Plant Archives Vol. 20, Supplement 2, 2020 pp. 2016-2025 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

IMMUNOHISTOPATHOLOGICAL AND IMMUNOLOGICAL EVALUATION OF LISTERIA NANOPARTICLE VACCINE DURING THE FIRST AND THIRD SEMESTER IN THE PREGNANCY RAT MODEL

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Abstract

Listeriosis considers one of the important health hazards is a foodborne disease that associated with a high percentage of morbidity and mortality, caused by *L. monocytogenes*, affect different species of animal pregnant and non-pregnant, the bacteria can reach to the placenta and cause many pathological conditions like fetal resorption, stillbirth, healthy offspring, development of novel vaccine give protection against this disease during pregnancy trimesters necessary to maintains fetal and animal intact. Uses of (Al_2O_3: Ag) nanoparticles with KWSLAgs as an adjuvant to eliminate the bacteria and strengthens the immune system (innate and adaptive) response by activation the APCs to produce cytokine, in turn, these inflammatory and proinflammatory cytokines activate cellular immunity by production memory and target cell can protect during pregnancy. All these stages fallowing by IHC detection of bacterial in pregnancy rat and fetal tissue.

Introduction

Listeria monocytogenes, a facultative intracellular pathogen, is the world's third leading cause of death from foodborne disease (Scallan *et al*., 2011), while Listeriosis, silage disease, circular disease, and meningoencephalitis are lethal infectious diseases caused by *L.monocytogenes* and primarily associated with septicemia and encephalitis in humans, poultry, fish and birds (OIE, 2014), High rate of fatality (20–30) per cent of cases), long incubation period and predilection for individuals with an underlying disorder leading to loss of T-cell-mediated immunity (Allerberger, 2003). In addition to abortion, septicemia, mastitis, encephalitis, listeriosis may be associated with endometriosis and frequent breeding in animals (Malik *et al*., 2002).

However, these pathogens can be transmitted from mother to fetus during peripartum and uterus following maternal bacteremia, also other uncommon possible ways include infected animals to humans through skin lesions especially veterinarians and nosocomial transmission(Abay *et al*., 2019; Goenka & Kneen, 2019).

During pregnancy cellular immunity is minimal due to the increased progesterone (Scheule, 2004), making pregnant particularly susceptible to intracellular microorganisms like *L.monocytogenes* (Leber, 2011), very few pathogens are capable of crossing the placental biological barrier with includes some viruses (Koi *et al*., 2001), and very rare bacteria including *Chlamydia psittaci*, *Coxiella burnetti*, *Anaplasma phagocytophilum* and *L.monocytogenes* (Pappas *et al*., 2005; Hamzah and Hasso, 2019). There is compelling evidence that the trophoblast plays a central role in vertical transmission of pathogens from mothers to the fetus (Le Monnier *et al*., 2005), these barrier function may circumvent by certain pathogens that can invade and replication within the fetus (Robbins & Bakardjiev, 2012), particularly partial intracellular life cycle pathogens such as *L.monocytogenes* which successfully targets and multiplies within the cells of the placenta and fetus that lead to the death of the fetus and abortion (Baud & Greub, 2011; Hasso and Al-Janabi, 2019). The principle protective immunity against *L.monocytogenes* is a cell-mediated immune response due to these pathogens is intracellular bacteria (Parmer, 2004), and killing these pathogens is mediated by activated macrophages by Th1 cytokine (IFNy) (Parmer, 2004). There are two arms of host immunity, innate and acquired immune responses, the mechanism efficiency in the early control *L. monocytogenes* infection is innate immune responses, in the murine the Listeria infection can reached the spleen and firstly localized in the macrophages in the marginal zone between area rich with T cells in white pulp and rich with B cells in the red pulp, focus of infection is began in the white pulp due to migration of infected cells to these area than expanded as a result cell to cell spread the pathogen (Conlan, 1996), innate immune response can activated specific adaptive immune response against *L. monocytogens* infection in which the main bridge between these two arms of immunity is DCs (Medzhitov, 2001), which activated by TLRs signaling cascade initiated after recognized bacterial products by these receptors DCs may greater expressed co stimulatory molecules and proinflammatory cytokines after activation by TLR signaling and it is became stronger T cell activator particularly CD8 and CD4 T cells response that form the most of the acquired immune response in addition to B cells and regulatory T cells , in addition to non-classical MHC T cells (Jung *et al*., 2002), CD4 T Cell and CD8 Cytotoxic T Cell are required to complete clearance of *L. monocytogens* infection after response of innate immunity in addition to memory CD 8 T cells that play effective role in mediating protection (Lauren *et al*., 2007). The vaccine can generate immunological memory cells that rapid response to a second exposure to the originating antigen (De Gregorio & Rappuoli, 2014), the effective immunity against intracellular pathogens such as *L. monocytogenes* are dependent on cell-mediated immunity that required activated T cells and type1 polarized dendritic cells (Kono *et al*., 2012), so effective vaccine against these pathogens can base on LLO important virulence factors of *L. monocytogenes* (Jahangir *et al*., 2011), in addition to, heat-killed bacterial strains, plasmid DNA and other listerial antigens can elicited immune response against *L. monocytogens* (OIE, 2014), innate and acquired immune response can be activated by Live attenuated vaccine (Young *et al*., 2018). On the other hand, nanoparticle molecules are

