Pathological and Immunohistochemical Assessment of *Salmonellatyphimurium* Pathogenicity During Oral Experimental Infection in Mice

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Abstract

Salmonella typhimurium is a Gram-negative zoonotic bacterium which causes a wide range of illnesses to both humans and animals. The aim of this research is to study the pathogenicity of *S. typhimurium in vivo*. A total of 40 adult white BALB/c mice were divided into 5 groups (8 animals each). Four groups were orally dosed by viable *S. typhimurium* (1 X 10^9 cfu/ml) suspended in phosphate buffer saline (PBS) by a stomach tube, while the fifth group was given PBS orally only (control group). Four mice were killed at 7, 12, 24, 48 hours after giving the infective dose plus one mouse from the control group. In addition, sera were collected after 2 weeks from animals of each group to detect the titer of antibodies. The viability of *S. typhimurium* was checked by culturing on SS agar after mice death. Slides were prepared for histopathological examination (to assess the lesions) and immuno-histochemistry (to detect cytotoxic T cells in the affected organs). The results included bacterial isolation from duodenum, jejunum, ileum and liver which were positive from the infected groups. Histopathological examination showed hepatic granulomatous lesions with severe infiltration of mononuclear cells (MNCs) in the liver parenchyma and within small intestine. Finally, to detect cytotoxic T cells in the slides, immunohistochemistry showed presence of CD8 T cells in the hepatic cells. Titers of antibodies were measured by ELISA where IgG antibodies were detected. The conclusion of this study could be summarized by addressing the severity of infection after 12 hours of oral dosing in the stomach while severe lesions were seen in the liver after 48 hours of oral administration.

Keywords: *Pathologyi; I mmunohistochemistry, Pathogenicity;S. typhimurium, IgG, CD-8 T cells.*

Introduction

Salmonella enterica serovar *Typhimurium* (hereafter *S. typhimurium*) is a Gram negative motile non spore forming encapsulated bacteria belongs to Enterobacteriaceae family that could cause a long list of infections (mainly diarrhea due to enteritis) to both humans and animals as a zoonotic virulent foodborne pathogen[1,2,3]. Therefore, *S. typhimurium* is responsible for being a major threat pathogen to public health globally as well as it causes huge economic losses in the field of veterinary medicine worldwide because of the biological damage to the intestine of infected animals which leads to poor absorption of digested food and weight loss[4,5]. Recent publications referred to the capability of *S. typhimurium* to develop a multidrug resistance to many antibiotics which worsen much more the economic losses in animals due to the added value of the cost of treatment[6,7,8,9].

In mice, *S. typhimurium* is responsible for bacterial diarrhea and considered as an animal model for human

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studies [10]. Infection of mice with *S. typhimurium* is mainly initiated due to oral-fecal route of transmission [11]. After ingestion of *S. typhimurium* with the contaminated food, the bacteria survive and colonize in the small intestine[12,13] Settling of bacteria in the small intestine leads to expansion in numbers through multiplication of these bacteria [14]. The clinical symptoms mainly characterized by anorexia, loss of appetite, and the most important clinical symptom is diarrhea (ranged from mild to bloody depending on the virulence of the *S. typhimurium* strain)[15].

After propagation of *S. typhimurium* in the intestine and establishing clear clinical symptoms, it is essential to interfere this microbial attack by giving antibiotics. However, *S. typhimurium* is sensitive to most antibiotics except a few emerged strains which gained resistance properties against antibiotics [6, 7, 8, 9].

The immune response against *S. typhimurium* differs according to the level of virulence of the strain[16]. Innate immunity against *S. typhimurium* represents by phagocytic activity of neutrophils and macrophages in the early stages of infection which almost always not biologically effective, thus adaptive immunity is required [17]. Adaptive immunity against *S. typhimurium* could be considered as the key role in the clearance of this bacterium through establishing production of more specific CD4+ and CD8+ T cells [18, 19, 20].

