GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

IMMUNOPATHOLOGICAL STUDY IN MICE REPRODUCTIVE SYSTEM INFECTED WITH *SALMONELLA ENTERITIS*

Salema, L.H
Dept of Pathology and Diseases, College of Vet. Medicine, University of Baghdad.

ABSTRACT
Infection with *salmonella* Enteritis was demonstrated immunohistologically in the mice reproductive tissues. Female BALB/c mice were infected with 5×10^8SE cells. After 10 weeks, the animals were sacrificed; uterus, ovary and oviduct were fixed and the slides were processed for IHC. Immunohistochemical staining identified numerous *Salmonella* Enteritis associated with the superficial surface of the mucosal epithelium, the lamina propria of the oviduct, as well as inside the endometrial glands and epithelium, and found in the tissue of the ovarian stroma, in the follicular wall including superficial and theca layers, and occasionally most often seen as diffuse or amorphous mainly in the macrophage, fibroblast and neutrophil, as brown finely granular or some time diffuse intra cytoplasmic staining.

KEYWORDS: mice, reproductive system, *Salmonella* Enteritis.

INTRODUCTION

Salmonella infections are zoonotic; they can be transmitted by humans to animals and vice versa. *Salmonella* Enteritis causes a wide range of infections in birds and mammals and food poisoning in humans (Tindall *et al.*, 2005). *Salmonella* are transmitted orally through the ingestion of contaminated food or water but can also be acquired through handling of pet reptiles and amphibians (Haraga *et al.*, 2008). Among characterized contaminated food sources are raw unpasteurized milk and milk products (AAP, 2014).

Salmonella is an intracellular bacterium. For many intracellular organisms including *Salmonella*, cell-mediated immunity plays a major role (George *et al*., 1987), and protection from Salmonella by humoral defense mechanism alone seems unlikely. Kaufmann, (1993) reported the acquired resistance to the infection with intracellular bacteria such as *Salmonella* is mediated by various cytokines, which are secreted by activated $CD4^+$ T lymphocytes. *Salmonella* produced changes in T lymphocytes, B lymphocytes and macrophages in the oviducts and ovaries. The relationship of decreased *Salmonella* recovery with elevated lymphocyte and macrophage numbers strongly due to local cell-mediated immunity is induced in controlling SE injection in the ovaries and oviducts. *(*Withanage *et al*., 2003).The My D88-dependent signaling pathway correlation with the transcription of a variety of cytokines and chemokines characteristic of ST infection (*e.g.* IL-6, Ify, TNF-a, and IL-12) (Eckmann and Kagnoff 2001). Different strains of mice show different levels of susceptibility to Salmonella infection (Hormaeche *et al.*, 1985). Therefore, mouse strains have been used successfully to study complex host–pathogen interactions. The lamina propria is the connective tissue underlying the epithelial cell monolayer. It consist from multiple immune effector cells including B cells, T-cells, dendritic cells, natural killer (NK) cells, macrophages, eosinophils, and mast cells. If enteric pathogens are capable of surmounting the barriers described above and penetrate the intestinal epithelium, a coordinated immune response utilizing these immune effector cells is activated. Sampling of luminal antigens occurs in specialized cells called M cells, which transport the antigens to a sub epithelial region where the antigen comes in contact with dendritic cells. Dendritic cells bound to antigen then migrate to the mesenteric lymph node to present the luminal antigens to naïve T-cells and B cells. These naïve lymphocytes then differentiate into several effector cells including CD8 cytotoxic T-cells, CD4 helper T-cells, regulatory T-cells, and antibody secreting B cells (Patel and McCormick, 2014). Rai *et al.* (2014) reported *Salmonella* cause a variety of adverse pregnancy complications and severe disease such as transplacental infections, abortions, chorioamnionitis neonatal and maternal septicemia. *Sallmonella* can be isolated from different organs including ovary and oviduct of the infected birds (Keller *et al*., 1997). The presence of immunocompetent cells, including antigen-presenting cells and T and B cells has been shown in the oviduct (Zheng *et al*., 2001) and ovary (Barua *et al.*, 2001; Barua and Yoshimura, 1999). The aim of the present study for detection of *Salmonella* Enteritis experimentally infected reproductive mice in the (ovary, oviduct and uterus) correlated with immune cells including T-cells, dendritic cells, macrophages by Immunohistochemical.

