# Evaluation of Gene Expression Levels of TLRs 2, 4, and 5 Genes in Patients with Chronic Suppurative Otitis Media (CSOM) and Relationship with Bacterial Infection

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**KEYWORDS**
Innate Immunity, Chronic Suppurative Otitis Media, Toll Like Receptors, Bacterial Infections

**ABSTRACT**
Toll-like receptors (TLRs), the building blocks of the innate immune response, are largely responsible for the development of otitis media. The illness may worsen due to mistakes or faults in TLR expression in immune cells found in the blood or middle ear tissue. People with various bacterial infections and chronic otitis media were evaluated for their TLR (TLR2, TLR4, and TLR5) gene expression. The results showed that higher expression levels of TLR2 were associated with Staphylococcus aureus, Streptococcus pneumoniae, and Pseudomonas aeruginosa in mucosal cells, whereas lower expression levels were associated with Moraxella catarrhalis and Haemophilus influenzae. The study found that TLR4 gene expression was higher in the mucosal cells of patients infected with Moraxella catarrhalis, Pseudomonas aeruginosa, Haemophilus influenzae and Streptococcus pneumoniae, compared to healthy controls, while Staphylococcus aureus was associated with lower expression. The study found that patients with infections caused by Haemophilus influenzae, Moraxella catarrhalis, and Staphylococcus aureus had no significant expression levels of the TLR5 gene in their mucosa cells, while patients with infections caused by Pseudomonas aeruginosa and Streptococcus pneumoniae had higher expression levels. TLRs2 mRNA expression levels increased in patients with chronic otitis media infected with Gram-positive bacteria (Staphylococcus aureus and Streptococcus pneumoniae), while TLR-4 and TLR-5 levels increased in patients infected with Gram-negative bacteria (Pseudomonas aeruginosa and Streptococcus pneumoniae).

## 1. Introduction

After an initial episode of acute otitis media, chronic suppurative otitis media (CSOM) develops. It is defined by a continuous discharge from the middle ear through a tympanic hole that lasts for at least two weeks. Tympanic membrane perforation is the primary cause of avoidable hearing loss and a major contributing factor, especially in developing nations [1, 18, 19, 20]. Tubotympanic ctitis media is a safe kind of inflammation of the mucoperiosteal lining of the middle ear, affecting part or all of it, Anticatitis media is a dangerous type (attico-antral) of inflammation, Severe deafness caused by ossicular chain discontinuity and round window mobility impairment of the tympanic membrane function are the typical causes of hearing loss, Conversely, sensorineural deafness encompasses all forms of deafness resulting from damage of the cochlea's "Organ of Corti" function or its central connections [14]. 39–200 million (or 60%) of the 65–330 million cases of CSOM that occur worldwide result in clinically severe hearing loss [2]. There are thought to be nine cases for every 100,000 persons [3]. Toll Like Receptor (TLR) function in innate immune activation [24, 25, 26]. The primary initiators of the innate immune system's initial line of defense against bacterial infection are toll-like receptors (TLRs), family pattern recognition receptors that recognize and respond to pathogen ligands [4, 21]. The non-specific response of immunology, which includes some pathogen recognition receptors (PRR), such as toll-like receptors (TLRs), is the first line of defense against invasive infections in humans [16, 17]. Animals' endoplasmic reticulum (ER) is where TLRs are produced. TLRs are then transported to the endosomal membranes or plasma, which are the cell's final destinations, Gram-positive bacteria's cell walls contain lipoproteins, peptidoglycan (PGN), and lipoteichoic acids (LTA). TLR2 regulates the host's reaction to this substance [5, 23]. Certain cells carry TLR2 on their surface, which aids in the recognition of foreign objects and in sending the right signals to the immune system's cells [22, 27]. Researchers have assessed the significance of TOOL like receptor 4 and other downstream signaling molecules for the lipopolysaccharide (LPS) driven production of immunity genes (mostly cytokines, including chemokines) and defense against various

infections using genetically manipulable organisms [6]. One of the primary inducers of sepsis, lipopolysaccharide (LPS, endotoxin), is a strong TLR4 agonist and an important component of the cell wall of Gram-negative bacteria. lipopolysaccharide (LPS) bounded to TOOL like receptor 4 activates the transcription factor necrosis factor beta, and signal transduction cascades can occur with or without the adaptor protein MyD88's assistance [7]. Toll-like receptor 4 (TLR-4) is a crucial element of storms. Co-infections and secondary infections can exacerbate the immune response, leading to an increase in cytokine production and potentially dangerous disease outcomes [15]. TLR5 can only be activated by bacteria that possess flagella since it is the only molecule capable of recognizing bacterial flagellin. Conversely, TLR2, TLR4, and TLR6 can all cause inflammatory reactions in response to different microorganisms. TLR5 and TLR9 are special because they can identify just one kind of microbial structure [8]. This study looked at the mRNA levels of Toll-like receptors (TLR2, TLR4, and TLR5) as well as gene expression in patients with chronic otitis media with effusion depending on the kind of bacterial infection in order to compare the results with those of healthy individuals.