The pathogenicity of *S. typhimurium* in mice was studied before more than 2 decades [21], but this study did not focus on the liver as an important organ involved in *S. typhimurium* infection. Therefore, and due to lack of studies in Iraq, this study is designated to spot the light on the major organs affected by *S. typhimurium* in murine intestine and liver through investigation of experimental infection which is evaluated by bacterial spread in the mentioned organs assessed by histopathological examination and immune response.

Materials and Method

A total of 40 adult white BALB/c mice were divided into 5 groups (8 animals each). Four groups were given an oral dose of viable *S. typhimurium* (1 X 10^9 cfu/ml) suspended in phosphate buffer saline (PBS) by a stomach tube, while the fifth group was given PBS orally only (control group). The bacteria were isolated from local Iraqi lambs by [22]. Four mice were killed at 7, 12, 24 and 48 hours after giving the infective dose plus one mouse from the control group. The viability of *S. typhimurium* was checked by culturing on SS agar after mice death. Slides were prepared for histopathological examination (to assess the lesions) and immunohistochemistry (to mainly detect cytotoxic T cells in the affected organs). Three mice were chosen from both infected and control groups and subjected to Widal test which was used to determine the presence of O and H antigens of *S. typhimurium* and the positive samples of Widal were further subjected to ELISA to confirm measuring the titer of the IgG antibodies in the serum against protein and LPS antigen of *S. typhimurium*.

Histopathological examination: The tissue specimens collected from liver, duodenum, jejunum and ileum were fixed in 10% formalin for 72 hour and processed for slide preparation and staining with Hematoxylin and Eosin (H and E) stain was done according to [23].Histopathological changes were observed under light microscope.

Immunohistochemistry: The kit used for this technique was purchased from "US Biological, USA" and the procedure was done according to [24]. The stain used in this technique was 3, 3′ Diaminobenzidin (DAB) stain which is a stable liquid substrate. DAB is the most common reagent employed for the immunohistochemical detection of horse radish peroxidase (HRP) probes. In the presence of HRP and hydrogen peroxide, DAB is oxidized to a brown polymer easily recognized by light microscope.

***Preparation of protein and lipopolysaccharide (LPS) antigens*:** *S. typhimurium* isolated from infected mice (biochemically and serologically proven by [22]), were grown overnight on Trypticase soy agar (TSA) (Difco, USA) at 37°C. After that, the bacteria were harvested in normal saline solution and mixed with three volumes of acetone for inactivation. Inactivated bacteria were centrifuged, and the pellet further washed from acetone. The LPS antigen of *S. typhimurium* was extracted from acetone-dried cells by the following hot phenol-water method [25]. The resultant protein antigen was obtained from the acetone dried cells by Veronal buffer extraction and purified by repeated precipitation with trichloroacetic acid[26]. The carbohydrate and protein content of the prepared mixturewas determined by Lowry and Anthrone method [27].

Preparation of O and H antigens for Widal test:

Preparation of O-antigen: Bacteria were grown on TSA agar at 37°C overnight, then harvested by adding

normal saline solution to each petri dish. Bacterial cells were brushed off from the agar surface by cotton swab. The bacterial suspension was centrifuged at 6000 rpm for 30 minutes and the supernatant was discarded. The bacterial cell pellet was washed three times and resuspended in normal saline. Bacterial cells were killed by heating at 100°C for 30 minutes, diluted with normal saline at appropriate dilution and kept at 4°C until later use.

Preparation of H antigen: Trypticase Soya (TS) broth (Difco, USA) was heavily inoculated with *S. typhimurium* and incubated at 37°C overnight. Bacterial cells were killed with formalin at a final concentration of 0.5% and harvested by centrifugation at 6000 rpm for 30 minutes. The bacterial cell pellet was washed three times before being resuspended in PBS. It was kept at 4°C until later use. Optimal concentrations was determined in carbonate-bicarbonate buffer (pH = 9.6).

