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Study of Illness Problems Caused By *Salmonella* and Relation with Typhoid

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Abstract

There is urgent need to address illness problems caused by *Salmonella* enteric serotype Typhibacteria. The bacteria are deposited in water or food by human carrier and are then spread to other people in the area. In this research,a blood specimens were collected from typhoid fever patients, andserum levels of IFN-γ and IL-6 during the chronic and acute phase in typhoid patients group was determined according protocol kit and calculation, results were higherlevels in chronic phase (137.187±0.703.427±206.545 pg/ml respectively) and in acute phase were 128.787±2.522, 137.733±23.424 pg/ml, respectively with highly significant (P≤0.01) than those in healthy control group. *Salmonella* infects hosts as diversified as human, animal, and plants.

Keywords: *Salmonellatyphi*; Serology; IL6; INF-γ; *Salmonella* infects hosts as plant

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Introduction

Typhoid fever is an enteric disease and one of the major health problems in the developingcountries, fostered by many interrelated factors, including increased urbanization, inadequate supplies of clean water, antibiotic resistance, and the variable efficacies of vaccine preparation [1]. *Salmonella* is a member of the family Enterobacteriaceae consist of more than 2500 serovars, and infections caused by *Salmonella* constitute a major public health problem worldwide [2]. These pathogens can affect both human and animals, causing food–borne disease ranging from mild gastroenteritis to life threating systemic infections, such as those caused by *Salmonella enterica* serovar *typhi* known as (*S. typhi*) [3]. *Salmonella* infects hosts as diversified as animal and plant. It is responsible for chlorosis on plant leaves causing death. Clinical studies demonstrated that *S. typhi* infection stimulates both an intestinal mucosal and systemic humoral and cellular immune response, which are play roles in controlling and clearing *S. typhi* infection [4,5], by increased levels of circulating proinflammatory and anti-inflammatory cytokines in patients with typhoid and a reduced capacity of whole blood to produce inflammatory cytokines in patients with severe disease [6,7]. Animals, biofilm formation on plant tissue observed in plant colonization by *Salmonella* often in association with plant pathogens.

Material and Methods

This study included 254 patients represented (124 males and 130 females) with age ranged from 6–60 years, and clinical suspected case of typhoid that came from Al-Kadhimiya Teaching hospital. At period from August to October 2017. Blood samples for culture, and serologic analysis were collected from all patients on the same day or within 1–2 days after the first consultation.

Bacterial isolates were identified by inoculation on *Salmonella* Shigella agar and tetrathionate broth as selective media and by biochemical tests, then incubation at 37°C for 24 hr. or by serological test and identification rapid system. Bacteria were cultured onto selective media include: XLD agar, Bismuth sulfate agar (BSA) and S-S agar media. Bacteria were inoculated into tubes containing TSI agar by streaking slant and stabbing butt. After incubation, the colony change on the slant and bottom were identified [8].

2 ml of defibrinated or anticoagulant-treated blood was taken and equal volumes of Hank balanced salt saline solution (HBSS) (final volume 4 ml) were placed into centrifuge tube [9]. The blood and buffer were mixed by inverting the tube several times, the lymphocyte separation medium bottle inverted several times to ensure mixing. And 3 ml of lymphocyte separation medium was added to the centrifuge tube, and then 4 ml of diluted blood sample was added carefully to the lymphocyte separation media solution without mixing the media solution with diluted blood sample. After that the sample was centrifuged at 400x g for 30 min. at 18-20°C. The upper layer containing plasma and platelets were drowning off using a sterile pipette, the layer of mononuclear cells was transferred to a sterile centrifuge tube

using a sterile pipette, and the volume of the transferred mononuclear cells was estimated. At least 5ml of RPMI-1640 medium was added to the mononuclear cells in the centrifuge tube. The cells suspended by gently drawing them in and out of a pipette and centrifuged at 400×g for 15 min at 18-20°C. The supernatant was removed; the mononuclear cells was suspended in 3 ml of RPMI-1640, and then centrifuged at 400× g (or 60 to 100 × g for removal of platelets) for 10min at 18-20°C, the supernatant was removed, and the cell pellet was re-suspended in HBSS for the application.

