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Measurement of IL-18, IFN-γ Levels in Iraqi Typhoid Fever Patients

Rasmiya A. Aburesha[1] , Rabab K. Seger[2]

[1]Department of Biology / College of science / University of Baghdad /Baghdad /Iraq / Central health laboratory.
[2]Consultant in Microbiology / Ministry of Health / Central public health laboratory.

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Abstract: Typhoid fever is an acute illness associated with fever caused by the *Salmonella enterica* serotype typhi bacteria. It can also be caused by *Salmonella paratyphi*, a related bacterium that usually causes a less severe illness. The aim of current study was estimated of IL-18, and IFN-γ level as a diagnostic tool. The level of IL-18, and IFN-γ was investigated in 254 blood specimens in patients with typhoid fever, 207(84.48%) bacterial isolates were obtained and isolated from three groups of patients, serum levels of IFN-γ and IL-18 during the chronic and acute phase in typhoid patients. The groups were determined according to clinical features and symptoms of disesase. The results have been shown higher levels in both IL-18, and IFN-γ (137.187±0.703, 377.357±106.585pg/ml respectively)in chronic phase while in acute phase 128.787±2.522, 137.833±23.424pg/ml respectively) with highly significant (0.01) than those in healthy control**.**

Keywords: IL-18; INF- γ; Typhoid fever.

Corresponding author: (Email: dr.rababalsegar@yahoo.com).

Introduction:

Typhoid fever is an enteric disease and one of the major health problems in the developing countries, 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).

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-c cytokines in patients with typhoid and a reduced capacity of whole blood to produce inflammatory cytokines in patients with severe diseases (6).

Some of the recently published methods for the detection of *S. typhi* in blood, water samples, and food are, lateral flow immunoassay (LFIA) using antibody-coated gold nanoparticles (7) uniplex and, multiplex PCR reverse transcriptase multiplex PCR (RT-MPCR) (8) and gel electrophoresis. The limit of detection (LOD) of these above

methods ranges between 500 to 10^4 CFU/mL. Besides, these methods are time-consuming, require numerous equipments including expensive thermocyclers and trained labour. Henceforth, there is a necessity for new methods that offer rapid, specific, and sensitive detection.(9).

Material and Methods:

Clinical specimens:

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, DNA extraction, and serologic analysis were collected from all patients on the same day or within 1–2 days after the first consultation.

Identification of bacterial isolates:

Bacterial isolates were identified by inoculation on Salmonella Shigella agar and tetrathionate broth as selsctive media and by biochemical tests, then incubation at 37^{o}C for 24hr. or by serological test and identification rapid system.

Identification of bacteria by selective media:

Bacteria were cultured onto selective media include: XLD agar, Bismuth sulfate agar (BSA) and S-S agar media.

Biochemical tests:

Production of H_2S and fermenting of sugars:

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 (10).

Cytokines levels assay in typhoid patients and cell culture:

Cytokines such as IL-18 and IFN-γ were measured in typhoid patients; and to study the ability of induced immune cells to produce of IL-18 and IFN-γ, measurement levels of these cytokines in serum of typhoid patients and cell culture compared to controls.

Isolation of PBMCs from blood:

Two ml of defibrinated or anticoagulant-treated blood was taken and equal volumes of Hank balanced salt saline solution (HBSS) (final volume 4ml) were placed into centrifuge tube. The blood and buffer were mixed by inverting the tube several times, the lymphocyte separation medium bottle inverted several times to ensure mixing. And 3ml of lymphocyte separation medium was added to the centrifuge tube, and then 4ml 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 30min. at 18-20ºC,. The upper layer containing plasma and platelets were drown off using a sterile pipette, the layer of mononuclear cells was transferred to a sterile centrifuge tube using a sterile pipette, 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 3ml 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.

Calculating of PBMNCs suspended concentration:

Lymphocyte cell suspension (0.1) ml was mixed with (0.9) ml of trypan blue, 50µl from mixture 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.