MATERIALS & METHODS

Tissue culture

Salmonella Enteritis serotype was obtained from Zoonoses Unit/ Veterinary Medicine/ Baghdad University, and the biochemical properties were tested depending on the method of (Quinn *et al*., 1998).

Experimental Design

Twenty females mice were used in this experiment. The females that divided into two groups:

1- The 1St group (n=10) (G1), was infected by Salmonella Enteritis bacteria through intraperitoneal route with the dosage 1×10^8 depending on the method of (Baron et al.,1998), and the mice of this group were killed after 20 day post challenge .The reproductive organs were taken for making the immunohistochemsitryl sections.

2- The 2ndgroup (n=10) was administrated orally with 0.3 ml of normal saline and considered as negative control group (G2).

Materials of Immunohistichemsitry CD+4 T cell

Primary antibody (Mouse anti CD4)/US Bioiogical (USA).

Immunohistichemsitry

A- Paraffin- embedded sections from each specimen were cut at 4 μm, mounted on glass and dried overnight at 37˚C.

B-To determine the signal specificity, negative and positive control slides were included for each immunohistochemical run

For the tissue collection, the mice were killed and then the ovary, oviduct, uterus, and vagina), were collected and fixed with 10% formaldehyde in PBS. The tissues were embedded inparaffin and sections of them (4-5µmin thickness, 10 sections for each tissue) were prepared and air-dried at room temperature.The Slides were dipped sequentially as followed:

- The tissue sections were deparaffinized in xylene (2×10min) and Re-dehydrated through graded alcohol as follow:

Two changes of xylene were used for 15 minutes.

-Two changes of absolute ethanol were used for 5 minutes.

- Ethanol 95% for 5 minutes.
- Ethanol 70% for 5 minutes.
- Immersion in distilled water for 5 minutes
- Endogenous peroxidase activity was blocked, using H_2O_2 (1.5 %) (1.5ml). H_2O_2+ 98.5 ml D.W.) for 5-10 minutes.

Then sections were incubated with primary antibody (1:30) 37c for overnight. Then sections were incubated with secondary antibody (biotin) 37c for 40min. Application of substrate–chromagen solution (DAB){ (0.75 ml +5 drops substrate+ 1drop DAB +1 drop proxidase)} for 30 min or until the brown color appeared

- Immersion in D.W for 5 minutes .

Washing in tap water for 2 minutes Counter staining with hematoxylin for 15 seconds. Washing in Tap water. After that Dehydration

We done the number of positive cells in the staining tissues, at least 10 fields were chosen randomly at a magnification of 40 as described by (Yasuda *et al.*, 2011).

RESULT

Immunohistohistochemical assays of CD4+ T cell.

In the tissue of infected animals in contrast to control negative groups

Table-(1shows that the infected mice appeared high level of CD4+ T cell in ovary, uterus and oviduct(80.21±0.11, 98.0.51±0.32, 78.32 ±0.17 respectively), compared with control negative groups (22.52 ±0.36, 21.34 ±0.71, 78.32 ±0.17 respectively).

TABLE 1: Density of CD4+ T cell in infected animals in contrast to control negative groups.

Group	Ovary		Uterus		Oviduct	
G1	80.21±0.11	A	98.0.51±0.32	A	78.32±0.17	A
G2	22.52±0.36	B	21.34±0.71	B	20.49±0.12	B

Means with different capital letters means significantly (P≤0.05) different

The IHC staining of the infected animals revealed the positive reaction products were located in the cells of each tissue. The tissues of the ovary, oviduct, Immuno histochemical staining identified numerous *S. enteritis* cells associated with the:-

Ovary

Immunohistochemical staining identified numerous *Salmonella* Enteritis associated with the preprimary follicle (Fig:1), pre secondary follicle (Fig:2,5,6), and ovarian stroma (Fig:3,4).