0.1 ml of lymphocyte cell suspicion was mixed with 0.9 ml of try panblue 50 µl from mixture solution was taken and putting in improved Neubauer chamber slide, the visible cells was counted in each of the four squares, the viable cell concentration/ml was counted by using the following formula: ($Ci=t\times tb\times 10^4$), When Ci: Initial cell concentration/ ml, t: the total viable cell count of four squares, tb: Correction for the trypan blue dilution, and 10^4: Conversion factor for counting chamber.

It was necessary to determine numbers of bacterial suspension which can be more stimulated the cultivated lymphocyte cell culture to produce of cytokines in more amounts *in vitro*.

2-6 colony of *S. typhi* were picked up, and then inoculated in 5 ml of BHIB then incubated at 37°C for 4-6 hr. until the inoculum turbidity is ≥ 0.1 OD at 620 nm or compared with McFarland standard. To ensure the turbidity measurement compared with McFarland standard solution, 0.1ml from turbid tube was transferred and compared to McFarland standard and then inoculated the nutrient agar plates by spreading inoculum over the surface of medium by spreader, and the plates were incubated at 37°C for 24 hr., the bacteria colonies were counted (in range 30-300 colony), and then the count numbers of bacterial suspension were calculated by using the following formula:No. of cells/ml=No. of colonies × Dilution factor. 5×10^6 CFU/ml was the typical live numbers of bacteria which induce the PMNCs culture to produce of cytokines *in vitro*.

Tubes containing 5 ml of growth media solution were inoculated by 50 µl of PBMCs suspend, and all tubes culture incubated at 37°C and 5% CO_2 for 72 hr., after pass 4 hr. the tubes were inoculated with diluents bacteria suspension, and then culture supernatant were collected at 1-72 hr. for cytokines assays.

Reconstitute the lyophilized recombinant protein to make a 10000 pg/ml of Il-6 solution by 1ml sample diluent buffer was added to a tube of lyophilized protein, the tube was kept at room temperature for 10min. and mixed thoroughly, 0.9 ml of the sample diluent buffer was aliquot into tube and labeled as 10000 pg/ml protein standard, 0.1 ml of the mixed 10000 pg/ml IL-6 solution was added to tube containing 0.9ml diluent buffer and mixed to make a 1000 pg/ml IL-6 solution, Labels 6 tubes with the protein concentration to be prepared by serial dilution: 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml and 15.6 pg/ml, 0.3 ml was aliquot of the sample diluent buffer to the labeled tubes, 0.3 ml was transferred from the 1000 pg/ml IL-6 solution to the 500 pg/ml tube and mixed thoroughly, Then 0.3 ml transferred from each tube to another to prepare series dilute the protein standards into their respectively and store at 4°C until uses.

0.1ml of the sample diluent buffer was aliquot into a control well to serve as the blank, 0.1 ml of the serial standard protein solutions werealiquot into empty wells of the pre-coated well plate. The sample (serum and supernatant of cell culture) was diluted by taking 50 µl of sample and 50 µl of diluent buffer and mixed thoroughly to prepared 1:2 from sample working dilution, 0.1ml of each diluted test samples was aliquot to empty wells, the wells plate were covered and incubated at 37°C for 90 min. During incubating, a stock biotinylated antibody working solution were prepared according to protocol kit, and the working solution used within 2 hr., the cover of the well plate was removed and plate well contents were discarded and the plate blotted onto paper towel, 0.1 ml of the biotinylated 1:100 antibody working solution was added to each well and the plate was incubated at 37ºC for 60 min. During incubation period, a stock of Avidin-Biotin-Peroxidase Complex (ABC) working solution was prepared according to kit protocol, pre-warm the ABC working solution at 37°C for 30 min. Before use with in1hr, the plate was washed 3 times with 0.3 ml of PBS (prepared in paragraph 2.6.6) and the washing buffer discarded and the plate blotted onto a filter paper No.1, 0.1 ml of ABC working solution was added to each well and the plate was incubated at 37°C for 30 min. During the incubation period, pre-warm TMB color at 37ºC for 30 min. before used, the plate was washed 5 times with 0.3 ml of PBS and the washing buffer was discarded and the plate was blotted onto a paper towel, 90 µl of TMB color developing agent was added into each well and incubated at 37ºC for 11 min. as show that in the Figure 1.

0.1 ml of TMB stop solution was added to each well to convert the color in well from blue to yellow as shown that in the Figure 2. The absorbance was read at 450 nm in a Micro plate reader within 30 min. after adding the stop solution, and then the relative OD was calculated by using the following formula: OD(relative)=OD450 (reading)– D450 (blank).

