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Molecular Detection of *Streptococcus pneumoniae* in Patients with Pneumonia in Diwaniyah City

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ABSTRACT

Objective: *Streptococcus pneumoniae is one of the major causative agents of pneumonia in the world. Toll-like receptor 4 (TLR-4) is essential to the innate immune response because it recognizes molecular patterns linked to pathogens. The study's goal was to identify the diagnostic and prognostic TLR-4 level in pneumonia patients and categorize them according to whether or not S. pneumoniae was present infection through the use of molecular detection (PCR).* ***Materials and Methods:*** *The total number of sample subjects was 100 and they were separated into three groups: patients with S. pneumoniae pneumonia (G1), non-S. pneumoniae pneumonia (G2), and a healthy control group. TLR-4 levels were measured, and PCR-based detection was performed on Genomic DNA extracted from blood samples. The statistical tests included multiple range comparisons, Duncan analysis, and independent T-tests.* ***Results:*** *The mean levels of TLR-4 were significantly higher in both pneumonia groups (G1: 483.19 ± 45.7; G2: 561.05 ± 52.7) compared to the healthy control group (265.17 ± 12.5) ($P < 0.05$). No significant difference was observed between the two pneumonia groups. ROC analysis revealed that TLR-4 has excellent diagnostic accuracy. For pneumonia with S. pneumoniae, the optimal cutoff value was 336.04 (AUC = 0.880), sensitivity = 87.2%, and specificity = 87.5%. For non-S. pneumoniae pneumonia, AUC = 0.912 at cutoff 378.48. TLR-4 levels did not significantly differ between males (518.90 ± 41.8) and females (504.81 ± 33.9) ($P = 0.794$).* ***Novelty:*** *TLR-4 is a strong biomarker in the clinical diagnosis of pneumonia, effectively distinguishing patients from healthy individuals, but not differentiating pneumococcal and non-pneumococcal etiologies due to its high sensitivity and specificity.*

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a facultative, Gram-positive anaerobe that is frequently found asymptomatically in the nasopharynx of 20–50% of people. Pneumococcus infections can range in severity from meningitis and invasive pneumonia to otitis media and mild sinusitis was initially discovered by Louis Pasteur in 1881 from the saliva of a rabies patient, and Friedlander and Talamon first documented the bacteria in 1883. Despite the fact that vaccination campaigns began as early as 1911, the United States did not produce the first conjugate vaccine until 2000 and the first pneumococcal vaccine until 1977 [1].

Focal lesions in the respiratory parts of the lungs with interalveolar exudation are the hallmark of pneumonia, an acute infectious disease. Pneumonia and other lower respiratory tract infections are the fourth most prevalent cause of mortality worldwide, according to the World Health Organization (WHO) [2]. Individual traits like age and long-term illnesses are linked to pneumonia. Smoking, obesity, alcoholism, and air pollution are the main risk factors for severe pneumonia [3].

One of the most effective techniques for analyzing gene expression and quantifying genes is real-time polymerase chain reaction (PCR) [4]. The application value is comparatively high, the sensitivity is high, the operation speed is quick, and the quantification is fairly accurate [5]. With a mean turnaround time of 24 hours as opposed to 64–72 hours, the PCR-based approach was substantially faster than the culture method [6]. Real-time PCR has the potential benefit of not requiring the microorganism to be viable in the sample in order for the test to be positiveReal-time PCR is more sensitive and specific than culture-based methods, according to a study on bacterial infections in chronic obstructive pulmonary disease.for identifying common bacterial species like Streptococcus pneumoniae, Moraxella catarrhalis, and Haemophilus influenzae [7].

The three pneumococcal genes that are most frequently used are lytA, ply, and psa. A that codes for surface adhesion, autolysin, and pneumolysin Among all the pneumococcal genes utilized in PCR, A [8], [9]. According to evaluations, the specificity levels for lytA, psaA, and ply are 100, 98, and 81%, respectively [10]. Other genes that are present in β-lactam-resistant Streptococcus, such as pbp1a and pbp2x, as well as the cpbA and wzy genes that are responsible for capsular genes, can also be named [8], [11], [12]. The cps and the box Housekeeping genes are A genes.

The identification of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) from damaged or dying host cells is the primary function of nonspecific immunity [13]. Toll-like receptors (TLRs), one type of PRR, are essential for controlling innate immunity. They are thought to be the first factors that influence immune system activation [14].

