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ORIGINAL ARTICLE

Polymorphism Effects of TLR2 and TLR4 genes on Different Otitis Media Bacterial Infections

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Key words:
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ABSTRACT

Background: *Otitis media (OM) is an infection or inflammation in the middle ear. OM can become chronic or cause difficulties if it is not recognized early in the middle ear or if sufficient immune response is not elicited.* ***Objective****: We investigated the role of SNP of Toll-like receptors (TLRs) in otitis media because of their importance in the early response to external antigens.* ***Methodology****: A case-control study was carried out from 27/9/2020 to 25/2/2021 on 120 Iraqi OM patients and 80 controls. They were collected from 50 females and 70 males with different ages ranging from 5 and 68 years. The VITEK 2 system was used to analyze bacterial isolates and identify bacterial species. For the extraction and purification of genomic DNA from blood samples, the Gene aid gSYNCTM DNA extraction kit fast technique.* ***Results:*** *(83.3 %) of patient swabs yielded positive cultures, indicating otitis media, and (16.7 %) yielded negative cultures. Two variants (SNPs: rs5743708 and rs121917864) of the TLR2 gene were investigated. The SNP rs5743708 allele and genotype frequencies differed significantly between patients' group and controls (p-values = 0.0002, 0.0203, and 0.0245,) respectively. Comparing Otitis media patients to control showed that the SNP rs121917864 had a similar allele and genotype frequencies distribution, and the p-values were insignificant. Two variants (SNPs: rs4986790 and rs4986791) of the TLR4 gene were investigated.* ***Conclusion:*** *There were a significant differences (p-value = 0.0008, 0.0109, and 0.0146) in the TLR4 gene SNP rs4986790 distribution of allele and genotype frequencies among patients to controls. However, when comparing patients to controls, significant differences in the distribution of allele and genotype frequencies of SNP rs4986791 were discovered (p-value = 0.0001, 0.0022, and 0.0318). In this study, SNPs (TLR2 gene: rs5743708; TLR4 gene: rs4986790 and rs4986791) were significantly associated with susceptibility to otitis media.*

INTRODUCTION

Otitis media (OM), often known as middle ear inflammation (ossicles and middle ear cavity inflammation), is a broad term that encompasses acute otitis media (AOM), otitis media with effusion (OME; 'glue ear), and chronic suppurative otitis media (CSOM) (CSOM)[1] . One of the most frequent illnesses among children is OM. It is also a prominent cause of medical consultations, antibiotic prescriptions, and surgery in high-income nations [2, 3] . Recurrent or chronic otitis media (COM) in children, in particular, causes hearing loss, which can lead to speech difficulties, delayed language acquisition, attention deficit disorders, and behavioral abnormalities [4]. The chance of repeated bouts of OM is greatly increased if the nasopharynx is colonized early by bacteria that are pathogens. The three most common bacterial pathogens recorded worldwide are *Streptococcus pneumoniae* or pneumococcus, *Moraxella catarrhalis* and non-typeable *Haemophilus influenza*[5] .

TLRs are polymorphic receptors that play a role in innate and adaptive immunity. It's not unexpected that single nucleotide polymorphisms (SNPs) in these genes have an impact on illness progression [6]. The single-nucleotide polymorphisms Arg753Gln and Arg677Trp have been identified as putatively functional in the human TLR2 gene (SNPs) and a microsatellite GT repeat polymorphism in intron [7]. TLR-2 has two non-synonymous SNPs that are linked to human illnesses rs5743708 and rs121917864. TLR abnormalities produced by SNPs may alter infection and inflammatory disease susceptibility [8]. TLR4 has been shown to be expressed in AOM, OME, and COM and linked to the pathogenesis of otitis media. The TLR4 299A/A genotype was shown to be linked to an otitis-prone condition after DNA samples were analyzed [9]. Reduced TLR4 levels in the middle ear mucosa create a long-

lasting inflammatory response and make the middle ear more susceptible to CSOM [10].The rs4986790 mutation changes the interaction between TLR4 and its ligand, causing it to lose some function and influence infection susceptibility [11].The present study was conducted to assessment of relationships between different genotypes in TLR2 and TLR4 with type and severity of bacterial infection.