## 2. Materials and methods

### *2.1 subjects' selection and sample collection*

110 clinical specimens (ear discharges) were collected under sterilized conditions by otorhinolaryngologists from outpatients and inpatients who visited the medical city, Ghazi Alhariri hospital's Ear, Nose, and Throat (ENT) consulting center for specialized surgeries in Baghdad during the period from August 2022 to April 2023. The specimens that were taken from people ranging in age from 15 to 55 were different. Using sterile cotton swabs, samples of ear discharge were acquired by rubbing the pus released from the ears following the auditory canal's cleaning with 70% ethanol for both the patient and the control.

### *2.2 ear swabs sample analysis*

The ear samples from both the patients and the healthy controls were placed in Eppendorf tubes with 300 µl of TRIzol (to preserve the RNA), well mixed, and then the swabs were discarded. This Eppendorf tube was used for gene expression and was kept in a freezer until the RNA was extracted by (New England Biolabs, USA, Monarch Total RNA Miniprep Kit). After extraction, measurement of the concentration of RNA by (Qubit 4.0, Invitrogen-ThermoFisher Scientific, USA), PCR was conducted using the cDNA as a template. Steps of the thermal cycler settings for cDNA reverse transcription utilizing (GoScript™ Reverse Transcription System, Promega, USA): First, 42°C in one hour, second, 70°C in fifteen minutes, and fourth, 4°C. These primers were supplied by Alpha Company in lyophilized form. Identification of TLR-2, 4, 5 expression was performed (Luna $^{R}$ Universal qpcr Master Mix, New England Biolabs, USA) using specific primers as shown in Table 1.

| **Gene** | **Specific Primers** | **Product size** (bp) | **references** |
|---|---|---|---|
| ***TLR-2*** | F 5`- TCCTGTGCCACCGTTTCCAT 3`<br>R 5`- AGGCATCCCGCTCACTGTAA3` | 138 | This study |
| ***TLR-4*** | F 5` TCTGACTTCCTGACGGGCAT 3`<br>R 5` TAAGGGCAGCCAGAGTGTGT) 3` | 119 | |
| ***TLR-5*** | F 5` AACACCACTGAGAGGCTCCT 3`<br>R 5` TTGGGCAGGTTTCTGAAGGC 3` | 152 | |
| ***GAPDH*** | F 5` ACGTGTCAGTGGTGGACCTGA 3`<br>R 5` AGGAGTGGGTGTCGCTGTTGA 3` | 162 | |

Table 1: Primer Sequence for mRNA Expression for *TLRs* genes (designed by NCBI).

In this technique, cDNA samples are taken from the patient and the control in the same run. Four PCR tubes—one for the target gene, TLR2, another for TLR4, a third for TLR5, and a fourth for GAPDH, which served as the study's housekeeping gene—were utilized for each sample. Quantity determination by the fluorescence power of the syber green. The components indicated in Table 2 are included in the reaction mixture in the proper proportions:

| Material | Volume (µL) |
|---|---|
| **M.M** | 10 |
| **Forward** | 0.5 |
| **Reverse** | 0.5 |
| **cDNA** | 4 |
| **D.W** | 5 |
| **Total** | 20 |

Table 2: Real Time Quantitative PCR (qPCR) material reaction.

The PCR tubes are used to collect the liquid and eliminate bubbles (one minute at 2000 Xg). Next, the proposed thermocycling method was used to create the Real-Time PCR application, as shown in Table 3.

| Cycle Step | Temperature | Time | Cycles |
|---|---|---|---|
| **Initial Denaturation** | 95°C | 15 seconds | 1 |
| **Denaturation Extension** | 95°C<br>60°C | 15 seconds<br>30 seconds<br>(+plate read) | 50 |
| **Melt Curve** | 60-95°C | 30 min | 1 |

Table 3: PCR protocol for TLR2, 4, 5 and GAPDH genes.