Enzyme-linked immunosorbent assay (ELISA): the kit (Biosource, USA) was purchased to measure IgG in the serum. Sandwich ELISA method was applied according to manufacturer instructions. Protein and LPS antigens were added into u-shape microtiter plates, then incubated at 37° C for 3 hours. After that, plates were washed three times by PBS containing (0.05% Tween20). Then a biotinylated detection antibody specific for IgG was loaded into the plates and Avidin-Horseradish Peroxidase (HRP) as a conjugate is added to each microplate well and incubated for 1 hour at 37 °C. A blue color was developed as a result of positive IgG titer. The colorimetric reaction was stopped by adding enzyme-substrate (sulphuric acid solution) and the color turned into yellow. The optical density (OD) was measured by ELISA reader (Varioskan™ LUX multimode microplate reader, ThermoFisher Scientific, USA) machine (spectrophotometer) at a wavelength of 450 nm.

The samples used for ELISA were incubated for one hour with optimal dilutions of serum samples (1:40) for protein antigen, and (1:20) for LPS antigen.

Statistical Method: T test was used to compare statistically between the titer of antibodies in infected and controls at a level of significance ($P<0.05$).

Results

The histopathological data obtained in this research showed hepatic granulomatous lesions scattered through its parenchyma consist of central foci of necrosis, associated with severe infiltration of mononuclear cells (MNCs), mainly macrophages and lymphocytes (Figure 1). In addition, sections illustrated dilated congested central veins and sinusoids, also inflammatory cells particularly neutrophils and macrophages were seen in lumen with large areas of necrotized hepatocytes replaced by RBCs (Figure 2). The results of immunohistochemical examination manifested by deposited brownish color in the intracellular area of hepatocytes within the cytoplasm of infected cell when stained by (3,3′-Diaminobenzidine) which were recognized in the liver tissues (Figure 3).

The histopathological lesion in the intestine of mice after orally administered by (0.2 ml containing 1 X 10^7cfu/ml of *S. typhimurium*), showed severe necrosis associated with massive infiltration of inflammatory cells particularly MNCs in the lamina propria and in mucosal glands, as well as severe congested blood vessels and edematous spaces appeared in the serosa layer of infected organ.

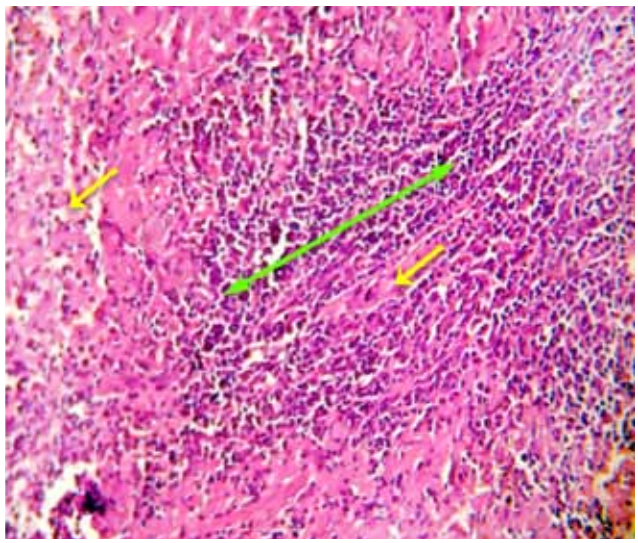

Figure 1: Histopathological section in liver of infected mouse after orally administered via (0.2 ml contain 1x10^7cfu/ml of *S. typhimurium*), shows granulomatous lesions characterized by, central of necrosis (yellow arrows) with severe infiltration MNCs mainly macrophages and lymphocyte in the liver parenchyma (green arrow) (H & E stain, 40X).

