Immunopathological evaluation of the immunity induced by whole bacteria sonicated antigen of *Brucella melitensis* in guinea pigs

Al-Oubaidy, S.S.(1); Alwan, M.J.(2); Al-Kaisei, B.I.(1)
(1) Department of pathology and poultry, (2) Zoonosis unit / Collage of Veterinary Medicine / University of Baghdad.

Summery:

In order to know the efficacy of whole Bacterial Sonicated Antigens (WBSAgs) of Brucella on immune response in guinea pigs. Eighteen (18) guinea pigs (both sex) were divided randomly to three groups: 1st group (n=6) was immunized with whole bacterial sonicted antigens (WBSAgs) of Brucella melitensis (0.5ml/ 1mg protein) S/C and repeated after 15 days in the same dose, the 2nd group (n=6) and the 3rd group (n=6) served as positive and negative control respectively.

Cell mediated and humoral immune responses were checked at day 27 and 30 post immunization respectively. After 30 days post immunization, the 1st, and 2nd groups were challenged I/P with 1ml containing 2x109 CFU/ml of viable virulent *B. melitensis* and 3rd group inculated with 1ml sterile normal saline.

Three animals from each groups were sacrificed on day 10 and 30 post-challenge.

The result showed that immunization with the WBSAgs of *B. melitensis* result in production of cellular and humoral immune response, as examined by delayed type hypersensitivity (DTH) and indirect heamagglutination test.

Abortion was found in the non-immunized infected animals, where as in the immunized infected animals was not reported.

Mild to moderate bacterial isolation and pathological changes were recorded in the target organs of the immunized infected animals at 10 days post infection, negative or very mild bacterial isolation with few splenic bacterial counts were obtained from the target organs of 1st with hyperplastic lymphoid tissue in their organs at 30 days post infection.

The control positive group showed very heavy bacterial isolation with intense sub-acute to chronic inflammatory reaction were noticed in the examined organs at 10 days post infection, and heavy bacterial isolation with high bacterial counts of spleen were investigated at 30 days post infection.

Mature to epithelioid granulomatous lesions were the main lesion in the target organs of non-immunized infected animals at 30 days post infection.

We concluded that the WBSAgs of *B. melitensis* induce a good protective immune response against the challenge with B. melitensis.

Introduction

Brucellosis is one of the five bacterial zoonoses, worldwide distribution, caused by organism belong to genus Brucella (Corbel, 1997). Brucellosis is recognized as a major cause of heavy economic losses of livestock industry and also poses serious human health and food safety problem in many countries, According to world health organization half million new human cases are reported each year world wide (WHO,1986).

Control of brucellosis in agricultural animals is prerequisite for prevention of the disease in human being, therefore, vaccination might efficiently protect against this disease.

Previously attenuated live vaccine (RevI) has been used to vaccinate small ruminant against *B. melitensis*, but this type of vaccine can cause disease to animals and human and can confuse differentiate diagnosis of active disease (Tabatabai *et al.*, 1989).Therefore, there is a need to research for improved possibly vaccine to induce protective immunity against Brucellosis. Little information's are available on immune response stimulated by whole sonicated brucella antigens. So here we evaluate the effects of the *B. melitensis* Ags in inducing immune response.

Materials and methods:

Organisms: *B. melitensis* virulent isolates obtained from Al-Nahda Veterinary Laboratories.

Culture media: Tryptic Soya broth, tryptic Soya agar and blood agar prepared according to the production manuals.

Microscopic slides for histopathological examination: prepared according to (Luna, 1968).

Pasie heameagglutination: were don according to (Herbert,1978).

Delayed type hypersensitivity were don according to (Hudson and Hay, 1980).

Bacterial isolation and counts from the spleen: Bacterial isolation and counting were carried according to (Pugh, *etal.*, 1989)

Experimental design:

Eighteen (18) guinea pigs were divided randomly into three groups:

1- **1st group:** (n=6) immunized with WBSAgs of *B. melitensis* 0.5ml/1mg protein S/C and repeated after 15 days in the same dose.

