Proinflammatory Cytokines Associated with *Inv*A and *Pag*C Genes of *Salmonella typhi* Isolated from Patients Undergoing Cholecystectomy

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ABSTRACT

Objective: Cholecystectomy is the surgical removal of the gallbladder. The gallbladder is often colonized by *Salmonella* during typhoid fever. The association between cytokine and virulence genes of *Salmonella* is not well known. The current study aims to identify the association between proinflammatory cytokines, TNF-α and IFN-γ, and two virulence genes, *Inv*A and *Pag*C, extracted from *S. typhi* isolates obtained from patients undergoing cholecystectomy.

Materials and Methods: One hundred and fifty clinical specimens, including gallbladder tissues and blood, were collected from patients undergoing cholecystectomy at AL-Sadder Medical City and AL-Furat General Hospital/ Iraq from December 2019 to September 2020. A Monoplex PCR technique was used to detect *Inv*A and *Pag*C, and an enzyme-linked immunosorbent assay was used to measure serum levels of TNF-α and IFNγ.

Results: From a total of 66 *S. typhi* isolates, the prevalence of *Inv*A and *Pag*C was (84.8% and 69.6%) respectively. Even after adjusting for potential confounding factors, serum levels of TNF-α and INF-γ were statistically significantly higher in *Pag*C detected in *S. typhi* isolates compared to *Pag*C not detected (p-value<0.001), but there was no significant difference between *Inv*A detected in *S. typhi* isolates and *Inv*A not detected (p-value > 0.05).

Conclusion: The virulence genes of *S. typhi*, especially *Pag*C, may be considered a potent inducer of proinflammatory cytokine secretion. Further studies are needed to present the patient's additional clinical course according to cytokine level.

Keywords: Typhoid; cytokine; TNF-α; IFN-γ; *Inv*A; *Pag*C (Siriraj Med J 2022; 74: 828-835)

INTRODUCTION

Cholecystectomy operations have been developed from open to laparoscopic surgery, which helps in reducing postoperative pain, returning faster to daily activities, and shortening the recovery period.[1,2] It remains the most effective treatment option for *Salmonella enterica serovar typhi (S. typhi)* carriers with gallstones.[3] *S. typhi* is a human-restricted pathogen that causes typhoid fever, a disease transmitted primarily through the fecal-oral route. Typhoid fever is considered to be one of the most important and under-reported diseases in the developing world. Based on the most recent global estimates, 9.9 and 24.2 million incident cases and 75,000–208,000 typhoid-related deaths occur annually.[4] In Asia, the incidence was more than 100 cases per 10^5 people per year, mostly among travelers; large outbreaks of *S. typhi* that are multidrug resistant have been observed in both urban and rural areas where access to sanitary facilities, food, and water are limited.[5] In Iraq, Mousa et al. estimated the incidence of typhoid fever to be 34.2 cases/10^5 individuals in 2012.[6]

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Typhoid fever is associated with a wide range of clinical symptoms from asymptomatic to severe, including fever, headache, malaise, and perforation of the ileum as complications of ileal ulceration. When some people are exposed to *S. typhi*, they become carriers, as they keep bacteria in the gallbladder that are responsible for storing and excreting bile from the liver to the small intestine.[7] Abdominal ultrasound screening showed an ulcerative inflammatory reaction with fluid accumulation and an obvious thickening of the gallbladder in more than 59% of patients with typhoid fever, which may be these features in response to a local infection.[8]

With the advent of molecular biology, including DNA fingerprinting and plasmid profiling, many vivo studies investigate gene variations of *S. typhi* that help bacterial organisms to adhere and survive in different environmental niches, as strains that lack the invasion machinery have drastically reduced capacity to colonize, spread[9] and/or drive inflammation[10] in mouse models. To our knowledge, live attenuated *S. typhi* strains delivered orally to humans or mice infected with *S. typhimurium*, which causes an illness similar to typhoid, have both been extensively used to study immune responses. These investigations showed a paradoxical phenomenon whereby the excessive synthesis of tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) by T cells reduces the protective immune response to *S. typhi* antigens.[11] The current study aims to identify the association between proinflammatory cytokines, TNF-α and IFN-γ, and two virulence genes, *Inv*A and *Pag*C, extracted from *S. typhi* isolates obtained from patients undergoing cholecystectomy.