Bacterial count:

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^{o}C for 4-6 hr. until the inoculum turbidity is ≥ 0.1OD at 620nm 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^{o}Cfor 24hr., 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*.

Produce of cytokines by peripheral blood cells:

Tubes containing 5ml of growth media solution were inoculated by 50µl of PBMCs suspend, and all tubes culture incubated at 37^{o}C and5% CO_2 for 72hr., after pass 4hr. the tubes were inoculated with diluents bacteria suspension, and then culture supernatant were collected at 1-72hr. for cytokines assays (11).

ELISA to measure IL-18 in serum and cell culture:

According the manufacturing company reconstitute the lyophilized recombinant protein to make a 10000pg/ml of Il-18 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, 0.9ml of the sample diluent buffer was aliquoted into tube and labeled as 10000pg/ml protein standard, 0.1ml of the mixed 10000pg/ml IL-18 solution was added to tube containing 0.9ml diluent buffer and mixed to make a 1000pg/ml IL-18 solution, Labels 6 tubes with the protein concentration to be prepared by serial

dilution: 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml and 15.6pg/ml, 0.3ml was aliquoted of the sample diluent buffer to the labeled tubes, 0.3ml was transferred from the 1000pg/ml IL-18 solution to the 500pg/ml tube and mixed thoroughly, Then 0.3ml transferred from each tube to another to prepare series dilute the protein standards into their respectively and store at 4^{o}C until uses.

Loading the samples into the micro-plate:

The sample diluent buffer was aliquoted into a control well to serve as the blank, 0.1ml of the serial standard protein solutions were aliquoted into empty wells of the pre-coated well plate. the sample (serum and supernatant of cell culture) was diluted by taking 50µl of sample and 50µl of diluent buffer and mixed thoroughly to prepared 1:2 from sample working dilution, 0.1ml of each diluted test samples was aliquoted to empty wells, the wells plate were covered and incubated at 37^{o}C for 90min., During incubating, a stock biotinylated antibody working solution were prepared according to protocol kit, and the working solution used within 2hr., the cover of the well plate was removed and plate well contents were discarded and the plate blotted onto paper towel, 0.1ml of the biotinylated 1:100 antibody working solution was added to each well and the plate was incubated at 37^{o}C for 60min. 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^{o}C for 30min. Before use with in1hr., the plate was washed 3 times with 0.3ml of PBS and the washing buffer discarded and the plate blotted onto a filter paper No.1, 0.1m of ABC working solution was added to each well and the plate was incubated at 37^{o}C for 30min., During the incubation period, pre-warm TMB color at 37^{o}C for 30min. before used, the plate was washed 5 times with 0.3ml of PBS and the washing buffer was discarded and the plate was blotted onto a paper towel, 90µl of TMB color developing agent was added into each well and incubated at 37^{o}C for 11min. as show that in the (Figure 1). 0.1ml of TMB stop solution was added to each well to convert the color in well from yellow to blue. The absorbance was read at 450nm in a Microplate reader within 30min. after adding the stop solution, and then the relative OD was calculated by using the following formula: OD(relative)= OD450 (reading) – OD450(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-18 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.

ELISA to measure IFN-γ in serum and cell culture:

The solution of vial item C was divided into two containers, for serum samples, 400µl assay diluent A was added in first container and 400µl of