When PAMPs and DAMPs bind to PRRs, innate immune signaling pathways are triggered, inflammatory cytokines and chemokines are produced, and effector cells are drawn in. The PRRs and their primary downstream signaling pathways will be discussed in this section [15].

When infectious pathogens enter the respiratory system, immune cells and epithelial cells release chemokines and cytokines that drive neutrophils to move into the lung through the pulmonary capillary walls [16]. Reactive oxygen species, antimicrobial proteins, and serine proteases aid neutrophils in phagocytosing and eliminating infectious agents. [17].

RESEARCH METHOD

Sample collection

Blood samples from pneumonia patients were taken at the Al-Diwaniyah Teaching Hospital in the city of Al-Diwaniyah. Al-Qadisiyah University's College of Science approved the study, and patients gave their informed consent. Ceptemper 2025 to December 2025 was the collection period.

One hundred pneumonia patients from various age groups make up the sample. Two milliliters of the five milliliters of aseptic venous blood were placed in an ethylenediamine tetraacetic acid (EDTA) tube and kept at -20 oC until they were needed for DNA extraction. After centrifuging the remaining three milliliters in a gel tube for five

minutes at 3000 rpm, the serum was extracted and stored at -20°C until it was needed for a serological test. were put in a gel tube and centrifuged for five minutes at 3000 rpm. The serum was then extracted and kept at -20°C until it was needed for a serological test.

Real Time PCR

Real-Time PCR was used for direct detection of Streptococcus pneumoniae according to Mosadegh.

Bacterial DNA Extraction

The PrestoTM Mini gDNA Bacteria Kit was used to extract genomic DNA from sputum samples:

Sample preparation

1 ml cultured cells were centrifuged at 10000 rpm for 1 min, and the supernatant was discarded.

Cell lysis

180 µl GT buffer was added, vortexed, and incubated at 60°C for 10 min (with 100 µl lysozyme for Gram-positive bacteria).

Then, 200 µl GB buffer was added and incubated again at 60°C for 10 min, with mixing every 3 min.

DNA binding

200 µl ethanol was added, and the mixture was transferred to a GD column and centrifuged.

Washing

Washed with 400 µl W1 buffer and 600 µl Wash buffer, followed by centrifugation and drying at 12000 rpm for 2 min.

Elution:

DNA was eluted using 100 µl pre-heated elution buffer after 3 min incubation, then centrifuged at 10000 rpm.

Estimation of extracted total DNA

A NanoDrop (Thermo Scientific NanoDrop Lite UV-Vis Spectrophotometer, USA) was used to measure the isolated DNA. to determine DNA concentration (ng/µL) and purity at (260/280 nm) as following steps:

1. The nucleic acid (DNA) application was selected in the NanoDrop software.
2. The measurement pedestals were cleaned, and the system was blanked using 2 µl nuclease-free water.
3. Then, 1 µl of the DNA sample was loaded and measured.

Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using **(GoTaq® Probe qPCR Master Mix) and** the qPCR master mix was prepared as following table (1):

Table 1. qPCR standard master mix protocol

qPCR master mix	Volume
DNA template (10ng)	5µL
Forward primer(10pmol)	1 µL

qPCR master mix	Volume
Reverse primer (10pmol)	1 µL
Probe	2 µL
qPCR Master Mix	10 µL
Nuclease free water	1µL
Total	20 µL

After that, these qPCR master mix components that mentioned above placed in qPCR white plate strip tubes and mixed by Exispin vortex and centrifuge for 5 minutes, then placed in CFX96 Real-Time PCR system.

qPCR Thermocycler conditions

qPCR Thermocycler conditions was done according to qPCR kit instruction as following table (2):

Table 2. qPCR Thermocycler conditions

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	10min	1
Denaturation	95 °C	20 sec	40
Annealing\Extension Detection(scan)	60 °C	30 sec	

RESULT AND DISCUSSION

The current study included 40 healthy control subjects and 60 pneumonia patients. The data was collected, compiled, analyzed, and presented using Microsoft Office Excel 2010 and the statistical program for social sciences (SPSS) version 26. After conducting the **Kolmogorov-Smirnov normality test** and determining which variables were normally and non-normally distributed, the mean and standard deviation of the numerical values were shown in figure (1).