## 2. Results and discussion

In patients with chronic otitis media who had different bacterial infections (*P. aeruginosa*, *M. catarrhalis*, *S. pneumoniae*, *S. aureus*, and *H. inflenzae*), the gene expression of TLRs (TLR2, TLR4, and TLR5) was evaluated using the delta-delta ct technique. The results were compared with those of a healthy control group. Adult infected people's middle ear mucosa cells expressed TLR mRNA at various levels. Table 4 summarizes the fold changes of TOOL like receptors in the gene expression data.

| Types of bacteria | Fold Change ( gene expression) | | |
|---|---|---|---|
| | **TLR-2** | **TLR-4** | **TLR-5** |
| **Control** | 1.00 ±0.00 c | 1.00 ±0.00 d | 1.00 ±0.00 d |
| ***Pseudomonas aeruginosa*** | 3.3 ±0.18 b | 6.1 ±0.41 a | 7.1 ±0.48 a |
| ***Moraxella catarhalis*** | 1.6 ±0.16 c | 4.8 ±0.33 b | 1.2 ±0.07 d |
| ***Streptococcus pneumoniae*** | 5.5 ±0.41 a | 3.7 ±0.28 c | 4.5 ±0.31 b |

| | | | |
|---|---|---|---|
| ***Staphylococcus aureus*** | 6.6 ±0.47 a | 1.8 ±0.15 d | 1.3 ±0.08 cd |
| ***Haemophilus influenzae*** | 1.5 ±0.09 c | 5.2 ±0.37 ab | 2.4 ±0.21 c |
| **L.S.D. (P-value)** | 1.067 ** (0.0001) | 0.983 ** (0.0001) | 1.1662 ** (0.0001) |
| Means having with different letters in the same column differ significantly. ** (P≤0.01). | | | |

Table 4. Fold change of TOOL like receptors 2, 4, and 5 gene expression in different types of bacteria.

The TLR2 gene expression data revealed that the mucosa cells from S. aureus infected patients expressed this receptor at greater levels than the healthy control (1.1 fold), followed by *S. pneumoniae* (5.5 fold) and *P. aeruginosa* (3.3). Lower expression levels of *M. catarrhalis* (1.6) and *H. influenzae* (1.5) were found (Figure 1).

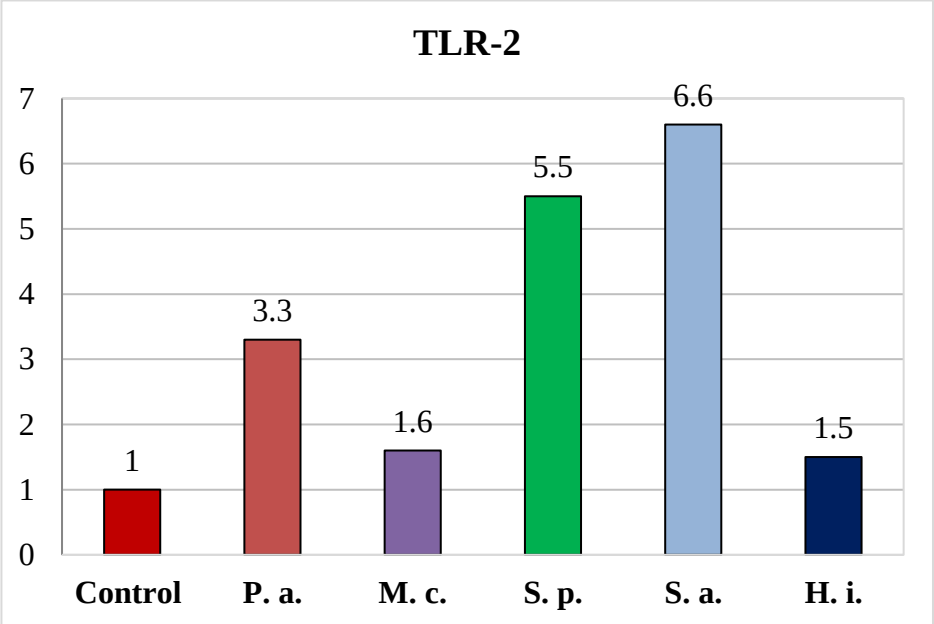



Figure 1: These patients' effusions with chronic otitis media infection were compared to the control group's effusions (*S. aureus, S. pneumoniae, P. aeruginosa, M. catarrhalis,* and *H. influenzae*.) to determine the TLR2 mRNA expression levels. The findings display the average of the five patients. When comparing levels of TOOL like receptors2 in *NTHi, S. pneumoniae*, and *M. catarrhalis*, the quantitative PCR data appeared that the mRNA expression of TOOL like receptors2 in the bacteria-positive group was higher than 100 times higher than that in the bacteria-negative group. Furthermore, compared to the rat nasopharynx or oral cavity, the expression of TOOL like receptors2 was found to be greater in the middle ear mucosa in animal tests [9]. The findings of TLR4 gene expression indicated that this receptor was elevated in mucosa cells for *P. aeruginosa* (6.1 fold) infected patients, followed by *H. influenzae* (5.2 fold), *M. catarrhalis* (4.8), and *S. pneumoniae* (3.7)

in contrast with the healthy control (1.1 fold). The lowest expression level was linked to S. aureus (1.8) (Fig. 2).

