Role of rs5743708 SNP in the risk of Bacterial Respiratory Infections in Children of Ramadi city/Iraq

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ABSTRACT
Background and Aim: Respiratory Infections are a major global health and financial burden, so among cause the five most common and deadly for respiratory illnesses is acute lower respiratory tract infections (RTIs), these infections claim over 4 million lives annually. Toll-like receptors (TLRs) are key components of the innate immune system that recognize pathogen which associated a molecular patterns) PAMPs) and initiate an immune response. Understanding the role of TLR polymorphisms in susceptibility to respiratory infections in children is important for developing strategies to prevent and treat these infections. So, the current study aims to "Investigate the potential association between TLR2 gene polymorphisms and susceptibility to respiratory infections in children". Methodology: The study included collect of 300 samples with respiratory infection from both sex with range ages(1-12) years, and other 50 healthy control samples. The samples were collected from general and private hospitals in Ramadi city, Iraq. PCR was carried out using primer for gene target and amplification of target gene. Sanger Sequencing conducted with both direction for all samples. Results and Conclusion: The result of sputum culture indicate that 100 of suspected samples were positive culturing while other demonstrate a negative growth. The results analysis of sequencing according to Query and subjected samples indicated that 4 of patient have SNP of rs5743708 G>A, while there is not found in control samples, and the results of genotype frequencies analysis it was found that TLR2 polymorphism was associated with increased risk for bacterial respiratory infections in children according to under study samples, but no statistical significant differences correlation of rs5743708 G>A and infection, with adjusted odds ratio [OD:5.3; 95%Cl: 0.3-101; P: 0.2].

1. Introduction

Respiratory tract infections are a serious threat to global health, causing significant illness, these infections responsible for over 4 million deaths each year, with children under 5 years being the most vulnerable (Tazinya et al., 2018). Bacterial infections consider second common cause of respiratory illnesses, and they can be more serious especially with infwction of Corynebacterium diphtheriae, Haemophilus influenzae, Acinetobacter baumannii, Mycoplasma pneumoniae, Streptococcus pneumoniae, Pseudomonas aeruginosa and Klebsiella pneumoniae (Liu et al., 2021).

TLRs act like sentinels in our body's first line of defense. They recognize signature molecules from invading germs and trigger an immune response (Sameer & Nissar, 2021). All Toll-like receptors are type 1 transmembrane proteins, and there are 10 of Toll-like receptors (TLRs) types in humans, each recognizing different of infection signatures called pathogen-associated molecular patterns (PAMPs) (Guo & Xia, 2015). Cell surface TLRs (1, 2, 4, 5, 6, 10) these guard the outside, recognizing infection molecules directly on the cell surface, while endosomal TLRs (3, 7, 8, 9) these patrol the inside, stationed within compartments called endosomes where they can detect pathogens that have been engulfed by the cell (Duan et al., 2022).

TLRs can be represent as alarm when they spot a harmful pathogen invading the human body (Vijay, 2018). This triggers are a chain reaction within the cells, which activating of transcription factors like (Tumor Necrosis Factor-alpha (TNFα) and Interleukin-1 (IL-1) which rally the troops by sending out signals to produce powerful chemicals signals called "pro-inflammatory cytokines" these cytokines act like megaphones, summoning more immune cells to the fight and helping to eliminate of the infection agents (Wyres et al. 2020).

The overall effect of a polymorphism on an individual's health will depend on a variety of factors, including the specific polymorphism, the individual's genetic background, and their environment (Salamaikina et al., 2022). Therefore, understanding the role of TLR gene polymorphisms in

susceptibility to respiratory infections in children is important for developing strategies to prevent and treat these infections. TLR gene polymorphisms can affect susceptibility to respiratory infections by altering the following:

Signaling pathway activation: Other polymorphisms affect downstream signaling pathways triggered by TLRs, leading to an altered inflammatory response and impaired pathogen clearance.

Protein structure and function: Certain polymorphisms may alter the protein structure of TLRs, affecting their ligand binding ability and function.

So, the current study aims to "Investigate the potential association between TLR2 gene polymorphisms and susceptibility to respiratory infections in children.