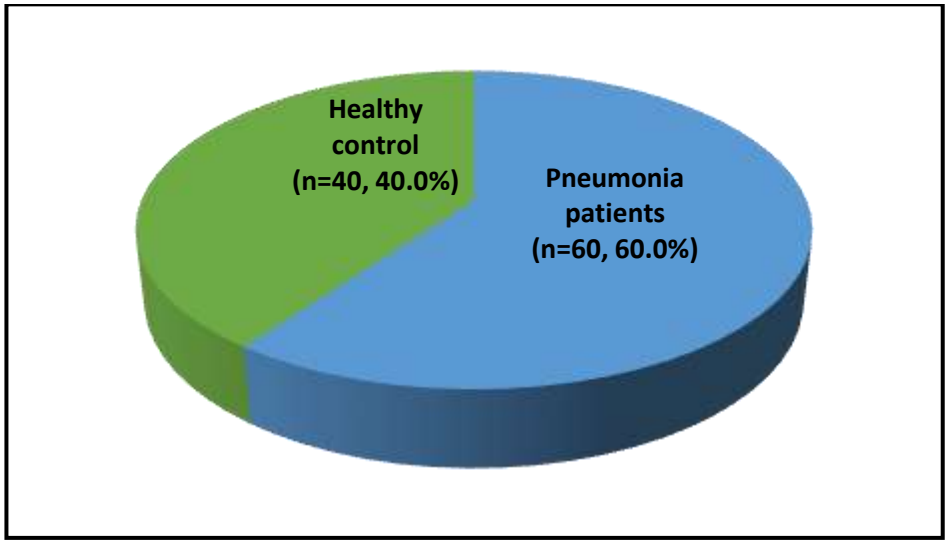

Figure 1. Subject groups

Demographic characteristics of patients with pneumonia and healthy control subjects

The mean age of pneumonia patients was 47.95 ± 11.27 years old, while the mean age of healthy control subjects was 46.15 ± 9.69 years old. The mean age difference between patients and control subjects was not statistically significant (P=0.565). Table (3) also displayed the frequency distribution of patients and control subjects by age group. Once more, the frequency distribution of patients and control subjects by age group did not differ significantly (P=0.214). However, as indicated in table (3), the majority of pneumonia patients enrolled in this study were over 60 years of age, with 18 (30.0%) of them falling into this age group. In terms of gender, the patients' group consisted of 24 (40.0%) males and 36 (60.0%) females, whereas the control group consisted of 20 (50.0%) males and 20 (50.0%) females. There was no discernible difference in the frequency distribution of sick and control subjects by gender (P=0.324). A prerequisite for preventing bias in such case control studies is the current finding, which is the absence of a significant difference in the distribution of individuals in both groups according to age and gender.

Table 3. Demographic characteristics of patients with pneumonia and healthy control subjects

Characteristic	Pneumonia patients *n* = 60	Healthy control *n* = 40	*P*
Age (years)			
Mean ±SD	47.95 ± 11.27	46.15 ± 9.69	0.565 †
Range	18–83 years	20– 67 years	NS
< 30, years, *n* (%)	9 (15.0%)	5 (12.5%)	
30-39 years, *n* (%)	14 (23.3%)	5 (12.5%)	
40-49 years, *n* (%)	6 (10.0%)	10 (25.0%)	0.214 ¥ NS
50-59 years, *n* (%)	13 (21.7%)	11 (27.5%)	
≥ 60 years, *n* (%)	18 (30.0%)	9 (22.5%)	
Gender			
Male, *n* (%)	24 (40.0%)	20 (50.0%)	0.324 ¥
Female, *n* (%)	36 (60.0%)	20 (50.0%)	NS

SD: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at P > 0.05.

Molecular detection of *Streptococcus pneumoniae in sputum samples among* patients with pneumonia

Molecular biology-based technology has become widely used to get around the issue of culture diagnosis and take advantage of the technique's sensitivity, specificity, and ease of use. Initially, PCR will be the gold standard for measuring other diagnostic techniques, microscopy, culture, antibody detection, etc.

In the current study, 39 sputum samples (65.0%) of pneumonia patients had positive results for S. pneumoniae, as shown in figure (2), following the extraction of DNA from sixty samples of pneumonia patients in order to detect and quantify Streptococcus pneumoniae using a Real Time PCR technique using specifictarget sequences primers for the (lyt A gene).

