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Genetic susceptibility associated with toxoplasmosis; genetic polymorphism, molecular and immunological study

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Abstract

In the present study 258 blood samples were gathered from the gynecology wards in Babylon hospital for children and maternity and out private clinic from Babylon and Baghdad province during the period from September 2014 to January 2015 for investigation on toxoplasmosis infections and the study effect of genes modifications on infection susceptibility by parasite. this research was carried out in laboratory of genetic researches in College of Science for girls- Babylon university .

Expression of many cytokines like TNF-α and IL- 1β and genes like *ALOX12* through infection with *Toxoplasma gondii* were revealed modifies as a result in this study. Genotypic and allelic frequency of IFN-γ +874 T/A were statistically differences in relation to the occurrence of the A allele in many cases of parasitic infection. Genetic frequency of *sag4* gene among patients was higher than control 10.7% and 1.3%, respectively. The genetic profiling of TLRs revealed that TLR11 and TLR12 was 21.1% and 21.7%, respectively, also the genetic profiling of TUB1 and FOL1 revealed that TUB1 and FOL1 was 19.2% and 16.4% respectively, while IL-6 and IL-1β genes was 14.8%, 12.9% in that order.

Keywords: Toxoplasmosis, *Toxoplasma gondii*, IFN γ, TLR, TUB1

Introduction

Toxoplasma gondii deliberate as intracellular protozoan which infects nearly 30% of the world's residents, The final hosts of *T. gondii* are cats and others animals like warm-blooded as well as humans. Infectious stages of life cycle of *T. gondii* are divided to three parts, which are: oocysts inclosing sporozoites, tachyzoites and bradyzoites contained in tissue cysts **(1)**. Human infection commonly follows through consuming eatable materials like contaminated drink or food with *Toxoplasma* (cysts from tissue undercooked meat and oocysts). There are other ways of infection like organ transplantation and Congenital transmission **(2)**.

There are three main genotypes of *Toxoplasma gondii*, labelled as type I, type II and type III that diverge in epidemiological configuration and their virulence effect. Strain of type I considered as main accompanying with mice virulence and has been recorded in patients with ocular toxoplasmosis **(3** and **4)**. Strain of Type II had the ability to generate chronic infection with perseverance cysts in tissue, it was commonly attendant with infections in human in Europe **(5)**. A nonvirulent strain for mice and less frequent than type II in Europe was Type III, and this Type well thought-out as most frequent strain from animals **(6)**. Additionally, both Types I and II strains have been recorded in patients with AIDS patients **(7)**.

Molecular investigates for *T. gondii* have shown a strangely stable nuclear genome with weight nearly 87 Mb which containing eleven chromosomes which screening slight disparity through each parasitic strains.in addition to that an extrachromosomal 35 kilobase (kb) circular DNA of *T. gondii* was revealed within plastid-like stuffs. Resident's revisions reveal that the minor quantity of distinction states only three clonal heredities which other criteria such as virulence (as phenotypes) are related with only one unique lineage. Arbitrary sequences of group with complementary DNA (cDNA) that imitative from diverse strains has emphasized most differences with genetic that underlying phenotypic variation which are observed. **(8** and **9)**. Coding capacity of extrachromosomal circular DNA genome proposes that nucleus encoded most of proteins responsible for organelle functions. The cooperated between these apparently unconnected genomic researches which delivered a countless considerate of pathogenesis for the parasite and recognized numerous novel marks for therapy **(10)**.

T. gondii have ability evade host immune system escapes early from macrophage killing machine then infect it. Infected macrophage is also a considered as target for T cell and that occurs because condensed expression of MHC class II, also induces the production of counter regulatory molecules by infection like cytokines or Interleukins. That defend host by regulating a possibly inflammatory response, also it will impede cytokines produced through Th1 and antimicrobial activity of macrophage **(11** and **12)**. Lately, a region of genome called Toxo1 which located in rats, revealed the associated with resistance to *T. gondii* **(13** and **14)**. Subsequent to this innovation, it was renowned that TOXO1 region present on human chromosome 17. These genes were revealed as genes of cell death, which containing the gene at the top of the region, ALOX12 **(15)**.