2. Methodology

Samples Collection and Study design:

The study included collect of 300 samples with respiratory infection from both sex with range ages(1-12) years, and other 50 healthy control samples. The samples were collected from general and private hospitals in Ramadi city, Iraq. At first, patient samples collect according to physicians recommendation as expected of bacterial respiratory infection by used of nasogastric (NG) tube to collect of sputum from baby, also collect of sputum with sterile cup by deep coughing from young children with sterile condition, then detection of isolates type that cause of bacterial infection by Vitek Com.2 devise as a final identification. The study involved collecting a small blood sample (5 milliliters) in special tubes to isolate DNA for genetic analysis. This research took place from June 2023 to May 2024.

Bacterial Identification:

Bacterial identification is a critical step in current study to distinguishes of bacterial respiratory infection from other illness. Samples were identified using traditional microbiological methods such as colonial morphology, Gram staining, biochemical tests, and the Vitek 2 Compact system (bioMérieux, France).

Molecular Study:

DNA Extraction:

Each frozen blood sample was thawed, centrifuged; genomic DNA was then extracted directly using the Norgen® blood DNA extraction kit (Ghatak et al., 2013).

Primer Design and Molecular screening of *rs5743708*:

PCR was performed using gene-specific primers listed in Table 1, with all reactions conducted on Applied Biosystems 2720 thermal cyclers, and amplification of target gene by using new set of primer to amplify **(560 bp)** for being use in sequencing portion, and target SNPs will be involved in this region. The 25 µl PCR amplification reaction consisted of 12.5 µl OneTaq (NEB®) mastermix, 3 µl DNA sample, 1.5 µl of each primer (10 pmol/µl), and 6.5 µl nuclease-free water. The reaction was performed under optimal conditions listed in Table 2.

Table (1): Sequence of TLR2 SNP primer and their size Gene:

Gene		Sequence of forward and reverse Primer (5´- 3´)	PCR Product Size bp	Annealing Temp.	Reference
TLR2 (rs5743708)	F	GGTATATGAAAATGATGTGGG	560 bp	48	NCBI
	R	GACATAAAGATCCCAACTAGA			

Table (2) PCR conditions for amplification of rs5743708SNP under study.

Step	Temperature(˚C)	Time	No.of cycle

Initial denaturation	95	5 min	**1**
Denaturation	95	30 sec	**35**
Annealing	48	48sec	
extension	72	50sec	
Final extension	72	7min	**1**
Hold temperature	**4**	-	-

Purified PCR DNA fragments of the target gene (forward and reverse) were sent for Sanger sequencing using ABI3730XL by Macrogen Company in Korea.

Ethics of research :

Ethical approval was obtained from the Al Anbar Medical Research University Ethics Committee (approval number: 3rd June 2023). Parental consent was given for all participants.

Statistical Analysis:

All statistical analyses were conducted using SPSS version 18.0 (SPSS Inc., NY, USA).Chi-square tests were used to calculate value of Hardy Weinberg Equilibrium for control samples of under study SNPs. MedCalc statistical software package used for data genetic analysis for Genotype frequencies of SNP, Allele Frequency of SNP and other features

3. Results and discussion

1Sampling and bacterial cultivation

Out of all specimens, the result of microbial culture indicate that 100(33.3%) were positive for culturing while 200(66.6%) negative for any growth. Some negative culture results could be caused by patients receiving antimicrobial chemotherapy before the cultures were taken (Lahij, H. F. & Almeani, S. A. L., 2024) or may back to another causes of microbial respiratory infection such viruses or fungal. The identification of bacterial species were be done according to colonial morphology and by VITEK Comp.2 system as a final identification, the results of microbial distribution show that 40 samples belong to *Klebsiella pneumoniae* and 20 samples to *Acinobacter baumanni*, 19 samples of *Streptococcus pneumonia*, 9 samples to *Enterobacter aerogens*, 8 samples to *Enterococcus faecium* and 4 samples belong to *Pseudomonas putida* (Fig.1).