The standard curve was plotted by using computer plot software, with relative of absorbance of each standard solution on the y- axis and standard concentration on the x-axis. The best fit straight line was drawn through the standard points. The IL-6 concentrations of samples were reported by multiply the interpolated standard curve by the dilution factor (sera x2) to obtain the target protein concentration in the samples. The normal range value detection was 15.6-1000pg/ml.

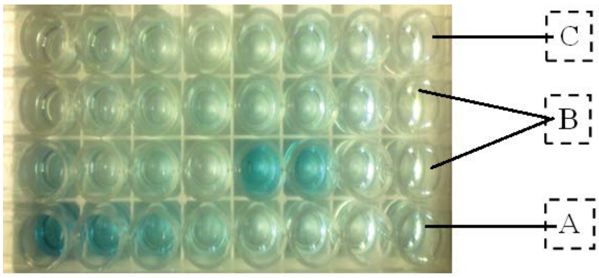

Figure 1: The blue color can be seen in the wells after adding of TMB solution, the deep blue color in (A) refers to solution with the most concentration protein standard solutions wells, but the light blue color or no obvious showed to the samples wells (B) for sera and (C) for cell culture.

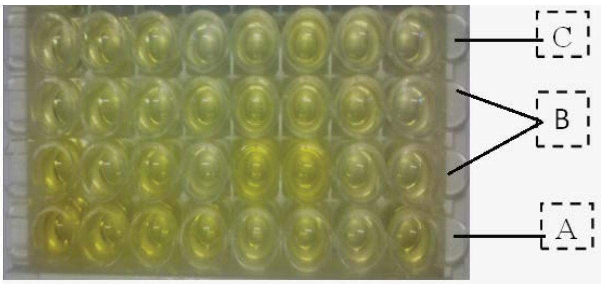

Figure 2: The acidic stop solution will change the mixture color in each well to yellow, the yellow intensity is proportional to the amount of target protein in (A) refer to standard solutions wells, the samples wells (B) for sera and (C) for cell culture.

The solution of vial item C was divided into two containers, for serum samples, 400 µl assay diluent A was added in first container and 400 µl of (1X) of assay diluent B for cell culture was added in second container to prepare a 50ng/ml standard and mixed thoroughly, 180 µl of IFN-γ standard from containers was added into two tubes, firstly tube containing 420 µl from assay diluent A, secondly tube containing (1x) of assay diluent B to prepare a 15000 pg/ml stock standard solution, 14 tubes were labeled and divided them into two groups with the IFN-γ concentration to prepare serial diluent: 5000 pg/ml, 1666.7 pg/ml, 555.6 pg/ml, 185.2 pg/ml, 61.7 pg/ml, 20.6 pg/ml, and 0 pg/ml, 400 µl was transferred from assay diluent A for firstly group tubes and 400 µl was transferred from (1x) of assay diluent B for the secondly group tubes, Then transfer 0.2 µl of 15000 pg/ml stock standard solution to first tube of groups to prepare 5000 pg/ml and 200 µl was transferred from 5000 pg/ml tube to second tube to prepare1666.7 pg/ml and transfer 200 µl from each tube to another tubes to prepare serial dilute standards into their respectively and mixed thoroughly, and then store at 4°C until uses.

100 µl of each of the serial standard solutions was added into empty wells of the pre-coated well plate, 100 µl from each samples test (serum or cell culture) was added into appropriate wells, well was covered, mixed gently and incubated at 4°C for overnight. Prepared wash buffer (1x) by dilute 20ml of wash buffer concentrate into 400ml D.D.W., and the solution was discarded and washed 4 times with 300 µl of (1x) wash buffer and the plate was blotted onto a filter paper No.1, 100 µl of prepared biotin antibody according to kit protocol was added to each well and incubate for 1hr. at room temperature with gentle mixing, the solution was discarded and the washing process repeated, 100 µl of prepared streptavidin solution according to kit protocol was added to each well and incubate for 45min. at room temperature with gentle mixing, the solution was discarded and the washing process repeated, 100 µl of TMB reagent was added to each well and incubated for 30 min. at room temperature in the dark with gentle mixing, 50 µl of stop solution was added to each well, the absorbance was read at 450 nm immediately.