The major elements involved in immune response to parasitic infection were Natural killer cells, T lymphocytes and Macrophages in combination with cytokines. Cytotoxicity and secretion of cytokines particularly IFN-γ consider as T cells role. Production of IL-12 and IFN-γ is essential to control infection by *T. gondii* which are a part of cell-mediated immunity the mechanism focused on limiting the parasite reproduction during the acute phase of infection **(16)**.

IFN-γ contemplate as extremely conserved, with rare genetic, Mutation that cause altered in genetic sequence called single nucleotide polymorphism (SNP). When SNPs positioned on first human intron gene for IFN γ at 5' adjacent boundary to both nucleotide C and A frequent repeat region which called IFN γ+874 polymorphism) that effects cytokine excretion and the case was read as fellow; individuals with low producers of IFN-γ that means it was loaded with A allele **(17)**, in addition to that, the variability in the production of IFN-γ related to this SNP susceptible to many pathogens (intracellular) **(18)**.

Earlier study has revealed the relationship between polymorphism of IFN γ+874T/A with ophthalmic toxoplasmosis. Genotype with AA allele displayed an amplified incidence in people with ophthalmic outcomes portentous relationship with proneness to RC9 development**(19)**.

There is insignificant character for tool like receptors (TLRs) which achieved in the preliminary reveal of microbes like parasite by recognition of distinctive pathogenic molecular patterns (PAMPs) **(20)**. Earlier researches have revealed that most cytokines produced by neutrophils macrophages and dendritic cells (DCs) like interleukin-12 (IL-12) in infected animals, was liable for the initiation of T helper 1 (Th1) cell responses and *T. gondii* clearance **(21, 22** and **23)**.

Most cytokines like IL-12 supports the induction of interferon-γ (IFN-γ) through parasitic infection, and IFN- γ is crucial for stimulation responses of T cell and *T. gondii* resistance **(24, 25** and **26)**. Most studies with animal models selectively pointing some divisions established that the package of specific cytokines especially interlukin12 which manufactured through parasitic infection (*T. gondii*) infection with tachyzoites **(27)**. Any removal of CD8, obviously specifies a defect of DC-independent component of the innate response to *T. gondii* **(28** and **29)**.

In the present study was planned to investigate the *Toxoplasma* infection and determinate some immunological markers in addition to genetic susceptibility of human to infect with this parasite using PCR in pregnant women. Also we aimed to estimate the correlation between the

IFN-γ genetic polymorphism in the coding at position +874T/A with people with AAT matched with clinical consequence.

Materials and Methods

Samples Source and DNA extraction

Overall of 258 blood samples were collected from the gynecology wards in Babylon hospital for children and maternity and out private clinic from Babylon and Baghdad governorate during the period from September 2014 to January 2015. Blood samples were occupied by 5 ml in a sterile tube with EDTA intended to genetic procedures (Nucleic acid extraction process). Blood samples were divided into three groups; one for immunological parameters second for hematological tests and the last one for DNA extraction, the extracted nucleic acid were stored at -80°C.

A total of 258 women were investigated for infection and polymorphisms in many genes which related to toxoplasmosis, these cases were incorporated in the study group contingent on earlier past of patient like having more than three pregnancy depletions, intrauterine deceases, preterm carriages, intrauterine growth obstruction and mysterious premature neonatal losses. DNA extracted from these samples using ultra script DNA extraction kit (Invitrogen\USA) according to the manufacturer procedure. Thermal cycler (Veriti® Thermal Cycler-Life Technologies\USA) was used for PCR reactions with Taq DNA polymerase (bioneer and promega USA).

PCR products were electrophoreses in 2% agarose gel prepared in 1X TBE buffer (boiled for 2 min in microwave then cooled to 55°C), after that adding staining dye “ethidium bromide” (0.8 µg/ml) to evaluated beneath ultra violet light. Specific DNA product for TORCH agents of each sample was determined by identifying the specific base pair amplified DNA bands in comparison with the 100-bp DNA ladder (Bioneer, USA), used as DNA size marker.

