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The correlation between NOD2 and anti-*Salmonella typhi* antibodies functions as a diagnostic marker for typhoid disease

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ABSTRACT

Typhoid fever is a life-threatening systemic infection caused by *Salmonella typhi,* which specifically infects humans. It remains a major public health concern in the developing countries. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a cytosolic receptor that detects pathogens and triggers an appropriate immune system response. This study investigated the diagnostic potential of serum NOD2 and anti-*Salmonella typhi* antibodies as biomarkers in typhoid fever. A total of 75 clinically and laboratory-confirmed typhoid fever patients and 50 healthy controls were enrolled in a case-control design. Blood samples were collected from all the participants. Serum NOD2 levels were measured, and anti-*Salmonella typhi* IgG and IgM antibodies were assessed via standard serological methods, including the Widal test. A receiver operating characteristic curve (ROC) was employed to evaluate the diagnostic potential of NOD2. The mean serum level of NOD2 in the patient group was 3.12 ± 0.39 ng/ml, which was significantly higher than that of the control group 3.6 ± 0.47. NOD2 showed a significant negative correlation with anti-*Salmonella typhi* IgM titer (r= 0.246, p= 0.022). According to the ROC curve analysis, the area under the curve was 0.827 (95% CI: 0.737–0.918; $p < 0.001$). The sensitivity and specificity of the test were 78% and 77%, respectively. The best cutoff value of soluble NOD2 was >3.3 ng/ml. A low serum level of NOD2 is associated with reduced immunological response to *Salmonella typhi* infection. The study's findings revealed that serum level of NOD2 could aid in the diagnosis of typhoid fever alongside serological tests.

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Introduction

Typhoid fever is a systemic infection caused by *Salmonella enterica* serotypes, primarily *typhi* and, less commonly, *paratyphi* A, B, and C. It is characterized by a fever of ≥38°C lasting at least three days and is confirmed through a positive bacterial culture from blood or bone marrow samples (Allam et al. 2018, Neupane et al. 2021, Hardany et al. 2025). The disease is transmitted primarily through the fecal-oral route. Once ingested, the bacteria multiply in the intestinal tract and spread into the bloodstream (Wangdi et al. 2012). Approximately 2–3% of individuals who recover from the acute phase of typhoid fever become chronic carriers, shedding *S. typhi* in their stools for years (Marchello et al. 2020). *Salmonella typhi* can invade, survive, and

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replicate within mononuclear phagocytes, eventually being released into the bloodstream (Barton et al. 2021).

Numerous obstacles hinder the conclusive identification of typhoid disease. This encompasses the necessity for adequate blood volume (often exceeding 7 mL), which fluctuates according to the patient's age owing to diminished bacteremia levels. Maintaining blood samples at ambient temperatures (15–40°C) during transport is also crucial. Although both procedures exhibit 100% specificity, the diagnostic sensitivity is quite modest, around 50% for blood cultures and 80% for bone marrow cultures. Supplementary obstacles encompass insufficient resources, including qualified workers and laboratory apparatus (Saha et al. 2023). According to the World Health Organization (WHO), an ideal diagnostic test for typhoid fever should achieve 100% sensitivity, specificity, and predictive accuracy (Neupane et al. 2021).

Salmonella strains express various distinct surface structures, including nucleotide oligomerization domain (NOD)-like receptors. These receptors are categorized as pathogen-associated molecular patterns (PAMPs) (Geddes et al. 2009). NOD-like receptors are a family of intracellular bacterial sensors, which include NOD1 and NOD2. These receptors detect specific microbe-associated molecular patterns present in bacterial peptidoglycan (Campbell et al. 2011). Among them, NOD2 plays a critical role in the immune system's response to bacterial infections. As a pattern recognition receptor, NOD2 detects bacterial components and initiates an immune response to eliminate the pathogen. Specifically, it recognizes muramyl dipeptide, a molecule found in the cell walls of many bacteria. Upon binding to muramyl dipeptide, NOD2 activates a signaling pathway that promotes the production of inflammatory cytokines and antimicrobial peptides. It also triggers the activation of immune cells, such as macrophages and dendritic cells, to strengthen the body's defense mechanisms (Salucci et al. 2008).