Uterus

Immunohistochemical staining identified numerous *Salmonella* Enteritis associated with the epithelial cell and utern gland (Fig: 7), in muscular layer (Fig: 8), the epithelial and stromal layer (Fig: 9), serosal layer (Fig:10), also mainly in the macrophage, fibroblast and neutrophil, as brown finely granular or some time diffuse intra cytoplasmic staining.

Oviduct

Large numbers of *S. enteritis* bacteria were confirmed by immunohistochemical staining in areas of inflammation in oviduct the inflammatory infiltrate was primarily mononuclear and present in the lamin propria. And adhered to the epithelium and desquamated cells, as well as inside macrophages (Fig: 11, 12). Nevertheless a strong positive reaction was found at the surface of the epithelium. No IHC reaction was observed in non-infected animal.

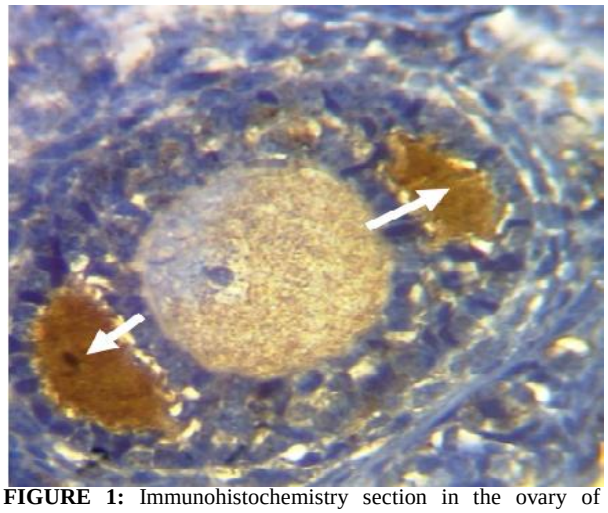

FIGURE 1: Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown color) positive preprimary follicle Stained by (DAB-chromogen (Brown color) immunostaining, X40).

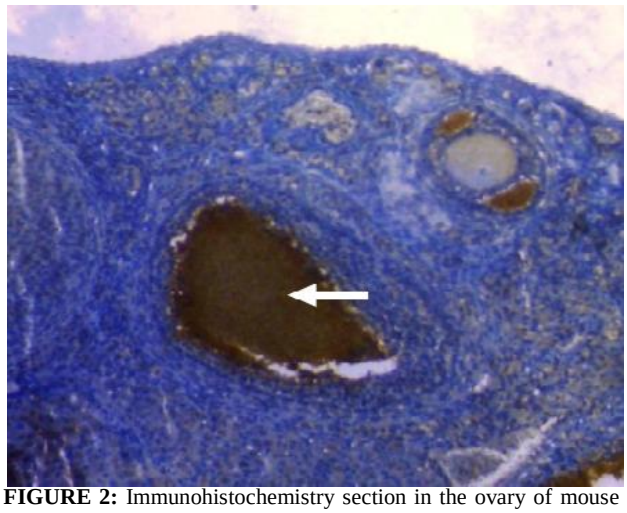

FIGURE 2: Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown pre secondary follicle Stained by (DAB- color) positive chromogen (Brown color) immunostaining, X10).

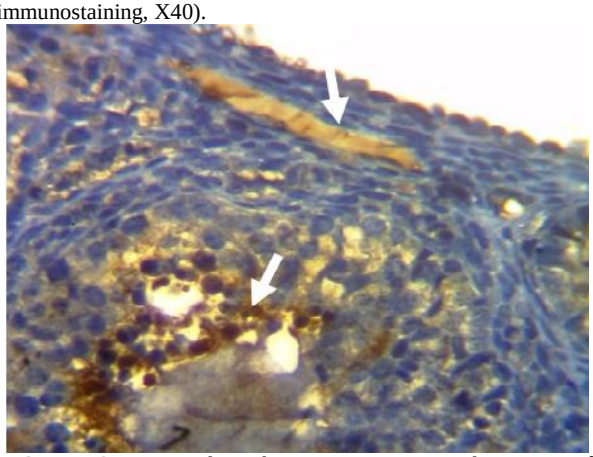

FIGURE 3: Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown color) positive pre follicle and ovary stroma Stained by (DAB-chromogen (Brown color) immunostaining, X40).