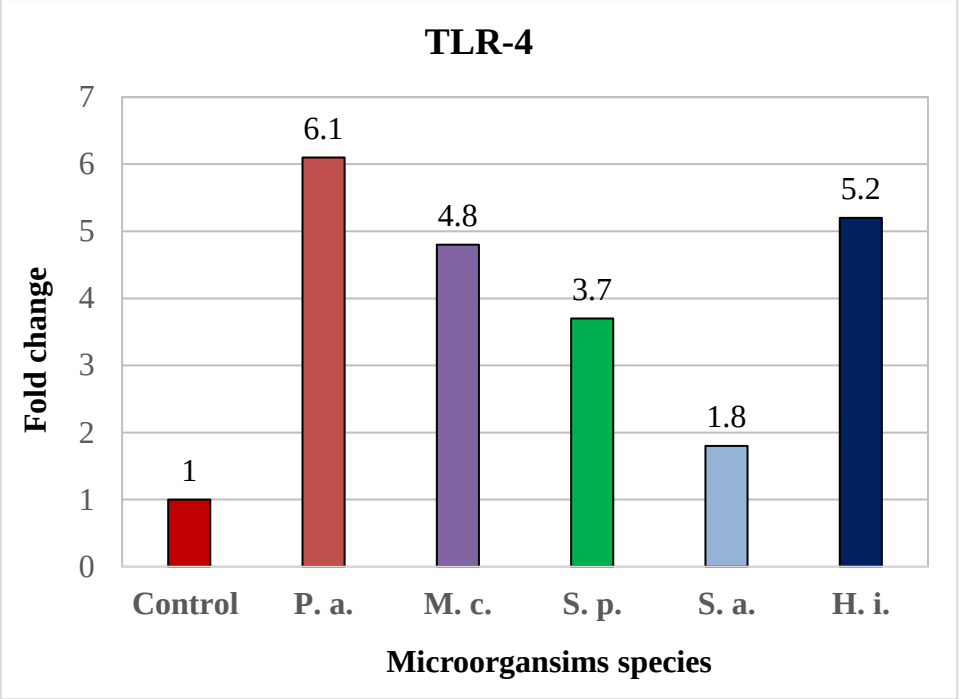
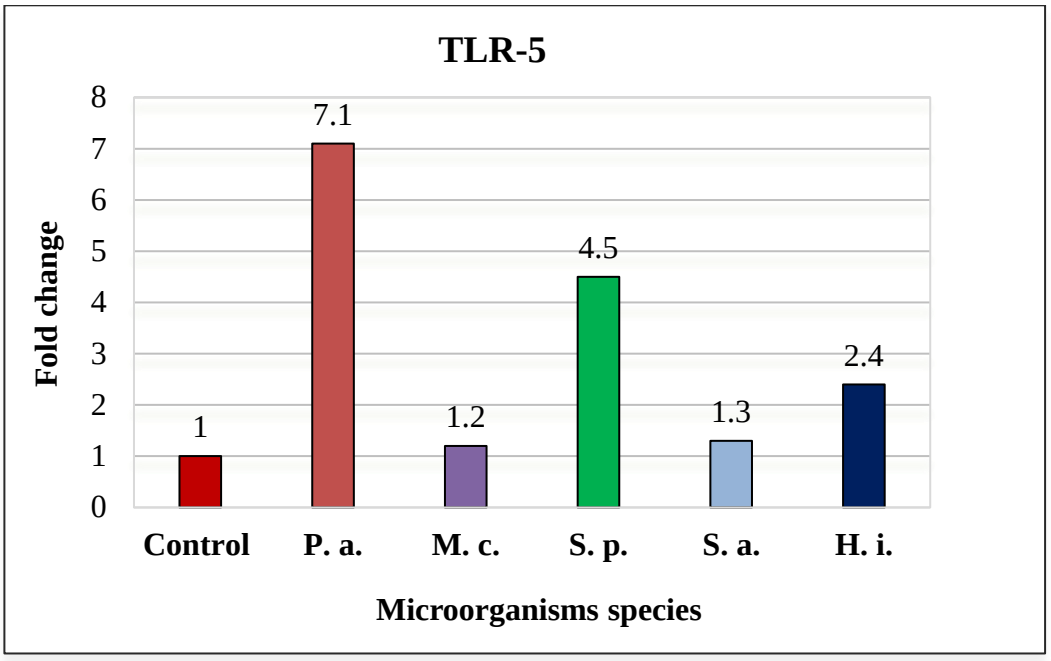