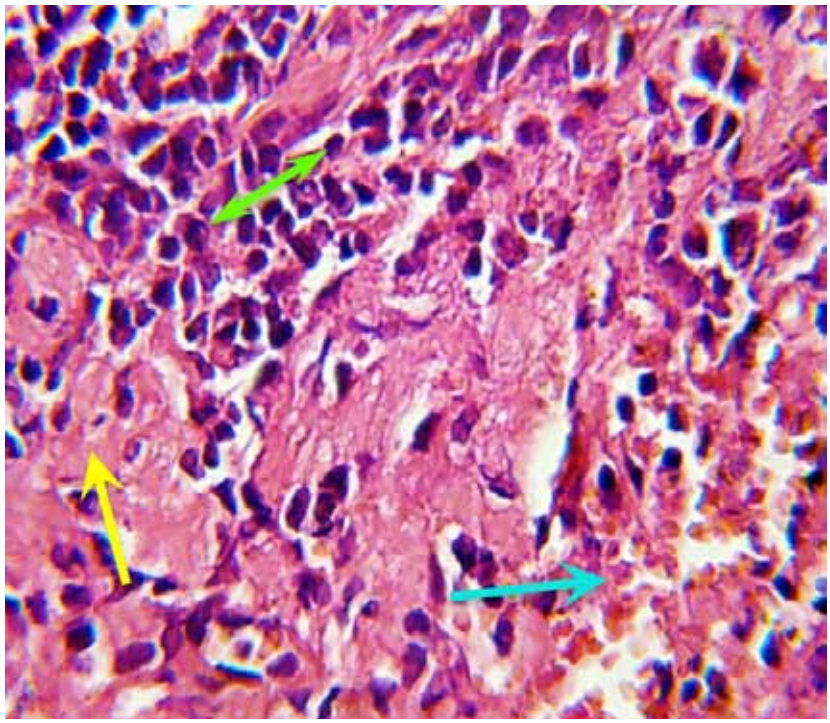

Figure 2: Histopathological section in liver of infected mouse after orally administered via (0.2 ml contain $1x10^7$cfu/ml of *S. typhimurium*), shows dilation of sinusoids (yellow arrow), neutrophils and macrophages infiltration (turquoise arrow) necrotic hepatocytes that replaced by RBCs (green arrow) (H & E stain, 40X).

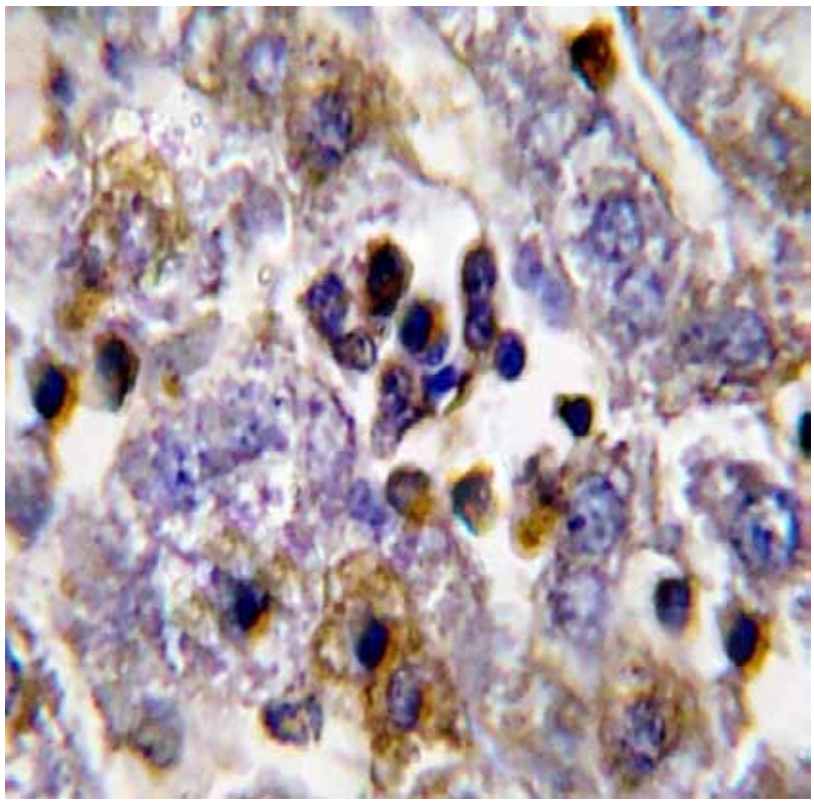

Figure 3: Immunohistochemistry section in liver of infected mouse after orally administered via (0.2 ml contain $1x10^7$cfu/ml of *S. typhimurium*), show infiltration of immunohistochemical staining positive cells of CD8, that appear as deposited brownish color in the intracellular within cytoplasm of infected cell (Diaminobenzidine staining, 40X).