MATERIALS AND METHODS

Following the acceptance of the Nursing Faculty's Ethical Review Board at the University of Kufa in Iraq (N: 10/05 on 02/September/2019) for our proposal, a case-control study was conducted in AL-Sadder Medical City and AL-Furat General Hospital/Iraq, from December 2019 to September 2020.

Subjects

One hundred and fifty patients undergoing either laparoscopic or open cholecystectomy were included as the study group. The patients were admitted to the hospital before 24 hours of surgery for preoperative investigations and assessment. Before surgery, no antimicrobial substances were prescribed while 3rd generation cephalosporins were given intra-operatively. Thirty individuals were included as the control group without suffering from acute fever or calcular cholecystitis. All subjects gave their written informed consent before enrollment.

Sample collection

A five mL blood sample was obtained from each participants by venous puncture and placed in a plain tube. After a blood clot, centrifugation at (3 x 10^3 rpm) for 20 minutes was used to separate the serum. All sera were stored at -20°C and had not been thawed before being analyzed. Gallbladder tissues taken from each patient after surgery were used for culture and immediately frozen at -20°C for DNA extraction.

Isolation and Identification of *S. typhi*

Sections of the gallbladder's tissue were injected into a Stewart transport medium (Hampshire, UK). In a sterile mortar, (1g) of gallbladder tissue was crushed and pestled in (1ml) saline, then it was cultured on selenite cystine medium (Hampshire, UK) and incubated for 24 hrs. at 37°C. For identification of individual colonies, they were subcultured on MacConkey agar, Salmonella Shigella agar, and Xylose Lysine Deoxycholate agar, as well as gram staining, IMViC test, triple sugar iron test, motility, lysine, and urease decarboxylase activities, were performed. The final identification was detected using an automated VITEK-2 compact system (BioMérieux –France). After these identification tests, we obtained 66 isolates of *S. typhi* out of 150 collected clinical specimens, and the remaining samples (84) were excluded from the current study.

By using the slide agglutination method, *Salmonella* antisera were performed for serotype and to determine corresponding antigens of *S. typhi* (somatic, flagellar, and virulence (Vi)) in the healthy control group according to the product's instructions.

Molecular Study

Genomic deoxyribonucleic acid (DNA) was extracted by using a DNA extraction kit (promega, USA), and a Monoplex PCR technique was performed to determine *Inv*A and *Pag*C. The forward and reverse primers used in this study for the InvA were 5'-ACA GTG CTC GTT TAC GAC CTG AAT-3' and 5'-AGA CGA CTG GTA CTG ATC GAT AAT-3', resulting in a 260-bp product.[12] *Pag*C forward and reverse primers were 5'-CGC CTT TTC CGT GGG GTA TGC-3' and 5'-GAA GCC GTT TAT TTT TGT AGA GGA GAT GTT-3', respectively, yielding a 454-bp product.[13]

For PCR reaction, a 20µL tube was used, which contained 5 µl of master mix (Taq DNA polymerase, Tris-HCL pH9, dNTPs, 30 mM KCL,1.5 mM MgCl2, and Track dye from Bioneer company-Korea), 5µl DNA template, 2.5µl form each forward and reverse primer, and 5µl deionized water. PCR programming conditions for *Inv*A were 35 cycles of initial denaturation at 94°C

for 2 min, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, elongation at 75°C for 1 min, and final elongation at 72°C for 10 min. PCR programming conditions for *Pag*C were 25 cycles of initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 66.5°C for 30 s, elongation at 72°C for 2 min, and final elongation at 72°C for 10 min.