2- **2nd group:** (n=6) injected with 0.5 ml of sterile PBS S/C served as positive control.

3- **3rd group:** (n=6) injected with 0.5 ml of sterile PBS S/C served as negative control.

At the 27 day skin test were done and at the 30 day blood sample were collected for passive heameagglutination test.

The 1st and the 2nd group were challenged with *B. melitensis* intra-peritoneally (IP) and the 3rd group injected with PBS IP.

At the 10 and 30 days post infection 3 animals from each group were sacrificed and samples for

bacteriological and others for histopathological examination were taken and fixed with 10% neutral buffered formalin.

Results

1- Cellular immunity (Delayed type hypersensitivity):

The mean of skin thickness against Brucellin of *B. melitensis* in animals which were immunized with WBSAg of *B. melitensis* (1.21± 0.07) was elevated as compared with skin thickness in the control group (Table:1).

2- Humoral immunity (The passive haemagglutination test (PHA)):

The Abs titers showed high value in animals were immunized with WBSAgs of *B. melitensis* (45.33 ± 8.68 (Table:2).

3- Clinical finding:

There is no clear clinical symptoms noticed on the experimental animals post infection with viable virulent *B. melitensis* through the course of the study.

Immunized infected female animals showed normal pregnancy with normal parturition, while non-immunized infected female showed abortion at 5,7,10,and 12 day post infection, and also there is a vaginal bleeding post infection with *B. melitensis* in the positive control group.

4- Bacterial isolation:

4-1- 10 days post infection:

The results showed that *Brucella* was isolated from the most of examined organs of immunized and non immunized animals but the level of bacterial isolates ranged between very mild to moderate in immunized animals as compared with very heavy isolates in the non-immunized animals. Spleen and liver showed more extensive bacterial isolation (Table: 3).

B. melitensis were isolated from fetuses and placenta of the aborted guinea pigs.

4-2- 30 days post infection:

4-2-1- bacterial counts from the spleen:

The bacterial count (CFU/ml) of *B. melitensis* were $21.50X10^{1}$ ± 10.2, and $231.25X10^{3}$ ± 15.2 in the 1st, and 2nd group respectively (Table:4).

4-2-2- Bacterial isolation from the internal organs:

Mild *B. melitensis* were isolated from liver and lung of two animals which were immunized with WBSAgs of *B. melitensis* but heavy bacterial isolates were recovered in the all examined organs of the non-immunized animals except heart and brain which showed very mild bacterial isolation (Table:5).

5-Histopathological examination:

5-1- 10th days post infection:

5-1-I- Control group challenged with *B. melitensis:*

- Lung: Histopathological examination revealed inflammatory cells infiltration in the interstitial tissue and in the lumen of the alveoli mainly macrophages, lymphocytes with few neutrophils (Fig: 1), in addition to the destruction of the alveolar wall and pulmonary collapse. Inflammatory cells infiltrations were seen in the wall of the bronchi and bronchioles with cystic dilation of the mucosal glands.
- Liver: The liver revealed severe centrilobular congestion and hepatocellular necrosis. The lumen of the blood vessels contain inflammatory cells mainly, neutrophils and macrophages. Multifocal granulomatous lesions consisting mainly from aggregation of macrophages scattered through the liver parenchyma were observed in addition to fibrin network and inflammatory cells present between hepatic cells (Fig: 2). In addition, mononuclear cells aggregation around the central vein and blood vessels were noticed.
- Kidney: The pathological picture of the kidney characterized by hypercellularity of glumerular tufts due to proliferation of the endothelial and mesangeal cells. Acute cellular degeneration of the epithelial lining of the renal tubules which characterized by vacuolation of their cytoplasm, in addition to leukocytic infiltration, both macrophages and lymphocyte around blood vessels and renal tubules were reported.
- Spleen: The major changes were acute congestion of the red pulp, infiltration of macrophages, plasma cells and few neutrophils through out white and red pulp as well as depletion of the splenic follicle.
- Heart: There was mononuclear cells infiltration around congested blood vessels between cardiac muscle fibers, their lumen contain inflammatory cells mainly neutrophils and macrophages.
- Brain: There were multiple small vacoules in the tissue poor staining separations of myelinated fiber (edema), inflammatory cells infiltration around congested blood vessels were present in menenges and brain parenchyma.
- Uterus: The microscopic lesions characterized by infiltration of the sub-epithelial layer of the endomaterium with inflammatory cells mainly neutrophils, macrophages, and lymphocytes together with dilation of uterine glands, there lumen were filled with neutrophils (Fig: 3).
- Testis: The histological section revealed thickness of tunica albugina due to inflammatory cells infiltration together with fibrous connective tissue formation as well as atrophy of the simenifrous tubules (Fig: 4)