METHODOLOGY

In the present study,120 otitis media patients were included blood and ear swab samples from Ramadi Teaching Hospital during the period from27/9/2020 to 25/2/2021. They were collected from 50 females and 70 males, with different ages ranging from 5 and 68 years. The selection of each case was established by the clinical examination done by otolaryngologists. Eighty apparently healthy volunteers without a previous history of middle ear infections consisting of 50 males and 30 females, were collected from Baghdad medical laboratory as a control group. The VITEK 2 system was used to analyze bacterial isolates and identify bacterial species. For the extraction and purification of genomic DNA from blood samples, the following Geneaid gSYNCTM DNA extraction kit fast technique. By Bioneer corporation, Specific primers were used for the detection of SNPs in the targeted Gene by the tetra arm technique (**Table 1&2).**

Table 1: Primers for detection of (rs5743708&rs121917864) in the human TLR2 gene

SNPS	SIZE(bp)	Primer	Sequence (5`-3`)
rs5743708	G allele: 169	IF	CAGCGCTTCTGCAAGCTTCA
	A allele: 122	IR	GGTAGGTCTTGGTGTTCATTATCTGCC
	two outer primers: 245	OF	AACTTTGTGAAGAGTGAGTGGTGCAA
		OR	GGAACCTAGGACTTTATCGCAGCTCT
rs121917864	C allele: 209	IF	CCCTTCAAGTTGTGTCTTCATACGT
	T allele: 146	IR	TTGCCAGGAATGAAGTCACG
	two outer primers: 310	OF	GGCCTGTGGTATATGAAAATGATGT
		OR	GTTCATACTTGCACCACTCACTCTTC

Table 2: Primers for detection of(rs4986790& rs4986791) in human TLR4 gene

SNPS	SIZE (bp)	Primer	Sequence (5`-3`)
rs4986790	A allele: 164	IF	CATACTTAGACTACTACCTCGAGGA
	G allele: 241	IR	GTCAAACAATTAAATAAGTCAATAAGAC
	two outer primers: 352	OF	TTCATAAGCTGACTTTAAGAAATAATT
		OF	AACTGTCCAAATTTACAGTTAACTAAT
rs4986791	T allele: 193	IF	CTCAAAGTGATTTTGGGACCAT
	C allele: 251	IR	CTCAGATCTAAATACTTTAGGCGGG
	two outer primers: 397	OF	AAAGACTTTTCTTATAATTTCGGATGG
		OF	GAGAAATGTCAAGGTAAATGAGGTTT

RESULTS

Our results showed that 100 (83.3%) of patient swabs yielded positive cultures, indicating otitis media, and 20 (16.7%) yielded negative cultures, but all 80 (100%) swabs from healthy people yielded negative cultures (Figure 1), the findings indicated that there were 72 (72 %) gram-negative bacteria and 28 (28 %) gram-positive bacteria (Figure 2). The most commonly identified gram-negative species, were 34(34 %) isolates identified as *Pseudomonas aeruginosa* , 10(10 %) isolates of *Proteus mirabilis* , 7(7 %) isolates of *Klebsiella pneumoniae*, while The most commonly identified gram-positive species were *Staphylococcus aureus* 18(18%) isolates followed by *Streptococcus pneumoniae* 7(7%) isolates (Figure 3).

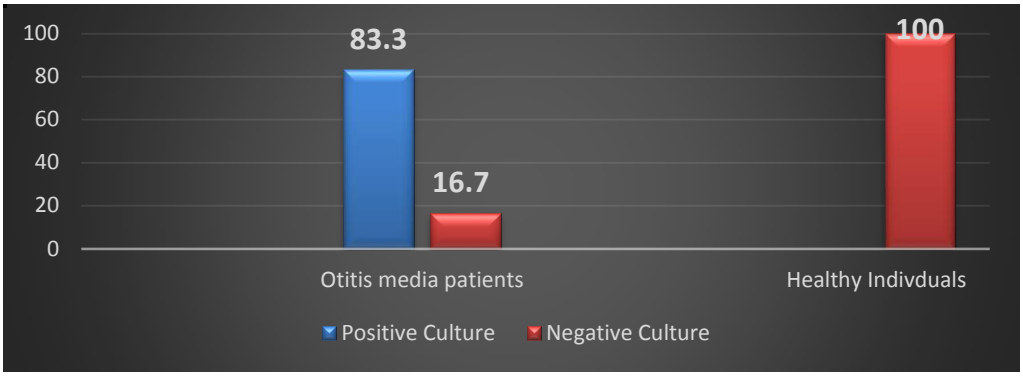

Fig. 1: Percentage of positive culture in the examined groups.