Proinflammatory cytokines

TNF-α was measured in serum samples using an enzyme-linked immunosorbent assay Kit (ab46087, UAS). IFNγ was measured in serum samples using an enzyme-linked immunosorbent assay Kit (ab174443, UAS). According to each standard curve and the dilution factor, the TNF-α and IFNγ concentrations in each plate were calculated.

Statistical analyses

A descriptive analysis of the qualitative variables was carried out, presenting the frequencies, median and interquartile range. A statistically significant difference was assessed by Chi- square test (X^2), student's t test, and Kruskal-Wallis test. For post hoc comparisons, the Dunnett C technique was applied. A general linear model analysis of covariance was conducted for adjusting for potential confounding factors. The statistical tests were executed using the GraphPad Prism version 8 software. Statistical significance was determined at a p-value < 0.05.

RESULTS

Demographic data of *S. typhi*- infected patients:

Demographic and clincal data of *S. typhi*-infected patients are summarized in Table 1.

The *S. typhi*-infected patients included 41 females and 25 males, with a mean age of 49.7±10.3 years (range, 32-65 years). The healthy individuals included 17 females and 13 males with a mean age of 50.6±8.7 years (range, 36-63 years). The clinical features of *S. typhi*-infected patients were 59 cases of pain, 51 cases of anorexia, 43 cases of nausea, 44 cases of vomiting, and 57 cases of fever, while the study did not find any of these features in healthy individuals.

For smoking and drinking alcohol, we found 27 cases of smoking and only two cases of drinking alcohol in patients, while there were six smokers and no drinkers in the control group. For other diseases, there were 15 patients who had other diseases and were taking different drugs. Furthermore, total white blood cells (WBCs) count were higher in patients than in the control group. As shown in Table 1, with the exception of age, gender, and drinking alcohol, the *S. typhi*-infected patients and control group significantly differed in demographic and clinical features (p-value <0.05).

Monoplex PCR Detection of Virulence Genes of *S. typhi* Isolates:

From a total of 66 *S. typhi* isolates, the prevalence of virulence genes was 56 (84.8%) for *Inv*A and 46 (69.6%) for *Pag*C (Fig 1).

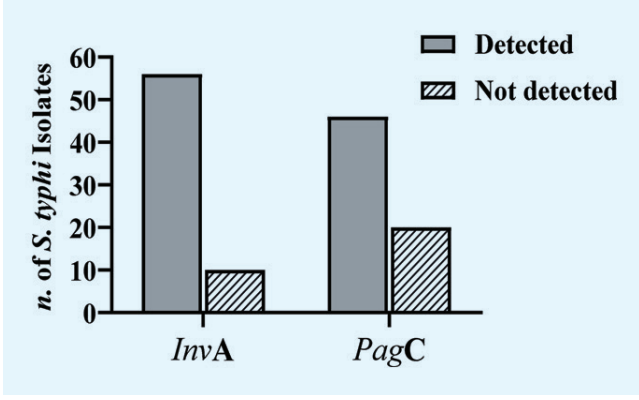

Fig 1. Prevalence of *Inv*A and *Pag*C, in *S. typhi* isolates which were collected from patients undergoing cholecystectomy by monoplex PCR assay.

By agarose gel electrophoresis, *Inv*A and *Pag*C were demonstrated in Fig 2A & 2B, respectively.