5-2-II- The 1st group: that immunized with *B. melitensis* WBSAgs and challenged with *B. melitensis*:

- Spleen: There were more pronounced hyperplasia in the white pulp together with muscular hypertrophy of the central arterioles.
- Lung, uterus, testes, and kidney: Lymphoid tissue hyperplasia was reported in these organs (Fig: ٥), in addition to lymphocytic cell infiltration around the blood vessels and between the muscle fibers of the heart.
- Brain: No significant lesions were observed in the brain.
- Liver: The liver showed proliferation of the kupffer cells with apoptotic cells (Fig: ٦) and lymphocyte aggregation around the central vein.

5-2- 30 days post infection:

5-2-I- Control group that challenged with *B. melitensis*:

The microscopic changes in the lung, liver, and spleen were more progressive than those observed in animals infected with *B. abortus*.

- Lung: There was widening of the interalveolar septa caused by severe inflammatory cells infiltration particularly macrophages and polymorph nuclear cells (PMN) cells together with congested blood vessels which surrounded by severe inflammatory cells aggregation . The alveoli had inflammatory cells in their lumen together with multifocal emphysematous and collapse areas as well as multiple granulomatous lesions.
- Liver: The liver has acute cellular degeneration with kupffer cells proliferation and hepatocyte necrosis. As well as inflammatory cells infiltration around the central vein and in the liver parenchyma , together with granulomatous lesions .
- Spleen: Amyloid like substance deposition in the red pulp lead to debilitation of the white pulp, in addition to mononuclear cells infiltration of the red pulp.
- Uterus: Sloughing of the epithelial layer of endometrium with marked inflammatory cells infiltration in the stroma and periglandular tissue together with granulomatouas lesions in sub-epithelial layer .
- Testes: Histopathological examination showed marked inflammatory cells infiltration in the tunica albuginea and between seminiferous tubules which showed degenerative changes necrosis of the seminiferous tubules epithelial cells also reported. The same reaction was seen in the epididymus.
- Other examined organs were showed similar lesions to those reported in animals infected with *B. abortus* but more severe (Fig:7).

5-2-II- The First group that immunized with *B. melitensis* WBSAgs and challenged with *B. melitensis*:

- Spleen: There was marked hyperplasia of white pulp which characterized by large multiple splenic corpuscles together with

proliferation of mononuclear cells around the sinuses of red pulp (Fig:8).

- Lung: No significant lesions were reported in the lung except lymphocytic aggregation around the blood vessels and air ways as well as in the interstitial tissue (Fig:9).
- Kidney: There was lymphocytic aggregation around the blood vessels and collecting tubules (Fig:10).
- Other examined organs showed no clear lesions.

Discussion:

1- The cellular and humoral immunity:

In the present study, we investigated the effect of the whole sonicated *B. melitensis* antigens against this organism in guinea pigs.

The result of skin test and indirect haemagglutination tests indicated that the whole bacteria sonicated antigens *B. melitensis* produced a cell mediated and humoral immune responses.