The average absorbance of each set of duplicate standards was calculated, control and samples. The standard curve was plotted to calculate the concentration of IFN-γ in serum and cell culture samples. The standard curve was plotted by using computer plot software, with standard concentration on the x-axis and absorbance on the y-axis. The best fit straight line was drawn through the standard points. The IFN-γ concentrations of samples were reported by multiply the interpolated standard curve by the dilution factor (sera x2) to obtain the target protein concentration in the samples. The normal range value detection was 82-103 pg/ml to serum samples and 84-104 pg/ml to cell cultures. The minimum detectable dose of IFN-γ is typically less than 15 pg/ml.

- Diagnostic test calculator Software program was used for statistical analysis the evaluation and comparison between diagnostic tests [10].

- Statistical calculator software was used to statistical analysis of significance value (in 0.01 value) of difference mean between two groups was assessed by Independent group's t-test between means.

- Statistical package of social science (t-Test) was used for statistical analysis of the results were shown as mean± standard deviation [11].

- Person correlation coefficient was used to analyses the correlation between serum cytokines levels in typhoid fever, or in PMNCs culture.

Result and Discussion

Agglutination tests were known anti-sera and unknown culture isolate is mixed, and the clumping occurred within few min. So the interpretation of results were, Granular "clumps" observed in the tube are regarded as a positive result for 'O' antigen identification, where as a more floccules appearance observed by using a bright light against a dark background is regarded as a positive result for 'H' antigen identification and from cultivation method on XLD and S.S agar media show growth of bacteria with bile colonies with black center also give black at bottom of TSI medium this means bacteria was produced H2S and gas and from biochemical test the result was shown no. of isolates belong to *S. typhi* and number of bacteria isolates give positive results to serological method was 168, and 69 bacteria isolates were gave negative these test was performed accordingly [12]. The results were appeared four layers after separation blood the first layer was plasma and the second layer was represented lymphocytes layer (Figure 3) which used for assay the cytokins.

In the present study, the concentrated levels of IFN-γ and IL-6 were determined to investigate their role in the pathophysiology of typhoid fever in acute and chronic humans infect and investigate their role on diagnosis of typhoid patients. Serum IFN-γ and IL-6 levels during the acute and chronic phases in typhoid patients compared to healthy control group (Table 1).

Serum IFN-γ and IL-6 level during the acute phase in typhoid patients group were high (128.787±2.522, 137.733±23.424 pg/m elyy) with highly significant (P≤ 0.01) than those in healthy control group. In addition, serum levels of IFN-γ and IL-6 during the chronic phase in typhoid patients group were higher levels (137.187±0.703, 427.206±545

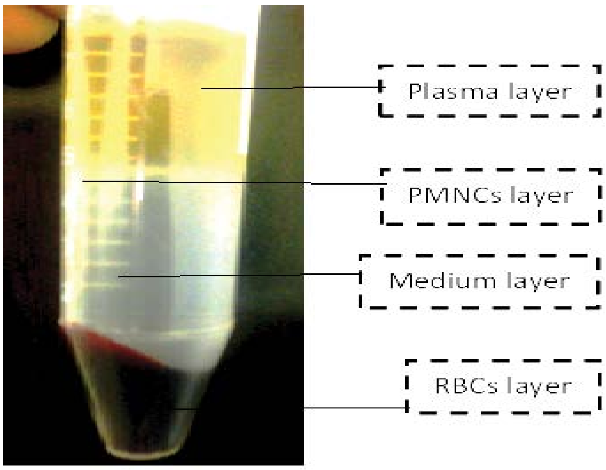

Figure 3: Four layers after separation by lymphocyte separation medium after centrifugation the sample at 400xg for 30 min.

Table 1: The mean levels of Cytokines (IFN-γ and IL-6) insera of healthy and typhoid Patients groups.

Cytokines	Mean ± SE			T- test
	Control	Patients groups		
		Acute Phase Patients	Chronic Phase Patients	
IFN-γ pg./ml	75.816±0.209	128.787± 2.522	137.187±0.703	77.51*
IL-6 pg/ml	10.333±1.958	137.733±23.424	427.20 ± 6545	34.978*

where:*P ≤ 0.01, SE: Standard error.

Pg/ml respectively) with highly significant (P ≤0.01) than those in healthy control group.