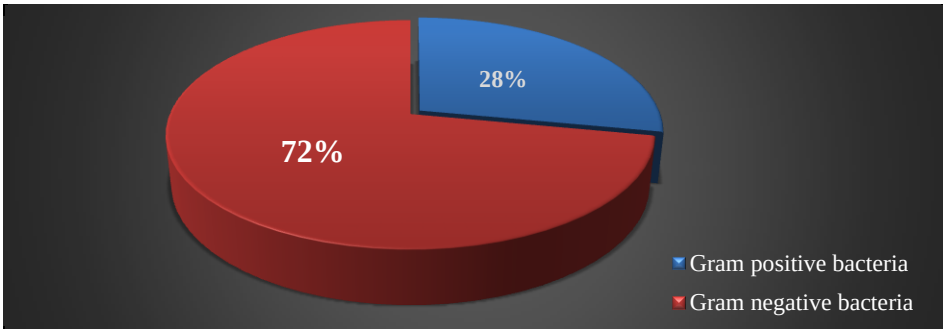

Fig 2: Percentage of Bacterial Group based on Gram stain.

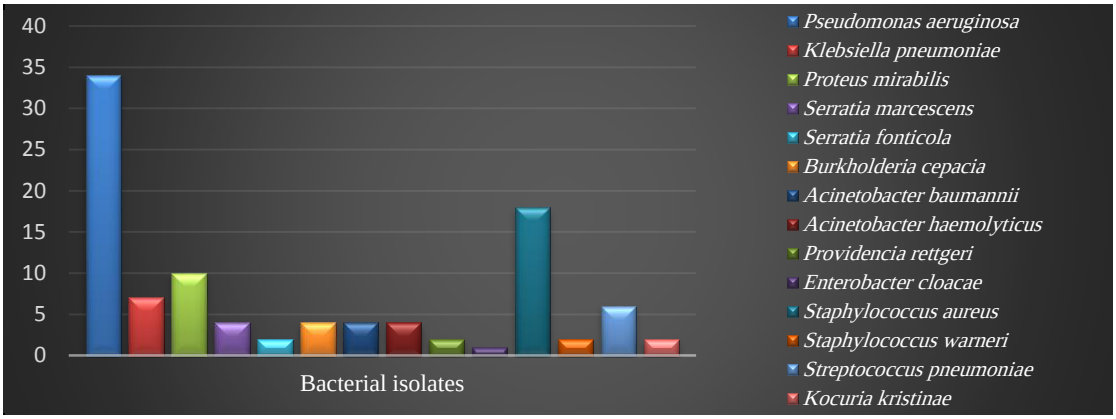

Fig. 3: Percentage of bacterial isolated

Figure 4 depicts a 2 percent agarose gel electrophoresis of the PCR-ARMS product of the rs5743708 gene polymorphism for patients' samples, with lane M 100 bp DNA ladder

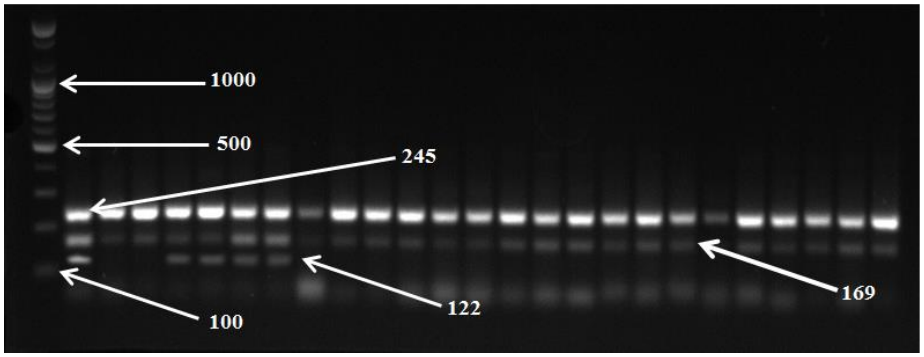

Fig. 4: Polymorphism of the TLR2 (rs5743708) genes in the O.M patients.