The Objective of the present study aimed to investigate the correlation between NOD2 levels and anti-*Salmonella typhi* antibody titers in patients with typhoid fever, with the goal of evaluating NOD2 potential as complementary diagnostic markers.

Materials and Methods

Over twelve months, from November 2023 to November 2024, 75 patients with confirmed typhoid fever were recruited in the case-control study. All patients were either outpatients or inpatients at Al-Kadhumiya Hospital in Baghdad and were diagnosed by a consultant physician based on clinical symptoms and laboratory findings. An additional 50 age- and sex-matched apparently healthy individuals were recruited as the control group. Participants with known autoimmune diseases were excluded from the study. Written consent was obtained from each participant after explaining the study's objectives. Each patient was given the complete unconditional choice to withdraw anytime. The confidentiality of data throughout the study was guaranteed, and the patients were assured that the data would be used for research purposes only.

Blood samples were collected from all participants using plain tubes and transported to the Medical Research Unit laboratory at the College of Medicine, Al-Nahrain University. Sera were separated by centrifugation and stored at -20ºC until being used. Demographic data, including age, sex, smoking status, and place of residence, were obtained through direct interview.

Widal test and anti-Salmonella IgG and IgM antibodies

The Widal test was performed using standardized *S. typhi* O and H antigens (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Serial serum dilutions were prepared starting at 1:100 using 0.9% saline solution. The tubes containing the sera and antigens were incubated at 37°C for 1 hour, followed by centrifugation. The samples were then examined for visible agglutination. Appropriate positive and negative control sera were included in the procedure. The test was carried out by laboratory technologists on rotation as part of the routine diagnostic services provided by the laboratory.

Determination of The Serum Level of NOD2

Serum concentration of NOD2 was measured using an indirect enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (Cusabio/China) following the manufacturer's protocols. The detection range of this kit is 1.0-400 ng/ml.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software (Chicago; version 25.0). Continuous data were subjected to a normality test (Shapiro Wilk test). Data with normal distribution were presented as mean $\pm$ standard deviation and analyzed with a student t-test. On the other hand, data with non-normal distribution were presented as median and range. Categorical variables were expressed as numbers and percentages and analyzed with the Chi-square test. The NOD2's diagnostic potential in terms of patient and control discrimination was assessed using a receiver operating characteristic (ROC) curve. Spearman's correlation test was performed to explore the possible correlation of serum concentration of NOD2 with antibody titers and age. A p-value less than 0.05 was considered to indicate a statistically significant difference.

The approval of Institutional Review Board of College of Science, Tikrit University was obtained before conduction of the study. A written consent from each participant was obtained prior to data collection after explaining the aim of study. The confidentiality of data throughout the study was guaranteed and the patients were assured that data will be used for research purpose only.

Results

Demographic Characteristics of The Study Population

The mean age of patients was 31.91 ± 12.97 years (range 13-73 years) compared to 35.5 ± 10.64 years (range 20-60 years) in the controls, with no significant difference between the two groups. Similarly, there were no significant differences in the sex distribution and smoking habits. Although ex/current smoking was more frequent among controls than patients (56% vs. 44%), the difference was not significant (Table 1).