Fig. 2: Chronic otitis media patient's infections were compared to the control group (*M. catarrhalis*, *P. aeruginosa*, *S. pneumoniae*, *S. aureus*, and *H.i.*) regarding the expression levels of TLR4 mRNA in their effusion. The results included a calculation of the five patients' average. With qRT-PCR, the expression of the TLR4 gene was measured. Regarding TLR4 gene expression, the healthy controls had ΔCt values of 2.29 (-1.63-4.85) whereas the sick had ΔCt values of 4.85 (2.61-8.55). [10] found that the TLR4 gene expression in the leukocytes of the CSOM group was significantly (P = 0.01) lower than that of the control group, dropping by almost 5.9 times. The TLR5 gene expression data revealed that the mucosa cells from *P. aeruginosa*-infected patients had higher TLR5 receptor expression (6.6 fold) compared to *S. pneumoniae*-infected healthy controls (4.5 fold) (1.1 fold). A moderate level of expression (2.4) was also seen for *H. influenzae* infection, but no appreciable elevation was observed with *S. aureus* (1.3) or *M. catarrhalis* (1.2) infections (Fig. 3).




The expression of mRNA levels TOOL like receptor 5 in the effusion from patients suffering from chronic otitis media were compared with those of the control group (*P. aeruginosa*, *S. pneumoniae, M. catarrhalis, S. aureus*, and *H.i.*) in Figure 3. The average of the five patients is represented in the results. The results of this investigation that chronic otitis media patients who were infected with Gram-positive bacteria (S. pneumoniae, S. aureus) had higher levels of TLRs2 mRNA expression, while patients with Gram-negative bacteria showed higher levels of TLR-4 expression and TLR-5 expression, which were correlated with two different bacterial pathogens (P. aeruginosa and S. pneumoniae).lipoteichoic acids, lipoproteins, glycosylphosphatidylinositol anchors, lipoarabinomannan, glycolipids and phenol-soluble modulin, are some of the pathogen structures that TLR2 is involved in recognizing. Furthermore, TLR2 plays a role in identifying particular structural variations of LPS that are present in Leptospira interrogans and Porphyromonas gingivalis. To recognize these variants, TLR2 must collaborate with either TLR6 or TLR1 and the resulting TLR6 with TLR2 or TLR2 with TLR1 heterodimers signal through the (PI3K) phosphoinositide 3-kinase pathway [11]. Acute otitis media, otitis media with effusion, chronic otitis media without cholesteatoma and chronic otitis media with cholesteatoma tissues, all expressed TLR, according to a study on expression of TLRs in otitis media disease in human. Additionally, the group with otitis media and inflammatory mucosa expressed more TLR2 than the group with normal control [12]. Investigators looked at TLR2 expression on granulocytes, monocytes, and lymphocytes in the blood of young individuals with recurrent acute otitis media or chronic otitis media, the findings showed that monocytes had strong TLR2 expression whereas lymphocytes had low TLR2 expression. According to [13], all of these data investigate that elevated expression of TLR2 in monocytes peripheral blood plays a part in the acute otitis media pathogenicity. hyaluronic acid, heat shock protein 60 (HSP60) and fibronectin are a few examples of endogenous ligands that are frequently generated in response to stressful situations. TLR4 allows gram-negative bacteria to detect LPS as well. Lipopolysaccharide (LPS) initiates TLR4 signaling by forming protein complexes mediated by the outside leucine-rich repeat domain (LRR) and the inner toll/interleukin-1 receptor (TIR) domain. Several proteins interact with TOOL like Receptor 4 to form complexes at the cell surface in response to LPS activation [14]. Acute otitis media, OME, chronic otitis media without cholesteatoma and chronic otitis media with cholesteatoma express TLR4. Its appearance is connected to otitis externa that is mediated, Peripheral blood was obtained from children with chronic or recurrent acute otitis media, and the expression of TOOL like Receptor 4 on their monocytes, granulocytes, and lymphocytes was assessed. The results demonstrated that whereas lymphocytes do

not express TLR4, monocytes do. According to [9], elevated peripheral blood monocyte TLR4 levels influence the pathogenesis of AOM.

## 3. Conclusion and future scope

The results of this investigation show the importance of TLRs in the pathophysiology and immune response to chronic otitis media, with the immune response depending on the kind of bacterial pathogen present because of differences in these TLRs' gene expression.

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## Author contributions

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