Typhoid fever is caused by the facultative intracellular gram negative bacillus *S. typhi,* the clinical features of typhoid fever confused with other febrile disease. Cytokines have been shown to play principal roles in the defense against *Salmonella* infection; IFN-γ is one of the representatives of cytokines involved in the clearance of intracellular pathogens. IL-6 is a Th1-inducing cytokine; IL-6 shares many of the biological properties and plays a critical role in the host defense against intracellular pathogens through T cell activation. In this study, IFN-γ and IL-6 are significantly increased in concentration levels in serum of typhoid patients compared the control, this result supports that IFN-γ and IL-6 are implicated in the pathogenesis of typhoid fever and agrees with the result of Acheson et al., [1] who reported that the levels of IFN-γ and IL-6 are elevated in typhoid patients compared to control[12].To determine which phase of typhoid fever (acute or chronic phase) is stimulate immune cells to produce and induce of these cytokines more than other phase in typhoid patients compared to control presented in Table 2.

Serum IFN-γ level was highly elevated in the chronic phase of typhoid fever compared to control with high significance (P ≤ 0.01), and elevated in the chronic phase compared to acute phase of typhoid fever with clearly significant (P≤0.01), and serum IFN-γ level was highly elevated in the acute phase of typhoid fever compared to control with highly significant (P ≤ 0.01).

In addition, the serum IL-6 level l was shown elevated in the chronic phase of typhoid fever compared to acute phase of typhoid fever with clearly significant (P≤0.01), the serum IL-6 level in the chronic phase showed more elevation compared to control with higher significance (P≤0.01), and serum IL-6 level was shown high elevation in the acute phase of typhoid fever compared to control with higher significance (P≤0.01) (Table 2). The present study showed significance of correlation coefficients between the maximum levels of cytokines in each typhoid fever patient, including both the chronic and acute phase when the IFN-γ level correlated significantly with IL-6 level (P=0.014 with statistical significance p<0·05). The present study has demonstrated the differences in the levels of cytokine responses between the acute and chronic cases of *S. typhi* infection by serum levels of IFN-γand IL-6 in typhoid fever patients compared to control, these results indicated a stronger IFN-γand IL-6 levels in the chronic typhoid patients more than acute typhoid patients compared to control; and agrees with the result of Mizunio, *et al*., [9] who reported that the levels of IFN-γ and IL-6 were elevated in systemic form of typhoid fever more than gastroenteric form compared to control. IFN-γ and IL-6 responses in the chronic typhoid fever returned to normal levels much later than those in the acute typhoid fever, it take around 6 weeks to eliminate even an attenuated virulent strain of *Salmonella* in mice, disseminate of *S. typhi* in systemic sites of chronic phase might result in prolonged survival of the bacteria and characteristic features of cytokine and cellular immune responses in the patients with systemic infection [13], that explained and supports the high levels of IFN-γ and IL-6in chronic phase more than in acute phase compared to control. The result in the present study agrees with the result of Sheikh et al., [14] who that reported and observed detection of a parallel cellular response against *S. typhi* (PagCAg.) during human infection, including both Interferon-γ and proliferative responses, and shows that responses in convalescence were higher than during acute stage illness. In figure 4 the mean of concentration of serum cytokine levels (IFN-γ, IL6) in the controls, 9acute and chronic phase. When *S. typhi* is a specific human-restricted intracellular pathogen and the cause of typhoid fever; Cellular immune responses are required to control and clear *Salmonella* infection. So the adaptive immune response also provides positive feedback to the innate immune system through the synthesis of cytokines that either increase effector-cell numbers or activate these cells to produce an increased antibacterial response [5]. The protective roles of IL-18 during *S. typhi* infections are primarily related to its ability to induce IFN-γ, which activates the microbicidal activity of macrophages through induction of nitric oxide production. That mean an adequate Th1 response is required to induce some cytokines pathway for eliminate the *S. typhi* such as Caspase-1 pathway to produce IL-6. But Mutate of the human genes of some crucial cytokines of this pathway, like IFN-γ, IL-12, and IL-6, greatly reduce the natural resistance to *Salmonella* infections and this hypothesis agree with the result in the present study [15], when high level of IFN-γ and IL-6 in typhoid patients compared to control. To evaluate the releasing and production of IL-18 and IFN-γ by human peripheral blood mononuclear cells (PBMC) in response to *S. typhi* were examined in PBMCs culture stimulated to live *S. typhi* bacteria compared PHA and control *in vitro*. IFN-γ and IL-18 production levels in supernatant of PMNCs culture after stimulation to live bacteria of *S. typhi* compared to PHA and control (Table 3).