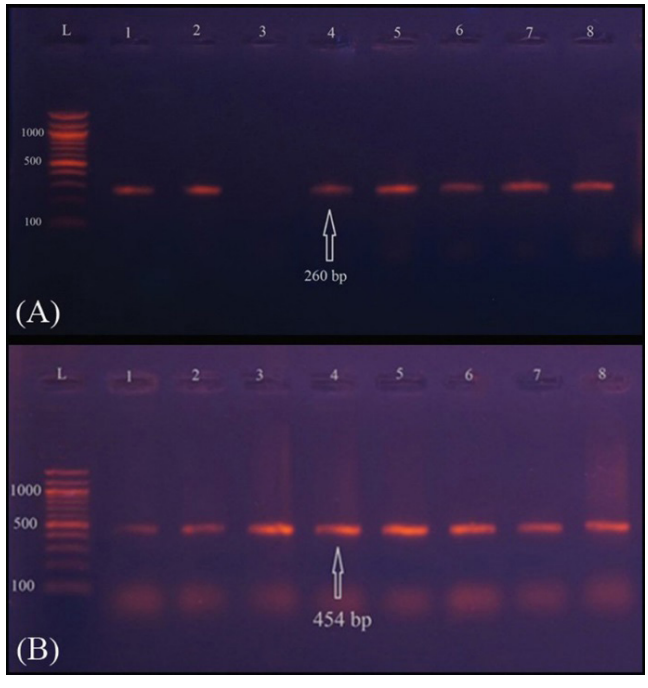

Fig 2. Ethidium bromide-stained agarose gel showing monoplex PCR for detection of the (A) *Inv*A and (B) *Pag*C of *S. typhi*. For both genes, lane 1: molecular weight marker and lanes 1 to 8: amplification products.

TABLE 1. Demographic and clinical data of participants (*S. typhi*-infected patients and healthy control).

	***S. typhi*- infected Patients n=66 Mean ± SD (rang)**		**Healthy Control n=30 Mean ± SD (rang)**		**Statistics**	***P* value**
Age groups (Years)	49.7±10.3 (32 - 65)		50.6±8.7 (36 - 63)		t = 0.415, df = 94	0.678
Total WBCs (x 10^3/µL)	7.3±1.71 (7 - 10)		5.2±1.32 (5 - 8.7)		t = 5.961, df = 94	<0.001**
	n	%	n	%		
Gender						
Male	25	37.88%	13	43.33%	X^2 = 0.257	0.612
Female	41	62.12%	17	56.67%	df = 1	
Abdominal Pain (RUQ tenderness)						
Yes	59	89.39%	-	-	X^2 = 69.58	<0.001**
No	7	10.61%	30	100%	df = 1	
Anorexia						
Yes	51	77.27%	-	-	X^2 = 49.45	<0.001**
No	15	22.73%	30	100%	df = 1	
Nausea						
Yes	43	65.15%	-	-	X^2 = 35.40	<0.001**
No	23	34.85%	30	100%	df = 1	
Vomiting						
Yes	44	66.67%	-	-	X^2 = 36.92	<0.001**
No	22	33.33%	30	100%	df = 1	
Fever						
< 38	9	13.64%	30	100%	X^2 = 4.514	0.034*
>= 38	57	86.36%	-	-	df = 1	
Smoking						
Yes	27	40.91%	6	20%	X^2 = 3.997	0.046*
No	39	59.09%	24	80%	df = 1	
Alcohol drink						
Yes	2	3.03%	-	-	X^2 = 0.928	0.566
No	64	96.97%	30	100%	df = 1	
Drugs						
Yes	15	22.73%	-	-	X^2 = 8.081	0.004**
No	51	77.72%	30	100%	df = 1	
Other Diseases						
Yes	15	22.73%	-	-	X^2 = 8.081	0.004**
No	51	77.72%	30	100%	df = 1	

Abbreviations: RUQ= Right upper quadrant; WBCs=White blood cells
*= significant; **= high significant

Proinflammatory cytokines findings

A general linear model analysis was conducted with age and total WBCs count as continuous predictors; gender, abdominal pain, anorexia, nausea, vomiting, fever, smoking, drinking alcohol, drugs, and other diseases as categorical predictors; and pro-inflammatory cytokines as dependent variables. Following their adjustment as covariates, the serum levels of TNF-α was statistically significantly different in *S. typhi*-infected patients and healthy control groups (X^2=50.46, df=2, *p*-value <0.001) (Fig 3).

Moreover, Fig 4 shows that, like TNF-α, the serum level of INF-γ was statistically significantly different in *S. typhi*-infected patients and healthy control group (X^2=46.99, df=2, *p*-value <0.001).