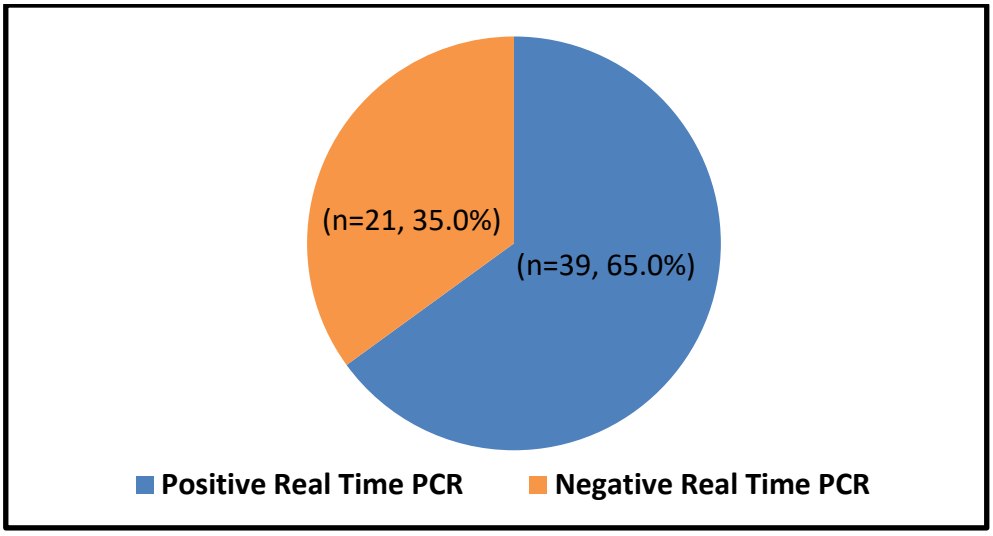

Figure 2. The frequency percentage of positive and negative results of Real Time PCR

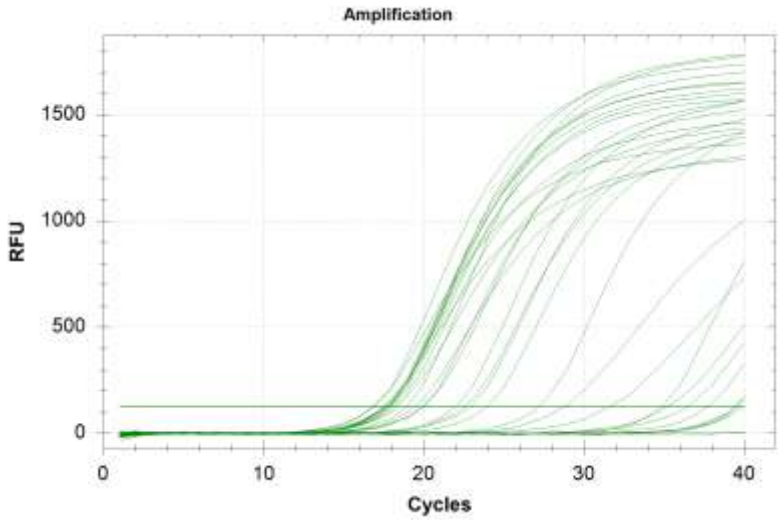

Figure 3. Real Time PCR amplification plots of *lyt A gene* for detection *Streptococcus pneumoniae* samples

Subjects' immunological parameters Analysis Results

Toll-like receptor-4 (TLR-4) level in study groups

Toll-like receptor-4 (TLR-4) levels in patients and control groups were compared, and the findings are shown in table (3-3). In pneumonia with S. pneumoniae infection, pneumonia without S. pneumoniae infection, and the healthy control group, the mean levels of TLR-4 were 483.19 ± 45.7, 561.05 ± 52.7, and 265.17 ± 12.5, respectively; both patient groups had higher mean levels than the healthy control group, and the difference was significant ($P < 0.05$). However, there was no statistically significant difference in the mean levels between the two patient groups (pneumonia with S. pneumoniae infection and pneumonia without S. pneumoniae infection) ($P < 0.05$).

Table 4. Toll-like receptor-4 (TLR-4) level in study groups

Groups		Toll-like receptor-4 (TLR-4) level
Pneumonia with *S. pneumoniae*	**Mean ± SE**	**483.19 ± 45.7^{A}**
	Range	148.03-769.75
Pneumonia without *S. pneumoniae*	**Mean ± SE**	**561.05 ±** 52.7^{A}
	Range	177.57-1061.37
Healthy control	**Mean ± SE**	**265.17 ±** 12.5^{B}
	Range	129.97-422.87
p-value		**0.001**** †

Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different.