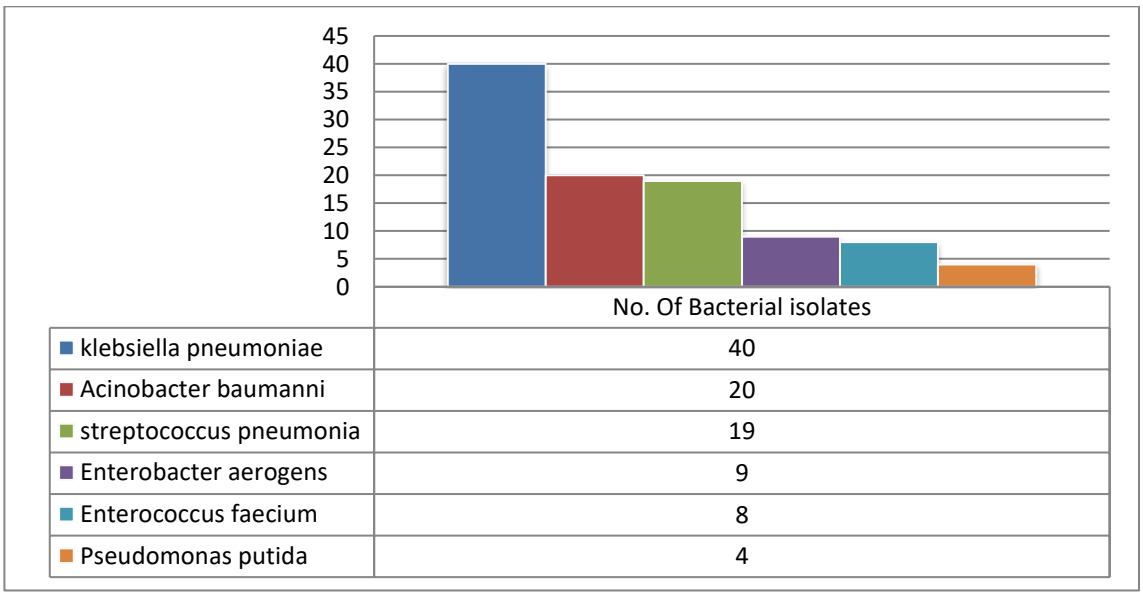

Figure (1): Samples distribution according to type of bacterial isolate infection.

The results of samples distribution according to type of bacterial isolate infection indicate that Gram-Negative bacteria infection more than Gram-Positive in respiratory infection of children, this may back to presence of outer membrane Gram-Negative bacteria that acts as a barrier against the immune

system and some antibiotics, this outer membrane can contain toxins like lipopolysaccharides (LPS) which can trigger a strong inflammatory response in the lungs, worsening respiratory infections (Chen *et al*., 2023), and also to adhesion factors: Many Gram-negative bacteria possess fimbriae or pili, which are hair-like structures that help them adhere to host cells in the respiratory tract which this allows them to colonize and establish an infection more easily (Werneburg & Thanassi, 2018).

There are several reasons that interpretation of sputum is best environment for infection such (a), direct exposure: the constant stream of air we inhale makes the respiratory tract a prime target for airborne pathogens, airway devices like ventilators and nebulizers further expose and potentially bypass natural defenses. (b), weakened defenses: Illness, medications, and intubation can compromise protective barriers like mucus and cilia, leaving the respiratory tract vulnerable to pathogen attachment and growth. (c) the habitat: The warm and humid environment inside the respiratory tract provides the perfect breeding ground for many bacteria and viruses, allowing them to thrive and multiply (Blot *et al*., 2022), (Revelas, 2012).

The more recurring isolates in respiratory infection were *K. pneumoniae*, that indicate to these bacteria was dominant of respiratory infection among AL-Ramadi hospital units, and several characteristics of *K. pneumoniae* contribute to its dominance in community and hospital-acquired infections such as (a) Optimal Habitat: (It readily colonizes human body sites like the respiratory tract, (b) Environmental Toughness: *K.pneumoniae* isn't a fragile creature which can survive on dry surfaces for extended periods, making transmission through contaminated objects (fomites) a real threat. (c) Antibiotic Armor: The emergence of multidrug-resistant *K. pneumoniae* strains (MDR-Kp) poses a major challenge. These "superbugs" are notoriously difficult to treat with conventional antibiotics, allowing them to flourish in healthcare environments (Russo & Marr, 2019), (Ahmed & Al Meani, 2019) In summary, a triple threat in hospitals: *K. pneumoniae* cunning adaptability, potent virulence, and the vulnerability of healthcare settings make it the king of gram-negative nosocomial infections.