Cell mediated type of hypersensitivity is the principle pattern of immunologic response to variety of intracellular pathogens, it is initiated by CD_4^+ T cells and direct cell cytotoxicity by CD_8^+ T cells (Ramzi *et al.*, 1994).

The WBSAgs containing all types of *Brucella* antigens. The main antigenic component of *Brucella* are Lipopolysaccharide and protein. Protective immunity against *Brucella* is confirmed by antibody to LPS and T cell mediated macrophages activate in triggered by protein antigen (Corbel, 1997).

Immunity can be achieved with some antigenic fractions extracted from *Brucella*. Elzer *et al.*, (1994) demonstrated that mice injected with killed whole cell *Brucella* stimulate the production of IgG2a and IgG3 to polysaccharide and IFN.γ response. Denoel *et al.*, (1997a) revealed that 18KDa OMP from *B. melitensis* and *B. abortus* induced T cell response, lymphocyte proliferation, IFN.γ production and delayed type hypersensitivity. Carlos *et al.*, (2002) showed that inoculated mice with plasmid DNA containing *Brucella* Lumazine synthesis elicited both Abs and Th1 cells mediated immunity response and confirms protection against *B. abortus*.

However, several investigators are making efforts to obtain immunity using antigenic structure of *Brucella* as sub-cellular vaccine based on DNA and RNA molecules (Rivers *et al.*, 2006).

2- Clinical signs

Normal pregnancy and normal parturition offspring's in immunized animals as compared with non-immunized animals which showed abortion, clearly indicate that the WBSAgs of *B. melitensis* induced a good protective immunity against challenge with *B. melitensis*.

3- Bacterial isolation

Results of bacterial isolation confirmed the results of immunological examination, *Brucella* antigen stimulate cellular immune response in laboratory animals model measured by lymphocyte blastogenisis and protective cytokines (Yifan and Christin, 1995) such as IFN.γ or TNFα. macrophages synthesis of IL-12 and TNFα in early infection results in a synergestic stimulation of natural killer cells to synthesis of IFN.γ (Hsich *et al.*, 1993), this IFN.γ activates resident macrophages to become bactericidal particularly through the production of nitric oxide and also induces Th0 cells to undergo differentiation to CD4+-Th1 cells, the Th1 cells synthesize additional IFN.γ thus positively amplifying the host response, by activated macrophages and have an increased ability to present bacterial antigens to these T cells resulting in their differentiated into armed effectors cells and the sterile eradication of the bacteria (Kaufman *et al.*, 1997), These evidence agreed with result of DTH in the present study. Denoel *et al.*, (1997b) explained that Th1 subsets of CD4+ T cells mediate acquired cellular response and DTH.

4- Pathological finding:

The complete clearance or marked decline of bacterial isolation from examined organs of immunized animals at 30 day post-infection, coinciding with marked lymphoid hyperplasia may be reflect the development of good protection to the challenge with *Brucella*. Maurice (1980) reported that mice which are vaccinated with BCG showed splenomegaly (due to hyperplasia of the white pulp) accompanied with resistance to intravenous challenge with *L. monocytogens*, also Alwan (1996) showed that hyperplastic lymphoid tissue in the internal organs of the immunized animals with *B. abortus* S19, accompanied with high resistance to intra-peritoneal challenge with *Nocardia asteroids*.

The presence of epithelioid granuloma in some examined organs particularly in animals infected with *B. melitensis* may indicate the strain of *Brucella* is highly virulent.

Vander-Gaag *et al.*, (1983) revealed that epithelioid granuloma is high-turnover reaction characteristic of virulent infectious agent in which the inflammatory cell population renewed by infiltration and local cell division. The mechanism by which *Brucella* cause epithelioid granuloma is not well understood. In many granulomatous diseases, the chemical structure of the etiological agents responsible for such lesion were not well known.

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Appendix

Table (1): The means* and standered error of different skin thickness at 27th day post immunization against Brucellin of *B. abortus* and *B. melitensis*.