SD: standard deviation; †: one way anova test; **: significant at $P < 0.05$

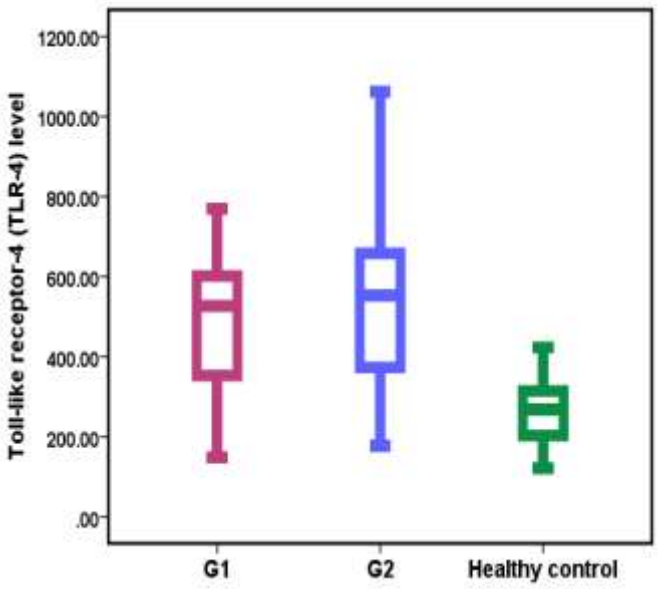

Figure 4. The means level of TLR-4 in patients and control groups; G1: pneumonia with *S. pneumoniae* infection; G2: pneumonia without *S. pneumoniae* infection

Diagnostic accuracy of TLR-4 level

To determine the predictive accuracy of utilizing TLR-4 concentrations to differentiate pneumonia with S. pneumoniae infection from healthy control participants, Receiver operating characteristic (ROC) analysis was carried out. An optimal TLR-4 cut-off value more than of 336.04 resulted in an AUC value of 0.880 (95% confidence interval [CI], 0.769-0.991, P=0.001), sensitivity of 87.2%, specificity of 87.5%, PPV of 87.2%, and NPV of 87.5%. The present results indicate TLR-4 is considered as a good prognostic marker to distinguish pneumonia with *S. pneumoniae* infection from healthy control.

Furthermore, pneumonia without S. pneumoniae infection could be distinguished from healthy control subjects using an ideal TLR-4 cut-off value of greater than 378.48, with a sensitivity of 90.5%, specificity of 90.0%, PPV of 82.6%, NPV of 94.7%, and 0.912 (0.804-0.998). According to the current findings, TLR-2 is thought to be a good prognostic marker for differentiating between healthy controls and pneumonia without S. pneumoniae infection.

Table 5. Roc curve of TLR-4 level

Characteristic	pneumonia with *S. pneumoniae* / control	pneumonia without *S. pneumoniae* /control
Cutoff value	> 336.04	> 378.48
P value	0.001	0.001
Sensitivity %	87.2 %	90.5 %
Specificity %	87.5 %	90.0 %
PPV %	87.2 %	82.6 %
NPV %	87.5 %	94.7 %
AUC (95% CI)	0.880 (0.769- 0.991)	0.912 (0.804- 0.998)

CI: Confidence interval, AUC: Area under curve.

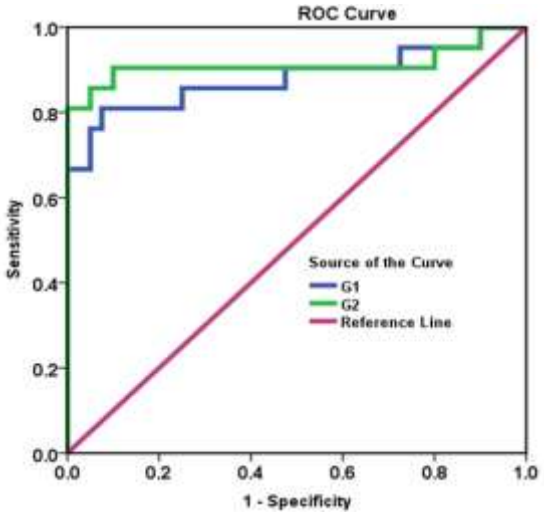

Figure 5. (Blue curve) Receiver operating characteristic curve for TLR-4 levels to distinguish pneumonia with *S. pneumoniae* infection from healthy control subjects. (Green curve) Receiver operating characteristic curve for TLR-4 levels to distinguish pneumonia without *S. pneumoniae* infection from healthy control subjects.

