermia (low spermatozoa count), (b) teratozoospermia (aberrant sperm), and (c) as then azoospermia (low sperm motility). This disorder is known as oligo as then oteratozoospermia 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].

Pattern-recognition receptors, that identify certain motifs, or pathogen-associated molecular patterns (PAMPs), generated by bacteria, virus, fungi, and protozoan pathogens [9]. And damage-associated molecular patterns, are required for the trigger of the innate immune system (DAMPs) [10].

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 [11]. Several TLRs react to distinct molecular patterns related to diseases, such as, lip peptides (TLR1, 2, 6), lipopolysaccharide (TLR4), double-strand RNA viruses (TLR3, 7, 8) and Chg.-rich UN methylated DNA (TLR9), bacterial flagella (TLR5) [12,13]. 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 [14]. Furthermore, these receptors can dimerize on the cell membrane, in which case two identical proteins he mo. dimerize or two distinct TLRs he terodimerize. Specificity in these receptors has improved via his tero dimerization [11]. on the surface of cells, TLR1, 2, 4, 5, and 6 were shown to be connected with external microorganisms, whereas TLR3, 7, 8, and 9 were found on the membranes of cytoplasmic organelles, such as endosomes, to sense pathogen-related nucleic acids [15].

TLR induction signaling pathways in the host as a defense against attackers and to heal injured tissue [16], causing the secretion of several inflammatory cytokines and immune mediator [17]. 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 [18].

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 [19]. In men, TLRs seem to have a role in both normal and pathological testicular steroidogenesis and spermatogenesis [20]. invasion of the testis or other regions of the reproductive organs by pathogens activates innate immune responses and TLRs [21]. 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 [22].

Methods

Samples sources: This cross sectional case control study was conducted between September 2021 and 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.

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 (20mg/ml)	1ml
Genomic 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.

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

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

Notes: the reaction was thoroughly mixed. Then, if required, a rapid spin was used to collect all liquid at the bottom of the tube. If utilizing a PCR machine without a heated cover, the sample was covered in mineral oil. The PCR tubes were moved to a PCR machine and thermo-cycling was initiated:

Table 3: Thermo-cycling conditions for a routine PCR

Genes	Temperature (˚C)/Time					Cycles No.
	Initial denaturation	Cyncling 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		

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

Results

DNA extraction

Figure 1 illustrate DNA that was extracted from human blood on agarose gel electrophoresis.

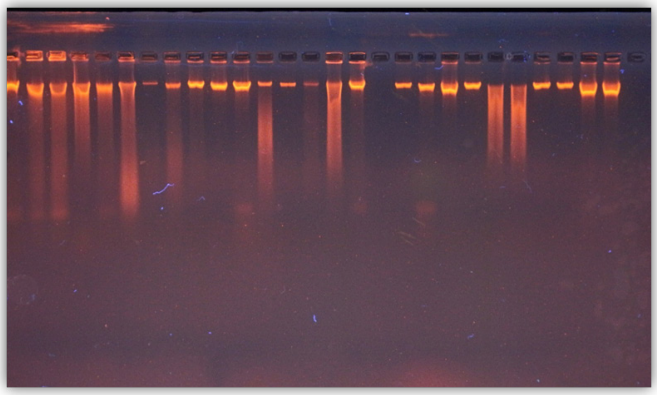

Figure 1: (Agarose 1%, 40min. at 110V) stained with Ethidium Bromide agarose gel electrophoresis appearance that reveals DNA that was extracted from (blood).

Optimization process

Figure (2) illustrate optimization process with different temperatures for PCR product of three primer sets (TLR2, TLR4 and TLR9) which show 154bp, 143bp and 177bp respectively. (Agarose 2%, 15min. at 110 volts then lowered to 75 volts for 60min.). Visualized under U.V light after staining with Ethidium bromide.

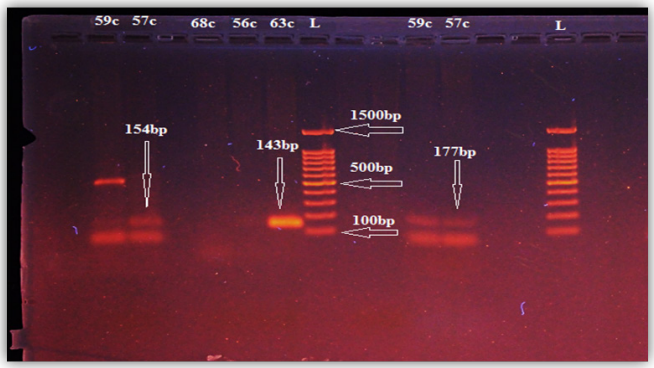

Figure 2: Gel electrophoresis for optimization process with different temperatures
Lane L: DNA ladder (100-1500) bp

TLR2 primer

Figure (3) show 154 bp Primer TM at 57C (Agarose 2%, 15min. at 110 volts then lowered to 75 volts for 60min.). Visualized under U.V light after staining with ethidium bromide.

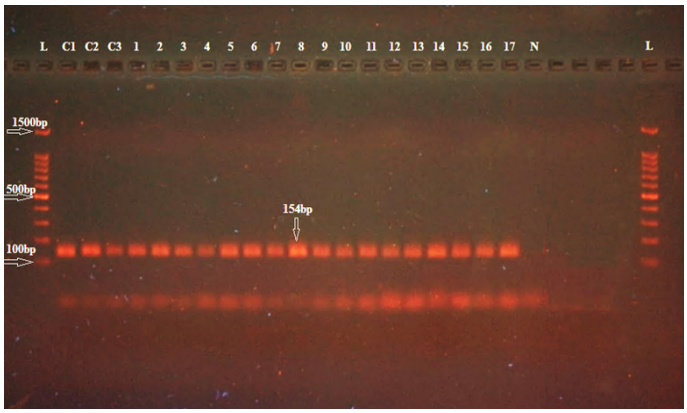

Figure 3: Gel electrophoresis for PCR product of (TLR2 primer) Lane L: DNA

Ladder (100-1500bp), Lanes (C1-17) represented positive results lane N represent negative control.