Group	Brucellin (*B. melitensis*)	
	24hr	42hr
I	**1.21 ± 0.07**	**0.78 ± 0.11**
II	**0.00**	**0.00**
III	**0.00**	**0.00**

* the mean of skin thickness of 6 animals.

Table (2): The means* and standered error of antibody titer at 30 days post immunization against Brucellin of *B. abortus* and *B. melitensis*.

Group	(*B. melitensis*) antigen
I	**45.33 ± 8.68**
II	**0**
III	**0**

- the mean of Abs titer of 6 animals.

Table (3): Bacterial isolation from the internal organs of guinea pigs challenged with *B. mlitensis* at day 10 post infection

Group	No.	spleen	Liver	Kidney	Lung	Heart	Brain
I	1	+++	+++	++	++	-	-
	2	+++	++	+	++	-	-
	3	++	+	+	+	-	-
II	1	+++++	+++++	++++	+++++	+	-
	2	+++++	+++++	++++	++++	+	-
	3	++++	+++++	+++	++++	+	+
III	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-

Table: (4): Bacterial isolation from spleen of guinea pigs challenged with *B. abortus* and *B. melitensis* (CFU/ ml) at day 30 post infection.

Group	Challenge *(B. melitensis)*
I	**$21.50*10^{1}$ +10.2**
II	**$231.25*10^{3}$ + 15.2**
III	**0**

- The mean of bacterial count of 3 animals.

Table (5): Bacterial isolation from the internal organs of guinea pigs challenged with *B. melitensis* day 30 post infection

Group	No.	Liver	Kidney	Lung	Heart	Brain
I	1	+	-	+	-	-
	2	+	-	+	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
II	1	++++	+++	+++++	+	+
	2	++++	+++	++++	+	-
	3	++++	++	++++	+	+
	4	++++	+++	++++	-	+
III	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-

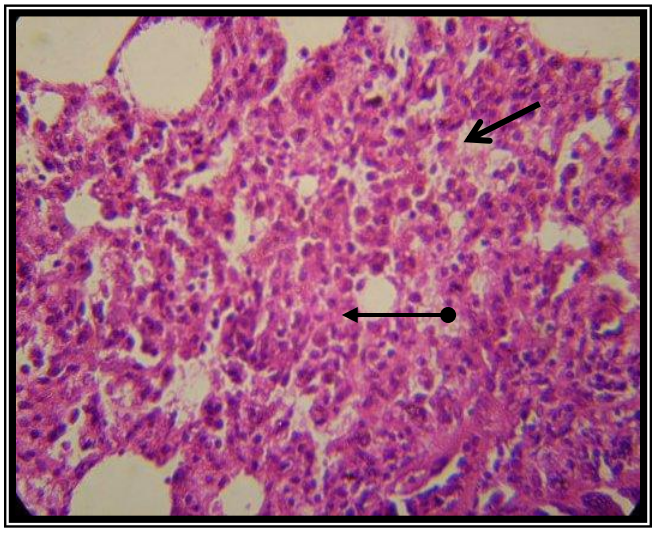

Fig 1: Histopathological section of guinea pig lung, 10 days post infection with *B. melitensis* showed severe inflammatory cells infiltration in the alveolar lumen (←) and interstitial tissue (←●). (H&EX 40).