The current study found that the age group (**≥ 60**) years old has the highest prevalence of ***Streptococcus pneumoniae***, accounting for 30.0% of the population, while age group (**40-49)** had the lowest frequency of ***Streptococcus pneumoniae*** was (10.0%), there was no statistically significant difference. The present result agreement with result of a study by Ochoa-Gondar et al which reported that pneumococcal pneumonia is significantly more prevalent among elderly individuals, who represent the highest-risk group for both infection and mortality [18]. Moreover, recent studies by Luo et al. (2025) corroborated the fact that community-acquired pneumonia by pneumococcus is still one of the primary contributors to morbidity and mortality among the individuals aged ≥60 years [19]. Similarly, another recent study demonstrated that individuals aged ≥65 years accounted for more than 70% of confirmed pneumococcal pneumonia cases, further supporting the strong association between aging and increased susceptibility [20]. Regarding gender, the present study showed no statistically significant differences between males and females, although a slightly higher proportion of females was observed. This finding is supported by a molecular epidemiological study which reported no significant gender-based differences in pneumococcal pneumonia incidence ($P > 0.05$) [21]. But other researches have shown minor differences in gender distribution. As an example, a massive epidemiological study has shown that the incidence of pneumonia increases with age in both males and females, although there can be gender-specific variations based on the population features and risk factors [22]. This vulnerability in older people can be attributed to age-induced immune dysfunction (immunosenescence), resulting in the loss of innate immune responses, pathogen recognition, and the effectiveness of immune receptors including Toll-like receptor 4. This leads to decreased production of cytokines and loss of ability to clear bacterial infections, contributing to the risk and severity of pneumonia. The current research showed that there was indeed a highly significant difference in serum TLR-4 levels between patients with pneumonia and healthy controls ($P \leq 0.001$) indicating the critical role of this receptor in the activation of the innate immune response towards bacterial infection especially by Streptococcus pneumoniae. Recent research has demonstrated that one of the most important pattern recognition receptors in the detection of bacterial constituents and triggering of inflammatory response is the Toll-like receptors and in particular TLR4 [23]. In addition, the reported increase in TLR-4 levels in pneumonia patients is justified by the evidence that pneumolysin, one of the primary virulence factors of Streptococcus pneumoniae, directly stimulates TLR4 signaling pathways, which results in the activation of immune cells and the increase of production of pro-inflammatory cytokines [24]. Moreover, experimental research has shown that TLR4 plays a role in host defense against Streptococcus pneumoniae by activating intracellular signaling pathways including JNK and p38, which in turn stimulate cytokine, and regulate inflammatory responses [25]. Recent findings also suggest that TLR4 plays a critical role in increasing the pulmonary resistance to pneumococcal infection with deficiency or impaired activity of this receptor resulting in increased bacteria burden and failure to clear the infection [26]. The absence of a strong difference between pneumococcal and non-pneumococcal pneumonia observed in the current study indicate that TLR4 activation is a generalized innate immune response to bacterial infection. This is in line with the evidence that TLRs react to widespread bacterial ligands, but not to a

pathogen-specific stimulus [27]. TLR4 stimulated before or after infection enhanced survival (90% vs. 0% in controls, $p<0.001$) by decreasing bacterial burden and cytokines, in a T-cell-dependent fashion, placing elevated TLR4 in the spotlight as an indicator of responsiveness [28]. TLR4 is directly bound by pneumolysin, causing respiratory epithelia to undergo programmed cell death, and TLR4 deficiency correlates with the ability to develop invasive disease [29], [30], [31].

CONCLUSION

Fundamental Finding: Toll-like receptor 4 (TLR-4) levels were significantly elevated in pneumonia patients compared to healthy controls, supporting its role as a diagnostic biomarker. This increase is attributed to the activation of TLR-4 by bacterial components, particularly from Streptococcus pneumoniae, which triggers inflammatory pathways and cytokine release. **Implication:** TLR-4 reflects host immune activation and may be useful in pneumonia diagnosis, although it lacks complete specificity. **Limitation:** The absence of infection in healthy individuals results in baseline TLR-4 expression, indicating that TLR-4 may not fully differentiate between various pneumonia etiologies. **Future Research:** Future studies should explore improving the specificity of TLR-4 as a biomarker for distinguishing between pneumococcal and non-pneumococcal pneumonia.

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