Table 1 Demographic characteristics of the study population

Variable	Patient (n = 75)	Control (n = 50)	*p-value*
Age (years)			
Mean ± SD	31.91 ± 12.97	35.5 ± 10.64	0.106
Range	13-73	20-60	
Gender			
Male	34(45.33%)	22(44%)	0.883
Female	41(54.67%)	28(56%)	
Smoking			
Never	51(68%)	30(60%)	0.359
Ex/current	24(32%)	20(40%)	
Residence			
Urban	42(56%)	22(44%)	0.189
Rural	33(44%)	28(56%)	

SD: Standard deviation. *: independent t-test, **Chi square test

Clinical Characteristics of The Patients

Data regarding anti-*Salmonella typhi* antibodies were found to be non-normally distributed. Therefore, the data were expressed as median and range. The median anti-*Salmonella typhi* IgG was 0.16 mIU/ml. Based on the manufacturer's instructions; there were 58 positive cases (77.33%). On the other hand, the median anti-*Salmonella typhi* IgG was 0.12 mIU/ml. Thus, there were 47 positive cases (62.67%), as shown in Table 2. Collectively, there were 60 positive cases for IgG or IgM (80%). As all included patients were positive for the Widal test, the sensitivity of the Widal test for detecting typhoid fever was 80%.

Table 2. The levels of Anti-*Salmonella typhi* IgG and IgM Among Patients

Variable	Value
Anti-*Salmonella typhi* IgG, mIU/ml	
Median	0.16
Range	0.08-0.73
Anti-*Salmonella typhi* IgG seropositivity	
Positive	58(77.33%)
Negative	17(22.67%)
Anti-*Salmonella typhi* IgM	
Median	0.12
Range	0.08-1.11
Anti-*Salmonella typhi* IgM Seropositivity	
Positive	47(62.67%)
Negative	28(37.33%)
Anti-*Salmonella typhi* IgG or IgM seropositivity	
Positive	60(80%)
Negative	15(20%)

Serum level of NOD2 protein

The mean serum level of NOD2 protein in patients was 3.12 ± 0.39 ng/ml (range= 1.45-4.47 ng/ml), which was higher than that of the controls (mean= 3.6 ± 0.47, range= 2.13-4.08 ng/ml), with a significant difference (Fig. 1).

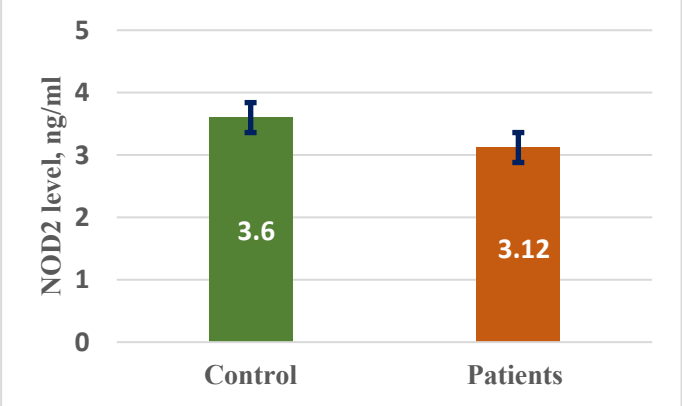

Fig 1. Mean serum level of NOD2 in patients and controls.

Diagnostic potential of NOD2 in the detection of typhoid fever

The receiver operating characteristic (ROC) curve was used to assess the diagnostic performance of NOD2 in distinguishing patients from controls. The area under the curve (AUC) was 0.827 (95% CI = 0.737- 0.918, p< 0.001). The sensitivity and specificity of the test at a cutoff value of soluble NOD2 = 3.3 ng/ml were 78% and 77%, respectively (Fig. 2).