Molecular Screening Study:

Molecular study subjected to find the frequency of very important SNP (***rs5743708* G>A**). Genomic DNA was extracted from the frozen blood of the **150** samples (100 samples of Bacterial Respiratory infection and 50 control samples). Primer pairs (Table1) was used to amplify a specific SNP region of *TLR2* gene (***rs5743708* G>A**) by using a conventional PCR technique. The results of PCR electrophoresis show that all sample gave a true amplification with expected PCR product size (560bp) (fig.2) of target gene for specific SNP.

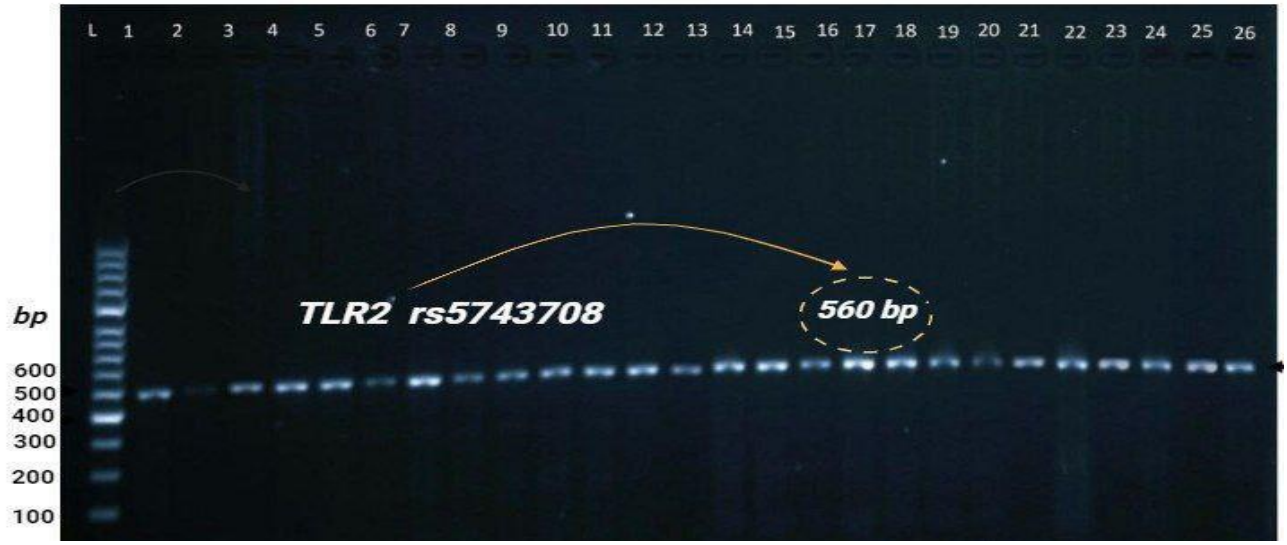

Figure 2: PCR amplification fragments for the detection of TLR2 gene (rs5743708 G>A) SNP (1.5%agarose, 7 V/cm^{2}for 90min). Lane L: 100-bp DNA ladder. Lane 1-26: *TLR2* gene

(*rs5743708* G>A) SNP bands with 560 bp.

All samples sent to Sanger sequencing with both direction (Forward and Reverse) to identify of single nucleotide polymorphisms (SNPs) within of gene interest. The results analysis of sequencing according to Query and subjected samples indicated that 4 of patient have SNP of ***rs5743708* G>A**, while there is not found in control samples, and the results of genotype frequencies analysis it was found that *TLR2* polymorphism was associated with increased risk in susceptibility to bacterial respiratory infections in children according to under study samples, but no statistical significant differences correlation of ***rs5743708* G>A** and infection, with adjusted odds ratio **[OD:5.3; 95%CI: 0.3-101; *P*: 0.2]** with their details listed with highlighted yellow color in (Table3). So, the genotype frequencies results of **SNP G>A** genotype considered a risk factor for some patients under study that alter wild type from G to A, but this risk factor not have a significant correlation with other genotype pattern in patient study.