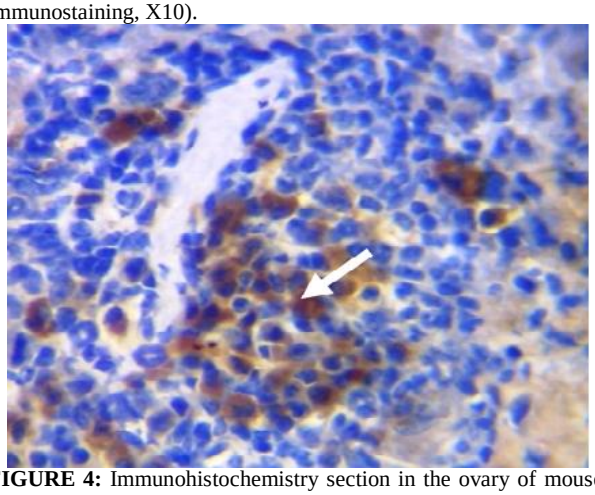

FIGURE 4: Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown color) positive ovary stroma Stained by(DAB-chromogen (Brown color)immunostaining,X40).

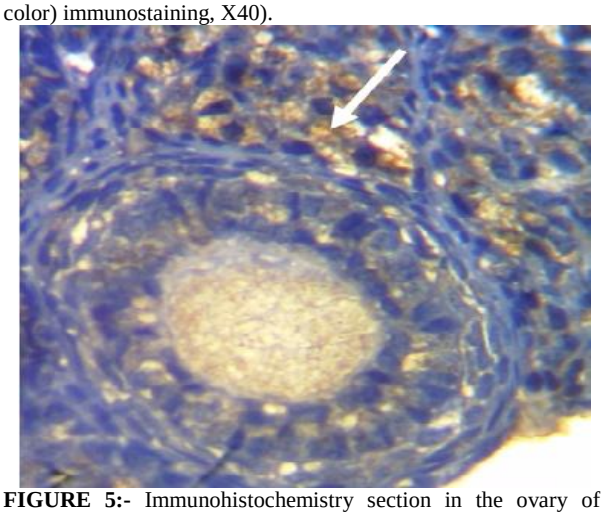

FIGURE 5:- Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown color) positive preosecondary follicle Stained by (DAB-chromogen (Brown color) immunostaining, X40).

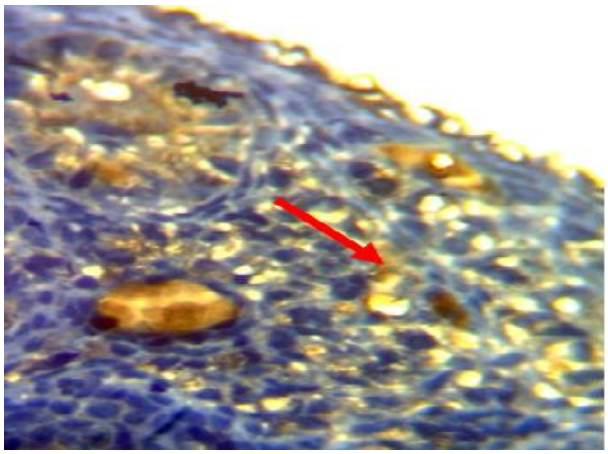

FIGURE 6:- Immunohistochemistry section in the ovary of mouse G1 showed CD8 Immuno cell (brown color) in the ovay stroma and pre follicle Stained by (DAB-chromogen (Brown color) immunostaining , X40)