According to *Inv*A gene detection, the post hoc comparsion revealed that the serum level of TNF-α was statistically significantly higher in *Inv*A detected in *S. typhi* isolates (143.1, (97.5-149.8)) compared to healthy control (85,1 (79.9-91.4)) (X^2=36.14, df=2, *p*-value <0.001), and it was statistically significantly higher in *Inv*A not detected (143.4, (136.4-148.1)) compared to healthy control (X^2=42.0, df=2, *p*-value <0.001), but there was no significant difference between *Inv*A detected and *Inv*A not detected (X^2=-5.88, df=2, *p*-value >0.05) (Fig 3).

Like TNF-α, the post hoc comparsion of *Inv*A revealed that the serum level of INF-γ were statistically significantly higher in *Inv*A detected in *S. typhi* isolates (21.2, (13.7-22.5)) compared to healthy control (11.6 (9.9-14.6)) (X^2=27.9, df=2, *p*-value <0.001), and it was

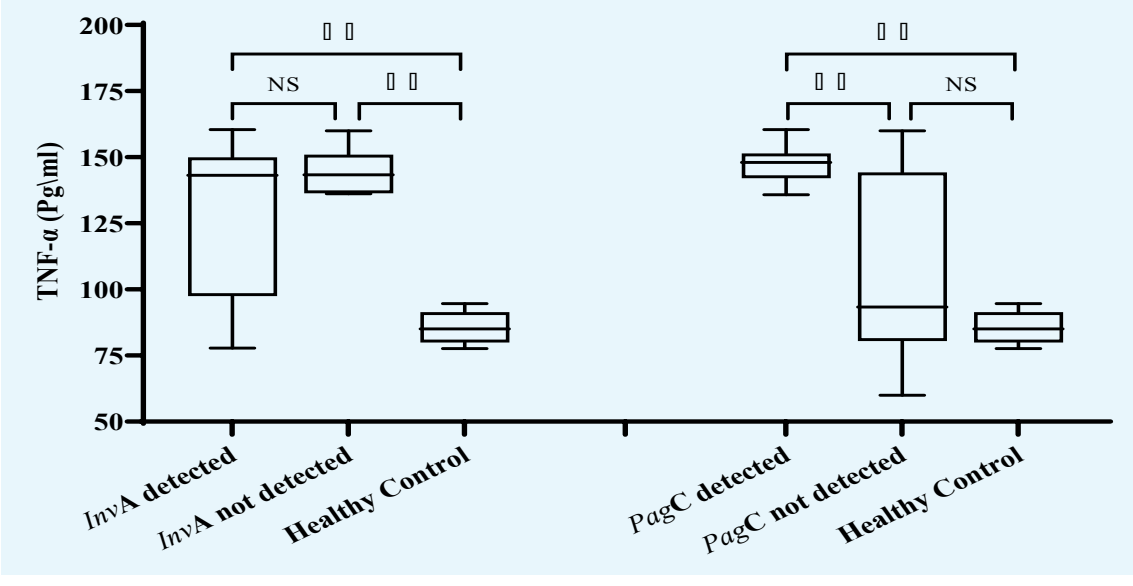

Fig 3. Boxplot of serum TNF-α levels in *S. typhi*-infected patients based on detection of virulence genes, *Inv*A and *Pag*C, and healthy control,**p*-value < 0.001.