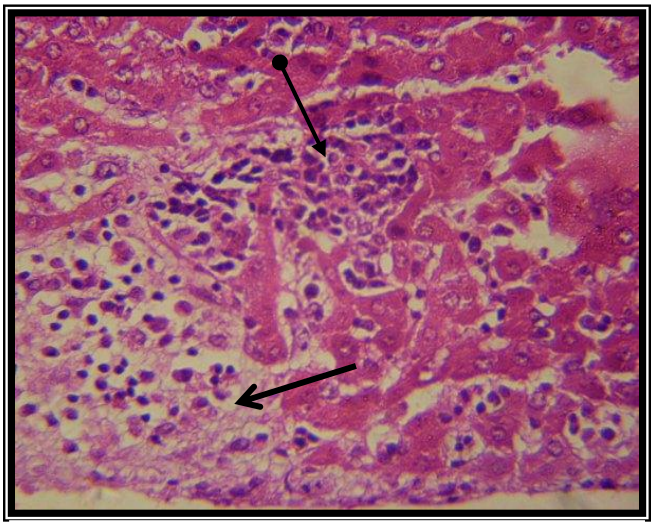

Fig 2: Histological section of guinea pig liver, 10 days post infection with *B. melitensis* notice fibrin network with inflamatory cells infiltration (←) with multifocal granulomatous lesions (←●). (H&EX40).

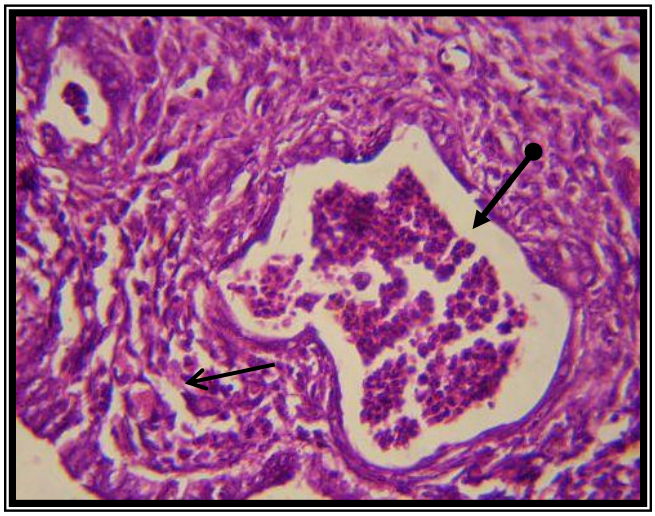

Fig 3: Histological section of guinea pig uterus, 10 days post infection with *B. melitensis* revealed inflammatory cells infiltration in sub-epithelial layer (←) and dilation of the uterine gland with neutrophils in their lumen (←●). (H&EX40).

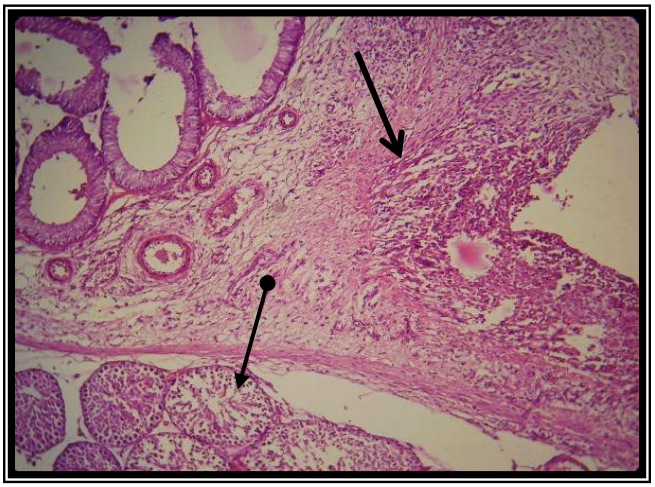

Fig 4: Histological section of guinea pig testis, 10 days post infection with *B. melitensis* revealed thick tunica albugina due to inflammatory cells infiltration together with fibrous connective tisse formation (←) as well as atrophy of the simenifrous tubules (←●). (H&EX40).