Table 3 reveals statistical analysis findings revealed a significant difference between genotype Frequency (%) TLR2 rs5743708 in Otitis media patients and control groups.

Table 3: Genotype Frequency (%) TLR2 rs5743708 in OM patients and control groups.

Genotype	Control n=80	Cases n=100	P-value	Odds Ratio	95% Cl
GG	70 (87.5%)	56 (56%)	----	1.0	----
GA	6 (7.5%)	24 (24%)	0.0203 *	5.00	1.2842 to 19.4668
AA	4 (5%)	20 (20%)	0.0245 *	6.25	1.2650 to 30.8783
Chi-squared	11.2501 **	10.0589 **			
P Value	0.0036	0.0065			
Allele frequency (%)					
Allele	**Control n=160**	**Cases n=200**	**P-value**	**Odds Ratio**	**95% Cl**
G	0.91 (146)	0.68 (136)	0.0002 **	4.908	2.096 to 12.60
A	0.09 (14)	0.32 (64)			

Figure 5 show a 2 % agarose gel electrophoresis of the rs121917864 gene polymorphism for patient's samples using lane M 100 bp DNA ladder.

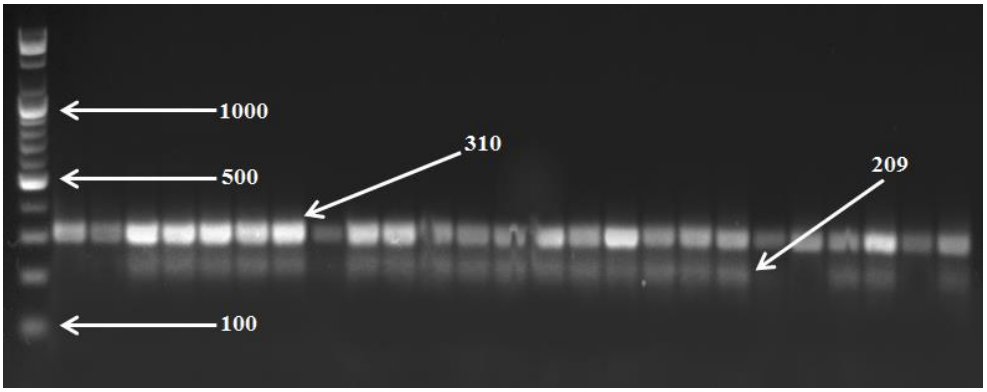

Fig. 5: Polymorphism of the TLR2 (rs121917864) genes in OM patients.

Table 4 reveals statistical analysis findings revealed a significant difference between genotype Frequency (%) TLR2 rs121917864 in Otitis media patients and control groups.

Table 4: Genotype Frequency (%) TLR2 rs121917864 in OM patients and control groups.

Genotype	Control n=80	Cases n=100	P-value	Odds Ratio	95% Cl
CC	48 (60%)	72 (72%)	----	1.0	----
CT	30 (37.5%)	28 (28%)	0.2977 NS	0.6222	0.2548 to 1.5196
TT	2 (2.5%)	0 (0%)	0.3653 NS	0.2237	0.0088 to 5.7207
Chi-squared	0.5803 NS	1.325 NS			
P Value	0.7481	0.5156			
Allele frequency (%)					
Allele	**Control n=160**	**Cases n=200**	**P-value**	**Odds Ratio**	**95% Cl**
C	0.79 (126)	0.86 (172)	0.2354 NS	0.6033	0.2863 to 1.333
T	0.21 (34)	0.14 (28)			

The result show in (Figure 6) a 2 percent agarose gel electrophoresis of the PCR-ARMS product of the rs4986790 gene polymorphism for patient samples, with lane M 100 bp DNA ladder.