considered better adjuvant that enhances antigen delivery or activating innate immune responses, Strength immunostimulation ability of nanocarrier vaccine were influence by certain factors such as particle size, charge nature, chemical composition and location of antigens and /or adjuvants within the carrier as well as the site of administration (Smith *et al*., 2013; Zaman *et al*., 2013), moreover, application of nanoparticles in the immune systems are considered an emerging field and the interaction between innate and adaptive immune responses with metal-based nanoparticles (Schrand *et al*., 2010).

Materials and Methods

1. Animals

Forty pregnant female rats aged between 8 months to 3 years (300±500 g) were used in this study. Twenty Males were placed in harem breeding units with 2:1 females. This arrangement allows the male to travel at will between the individually-housed females. Females are collared to prevent their entry into the common male runway, thus avoiding their access to other female cages. The females were housed individually in stainless-steel cages. The rats were fed with pellets. Clean water was given daily in glass bottles. Rats were kept in a well-aired environment with a temperature between 10±18 C. They were hot exposed to direct sunlight but kept in a room, which was illuminated for 12 h and dark for 12 h a day.

2. *Listeria monocytogenes* strains:

Listeria monocytogenes Was obtained from the MEDIA diagnostic center in Erbil, then transported to College Vet. Medicine in Baghdad in icebox under suitable condition, after that the bacteria were cultured on routine culture media and confirmed diagnosis again by:

Polymerase Chain Reaction (PCR):

Preparation of *Listeria monocytogenes* Antigens:

Killed whole Sonicated *Listeria monocytogenes* Ag. (KWLS Ag):

It was prepared as follow **(Mitove *et al*., 1992):**

1. *Listeria monocytogenes* cultured on nutrient agar, incubated at 37 °C for 24 hrs. and harvested by PBS 7.2, centrifuged at 3000 rpm 4 °C /30 minutes then washed the precipitate three times with PBS, and the precipitate was re-suspended with PBS and put in the universal tube.
2. Sonication: the universal tube that contained *Listeria monocytogenes* **s**uspension was placed in the ultrasonicator (type Karl Klob – Germany) at 12 Peak with 2 minutes intervals between them, for 30 minutes in a cold environment (ice).
3. The sonicated suspension was centrifuged at 23000 rpm for 30 minutes.
4. The supernatant fluid was examined by gram stain and culture on blood agar to confirm the sterility of these antigens.
5. The total protein concentration of this antigen, which was measured according to Biuret procedure 23.4 mg/ml and it was diluted to become 0.5 mg/ml this antigen was considered as killed whole sonicated *L. monocytogens* antigens (KWSL Ag).

To prepare the killed whole soluble sonicated *L. monocytogens* antigens (KWSLAg), the homogenate was centrifuged twice at 14000 rpm for 30minutes each time to remove cellular debris. The supernatants passed through a 0.22µm Millipore filter and stored at -20C until used. The filtered fluid was examined by Gram stain and cultured on nutrient agar and Listeria oxford agar to confirm sterility of this antigen. The total protein concentration of this antigen, was measured according to Biuret procedure 0.5 mg/ml. These antigens were considered as killed whole soluble sonicated L.monocytogens antigens (KWSLAg), it is used for delayed-type hypersensitivity test.