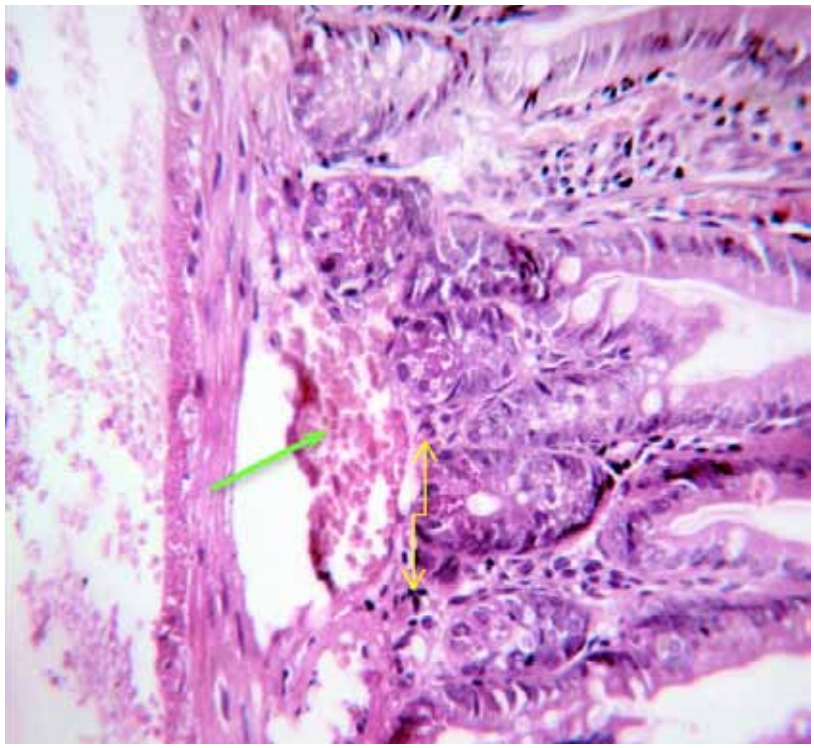

Figure 4: Histopathological section in intestine of infected mouse after orally administered via (0.2 ml contain 1x10^{7}cfu/ml of *S. typhimurium*), shows severe infiltration of inflammatory cells within and between the mucosa intestinal gland (green arrow) as well as severe congestion that appear in the serosa layer (yellow arrow)(H & E stain, 40X).