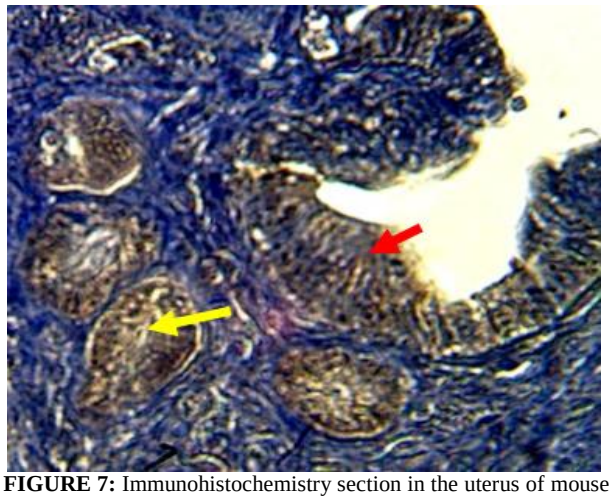

FIGURE 7: Immunohistochemistry section in the uterus of mouse G1 showed CD8 Immuno cell (brown color) positive in epithelial cell and utern gland Stained by (DAB-chromogen (Brown color) immunostaining, X40).

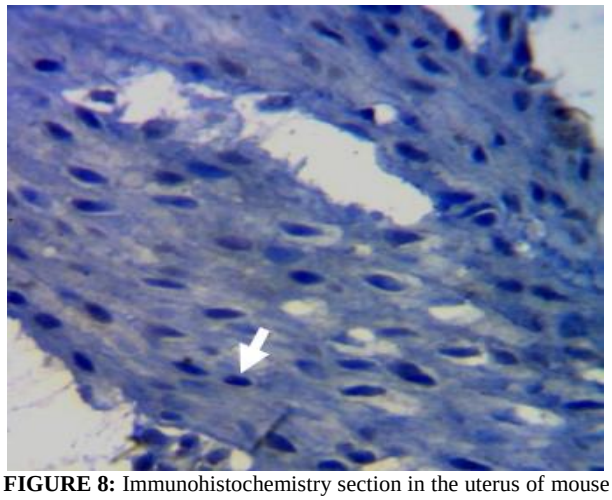

FIGURE 8: Immunohistochemistry section in the uterus of mouse G1 showed CD8 Immuno cell (brown color) positive in muscular layer Stained by (DAB-chromogen (Brown color) immune staining, X 40).

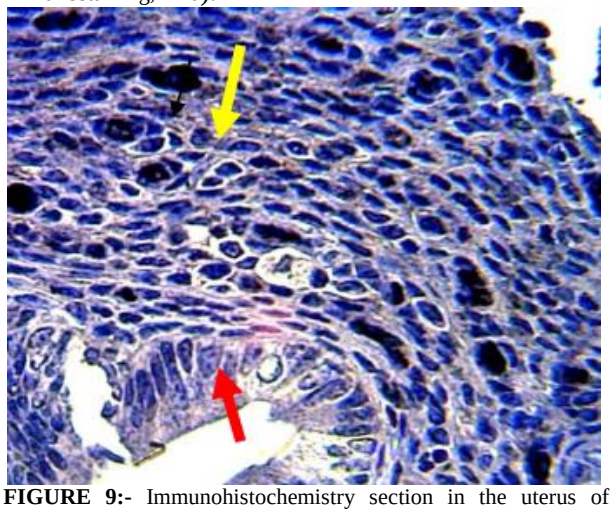

FIGURE 9:- Immunohistochemistry section in the uterus of mouse G1 showed CD8 Immuno cell (brown color) positive in the epithelial and stromal layer Stained by (DAB-chromogen (Brown color) immunostaining, X40).