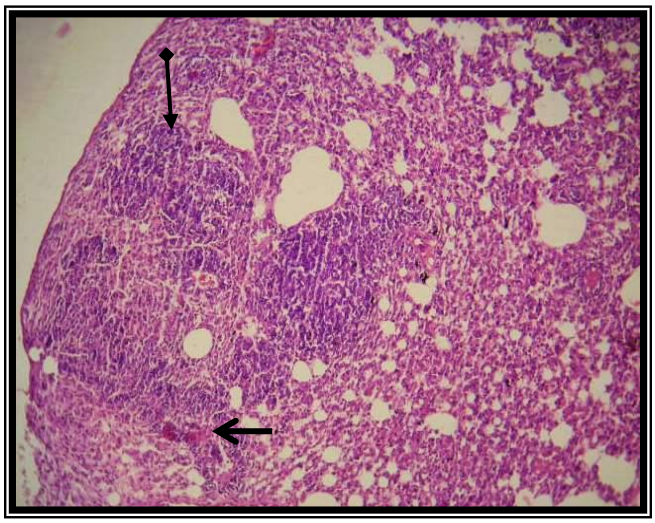

Fig ٥: Histological section of *B. melitensis* WBSAgs immunized guinea pig lung, 10 days post infection with *B. melitensis* notice lymphocytic cells infiltration around the blood vessels(←) and aggregation of lymphocytic cells in the lung parenchyma (←●). (H&EX10).

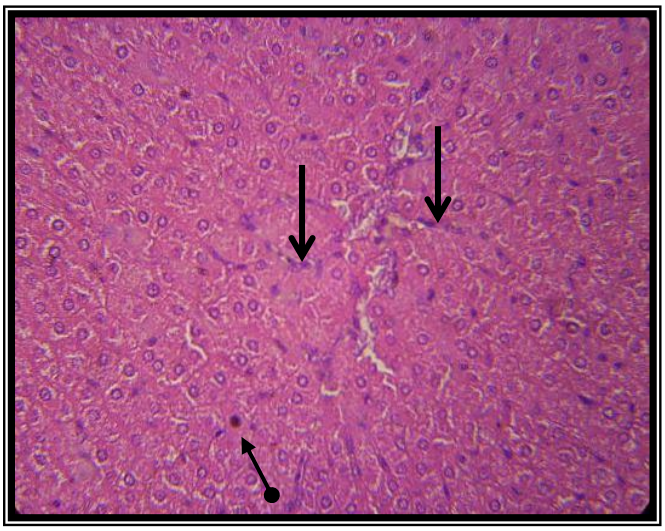

Fig ٦: Histological section of *B. melitensis* immunized guinea pig liver, 10 days post infection with *B. melitensis* notice the proliferation of the Kupher cells (←) and apoptotic cells (←●). (H&EX40).

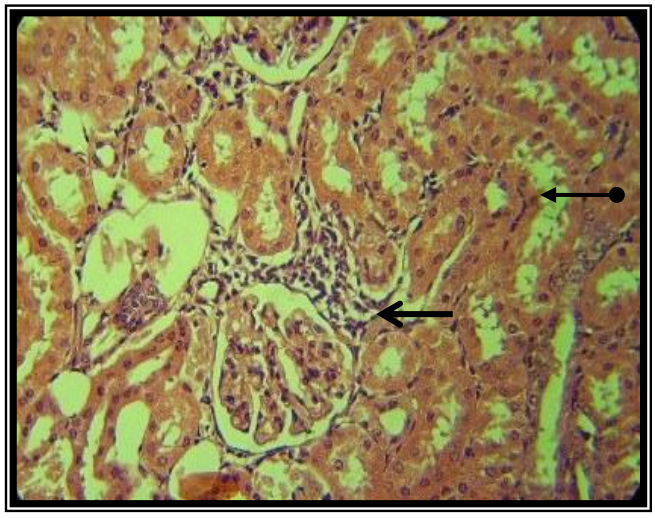

Fig 7: Histological section of non-immunized guinea pig kidney, 30 days post infection with *B. melitensis* showed periglomerular infiltration inflammatory cells and in the interstitial tissue (←●) as well as acute cellular degeneration (←●). (H&EX40).