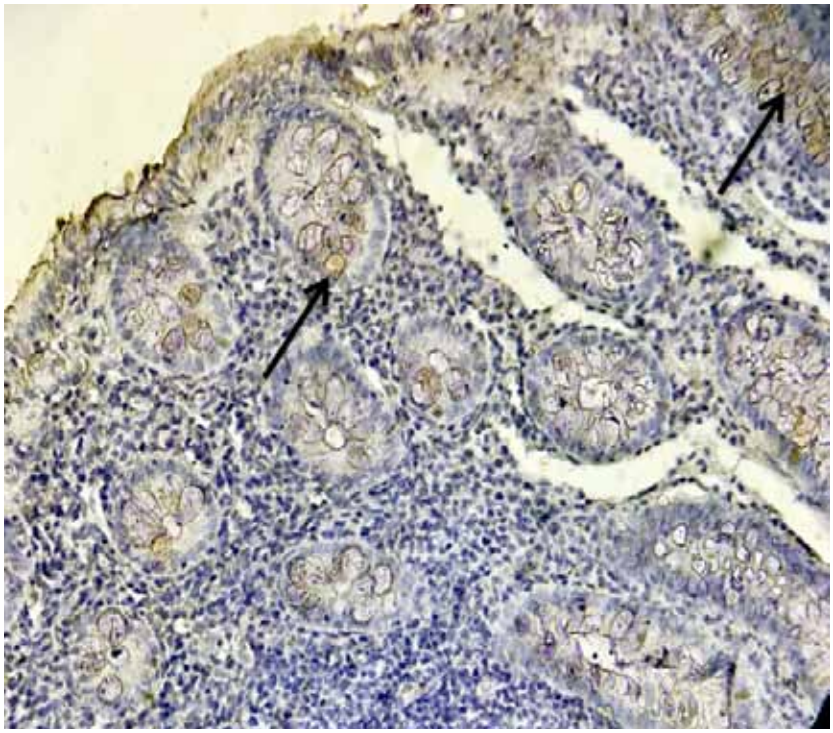

Figure 5: Immunohistochemistry section in intestine of infected mouse after orally administered via (0.2 ml contain 1x10^{7}cfu/ml of *S. typhimurium*), shows infiltration of immunohistochemical staining positive cells of CD8, that appear as deposited brownish intracellular color within and between the mucosa gland and intestinal villi (Diaminobenzidine staining, 40X).

Mean quantities of CD8+ T lymphocytes marked (table 1) with specific antibody by immunohistochemistry in tissue section of the Liver and Intestine (duodenum, jejunum, and ileum) of infected mice after orally administered via (0.2 ml contain $1x10^7$cfu/ml of *S. typhimurium*).

Table 1: Quantities of CD8+ T cells in detected in liver and small intestine

Organ	Control group Mean±SE	Infected group Mean±SE
Liver	9.74 ± 0.69	24.25 ± 0.83*
Duodenum	8.70 ± 0.55	26.39 ± 0.86*
Jejunum	8.45 ±0.44	24.97 ±0.55*
Ileum	8.25 ± 0.60	22.95 ± 0.71*

*=Presence of significant differences between groups ($P \leq 0.05$).

The results of bacterial isolation from internal organs revealed positive isolation from duodenum and jejunum after 12 hours of giving the infective dose of *S. typhimurium* orally, while the bacterium was seen after 24 hours post infection. However, invasion of *S. typhimurium* to the liver was detected after 48 hours (table 2).

Table 2: Bacterial isolation from internal organs at different time points.

Time	Organ			
	Duodenum	Jejunum	Ileum	Liver
7 hours	-	-	-	-
12 hours	+	+	-	-
24 hours	+	+	+	-
48 hours	+	+	+	+
Control	-	-	-	-

The serological results revealed detection of fair levels of antibodies against oral dosing of pathogenic *S. typhimurium* measured by Widal test after 2 weeks post infection (table 3). The antibody titer was 71.11 pg/ml against *S. typhimurium* O-antigen whereas, it was 94.22 pg/ml against *S. typhimurium* H-antigen. The antibody titers measured by Widal test were re-tested by ELISA and they were 150.83 pg/ml and 95.44 pg/ml for *S. typhimurium* protein and LPS antigens respectively (table 4).

Table 3: Mean quantities of antibodies measured by Widal test for (O and H) antigens (pg/ml).

Time	Mean quantity of antibodies against O-antigen (pg/ml)	Mean quantity of antibodies against H-antigen (pg/ml)
Infected	94.22	71.11
Control	0	0

No significant differences between groups (P>0.05)

Table 4: Mean quantities of IgG antibodies measured by ELISA test for both protein and LPS antigens (pg/ml).

Time	Mean quantity of IgG against protein antigen (pg/ml)	Mean quantity of IgG against LPS antigen (pg/ml)
Infected	150.83*	95.44
Control	0	0

* Presence of significant differences between groups (P<0.05)

Discussion

The genus *Salmonella* in general was extensively studied in Iraq in both animals and humans [28, 29, 30, 31, 32]. However,there was no research done with regards to immunopathological studies on *S. typhimurium* in Iraq. There was one study in Iraq[33] who isolated this bacterium from chicken meet.

The histopathological lesions caused by oral dosing of *S. typhimurium* in mice in this study revealed massive destruction to the epithelium of intestine accompanied by mononuclear cells (MNCs) infiltration as well as invasion of these bacteria into liver which caused inflammatory reactions represented by infiltration of MNCs into the hepatic tissue which developed into a hepatic necrosis to wide areas of liver in addition to scattered hemorrhages in the hepatic tissue. These findings are in line with previous studies [34, 35, 36] who reported microbial invasion and colonization of *S. typhimurium* to the enteric canal (GIT) of mice causing diarrhea (ranged from mild to severe bloody type). However, further research similar to we have found with regards to spreading of *S. typhimurium* into hepatic tissue was investigated by [37, 38] who described the penetrative ability of *S. typhimurium* to infect liver.