Genotype distribution in the control group, it was found within "Hardy Weinberg Equilibrium" which recorded between observed and expected genotype. The result of allele frequencies of (G,A) also, haven't a statistic significantly differences between patients and control (Table 3), but **G** allele indicate a risk factor under study samples of children respiratory infection.

Table(3): Genotype and Allele frequencies of SNP (rs5743708) among patients and control

Genotype frequencies					
Genotype	Patient No. (100)	Control No. (50)	Odds. Ratio	95%Cl	*P* value
G:G	80	40	1.0	0.4 to 2.3	1.0
G:A	4	-	5.3	0.3 to 101	0.2
A:A	16	10	0.7	0.3 to 1.2	0.6
Allele Frequency					
G	164	80	1.1	0.6- 2.0	0.4
A	36	20	0.8	0.4-1.6	0.6

On the other hand, the study results showed no significant difference between patients and controls (P > 0.05) under both dominant and recessive models (Tables 4 and 5).

Table 4: Distribution of SNP in the study population under recessive model.

Genotype	**Patients N= 100**	**Control N = 50**	**Odds ratio**	**95% CI**	**P value**
GG + GA	**84**	**40**	**0.8**	**0.3 to 1.8**	**0.7**
AA	**16**	**10**			

Table 5: Distribution of SNP in the study population under dominant model.

genotype	**Patients N = 100**	**Control N = 50**	**Odds ratio**	**95% CI**	**P value**
GG	**80**	**40**	**1.0**	**0.4 to 2.3**	**0.2**
AA+ GA	**20**	**10**			

TLR2 (rs5743708 G>A) SNP consider a missense SNP mutations which is very important because they can alter the code of amino-acid then alter codes for proteins synthesis that are essential molecules that carry out many functions in the body, and even a small change in a protein's structure can affect its function (Guo & Xia, 2015). So, any SNP as a level of ***rs5743708* G>A** may tend to make *TLR2* change in core function whether increase or decrease of activating transcription factors like (Tumor Necrosis Factor-alpha (TNFα) and Interleukin-1 (IL-1) which rally the troops by sending out signals to produce powerful chemicals signals called "pro-inflammatory cytokines" these cytokines act like megaphones, summoning more immune cells to the fight and helping to eliminate

of the infection agents (Wyres *et al*. 2020). *TLR2* recognizes bacterial lipopolysaccharides and induces chemokine-dependent cellular migration, essential for the host's innate response to certain pneumonia-causing bacteria (Le *et al*., 2023).

It's important to note that of current study subjected of random samples who presented in local hospitals, and these genetic interpretation may by not contracted with other local study in future because that depend on type and number size of population, but we would to explain that the current study consider the first study focused on SNP with bacterial respiratory infection in Ramadi city, Iraq. On the other hand, understanding the role of TLR gene polymorphisms in susceptibility to respiratory infections in children is important for developing strategies to prevent and treat these infections.

Association between *TLR2* gene polymorphism *rs5743708* G>A and Leucocyte:

The analysis results of *TLR2* gene polymorphism *rs5743708* G>A indicated there is a statistic significantly differences between white blood cells count (WBC) and *rs5743708* G>A SNP because all samples which find have of these SNP were in normality with WBC, that means of these SNP maybe effectance on response the innate immune system, because TLR2 in this case which is not inform of human body when they spot a bacterial respiratory infection invading so, the WBC counts which is not increased. This triggers are a chain reaction within the cells, which activating of transcription factors like (Tumor Necrosis Factor-alpha (TNFα) and Interleukin-1 (IL-1) which rally the troops by sending out signals to produce powerful chemicals signals called "pro-inflammatory cytokines" these cytokines act like megaphones, summoning more immune cells to the fight and helping to eliminate of the infection agents (Wyres *et al*. 2020).

4. Conclusion and future scope

The study conclude that Gram-negative bacteria infection more than Gram-Positive in respiratory infection of Ramadi city children. Genotype G:A consider a risk factor under study samples with SNP rs5743708. There are not statically significantly correlation among TLR2 (rs5743708) SNP and bacterial respiratory infection with P value > 0.05.

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