Sets of primers

In the present study were used many primers for detection of various genes which is important and related to *Toxoplasma* infection and to the pregnancy situation in addition to the fetus life. These primers was listed in table 1.

PCR Condition

***ALOX12*;**

Amplification steps involved of denaturation at 94°C for 3 min, followed by 42 cycles, with denaturation for 45 s at 94°C, annealing for 1.30 min at 62°C. Lastly extension for 50 s at 72°C.

***IFN- γ +874*;**

An amplification headstrong mutation (ARMS-PCR) approved for detection of polymorphism, total reaction volume of mix was (25µl) used for of IFN- γ +874 polymorphism gene was in the form of whole size of 20µl comprising 2.5 µl of reverse primer : 100 pmol/µl; for sense primer, 1 µl of specific antisense (**A**) primer :100 pmol/µl, or 1 µL of specific antisense (**T**) primer : 100 pmol/µl, 4 µl of dNTPs (2mM), 2.4 µl of Mgcl2 (25 mM), 3µl of buffer (10x), 0.5µl of Taq DNA polymerase (5 U/µl), 5.1 µl of sterile Mili Q H2O and 3µl of genomic DNA.

PCR was performed with the cycles: 94 º C (2min), 25 cycles of 94 ºC (35 s), 63 ºC (1 m.) and 72 ºC (55 s), then by 35 cycles of 94 ºC (45 s), 58 º C (50 s.), 72 º C (2 min) then final series of 72 ºC (5 min). Amplicon were subjected to 2 % agarose for electrophoresis, stained with 0.6 µl ethidium bromide and visualized on an ultraviolet transilluminator.

SAG4;

Subsequently preliminary denaturation step at 95 ºC for 3 min.; followed by 30 cycles of denaturation for 50 s. at 95 ºC, annealing for 1 min. at 59 °C, extension for 3 min. at 72 ºC and the ending extension for 7 min. at 72 °C.

TUB1;

Thermal cycling for this gene was as followed; for 4 min at 94°C as denaturation step, then PCR intensifications for 30 cycles were achieved along these lines: 1 min at 95°C for denaturation, 1.30 min. at 60°C for annealing and 1 min. at 72°C for extension, formerly the procedure were followed by the final extension for 4 min at 72°C.

FOL1;

Preliminary denaturation for 4 min at 95°C; after that 40 cycles of denaturation for 55 s at 95°C, annealing for 2 min at 65°C, extension for 80 s. at 72°C and a final extension for 7 min at 72°C.

TLR 11, 12

Thermal cycling for PCR was began with original denaturation for 3 min at 95 °C and a subsequent series of 40 cycles of for 50 sec at 95 °C as denaturation, for 2 min. at 55 °C as annealing and for 2 min at 72°C as extension. The finishing extension was carried out for 7 min at 72°C.

IL-6

At the first step; denaturation process at 93 ºC for 4 min.; followed by 35 cycles of denaturation for 1.30 min. at 94 ºC, annealing for 2 min. at 62 °C, extension for 2 min. at 72 ºC and the ending extension for 5 min. at 72 °C.

IL-1β

Amplification stages began with denaturation at 95°C for 3 min, followed by 35 cycles, with denaturation for 40 s at 95°C, annealing for 2 min at 58°C. Lastly extension for 1.5 min. at 72°C. Ending of extension with 5 min. at 72 °C.