TLR4 primer

Figure (4) show 143 bp Primer TM at (63C), (Agarose 2%, 15min. at 110 volts then lowered to 75 volts for 60min.). Visualized under U.V light after staining with ethidium bromide.

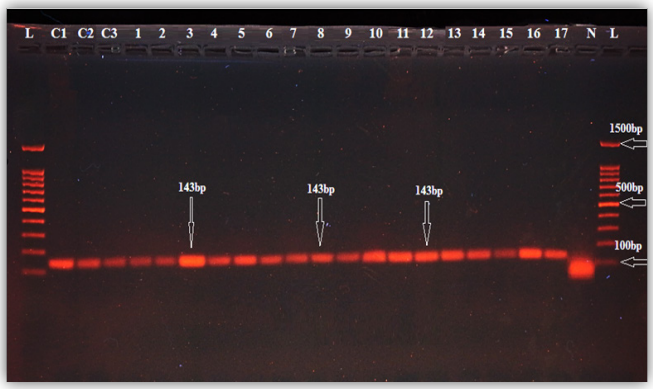

Figure 4: Gel electrophoresis for PCR product of (TLR4 primer). Lane L: DNA

Ladder (100-1500bp), Lanes (C1-17) represented positive results lane N represent negative control.

TLR9 primer

Figure (5) show 177bp results Primer TM at (59C). (Agarose 2%, 15min. at 110 volts then lowered to 75 volts for 60min.). Visualized under U.V light after staining with ethidium bromide.

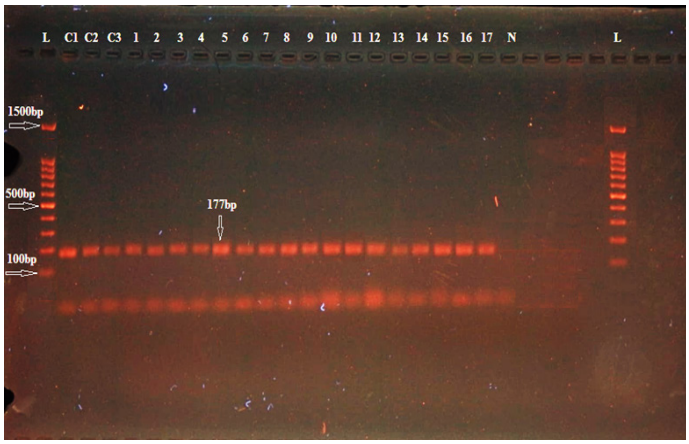

Figure 5: Gel electrophoresis for PCR product of TLR9 primer. Lane L: DNA ladder (100-1500bp), Lanes (C1-17) represented positive results lane N represent negative control.

Table 5: TLRS genes number and percentage for patients and control group.

Gene	Patients				Control			
	Total No	%	Positive No	%	Total No	%	Positive No	%
TLR2	17	100	17	100	3	100	3	100
TLR4	17	100	17	100	3	100	3	100
TLR9	17	100	17	100	3	100	3	100

Discussion

The Polymerase Chain Reaction (PCR) is a molecular biology technique which amplifies one or several copies of a part of DNA in several magnitudes, able to generate thousands to million copies of a certain DNA sequence advanced by Cary Mullis in 1983 [23].

The DNA was extracted from human whole blood on agarose gel electrophoresis. The DNA quality and integrity were estimated through remarking the DNA bands electrophoresis as shown in the present study and optimization process was done at different temperature for PCR product of three primer sets (TLR2, TLR4 and TLR9) which show 154bp,143bp and 177bp respectively to choose the best temperature for each primer.

In addition, clear and well-focused DNA band can be observed, and this indicates the presence of TLRs genes in a high concentration, and the percentage of the detection was 100% for patients and control groups as shown in our re-

sults. In a study that showed the expression of TLRs mRNAs in the male rat reproductive system, RNA was extracted and analyzed by RT-PCR using primer, m RNA for a most of TLR family members. In particular, TLR1 through TLR9 were observed and widely expressed in the testis, vas deferens and epididymis, whereas a low level of TLR10 was detected in the test and epididymis [24] In addition, other study showed the expression of TLRs in mice ley dig cells and peritoneal macrophage from the same mice as control. Several TLRs, such as TLR2, TLR3, TLR4, TLR9, and TLR12, were found in ley dig cells by Q-PCR. TLR3 and TLR4 were expressed in ley dig cells at relatively high levels as compared to the other TLR members, which were comparable in macrophages. Steroidogenesis is the primary function of Ley dig cells. In order for the testis to sustain spermatogenesis and the peripheral circulation to extra testicular androgen target organs, the testosterone generated by Ley dig cells is critical. These findings support the hypothesis that the activation of the TLR3 or TLR4 receptors in mouse Ley dig cells inhibits testosterone production [25] Finally, Another study on TB patients in Basrah province showed the results of gene detection revealed that the amplified DNA (PCR product) of TLR2 was 85.2% of the overall amplified sample, whereas TLR4 (100% of the amplified) and TLR9 in 98.8% of the total amplified sample [26].

Conclusion

TLRs are a novel topic, and to achieve definitive results about them further studies are needed. This study is preliminary, and we urge further research to be focused on understanding the fundamental mechanisms of TLRs in male reproductive biology and sperm function.

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