3. Synthesis of Al_2O_3: Ag nanoparticles

Aluminum nitrate, Silver nitrate, and trisodium citrate were used as starting materials for the preparation of Al_2O_3**:**Ag nanoparticles. The Al_2O_3**:**Ag colloid was prepared by using a chemical reduction method. All solutions of reacting materials were prepared in doubled distilled water. In typical experiments 100 ml of 0.001 M $Al(NO_3)_3 \cdot 9H_2O$ and $AgNO_3$ were heated to boil. To this solution 10 mL of 1% trisodium citrate was added drop by drop. During the process, solutions were mixed vigorously and heated until the change of color was evident. Then it was removed from the heating device and stirred until cooled to room temperature.

Aluminium nitrate, Silver nitrate and trisodium citrate: this substance was obtained from the Iraqi lab, dissolve 45 mg in 100 ml deionized water for 24 hours, which is considered as an adjuvant.

4. Experimental Design:

40 females' white rat, average age 10 to 12 weeks were divided randomly into 5 groups and treated as follows

1. 1st group was immunized with 0.5 ml of KWSLAgs with protein concentration 0.5 mg/ml, I\P two-dose, 2weeks intervals.
2. 2nd group was immunized with 0.5 ml of mixed group texture consist of 0.5 ml of KWSLAgs and 0.5 ml of nanoparticle adjuvant.
3. 3rd group will be served as a control positive group.
4. 4th control negative group.

At 28 days post-immunization, cell-mediated immunity was determined by skin test in the 1st, 3rd and 5th group, and at 30 days post-immunization, blood samples were taken from1st, 2nd, 3rd and 5th groups to determine humoral immune responses, IgG, TLR2 (toll-like receptor) and IL2 (interleukin 2) by ELISA assay.

Then all animal groups were mixed with normal males at ratio 1 male: 2 female for 10 days then it was removed the males and determine the pregnant animals, then all pregnant animals in each group were divided into 2 subgroups A and B, subgroup A was inoculated I/P with effective dose ($1x10^7$ cfu/ml) of virulence *L.monocytoges* at (5) days day after separated from the males and B subgroup will be inoculated I/P with an effective dose of virulence *L.monocytogen* at day (15) days post separated from the males, it was determined number of an aborted fetus, the number of abnormal and healthy offspring after infection by *L.monocytogens*, as well as pathological changes in internal organs and genital tracts after infection and immunohistochemistry technique for detection of Bactria Ag in tissue section.

5. Delayed-Type Hypersensitivity Test (DTH):

This test was carried out at the 28th-day post 1st immunization and the right footpad of Five (5) animals from each immunized groups and control negative group were injected intradermally with 0.01 ml of KWSLAgs (0.5 mg/ml protein concentration) and the left footpad of the same animals was intradermal injected with 0.01ml of PBS. The mean thickness of the skin on both sides was measured at 24, 28and 72 hrs using standard vernier.

Determination of ELISA Kit

1- Rat IgG (Immunoglobulin G)

2- Rat TLR-2(Toll-like Receptor 2)

3- Rat IL-2(Interleukin 2)

Assay procedure

1. Add the Standard working solution to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37ºC. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall, and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of wash buffer to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3Btimes. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at37°C.
5. Aspirate or decant the solution from each well, repeat the wash process five times as conducted in step 3.
6. Add 90 μL of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Add 50 μL of Stop Solution to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

6. Immunohistochemistry by detection of bacteria(*Listeria monocytogenes*)Ag in tissue section, by an indirect method.

Immunohistochemical Examination:

Immunohistochemistry is a method for revealing the intracellular or membranous bound location and distribution of specific target antigens in cells and tissues by staining with an antigen-specific antibody. In the current study we obtained specialized Kit (anti-*Listeria monocytogenes* antibody) (ab35132) ABCAM USA company for detection *Listeria monocytogenes* Ags in tissue by using Immunohistochemical technique according to (Ana Catarina *et al*., 2018).

Tissue Sectioning and Handling

The thickness of the slide was made approximately 4-5 microns, to obtain an optimal resolution with staining. Moreover, the section was put on the water bath at 47 ºC to relax the section from folding due to sectioning. The section was placed on a positively charged slide as flat and wrinkle-free as possible to optimize stain contact with tissue. Then left to dry at room temperature.

7. Pathological study:

Macroscopic Examination : Postmortem examination was done for all animals, the macroscopic appearance was recorded to detect any abnormal gross change in the internal organs, including location, color, size, shape, consistency, and appearance of the cut section.