(1X) of assay diluent B for cell culture was added in second container to prepare a 50ng/ml standard and mixed thoroughly, 180µl of IFN-γ standard from containers was added into two tubes, firstly tube containing 420µl from assay diluent A, secondly tube containing (1x) of assay diluent B to prepare a 15000pg/ml stock standard solution, 14tubes were labeled and divided them into two groups with the IFN-γ concentration to prepare serial diluent: 5000pg/ml, 1666.7pg/ml, 555.6pg/ml, 185.2pg/ml, 61.7pg/ml, 20.6pg/ml, and 0pg/ml, 400µl was transferred from assay diluent A for firstly group tubes and 400µl was transferred from (1x) of assay diluent B for the secondly group tubes, Then transfer 0.2µl of 15000pg/ml stock standard solution to first tube of groups to prepare 5000pg/ml and 200µl was transferred from 5000pg/ml tube to second tube to prepare1666.7pg/ml and transfer 200µl from each tube to another tubes to prepare serial dilute standards into their respectively and mixed thoroughly, and then store at 4^{o}C until uses. 100µl of each of the serial standard solutions was added into empty wells of the pre-coated well plate, 100µl from each samples test (serum or cell culture) was added into appropriate wells, well was covered, mixed gently and incubated at 4^{o}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 300µl of (1x) wash buffer and the plate was blotted onto a filter paper No.1, 100µl 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, 100µl 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, 100µl of TMB reagent was added to each well and incubated for 30min. at room temperature in the dark with gentle mixing, 50µl of stop solution was added to each well, the absorbance was read at 450nm 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 IL8 and 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 82-103pg/ml to serum samples and 84-104pg/ml to cell cultures. The minimum detectable dose of IFN-γ is typically less than 15pg/ml.

Statistical analysis:

Diagnostic test calculator Software program was used for statistical analysis the evaluation and comparison between diagnostic tests (9).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(12).

Result and Discussion:

Identification of *S. typhi* by serological test and rapid identification system:

Identification of *S. typhi* by serological test:

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 centre also give black at bottom of TSI medium this means bacteria was produced H2S and gas and the result was shown number 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 according to(13)

Separation of PMNs layers:

The results was appeared four layers after separation blood the first layer was plasma and the second layer was represented lymphocytes layer (Fig 1)which used for assay the cytokines.

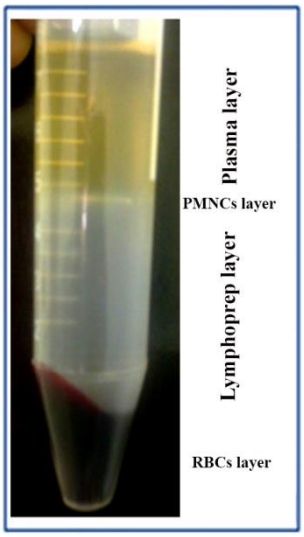

Figure (1): Seperation of monolyers cells after centrifugation the samples at 5000 rpm for 10 min.

Determining the concentration levels of cytokines in sera of typhoid patients:

In the present study, the concentrated levels of IFN-γ and IL-18 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-18 levels during the acute and chronic

phases in typhoid patients compared to healthy control group (Table 1). Serum IFN-γ and IL-18 levels during the acute phase in typhoid patients group were high (128.787±2.522, 137.833±23.424pg/ml respectively) with highly significant (P≤ 0.01) than those in healthy control group. In addition, serum levels of IFN-γ and IL-18 during the chronic phase in typhoid patients group were higher levels (137.187±0.703,377.357±106.585pg/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 (14). 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-18 is a Th1-inducing cytokine; IL-18 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-18 are significantly increased in concentration levels in serum of typhoid patients compared the control, this result supports that IFN-γ and IL-18 are implicated in the pathogenesis of typhoid fever and agrees with the result of (15) who reported that the levels of IFN-γ and IL-18 are elevated in typhoid patients compared to control(16).

Table (1): Mean level of IFN-γ and IL-18 in patients and control group.

Cytokines	Control	Patients groups		T- test
		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-18 pg./ml	10.333±1.958	137.833±23.424	377.357±106.585	34.978*

Where: *P ≤0.05, SE: Standard error.

Comparison between typhoid fever phases in inducing IFN-γ and IL18:

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 the (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-18 level 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-18 level in the chronic phase showed more elevation compared to control with higher significance (P≤0.01), and serum IL-18 level was shown high elevation in the acute phase of typhoid fever compared to control with higher significance (P≤0.01) (Table 2).