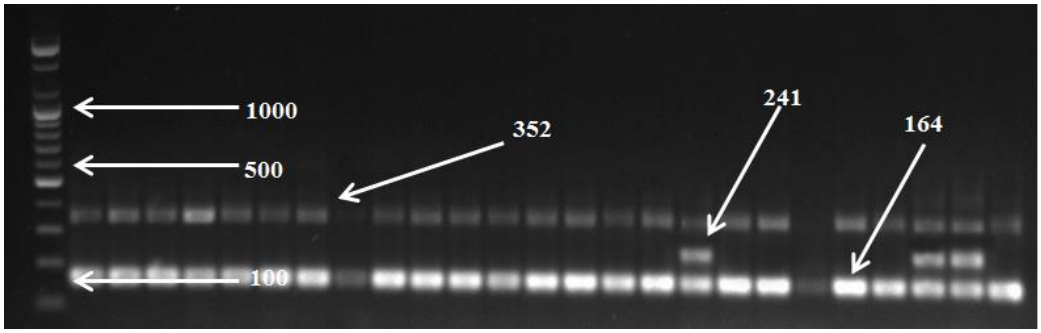

Fig. 6: Polymorphism of the TLR4 (rs4986790) genes in OM patients.

Table 5 indicated to statistical analysis findings revealed a significant difference between genotype Frequency (%) TLR4 rs4986790 in OM patients and control groups

Table 5: Genotype Frequency (%) TLR4 rs4986790 in OM patients and control groups.

Genotype	Control n=80	Cases n=100	P-value	OddsRatio	95% CI
AA	54 (67.5%)	34 (34%)	----	1.0	----
AG	20 (25%)	44 (44%)	0.0109 *	3.491	1.3341 to 9.1514
GG	6 (7.5%)	22 (22%)	0.0146 *	5.823	1.4168 to 23.9369
Chi-squared	1.9141 NS	0.574 NS			
P Value	0.3840	0.7505			
Allele frequency (%)					
Allele	**Control n=160**	**Cases n=200**	**P-value**	**Odds Ratio**	**95% CI**
A	0.8 (128)	0.56 (112)	0.0008 **	3.143	1.614 to 5.998
G	0.2 (32)	0.44 (88)			

Figure 7 show a 2 percent agarose gel electrophoresis of the PCR-ARMS product of the rs4986791gene polymorphism for patient samples, with lane M 100 bp DNA ladder

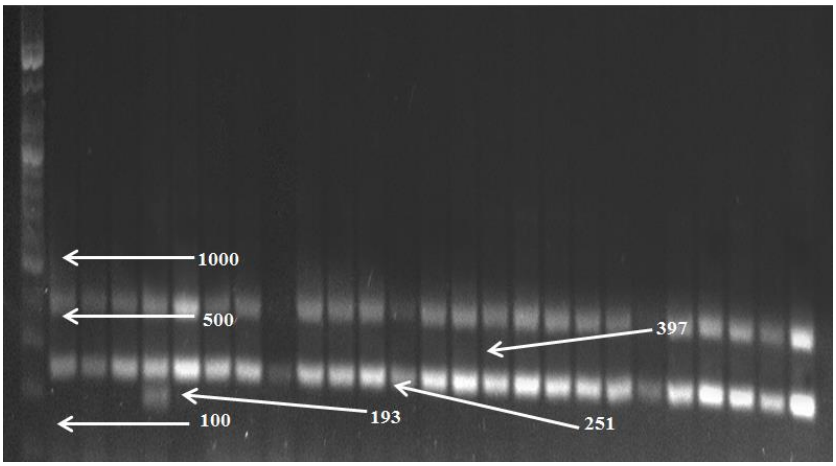

Fig. 7: Polymorphism of the TLR4 (rs4986791) genes in OM patients

The results shoe in (Table 6) reveals statistical analysis findings revealed a significant difference between genotype Frequency (%)TLR4 rs4986791in Otitis media patients and control groups.

Table 6: Genotype Frequency (%) TLR4 rs4986791 in OM patients and control groups.