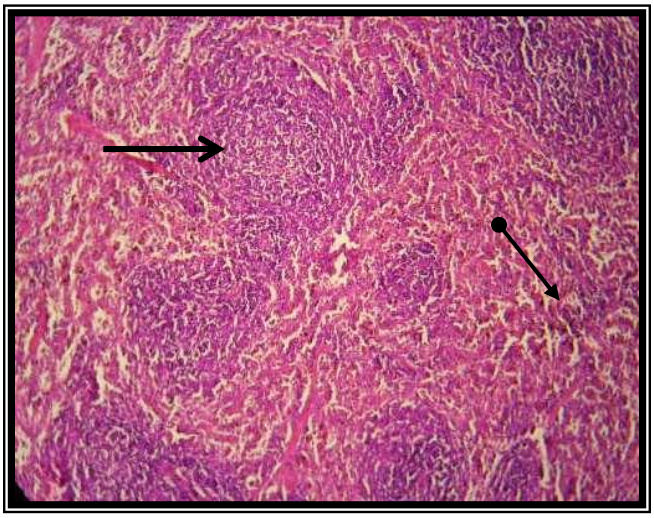

Fig 8: Histological section of *B. melitensis* immunized guinea pig spleen, 30 days post infection with *B. melitensis* showed white pulp hyperplasia(←) with mononuclear cells proliferation around sinuses of the red pulp (←●).(H&EX10).

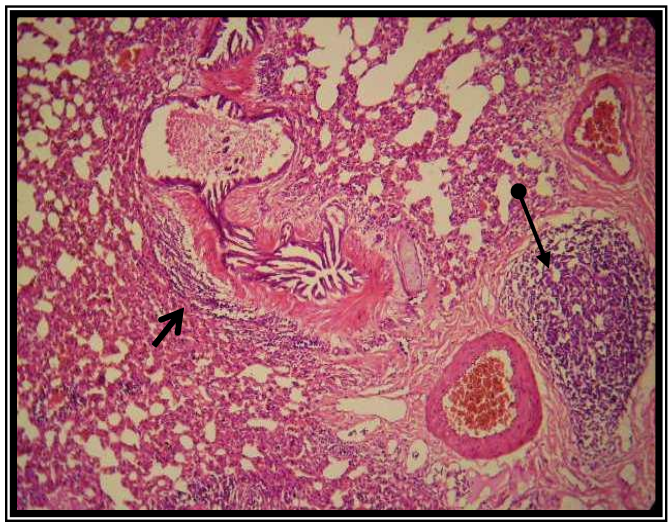

Fig 9: Histological section of *B. melitensis* immunized guinea pig lung, 30 days post infection with *B. melitensis* showed lymphocytic aggregation around the blood vessels and air ways(←) and in the interstitial tissue (←●) . (H&EX40).

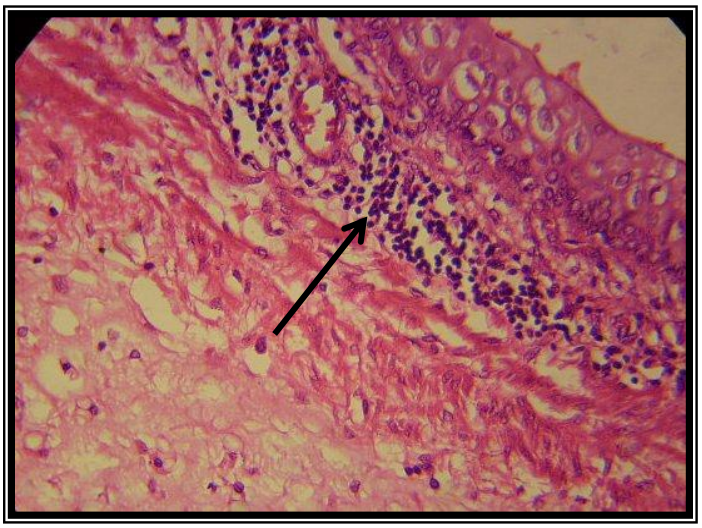

Fig 10: Histological section of *B. melitensis* immunized guinea pig kidney, 30 days post infection with *B. melitensis* revealed lymphocytic aggregation around the blood vessels and collecting tube (←). (H&EX40).