FN-γ production levels in supernatant of PMNCs culture after stimulation to PHA and a live bacteria of *S. typhi* were higher levels (9347.037±485.736, 8187.777±375.319 pg/ml respectively), with higher significance (P≤ 0.01) during PBMNCs culture stimulation to PHA and a live bacteria of *S. typhi* than those in control respectively, but non-significant of IFN-γ levels production to stimulate of PBMNCs culture between PHA and *S. typhi* (P≤0.01). In addition, IL6 production levels in supernatant of PMNCs culture after stimulation to a live *S. typhi* bacteria was high levels (66.444±4.177 pg/ml) compared to PHA and control with higher of statistical significance (P≤ 0.01). And IL-6 production levels during the stimulation of PBMNCs culture to PHA is significant compared to control (P ≤ 0.01) (Table 3).

The limit evaluation of cellular responses in humans to wild-type *S. typhi,* with no animal model fully replicates host pathogen interactions and immunologic events that occur during this human-restricted infection, in additional evaluation in humans has largely focused on characterizing responses in recipients of attenuated vaccine strains of *S. typhi* [16]. So this study presented some of defense mechanism against *Salmonella* infection by ability of PMNCs culture to releasing

Table 2 Comparison between two phase of typhoid fever disease in inducing IFN-γ and IL-6.

Phase	Cytokines			
	IFN-γpg/ml		IL-6 pg/ml	
	T-test	Probability	T-test	Probability
Control(1) *vs.* Acute(2)	20.93	0.01***	5.43	0.01**
Chronic(3) *vs.* Control(1)	83.61	0.01***	3.44	0.01***
Chronic(3) *vs.* Acute(2)	3.21	0.01*	2.20	0.01*

where: (1) is Control healthy group, (2) Acute phase typhoid patients group, (3) is Chronic phase typhoid patient group, *: is clear significant (P≤0.01), **: is highly significant (P≤0.01), ***: is higher significant (P≤0.01).

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Table 3: The mean levels of Cytokines (IFN-γ and IL-6) in PMNCs culture in response to *S. typhi*, PHA and compared to control.

Cytokines	Stimulate by	Mean ± SE	T- test value		Probability
IFN-γ pg./ml	PHA(1)	9347.037±485.736	1vs2	1.889	0.01^{*}
			1vs3	14.592	0.01^{***}
	S. typhi (2)	8187.777±375.319	2vs3	15.320	0.01^{***}
	Control (3)	1713.70±194.189			
IL-6 pg./ ml	PHA (1)	42.317±2.289	1vs2	8.424	0.01^{***}
			1vs3	2.526	0.01^{**}
	S. typhi (2)	66.444±4.177	2vs3	8.400	0.01^{***}
	Control (3)	28.827±4.826			

where: $^{*}P \leq 0.01$(non-significant), $^{**}P \leq 0.01$(significant), $^{***}P \leq 0.01$ (high significant).

and production of IL-6 and IFN-γ which are circuits to activate of many immune cells in typhoid fever infection. In peripheral blood, Cellular immune responses (T-helper cells) mediated produce Th1 cytokines such as IL-6 and IFN-γ in response to *S. typhi* infections. The inflammatory processes trigger various types of cells, macrophages and monocytes, to release many cytokines. The released cytokines trigger other cells and initiate the cascade of cytokine release which can contribute to activating of appropriate host defenses [17]. IL-6 is important for the induction of IFN-γ, and these cytokine is central for successful host defense against *Salmonella* infection; because neutralization of IL-6leads to increased bacterial numbers in spleen and liver and decreased host survival, while IL-18 treatment decreases bacterial counts in spleen and liver and increases host survival. This shows that IL-6 plays an important role in host defense against *Salmonella*. This role is effective to be mediated of IFN-γ production [12,18]. IFN-γ production by PMNCs following stimulation with *Salmonella* was significant inhibited by anti-IL- 6 monoclonal Ab. (P<0·05).

Conclusion

The IFN-γ level correlated significant with the IL-6 level in the present study, suggesting a possible involvement of IL-18 to induce IFN-γ against human *Salmonella* infection *in vivo*. Higher levels of production of IL-6 and IFN-γ *in vitro* stimulated by live *S. typhi* than control might reflect the *in vivo* activation for producing of these cytokine from cells.

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