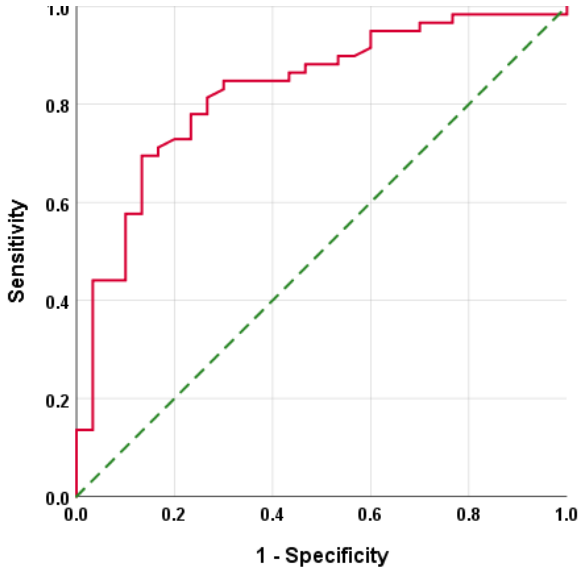

Fig 2. Receiver operating characteristic curve for NOD2 in the context of discrimination between patients with typhoid fever and healthy controls

Correlation of NOD2 with Antibody Titers and Age

Spearman's correlation test was used to explore the possible correlation of serum concentration of NOD2 with antibody titers and age. NOD2 protein demonstrated a significant negative correlation with anti-*Salmonella typhi* IgM titer ($r = 0.246$, $p = 0.022$), as illustrated in Figure 3. On the other hand, there was no significant correlation of NOD2 with anti-*Salmonella typhi* IgG or with the age of the patients (Table 3).

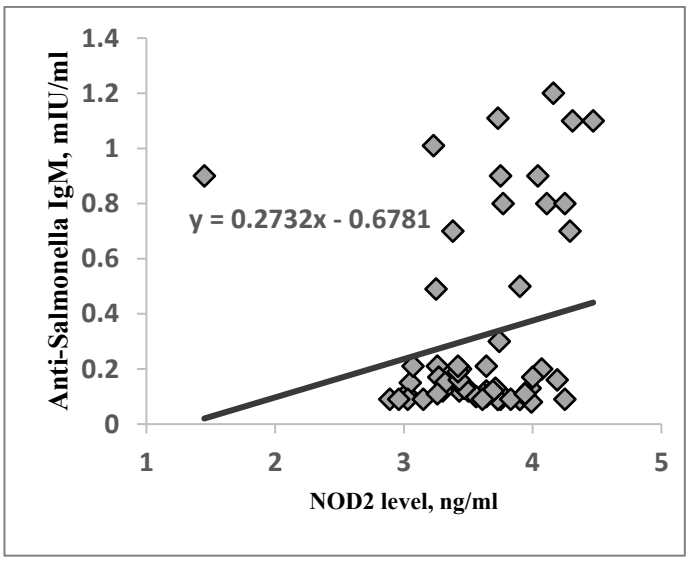

Fig 3. Scatter plot and regression line between NOD2 and anti-*Salmonella typhi* IgM concentration in patients with typhoid fever.

Table 3 Spearman's correlation of NOD2 with antibody titer and age of the patients with typhoid fever

Variable	Correlation Coefficient	*p-value*
Age (years)	-0.151	0.158
IgG (mIU/ml)	0.100	0.453
IgM (mIU/ml)	0.264	0.022

Discussion

The present study aimed to evaluate the diagnostic potential of NOD2 in patients with typhoid fever. The main findings demonstrated an elevated serum level of NOD2 in patients compared with the controls, along with a significant negative correlation between NOD2 and anti-*Salmonella* IgM antibodies. These findings are in accordance with some published studies. The first attempt to characterize the role of NOD2 in *Salmonella* infection was performed in human dendritic cells (DCs) carrying a homozygous loss-of-function NOD2 L1007fs mutation and showed a decreased production of proinflammatory cytokines compared to DCs from healthy individuals (Salucci et al. 2008). Most other studies were conducted on mouse models. Geddes et al. (2009) reported that mice deficient in both NOD1 and NOD2 exhibited attenuated inflammatory pathology, lower levels of inflammatory cytokines, and increased mucosal tissue colonization. Furthermore, mice lacking NOD2 and NOD1 have reduced overall pathology and cytokine production, accompanied by an increased bacterial burden in the mucosal tissue (Geddes et al. 2010).