Genotype	Control n=80	Cases n=100	P-value	Odds Ratio	95% Cl
CC	58 (72.5%)	22 (22%)	----	1.0	----
CT	20 (25%)	38 (38%)	0.0022 **	0.1996	0.0710 to 0.5611
TT	2 (2.5%)	40 (40%)	0.0318 *	10.526	1.2269 to 90.3150
Chi-squared	0.0154 NS	2.3016 NS			
P Value	0.9923	0.3164			
Allele frequency (%)					
Allele	**Control n=160**	**Cases n=200**	**P-value**	**Odds Ratio**	**95% Cl**
C	0.85 (136)	0.41 (82)	<0.0001**	8.154	3.870 to 16.74
T	0.15 (24)	0.44 (118)			

In the current study bacterial species were classified based on genotype as shown in the (Figure 8).

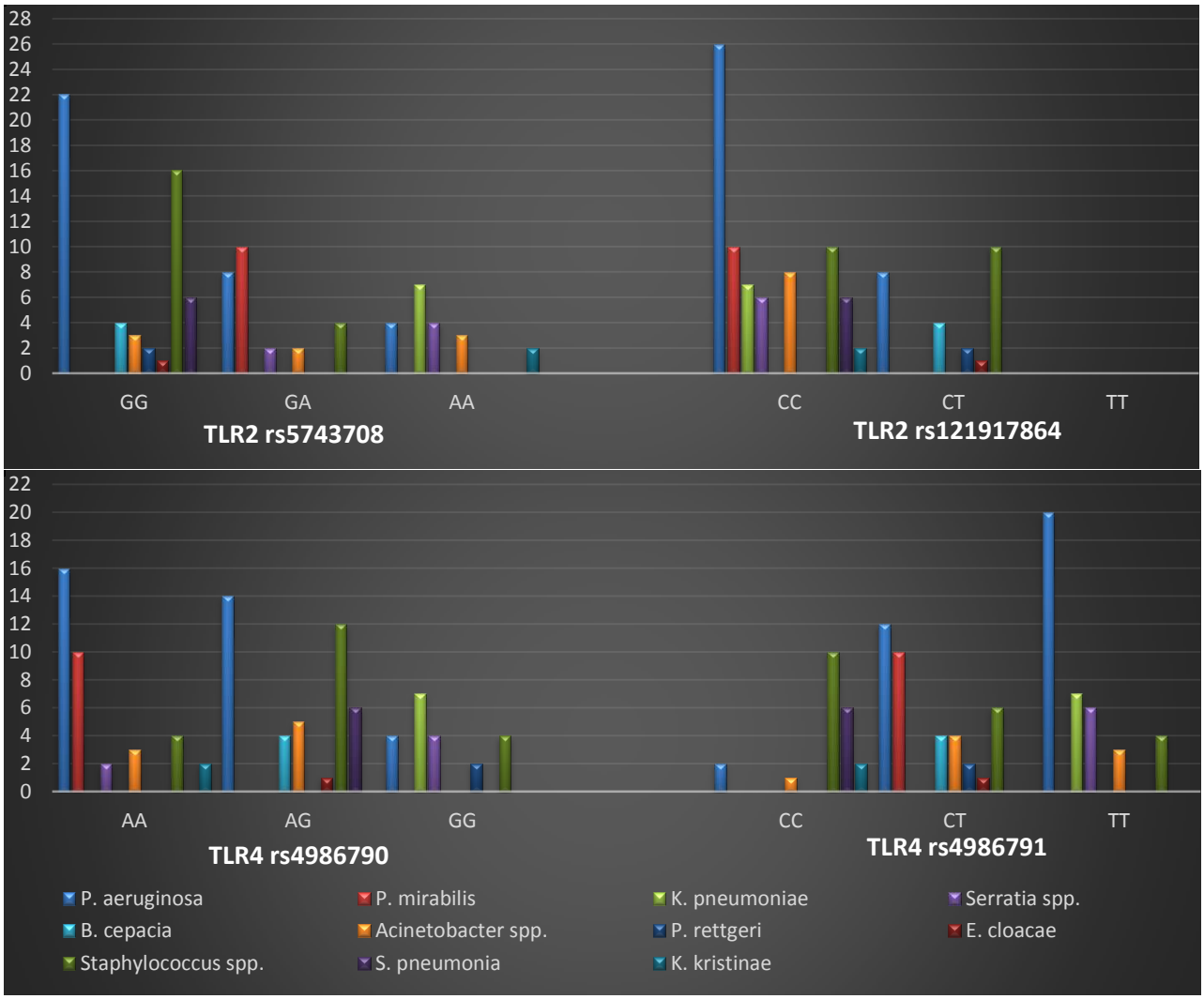

Figure 8: Classification of otitis media bacteria genotype

DISCUSSION

Comparison of the prevalence of SNPs in TLR2 and TLR4 genes between the OM case and the control groups did not show any significant differences between these populations. TLR2 rs5743708 genotype in OM and healthy participants revealed that 24% of OM patients and 7.5 % of healthy subjects were heterozygous(GA) for the Arg753Gly gene, while 20% of OM and 4% of healthy subjects were homozygous mutant(AA) for the same gene.

According to these findings, the presence of allele A is a risk factor that impacts the risk of developing OM.The presence of Arg753Gln reduces TLR2/TLR1 signaling and thereby protects against the development of late-stage Lyme disease (LD).(8) The probability of contracting tuberculosis increased 1.60-fold in those with heterozygous genotypes (GA) but increased 6.04-fold in people with homozygous mutant genotypes (AA)[12].

TLR2 rs121917864 genotype in OM and healthy participants revealed that 28 percent of OM patients and 37.5 percent of healthy subjects were heterozygous for the Arg677Trp gene, whereas 0 percent of OM and 2.5 percent of healthy subjects were homozygous mutant for the same gene, according to our research. When compared to the wild genotype CC, which has a high risk of acquiring otitis media, our findings suggest that the presence of the T allele helps to protect against the risk of developing OM. In a previous investigation, no indication of a link between the TLR2 Arg677Trp polymorphism and the incidence of pulmonary tuberculosis was founded [13], this agrees with our finding of no association between TLR2 Arg677Trp polymorphism and OM risk. Despite the lack of a substantial link between the Arg677Trp polymorphism and the likelihood of OM infection, additional changes in the host's TLR2 gene may contribute to greater OM susceptibility.