TABLE 1. Oligonucleotide primers used in this study

No.	Primers and application	Oligonucleotide sequence, 5′ to 3′	(bp)
1.	*ALOX12*	5′ CACCAAAGCTGTGCTAAAAATCTGTTCGGAATTGGTTTAGCACAGC-3′	892
		5′ AAAAGCTGTGCTAAACCAATTCCGAAGATTGGTTTAGCACAGCTTT-3′	
2.	*IFN- γ +874*	5′ TCA ACA AAG CTG ATA CTC CA-3′	285
		5′ TTC TTA CAA CAC AAA ATC AAA TCA-3′	
		5′ TTC TTA CAA CAC AAA ATC AAA TCT-3′	
3.	*SAG4*	5- AATTTGAGCATTCGTGTCCATTCT-3	1024
		5- AATGCCCTTGAATTAAATGGACT-3	
4.	*TLR 11*	5′-AAGTCGACGCCACCATGGGCCGCTACTGGCT-3′	298
		5′-AAGGATCCTTTAAGTTCCAGAGTTTG-3′,	
5.	*TLR 12*	5′-AAGTCGACGCCACCATGCCCCGCATGGAGCG-3′	584
		5′-AAGGATCCCTCTGTTCCATGCGGACAATT-3′	
6.	*TUB1*	5′ GGG TTT TGC CTG CAT ACG TAA TTA-3′	312
		5′CCT AAA AGC AAT TCT AAA CCT CCA G-3′	
7.	*FOL1*	5′ ATG CAC TTC AGA GAT ACG G-3′	800
		5′ CCT CTC TCC ACA AAA CC A ACA-3′	
8.	*IL-6*	5′ AGAAGATTCCAAAGATGTA3′	420
		5′ TCACTACTCTCAAATCTGTT-3′	
9.	*IL-1β*	5′ -TGGACAAGCTGAGGAAGATGCTGGT-3′	504
		5′ -AGGACATGGAGAACACCACTTGTTGCT-3′	

Results and Discussion

ALOX12

Congenital toxoplasmosis is associated with many genes which influence on the severity of infection, from these genes ALOX12. Association of allelic variants of ALOX12 in humans ALOX12 allelic variants with congenital toxoplasmosis was revealed by genotyping SNPs of ALOX12 genes of patient. Linkage disequilibrium (LD) with that exacting signs considered as

an etiological variant of SNPs, in addition to that; SNPs within the ALOX12 gene can association with susceptibility to congenital toxoplasmosis **(30** and **31)**.

The critical role of ALOX12 reduces the sustainability of monocytic cells which infected with *T. gondii*. Throughout *T. gondii*, the outcome of devastation ALOX12 gene expression in human monocytic cells **(32)**, the impracticality of ALOX12 expressing in infected cells with *T. gondii* strain relative to those of wild-type **(33)**.

Increase of ALOX12 *T. gondii* proliferation in monocyte cells during infection, we can validate the consequence of ALOX12 on the comparative development rate of parasites among monocyte and that agreed with other studies **(34** and **35)**. To imagine ALOX12 properties of knockdown on the production of parasite. Expression of many cytokines like TNF-α and IL- 1β through infection with *T. gondii* the genes like ALOX12 will modifies as a result **(36** and **37)**. The electrophoresis results was showed in **figure 3-G** (892 bp).

IFN-γ

Many cytokines like IFN- γ consider as genetically highly preserved, with few allelic deviations. The secretion of this cytokine influenced and related with expression of human gene intron with single nucleotide polymorphism for IFN-γ at the extremity 5' adjacent to the CA repeated region (IFN-γ +874 T/A polymorphism). Individuals who loud the **A** allele deliberate as low producers of IFN- γ **(38, 39** and **40)**. Genotype with AA alleles presented highly occurrence in individuals with pathogenic outcomes and that lead to the concept that association with predisposition to the RC expansion **(41)**.

Hardy-Weinberg equilibrium used to analysis disclosed Genotype distribution and allele's group of characteristic/controls cases. Existence of parasitic infection was correlated with IFN-γ +874 T/A polymorphism as showed in **figure 1**. Among the 258 toxoplasmosis patients, all females with an age mean of 25.9 years. Analysis of this variable in response to genotype (AA, TT and AT) and it was showed statistically significant results; conversely, homozygous persons with the AA allele had a greater probability to emergent extended complaint than other individuals with the T allele importers as revealed in **figure 1**, and the genetic results of amplicon was revealed in **figure 3-F** (285 bp).