Histopathological Examination : Specimens with dimensions 1x1x1 cm were taken from internal organs including spleen, liver, lung, heart, brain, and kidney, the tissue was fixed in 10% buffer formaldehyde solution immediately after removal. After 72 hrs of the fixation, the specimens were washed with tap water and then processing was routinely done with a set of upgrading alcoholic concentration from 70% to absolute 100% for 2 hrs in each concentration to remove water from the tissues, then clearance was done by xylol, then the specimens were infiltrated with semi-liquid paraffin wax at 58 Ċ on two stages, then blocks of specimens were made with paraffin wax and sectioned by rotary microtome at 5μm for all tissues. All tissues were stained with Hematoxylin and Eosin (H & E) stain and the histopathological changes were observed under a light microscope (Luna, 1968).

8. Statistical Analysis:

Statistical analysis of data was performed by using Statistical Package for Social Science, and for determination of significant differences using ANOVA one way and two way. Group differences were determined using the least significant difference (LSD) test at $P<0.05$ (SAS, 2010).

Results

1. Immune study Skin test

The results showed that the mean in duration of the skin at the site of inoculation was 2.10 ± 0.19, 2.22 ±0.07 and 1.18±0.008 in 1st, 2nd and 4th groups respectively at 24hr post examination and these values were decline at 48hr post-test as following 1.38 ± 0.03, 1.89 ± 0.02 and 0.86±0.19 in 1st, 2nd and 4th groups respectively.

2. Serum levels for detection of TLR2, IL2 and IgG

At 28 days post-immunization with killed whole Sonicated *Listeria monocytogenes* Ag, the mean serum levels of IgG titers in the 1st,2nd and 4th and groups were 12.78±0.17b, 11.14±0.27c, 15.61±0.37a, and 9.72±0.26d respectively, and the mean serum levels of IL 2 in the 1st,2nd and 4th groups were 165.99±1.85, 135.64±1.90, 187.50±2.20, and 102.88±3.49 respectively. While the mean serum levels of TLR2 were high in the 2nd group (18.27±0.42), as compared with those values 14.82±0.46, 12.50±0.16 and 8.25±0.79 in 1st and 4th groups respectively.

3. **Determined number of aborted fetus, abnormal and healthy offspring in (5) and (15) days of gestation**
4. **Histopathological examination :**
5. **Immunohistochemistry detection of Bactria Ag (*Listeria monocytogenes*) in tissue section.**

Discussion

The present result of skin test indicated that the killed whole sonocated *Listeria monocytogenes* antigens can stimulated cell mediated immune response since DTH that considered one arm of cell mediated immune response that is dependent on the activity of Th1 cell cytokine production which result from activated CD 4 T cells in addition to activated CD8 T cells, these idea was agreement with observation of Awni *et al*., (2017), who recorded increased in the levels of CD4 and CD8 T cells in animals immunized bacterial antigens, also Mielke *et al*. (1997) showed that protective immunity against intracellular facultative pathogens is mediated by activated CD Tcells which differentiated into Th1 cells that produced TNFa and IFN y, these cytokines are responsible for DTH reaction, also these observations were supporting by (AL-Bayaty, 2012), who proposed that the causes of skin indurations may be due to the small molecular weight of killed whole sonocate *Staphylococcus aureus* antigens that are characterized by antigenenicity but no immunogenicity, induced the effectors phase Th1 cell secretion a variety of lymphokines that activating macrophages to produce monokines that facilitate extravasations and infiltration of inflammatory and immune cells, edema and congestion blood vessels in the dermis and subcutaneous tissues and induced indurations and swelling of the inoculation site during (24-48) hrs post-antigen exposure. Moreover The current result revealed that the mean values of DTH in immunized animals with Aluminium nitrate, Silver nitrate and trisodium citrate (Al_2O_3: Ag)NPs adjuvant were higher than those value in other groups, these result may indicated that Al_2O_3:Ag NPs can augment the immune response, these evidence was in consistent with Zhao *et al*. (2014), who demonstrated that the Nano adjuvants can used in vaccine formulation to improve protective immune response also the present result was coincident with high levels of serum TNF, In addition to, in current result may indicated that these type of immunization can better stimulated both humoral and cell mediated immune responses, KWSLAg containing all type of bacterial antigens such as proteins and polysacharide, these molecules can stimulated both cell mediated immune and humoral responses and Aluminium nitrate, Silver nitrate and trisodium citrate (Al_2O_3:Ag) NPs can strength these responses, these idea was agreement with Xu *et al*. (2013), demonstrated that Ag NPs adjuvant expressed good stimulator ability for both cell mediated and humoral immune responses and they revealed that these substances can activated CD4 and CD 8T cells to produced immune cytokines such as TNFα and IFNy which associated with class switch immunoglobulin to Ig2a.