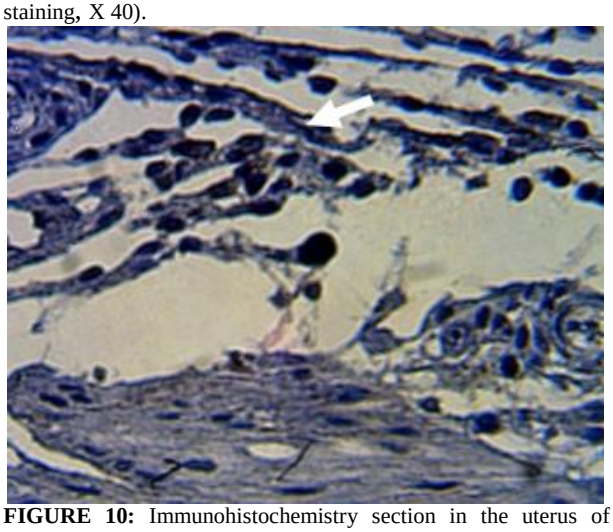

FIGURE 10: Immunohistochemistry section in the uterus of mouse G1 showed CD8 Immuno cell (brown color) positive in serosal layer Stained by (DAB-chromogen (Brown color) immunostaining, X40).

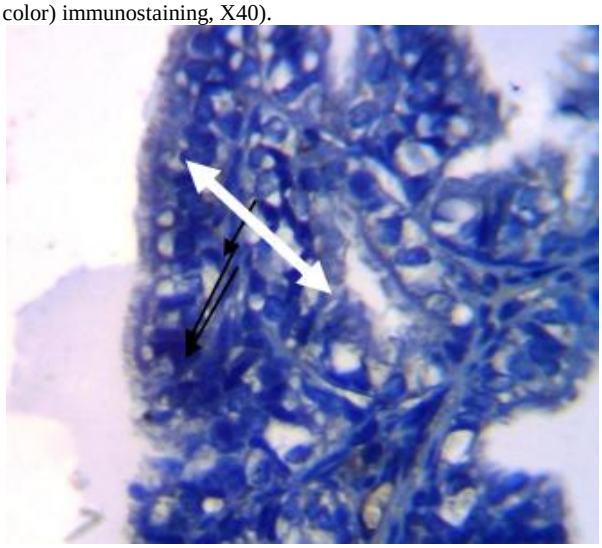

FIGURE11: Immunohistochemistry section in the oviduct of mouse G1 showed CD8 Immuno cell (brown color) positive in epithelial cell and lamina properia Stained by (DAB-chromogen (Brown color) immunostaining, X40).

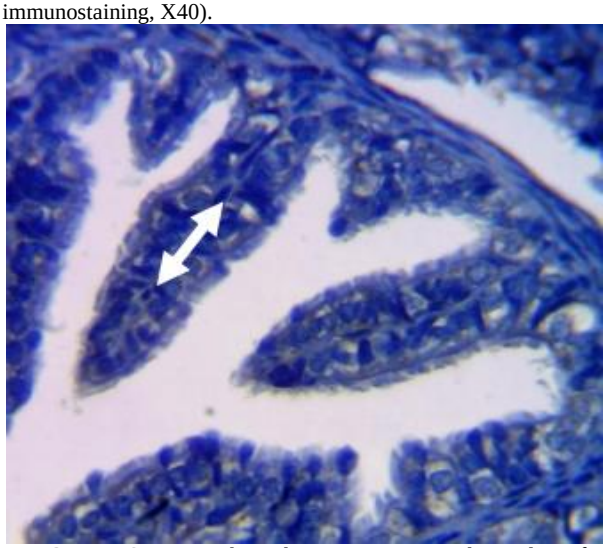

FIGURE 12: Immunohistochemistry section in the oviduct of mouse G1 showed CD8 Immuno cell (brown color) positive in epithelial cell and lamina properia Stained by (DAB-chromogen (Brown color) immunostaining, X10).