Genotypic and allelic frequency of IFN-γ +874 T/A was bare statistically variances in relation to the occurrence of the A allele in many cases of parasitic infection, and that was agreed

with other studies which proposed that persons with this allele in its homozygous form have a tendency to develop disease **(39)**.

sag4 gene

A bradyzoite specific surface protein with 18 k.Da. of *T. gondii* has been distinguished using PCR technique with a precise primers. The sequences of N-terminal and internal amino acid which obtained using microsequencing qualified us to design perverted nucleotides. The genetic part of 1024 bp was amplified by thermal cycler as exposed in **figure 3- E**. As showed in **figure 2** that genetic frequency among patients was higher than control 10.7% and 1.3% respectively.

TLR12 and *TLR11*

TLRs performance essential role in influential access to ligands and downstream motioning molecules according to their cellular localization of **(42)**. Chaperone-like protein and Nucleic acid coding for TLRs are restricted intracellularly, and it is necessary for the suitable function of all RNA and DNA TLRs **(43, 44** and **45)**.

Regulation of the IL-12 response was influence with TLR12 which is involved in the in response to *T. gondii* profiling, furthermore IRF8 who regulates TLR11 and TLR12 play essential role of dependent IL-12 responses, and initiation of IL-12 is the decisive stage for the stimulation of the immune system which is necessary for host defense against *T. gondii* **(46)**. Cytokine especially like IL-12 is crucial for eliciting creation of IFN-γ by T cells **(47)**, any disrupted to produce any of them (IFN-γ and IL-12) lead to consequences which cause acute susceptibility to the parasitic infection (*T. gondii*) **(48)**. Neutrophils, macrophages, proinflammatory monocytes and DCs which are innate immune cells, have the ability to producing IL-12 as a feedback response to parasitic infection with *T. gondii* **(49, 50** and **51)**. Moreover, in the deficiency of CD8+ the exposure to *T. gondii* capable to release by treatment with recombinant cytokines, representing that the main purpose of these DCs is TLR dependent gratitude the parasite and production of cytokines like IL-12 **(52** and **53)**. Electrophoresis result was exposed in **figure 3- D** (TLR11 298bp and TLR12 584). The genetic profiling of TLRs revealed that TLR11 and 12 was 21.1 and 21.7% respectively as describe in **figure 2.**

TUB1 and *FOL1*

Diversity of allele was noticed for most of genotypic and phenotypic markers is little, the case for isoenzyme markers was six polymorphic enzymatic systems (amylase, glutathione reductase, aspartate aminotransferase, acid phosphatase, propionyl, esterase glucose phosphate

isomerase) and these exhibit only less than three isoforms in a residents of 83 isolates of *Toxoplasma* **(54)**. there is also allelic diversity detected by PCR other genes which coding for main *Toxoplasma* antigens like cyst matrix and surface antigens and genes coding for other Toxoplasma products (dihydrofolate reductase-thymidilate synthase, α-tubulin, β-tubulin, actin, DNA polymerase alpha and nucleoside triphosphatase **(55** and **56)**. The higher polymorphism (less than 3 alleles) is identified some microsatellites which are positioned in the intron genes that coding for myosin-A (TgM-A) and these which coding for β-tubulin (TUB2) **(54)**. The genetic profiling of TUB1 and FOL1 revealed that TUB1 and FOL1 was 19.2% and 16.4% respectively as revealed in **figure 2**, and the genetic analysis for these genes was exposed in **figure 3- B** for Fol1 (800 bp) and **figure 3- C** for Tub1 (312 bp).

IL-6 and IL-1β genes

Parasitophorous vacuoles (PV) formed by Toxoplasma which represent as specialized non fusogenic compartment in invasive host cell. Micronemes, rhoptries and dense granules create as a result of invasion process and consecutive liberation of parasitic proteins from secretory organelles **(57)**. Attachment and invasion of parasite was depending on micronemes proteins which plays as dense granule change the host cell into an appropriate atmosphere for organism development by curbing a variability processes of host cells **(58)**, and numerous polymorphic proteins in the genomic of *T. gondii* are concealed elements which cooperate with cells of the host **(59** and **60)**. NK and T cells was activate by IL-12 to secrete IFNγ, concluding activates induce mechanisms for intracellular purging of *T. gondii*, containing the stimulation of interferon-regulated GTPases (IRGs) **(61** and **62)**, stimulation intermediates of reactive nitrogen, autophagy and tryptophan degradation in human cells **(63** and **64)**. The inflammasome has recently extended consideration, any faults in this path will connected with unrestrained growth and developing of parasite **(65, 66** and **67)**, the subtle equilibrium between signals of pro and anti-inflammatory is essential to assurance existence of host and parasite. The results of lL6 and IL-1β genes was revealed in **figure 2,** and the genetic picture of these genes was revealed in **figure 3- A**