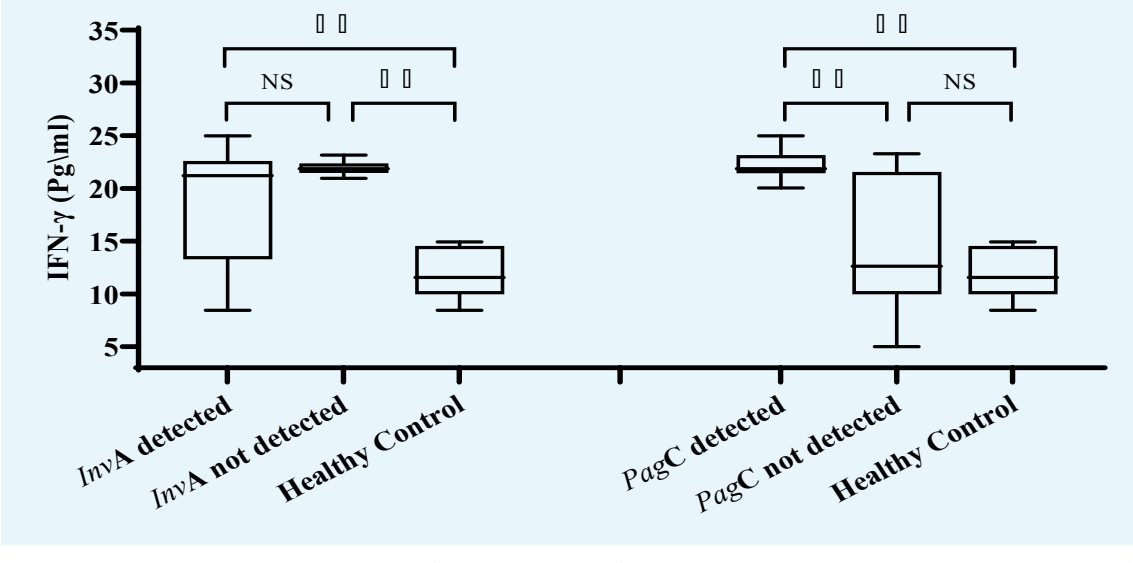

Fig 4. Boxplot of serum INF-γ levels in *S. typhi*-infected patients based on detection of virulence genes, *Inv*A and *Pag*C, and healthy control, **p*-value < 0.001.

statistically significantly higher in *Inv*A not detected (21.9, (21.6-22.1)) compared to healthy control (11.6 (9.9-14.6)) (X^2=41.2, df=2, *p*-value <0.001), but there was no significant difference between *Inv*A detected and *Inv*A not detected (X^2=-13.24, df=2, *p*-value >0.05) (Fig 4).

According to *Pag*C gene detection, the post hoc comparsion revealed that the serum level of TNF-α was statistically significantly higher in *Pag*C detected in *S. typhi* isolates (148.1, (142.1-151.2)) compared to *Pag*C not detected (93.4, (80.4-144.2)) (X^2=31.75, df=2, *p*-value <0.001), and it was statistically significantly higher in *Pag*C detected compared to healthy control (85,1 (79.9-91.4)) (X^2=48.78, df=2, *p*-value <0.001), but there was no significant difference between *Pag*C not detected and healthy control with (X^2=17.03, df=2, *p*-value >0.05) (Fig 3). Like TNF-α, the post hoc comparsion of *Pag*C revealed that the serum level of INF-γ was statistically significantly higher in *Pag*C detected in *S. typhi* isolates (21.8, (21.5-23.2)) compared to *Pag*C not detected (12.6, (9.9-21.5)) (X^2=31.78, df=2, *p*-value <0.001), and it was statistically significantly higher in *Pag*C detected compared to healthy control (11.6 (9.9-14.6)) (X^2=46.3, df=2, *p*-value <0.001), but there was no significant difference between *Pag*C not detected and healthy control (X^2=14.5, df=2, *p*-value >0.05) (Fig 4).

DISCUSSION

This study detect two virulence genes, *Inv*A and *Pag*C, in *S. typhi* isolates which were collected from patients undergoing cholecystectomy. Out of 66 *S. typhi* isolates, the findings of monoplex PCR of studied virulence genes were 56 (84.8%) *Inv*A detected, and 46 (69.6%) *Pag*C detected. A previous study showed that *S. typhi* isolates possess several genes for pathogenicity, such as *Inv*A, *Pag*C, *Spa*N, *Org*A, *Prg*H, *Inv*J, which give specific virulence traits and may deviate from the typical pattern of *S. typhi*, because they may be gained by horizontal transfer from another organism.[14]