DISCUSSION

We have examined how the SE colonizes in the reproductive organs of mice that were intraperitoneally inoculated with SE by immunocytochemistry. The significant finding of this study is that the SE reaction products were localized in the tissues and cells of ovary, oviduct and uterus. The localized reaction in the ovarian stroma, in the follicular wall including superficial and theca layers suggest that the SE in the invaded peritoneal cavity and pass through superficial epithelium surrounding lamina properia of oviduct these result agree with (Popiel and Turnbull, 1985), revealed the passage of Salmonella organisms from epithelium to lamina propria of chick ileocecal mucosa, as well as (Keller *et al.*,1995), described that the SE appeared in the medulla of ovary, cortical stromal tissue of atretic follicle , and mucosal epithelium of oviduct. The result showed size differences between the variably stained bacteria could be due to combination of the polysaccharidal bacterial capsule antigens with the specific antibody, resulting in an apparent capsule swelling. These result agreed with Weintraub (2003) as well as an antigens in particular vary during the journey through the body, to avoid adverse immune reactions and long term persistence (Woude, 2004). The result recorded *Salmonella*-infected macro phages that undergo apoptosis. These results consistent with results obtained by studies with mice which recorded *Salmonella* grows in phagocytic cells that reside in well-defined pathological lesions are activated by cytokines and control the growth of intracellular bacteria using oxygen and nitrogen derivatives, and Salmonella growth in the tissues results in the spatial segregation of bacterial populations and in their continuous distribution to new phagocytes**. (**Mastroeni and Sheppard 2004 **;** Sheppard *et al*., 2003).

The colonization of *salmonella* within epithelial cells correlated with the levels of cytokine production. A Th1-cytokine reaction was observed together with a strong T-cell influx in the tissue. Remarkably, mRNA expression of IL-2 and IL-2Rα and the CD4+T cell influx seemed to be dependent on the infection of epithelial cells (Rieger *et al*., 2015).

Also Monack *et al.,* (2004b), recorded one year after infection most of the bacteria (85% of infected cells) are visualized in macrophages (MOMA-2-positive cells) located in the MLN. These macrophages also carry an average of three to four bacteria.

The current result showed that the mean values of CD4+Tcell in the infected animals with *Salmonella* was significant (P≤0.05) higher than those values in the 2rd group (control group), this could indicate that *Salmonella* infection activates inflammatory pathways in response to infection with *S. enteritis*. The binding of TLRs and NOD1/NOD2 proteins to their respective ligands activates the NF-kB pathway leading to production of pro-inflammatory cytokines and chemokines, secretion of the cytokine IL-8 recruits neutrophils and is necessary for PMN migration into the sub epithelium. Additional chemokines, such as CCL20, plays a role in attracting immature dendritic cells, which upon exposure to antigen, can mature and present antigenic peptides to naïve B and T-cells in the mesenteric lymph nodes (Murphy, 2012**),** as well as *Salmonella* react with TLRs on macrophages in the subepithelial region after being transcytosed through M cells, thereby activating and inducing them to also produce cytokines and chemokines. Cytokines produced by these activated macrophages include IL-1, IL-6, IL-23 (Godinez I *et al.,* 2009). And IL-18 and IL-12, both of which drive the IFN-gamma-dependent production of antigen-specific T cells (Murphy, 2012).

In conclusion, the current results suggest that the SE in the peritoneal cavity can invade and colonize the ovarian and oviductal tissues, as well as *sallmonella* infection causes high levels of CD4+Cell compared with negative control group.

REFERENCES

American Academy of Pediatrics (2014) Consumption of raw or unpasteurized milk and milk products by pregnant women and children, Pediatrics; 133(1):1759.

Baron, E.J., Gilman, J.P.W. & Mcsherry, B.J. (1994) Bailey and Scott's Diagnostic Microbiology, 9thed., Mosby, St.Louis,pp:761-766.

Barua, A. and Yoshimura, Y. (1999) Effects of aging and sex steroids on the localization of Tcell sub sets in the ovary of chicken, *Gallus domesticus.* Gen. Comp. Endo crinol.114:2835.

Barua, A., Michiue, H. andYoshimura, Y. (2001) Changes in the localization of MHC classII positive cells in hen ovarian follicles during the process off ollicular growth, post ovulatory regression and atresia. Reproduction, 121:953–957.

Eckmann, L. and Kagnoff, M.F. (2001) Cytokines in host defence against Salmonella. Microbes Infect. 3: 1191-1200.

George, A., Nair, R., Rath, S., Ghosh, S.N. and Kamat, R. S. (1987) Regulation of cell mediated immunity in mice immunized with *Salmonella* enteritidis. Journal of Medical Microbiology, 32: 239–24۴.