Table (2): Different between IFN-γ and IL-18 in patients and control group.

Phase	Cytokines			
	IFN-γ pg/ml		IL-18 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≤0.05), **: is highly significant (P≤ 0.01), ***: is higher significant (P≤0.001).

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-18 level(*P*=0.014 with statistical significance p<0·05) as shown in (Figure 2). The current 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-γ and IL-18 in typhoid fever patients compared to control, these results indicated a stronger IFN-γ and IL-18 levels in the chronic typhoid patients more than acute typhoid patients compared to control; and agrees with the results of (17).

Haque *et al.,* (18) reported that the levels of IFN-γ and IL-18 are elevated in systemic form of typhoid fever more than gastroenteric form compared to control which agree whith the results of current study as in (Figure 3). IFN-γ and IL-18 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 (18), that explained and supports the high levels of IFN-γ and IL-18 in chronic phase more than in acute phase compared to control. The result in the present study agrees with the result of(18) 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. 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(19). 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(20). 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-18 .But Mutate of the human genes of some crucial cytokines of this pathway, like IFN-γ, IL-12, and IL-18, greatly reduce the natural resistance to *Salmonella* infections.

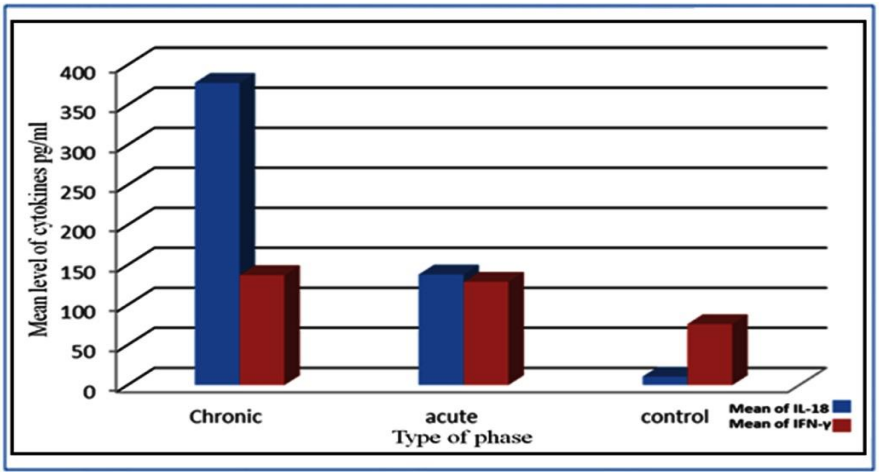

Figure (2): Mean of IL-18, and IFN-γ level in patients and control group.

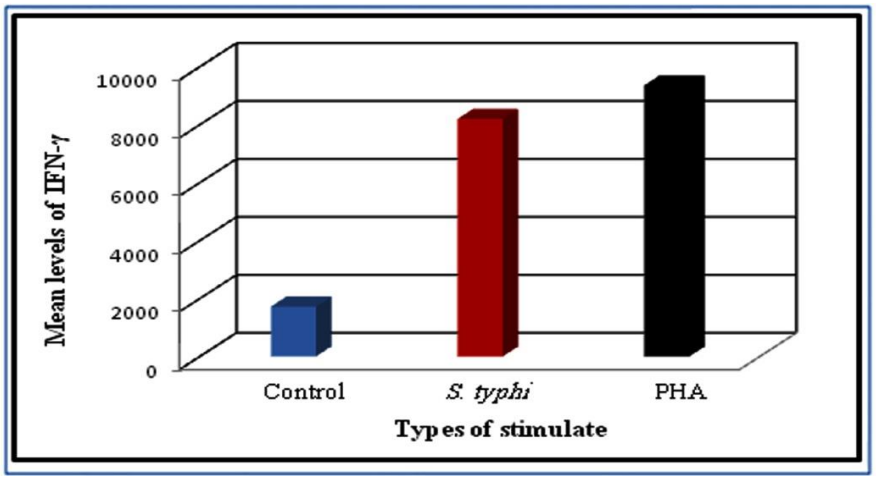

Figure (3): *In-vitro* mean of IFN-γ production following stimulated to live bacteria and PHA.