In most strains of *S. typhi* isolated from humans and animals, the *Inv*A has been reported to be very important for pathogenicity as this gene encodes a protein responsible for adhesion and invasion of *S. typhi* into epithelial cells of the infected host.[15] In addition to the above virulence gene, the *Pag*C is also very important for bacterial adhesion, toxin transfer, and surviving with infected host cells through stimulating bacteria to form a vesicle called outer membrane vesicles (OMVs), spherical membranous compounds that are secreted from the surfaces of virtually all gram-negative microorganisms. High expression of *Pag*C accelerates *S. typhi*-OMVs formation.[16] In consistency with the rseults of our study, previous studies have observed a high prevalence of *Inv*A and *Pag*C in *S. typhi*.[17,18] On the basis of the results of this study, these two virulence genes, *Inv*A and *Pag*C, could be very useful genes for the fast determination of *Salmonella* pathogen.

Furthermore, despite *S. typhi* infection, our findings show that TNF-α release was significantly higher in *Pag*C detected in *S. typhi* isolates than in *Pag*C not detected, and INF-γ release was also significantly higher, but there was no significant difference between *Inv*A detected in *S. typhi* isolates and *Inv*A not detected, indicating that this phenomenon extends to other cytokine molecules. These findings could be attributed to strong defense responses, such as immune cell proliferation and excretion of T helper 1 (Th1) cytokines after expression of *Pag*C. This supports the findings of two studies from Iraq and Indonesia.[19,20] Following in vitro incubation of the peripheral blood mononuclear cells (PBMCs) with isolated flagella from *S. typhi*, Wyant et al.[21] have demonstrated a decrease in the number of cells expressing the lipopolysaccharide (LPS) receptor CD14. According to the same research group,[22] PBMCs that have been coincubated with flagella of *S. typhi* have a diminished response to mitogens and antigens. Patients with typhoid fever and cholecystitis show alterations in the total number of circulating white blood cells (WBCs).[23,24] However, the majority of participants in the study described here had total WBCs that were within the normal range at the time of enrollment. It remains to be proven whether this process manifests in vivo during typhoid fever and is the cause of the reported reduction in proinflammatory cytokine release. The serum of typhoid fever patients may, however, include soluble molecules that prevent or decrease *Pag*C-induced cytokine release, as has been observed for LPS-induced TNF-α release in mycobacterial infections.[25] LPS-induced IL-1beta and TNF-α production were inhibited by IL-6 and IL-1 receptor antagonist in vitro, and these two cytokines can both be increased in the blood of individuals with typhoid fever.[26]

TNF-α is a proinflammatory cytokine that is released when bacterial engulfment by macrophages occurs. Infected macrophages become *S. typhi* antigen presenters to Th1, which secretes IFN- γ and interleukin-12. IFN-γ stimulates many effector activities of mononuclear phagocytes, such as monocytes and macrophages, in order to kill intracellular pathogens.[27] A study discovered that incubating peripheral blood mononuclear cells for four days with *S. typhi* flagella raises TNF-α to 2000 pg/ml.[28] Depending on *Salmonella* flagellum expression by fliC gene, the ability of *salmonella* to enhance production of

TNF-α and IFN-γ by human monocytes was shown.[29] So, the LPS is not the only component of gram-negative microorganisms that enhances cytokine production, but also on the bacteria's genes. According to the results of this study, infection by *Pag*C -involved *S. typhi* is more harmful than infection by *Pag*C -not involved *S. typhi*, because excessive production of inflammatory cytokines, such as TNF-α, and the subsequent release additional inflammatory mediators might lead to decreased blood pressure, shock, and even death.[30] The present findings have limiting factors; the sample size was small to draw any firm conclusions on the relationship between cytokines and virulence genes, and due to a deficiency of laboratory supplies, we were unable to complete all analyses with other virulence genes.

CONCLUSION

The virulence genes of *S. typhi*, especially *Pag*C, may be considered a potent inducer of systemic inflammatory cytokine secretion. Further studies are needed to present the patient's additional clinical course according to cytokine level.

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Conflict of interest: none

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