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Figure 1: Distribution of genotypes and alleles for IFNγ+874 T/A between cases toxoplasmosis (SD)

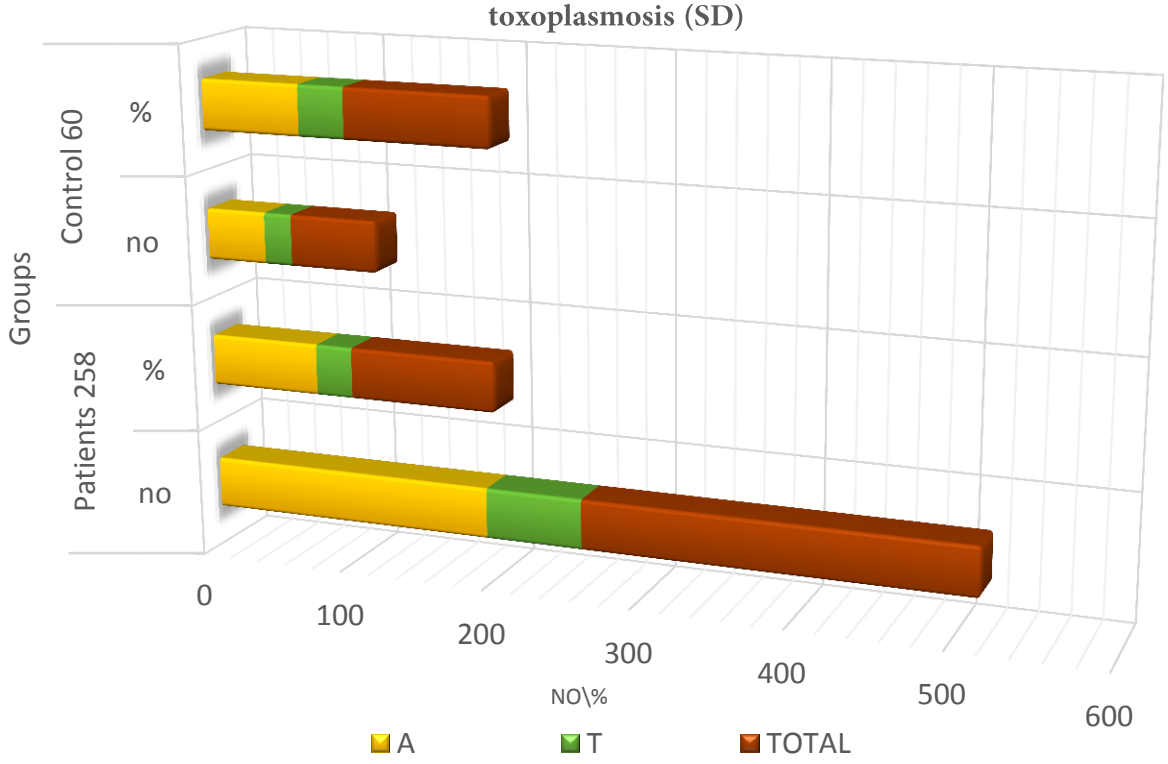
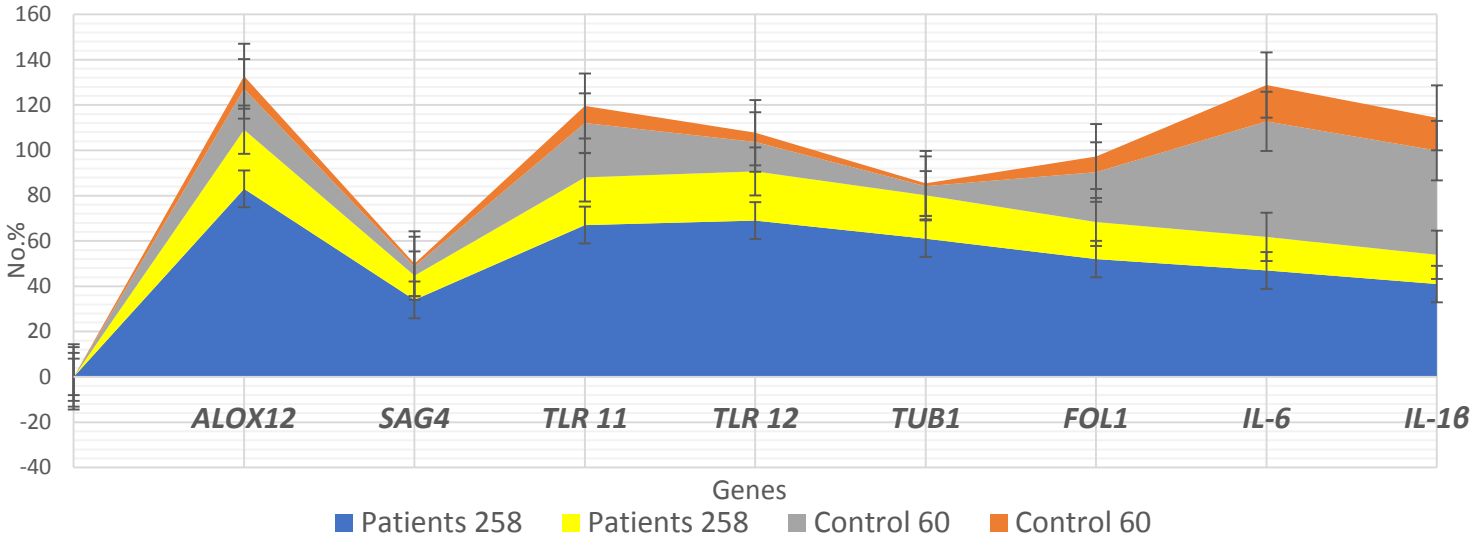