Determining of the concentration level of Cytokines in supernatant of PMNCs cultures:

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 phytohaemagglytinin PHA and control *in vitro*. IFN-γ and IL-18 production levels in supernatant of PMNCs culture after stimulation to live IFN-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±375.319pg/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, IL-18 production levels in supernatant of PMNCs culture after stimulation to a live *S. typhi* bacteria was high levels (82.444±4.177pg/ml) compared to PHA and control with higher of statistical significance (P≤ 0.01). And IL-18 production levels during the stimulation of PBMNCs culture to PHA is significant compared to control (P ≤ 0.01) (Table 3).

Table (3): Mean level of IFN-γ and IL-18 in PMNs culture following treated to live bacteria and PHA.

Cytokines	Stimulate by	Mean ± SE.	T- test value		Probability
IFN-γ pg/ml	PHA(1)	9347.037±485.736	1vs2	1.889	0.01*
	S. typhi(2)	8187.777±375.319	1vs3	14.592	0.01***
	Control(3)	1713.70±194.189	2vs3	15.320	0.01***
IL-18 pg/ml	PHA(1)	42.317±2.289	1vs2	8.424	0.01***
	S. typhi(2)	82.444±4.177	1vs3	2.526	0.01**
	Control(3)	28.827±4.826	2vs3	8.400	0.01***

Significant amount of IL-18 level was produced by PMNCs cultures from *S. typhi* stimulation compared following either PHA or control (Figure 4). On the other hand, significant amount of IFN-γ was produced only upon PHA and *S. typhi* stimulation of PMNCs compared to control group. 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* (21). So this study presented some of defense mechanism against *Salmonella* infection by ability of PMNCs culture to releasing and production of IL-18 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-18 and IFN-γ in response to *S. typhi* infections.

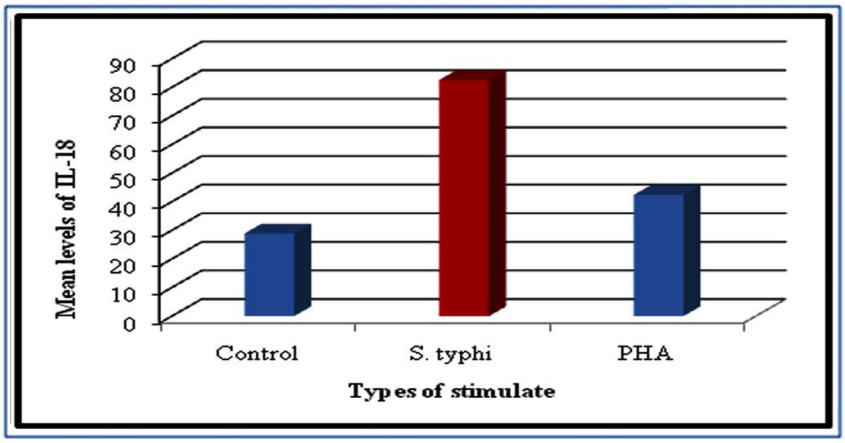

Figure (4): *In-vitro* mean of IL-18 production following stimulated to live bacteria and PHA.

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(21). IL-18 is important for the induction of IFN-γ, and these cytokine is central for successful host defense against *Salmonella* infection; because neutralization of IL-18 leads 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 (22). This shows that IL-18 plays an important role in host defense against *Salmonella*. This role is effective to be mediated of IFN-γ production (23). A study by (24) demonstrated that the IFN-γ production by PMNCs following stimulation with *Salmonella* was significant inhibited by anti-IL- 18 Monoclonal Ab. ($P < 0{\cdot}05$). Consistent with the above results, the IFN-γ level correlated significant with the IL-18 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-18 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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