Several hypotheses have been proposed to explain this effect of NOD2 on *Salmonella* infection. NOD2 has been reported to recognize muramyl dipeptide (MDP), a molecular motif found in the cell walls of both Gram-negative and Gram-positive bacteria (Girardin et al. 2003). Upon detecting its specific ligand, NOD2 associates with a common adaptor protein known as Rip2. This interaction triggers a signal transduction cascade that ultimately activates nuclear factor kappa B (NF-κB), leading to the production of proinflammatory mediators such as interleukin (IL)-6 and IL-8 (Ogura et al. 2001). Another hypothesis suggests that *Salmonella enterica* utilizes a type III secretion system (T3SS-1) to induce intestinal inflammation. The primary role of T3SS-1 is to deliver effector proteins into host cells. Five key T3SS-1 effectors- SipA, SopA, SopB, SopD, and SopE2, work together to induce changes in the host cell actin cytoskeleton, facilitating epithelial invasion and triggering intestinal inflammation (Raffatellu et al. 2005). Keestra et al. (2011) demonstrated that the proinflammatory effects of SipA are dependent on NOD1

and NOD2 activity. *In vitro* studies further revealed that SipA alone is both necessary and sufficient to activate NOD1/NOD2-dependent NF-κB. These findings suggest that cytosolic detection of SipA by the pattern recognition receptors NOD1 and NOD2 acts as a signal for cytosolic access by the invasion-associated T3SS-1 of *S. typhi murium*, thereby amplifying mucosal inflammatory responses.

However, not all studies have identified an association between NOD2 and *Salmonella* infection. Bruno et al. (2009), for instance, investigated the role of NOD1 and NOD2 in a *Salmonella* colitis model and concluded that NOD1/2 signaling does not contribute to the inflammatory response in this context. This discrepancy may be related to the growth conditions of *Salmonella* during experimental infections. When cultures are prepared under conditions favoring SPI-1 expression (log-phase growth), rapid macrophage death is observed. In contrast, under conditions favoring SPI-2 expression (stationary-phase growth), delayed cell death occurs (Wang et al. 1991). Experimental evidence supports a role for NOD1 and NOD2 in host defense. In mice infected with a bacterial mutant lacking SPI-1 or with *Salmonella* cultured to favor SPI-2 expression (Raffatellu et al. 2005), a significant bacterial burden and progressive infection were observed. These findings suggest that the contribution of NOD1 and NOD2 to the host immune response against *Salmonella* is contingent on the expression of specific *Salmonella* virulence factors.

In the human body, SPI-1 has a role in the invasion of bacteria into intestinal epithelial cells, while SPI-2 is essential for the survival and replication of bacteria within phagocytes (Neupane et al. 2021). Thus, it is reasonable to assume the relative NOD2 deficiency in patients with typhoid fever could facilitate the infection. Collectively, these findings strongly indicate the role of NOD2 in the defense mechanism against *Salmonella* infections. Further studies on the genetic level are required to illustrate the effects of a genetic alteration in the NOD2 gene on susceptibility to *Salmonella* infection. The PCR technique has been applied in biological fields (Muhammed et al. 2024, Ibrahim & Laftaah 2024, Al-Khafaji et al. 2025, Abbas et al. 2025).

Conclusion

This study links higher serum NOD2 levels to typhoid disease, proposing its use as a biomarker. NOD2 had good sensitivity and specificity in distinguishing patients from healthy controls, according to ROC curve analysis. NOD2's negative connection with anti-*Salmonella typhi* IgM titers suggests an immunoregulatory involvement during acute infection. These findings show that NOD2 protein measurement and serological testing improve typhoid disease diagnosis. Further genetic study and larger sample sizes are needed to confirm these findings and clarify NOD2's role in host defense against *Salmonella typhi*.

Authors' contributions

The authors planned the study, analysed the information, and contributed to the manuscript's drafting. All authors reviewed and permitted the final manuscript.

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Conflicts of interest

There is no conflict of interest.

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