The immune response against *S. typhimurium*was measured in this study by three method (Immunohistochemistry "IHC", Widal test and ELISA). The cellular immune response was determined by IHC method to search for cytotoxic T cells (CD8+ T cells) in the liver which were prevalent in the slide sections. This finding is in line with previous studies [39, 40, 41] who demonstrated that CD8+ T cells (T cytotoxic cells) are the predominant cell type in the immune response to *S. typhimurium* and explained how these lymphocytes killed the bacterium through production of bactericidal cytokines such as interferon gamma (IFN-γ) and interleukins (IL-17 and IL-23).

Humoral immune response was also assayed to measure the levels of antibodies in the sera against *S. typhimurium*. Initial attempts were made to measure the titer of antibodies through Widal test and then confirmed by ELISA (more specific and sensitive).IgG titers were relatively low in this study against *S. typhimurium* which was (150.83 pg/ml) against protein antigen and (95.44 pg/ml) against LPS. The titers of IgG were much higher in a study to [42] who measured IgG in the blood of mice orally dosed with bovine lactoferrin and infected (challenged by) pathogenic *S. typhimurium*. The titer of IgG measured by ELISA ranged from about 300 to 3400 pg/ml measured at multiple time points (7 days, 14 days and 21 days) post challenge.

Another study in Iran was performed to study the effect of alum as an adjuvant while vaccination against endotoxin-removed lysates of *S. typhimurium* in mice. They used ELISA to measure the titer of IgG which was significantly higher in the vaccinated groups by comparison with controls (given PBS only) at a level ($P<0.05$) plus a significant increase in the leucocytes count (mainly T helper 1 cells) compared with controls [43] which is approximately in line to what we found.

Further research done by [44] was applied to study antibodies' titers (IgA, IgM and IgG) against African O antigen of *S. typhimurium* and found that IgG was increased 4 logs in the vaccinated mice by comparison with controls after two weeks of immunization. This finding is much higher than our results.

A contemporary study to [45] found that *Rag1*$^{-/-}$ mice has the ability to eliminate *S. typhimurium* from the gut through the antibacterial activity of IgG in the mucus of GIT that immobilize bacteria which explains the beneficial role of humoral immunity against this bacteria and this is in agreement to the findings of our study.

Further serological research to compare between pIgR knockout mice and wildtype mice was done to detect both IgA and IgG in the sera samples and stool specimens in response to oral dosing and intravenous injection of pathogenic *S. typhimurium*. The concentration of IgA and IgG measured by ELISA demonstrated significant increase of IgA and IgG ($P<0.05$) in the sera of pIgR knockout mice by comparison with the wildtype mice which resulted in elevation of survival rates in these mice against *S. typhimurium* infection [46] with which we agree.

Finally, a recent study to [47]discovered an essential role of core fucosylation in the immunological mechanism of Fut8$^{+/+}$ and Fut8$^{-/-}$ mice against *S. typhimurium* infection. They demonstrated significant lower concentrations of IgA and IgG ($P<0.05$) in Fut8$^{-/-}$ mice plus remarkable fall ($P<0.05$) in lymphocytes count (both B and T cells) which is in contrast to what we found.

In conclusion, pathogenesis of oral dosing with *S. typhimurium* was studied in mice which spread into intestine and liver within 48 hours and this was examined by bacterial isolation from the infected organs and histopathological pictures. IgG antibodies against *S. typhimurium* were also detected.

Declaration of Interest: This research article is self funded by the authors. The authors did not receive any fund or financial support to accomplish this work.

Conflict of Interest: The authors declare no conflict of interest.

Ethical Approval: Ethical Clearance was taken from the Committee of College of Veterinary Medicine, University of Baghdad.

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