Godinez, I., Raffatellu, M., Chu, H., Paixão, T.A., Haneda, T., Santos, R.L. (2009) Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype typhimurium in the intestine. Infect Immun., 77(1):387–9810.

Haraga, A., Ohlson, M.B., Miller, S.I. (2008) Salmonellae interplay with host cells. Nature Reviews Microbiology; 6(1):53-6

Hormaeche, C.E., Harrington, K.A. and Joysey, H.S. (1985) Natural resistance to salmonellae in mice: control by genes within the major histocompatibility complex. J. Infect. Dis. 152:1050–1056

Kaufmann and Kaufmann (1993) Immunity to intracellular bacteria. Annual Review of Immunology, 32: 129–163.).

Keller, L.H., Benson, C.E., Krotec, K. and Eckroade, R. (1995) *Salmonella* enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. Immun.63:2443–2449.

Keller, L.H., Schifferli, D.M., Benson, C.E., Aslam, S. and Eckroade, R.J. (1997) Invasion of chicken mreproductive tissues and forming eggs is not unique to *Salmonella* enteritidis.AvianDis.41:535–539.

Mastroeni, P. & Sheppard, M. (2004) *Salmonella* infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. Microbes Infect. 6:398-405.

Monack, D.M., Bouley, D.M. and Falkow (2004) *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFN gamma neutralization. J. Exp. Med. 199:231-241.

Murphy K. (2012) Janeway's Immunobiology. New York, NY: Garland Science.

Patel, S. and McCormick, B.A. (2014) Mucosal Inflammatory Response to *Salmonella typhimurium* Infection. Front Immunol. 5: 311.

Popiel, I. and Turnbull, P.B. (1985) Passage of *Salmonella enteritidis* and *Salmonella Thompson* through chick ileocecal mucosa. Infect. Immun. 47:786–792.

Quinn, P.J., Carter, M.E., Markey, B. & Carter, C.R. (1998) ClinicalVeterinary Microbiology.Pp 261-267.M. Wolfe. London.

Rai, B., Utekar, T., Ray, R. (2014) Preterm delivery and neonatal meningitis due to transplacental acquisition of non-typhoidal Salmonella serovar Montevideo. BMJ Case Reports; 2014:13.11

Rieger, J., Janczyk, P., Hünigen H. and Plendl, J. (2015) Enhancement of Immunohistochemical Detection of *Salmonella* in Tissues ofExperimentally Infected Pigs Eur J Histochem. 59(3): 2516.

Sheppard, M., Webb, C., Heath, F., Mallows, V., Emilianus, R., Maskell, D. and Mastroeni, P. (2003) Dynamics of bacterial growth and distribution within the liver during *Salmonella* infection. Cell Microbiol.5:593-600.

Tindall, B.J., Grimont , P.A., Garrity, G.M. and Euzeby, J.P. (2005) Nomenclature and taxonomy of the genus Salmonella . In: Int. J. Syst. Evol. Microbiol. Bd. 55, p. 521–524.

Weintraub, A. (2003) Immunology of bacterial polysaccharide antigens. Carbohydrate Research, 338: 2539- /2547.

Withanage, T. (2003) Increased lymphocyte sub populations and macrophages in the ovaries and oviducts of laying hens infected with *Salmonella* enterica serovar Enteritidis. Avian Pathol., 32(6):583-90.

Woude, M.W. Van der, Bäumler, A.J. (2004) Phase and antigenic variation in bacteria. ClinMicrobiol Rev .17:581-611.

Yasuda, K., Nirei, T., Sunami, E., Nagawa, H. and Kitayama, J. (2011).Density of CD4 (+) and CD8 (+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. Radiation Oncology, 6:49.

Zheng,W.M., Nishibori, M., Isobe, N., Yoshimura, Y. (2001) Aninsitu hybridization study of the effects of artificialin semination on the localization of cells expressing MHC class II mRNA in the chicken oviduct. Reproduction, 122:581–586.