Figure 2: Distribution of genes in this study related to *Toxoplasma* infection copmared with control (SD)



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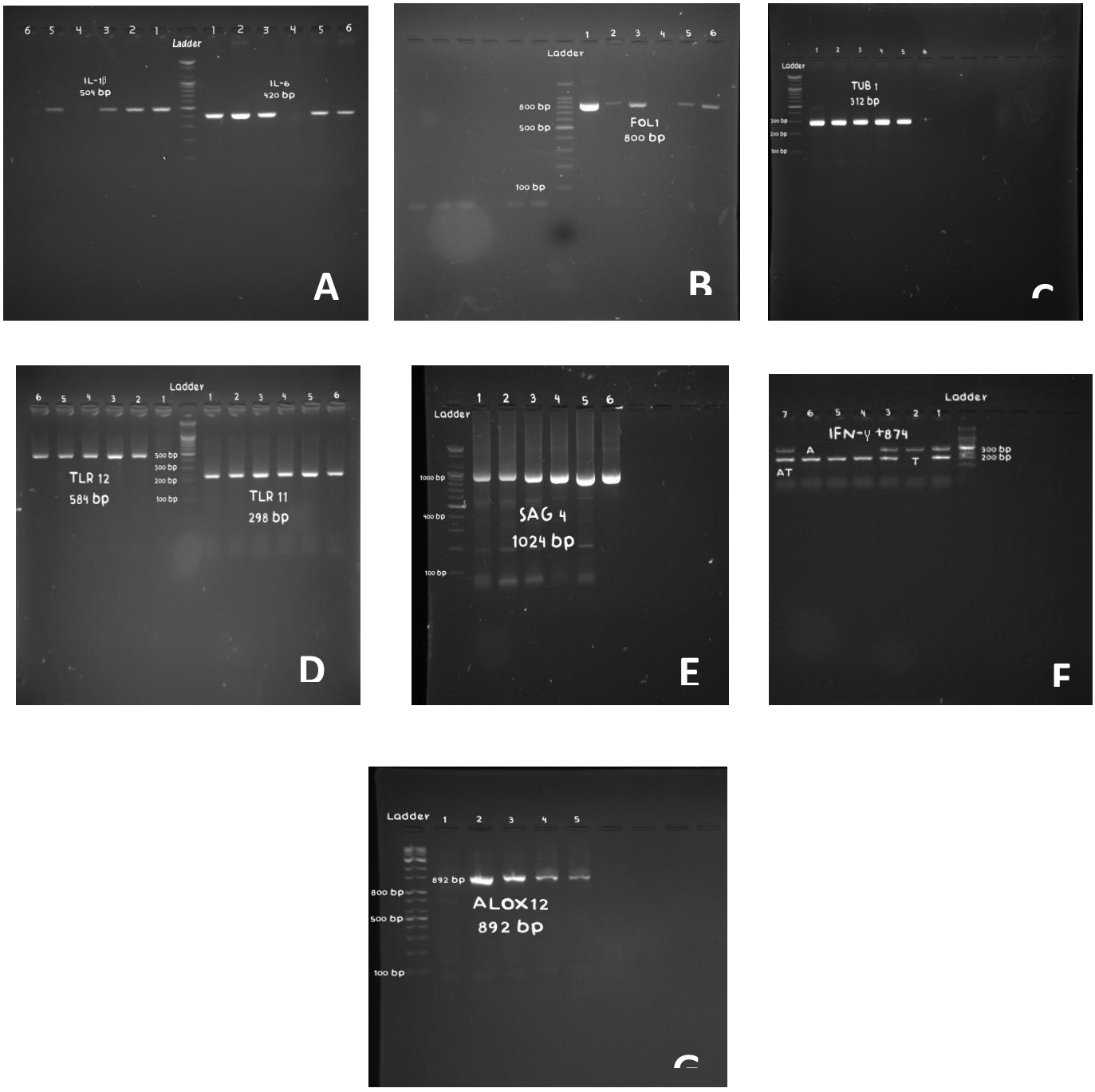

Figures 3 (from A-G) Ethidium bromide-stained Agarose Gel Electrophoresis of PCR-amplified products from extracted (1.5%) patterns showing typical PCR amplification products in multiplex PCRs for all figures including ***IL-1β, IL-6, FOL1, TUB1, TLR 11, TLR 12, SAG4, IFN- γ +874*** and ***ALOX12*** genes. Lane L, was DNA ladder (bioneer 25/100 Mixed DNA ladder and 100 DNA ladder)

FIGURE 3-A; left electrophoresis showed ***IL-1β*** gene (504 bp.) and at the right side of Ladder ***IL-6*** gene (420 bp.) with 100 DNA ladder. Sample No. 4 was negative control.

FIGURE 3-B; showed ***FOL1*** gene (800 bp.), at the right side of Ladder it was ***FOL1*** gene with 100 DNA ladder, while Sample No. 4 was negative control.

FIGURE 3-C; showed ***TUB1*** gene (312 bp.), at the right side of Ladder it was ***TUB1*** gene (312 bp.) and with 100 DNA ladder, while Sample No. 6 was negative control.

FIGURE 3-D; showed ***TLR 11*** gene (298 bp.) and ***TLR 12*** gene (584 bp) with 100 DNA ladder. at the right side of Ladder ***TLR 11*** gene while ate the left side of ladder was ***TLR 12.***

FIGURE 3-E; showed ***SAG4*** gene (1024 bp.) with 25/100 Mixed DNA ladder.

FIGURE 3-F; showed ***IFN- γ +874*** gene (285 bp.) with 100 DNA ladder, samples No. 1 and 3 was with both allele **A**/**T** , sample No. 2 was with only one allele (**T**), samples No. 6,5,4 was with only one allele (**A**).

FIGURE 3-G; showed ***ALOX12*** gene (892 bp.) with 100 DNA ladder. Sample No. 1 was negative control.

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