TLR4 rs4986790 in OM patients and healthy subjects, showed that 44 % of OM patients and 25 % of healthy subjects were heterozygous for the Asp299Gly gene, while 22 % of OM and 7.5 % of healthy subjects were homozygous mutant for the same gene. It was also discovered that the presence of the G allele is a risk factor for developing otitis media in 66% of the cases. The discovery that osteomyelitis was related to the GG genotype of the (Asp299Gly) polymorphism was reported in a research evaluating TLR4 gene polymorphisms [14]. According to research comparing TLR4 gene polymorphisms in chronic periodontitis (CP) patients and healthy persons, 11 %of CP patients and 7% of healthy subjects were heterozygous for the Asp299Gly gene [15].

TLR4 rs4986791 gene polymorphisms in OM and healthy persons were found to be heterozygous for the Thr399Ile gene in 38 %of OM patients and 25 %of healthy subjects, whereas 40 %of OM and 2.5 %of healthy participants were homozygous mutants for the same gene in our study. In contrast to the TLR4 wild-type group, those having the minor T allele on SNP rs4986791 had a 78 % greater risk of otitis media (P = 0.0001**).Human TLR4 gene variants Asp299Gly and Thr399Ile have been linked to functional alterations that predispose persons to be less sensitive to LPS and have a higher risk of severe infection susceptibility to pathogenic bacterial infections [16].

CONCLUSIONS

Our study extended the evaluation of TLR2 and TLR4's role in the etiology of OM by examining some single nucleotide polymorphisms (SNPs) of *TLR2* and *TLR4* genes. Such a genetic approach enriched the contribution to the etiology and pathogenesis of OM, as some SNPs (*TLR2* gene: rs5743708; *TLR4* gene: rs4986790 and rs4986791) were significantly associated with susceptibility to this OM.

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Ethical approval

The study was approved by Ethics Committee of the Al Anbar Medical Research University (approval number 32, June. 22, 2020). All individuals have given consent to participate in the current study.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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