Serum levels for detection of (IgG, TLR2, IL2)

In current study increase value of IgG level in serum of immunized animal with KWSLAg and Al_2O_3:Ag NPs as compared with other groups in recent study indicated that KWSLAg vaccine can activated both innate and adaptive immune response and WSKAgs contain all *L.monocytogens* structures that can stimulated innate immune cells, phagocytic cells to secrete proinflammatory cytokines such as TNFa and IL12 which play essential role in initiating acquired immune responses to give, in addition to acquired immunity responsible for preparation special defense mechanism can directly eliminate the pathogen infection from the body as target cell like immunoglobulin or memory cell with interact with second infection by same pathogen in future, these idea was in agreement with Xu *et al*. (2013), who explained that application of non-viable bacterial structures in immunized animals can avoid risk of bacterial translocation and its ability to return their virulent factors in addition immune cells can directly interacted with these particles giving rise immune response through interaction between specialized pattern recognition receptors PRR such as toll like receptors TLR, nucleotide binding domain proteins NOD that recognized and interacted with microbe associated molecular patterns MAMP such as peptidoglycan and lipopolysacchride of gram negative bacteria result in production proinflammatory cytokines including Tumor necrosis factor alpha (TNF a), interleukin six and IL12, agreement with Kono *et al*. (2012), reported that effective vaccine against these pathogen can based on LLO important virulence factors of *L.monocytogenes*, furthermore (Jahangir *et al*. 2011) showed that heat killed bacterial strains, plasmid DNA and other listerial antigens can elicited immune response against *L.monocytogens*, also Qingmei *et al*. (2017), was recorded that immunized animals by combination of recombinant p60 and LLO expressed high levels of antibodies against *L. monocytogenes*, furthermore the current result, the increasing serum levels of IgG in immunized animals with adjuvant as compared with those levels in immunized animals only, these may support idea that adjuvant strengthen immune response Agger, (2016), demonstrated that Adjuvant molecules are play essential role in increasing immunogenicity of antigens and efficiency of both innate and acquired immune response.

Moreover, metal based nanoparticles such as Al_2O_3:Ag can stimulated maturation of DCs and increase their ability to express co-stimulatory molecules that provide second signal for prime naïve cells proliferation and differentiation (Ghoneum *et al*., 2010), also high levels of TNFa in the present result may due to Al_2O_3:Ag NPs activated Th1 immune response that secreted these cytokine, also these idea was agreement with Gregory *et al*. (2013), reported nanoparticles can interacted with APCs such as B cells, monocytes/ macrophages in the blood circulation and to DCs which considered specialized APCs that engulf and processes the antigen and transmitted them to regional lymph node in order to the present the antigens to T and B lymphocytes therefore these cells are considered bridge between innate and adaptive immune responses, immature DCs can efficiency engulf the antigen then became mature type which express costimulatory molecules that supply the second signal associated with proliferation and differentiation of CD4 T cells either to Th1 that mediated cellular immunity and regulation inflammatory reaction, or CD4 T cells can differentiated into Th 2 that promote humoral immune responses that's explained by (Elsabahy & Wooley, 2013). The double effect of (Al_2O_3: Ag NPs) with (KWSLAg) give more influence to immune response (two arms) that lead to increase the ability of APCs to recognize (PAMP) and presenting (TLRs) that means more production of IL2 and more activation of others immune cell-like T cell, CD4 T cell, and CD8 cytotoxic T cell the latest two cell responsible

for the production of IL2 cytokine which answerable for activation of T cell and this idea predicted by Prego *et al*. (2010), how investigated that intranasal administration of chitosan NPs loaded with tetanus toxoid lead to increase in the humoral and mucosal responses.

Determined number of aborted fetus, abnormal and healthy offspring in (5) and (10) days of gestation According to the table (5) and (6) in the current study the results showed affinity of *L.monocytogenes* to expressed high susceptibility of infection to an individual such as pregnant and causes many differences cases like an aborted fetus, healthy offspring, stillbirth, and fetal resorption, in all groups of the current study the ratio of all cases vary from one group to another and also vary from period to time (5,10days) during gestation.

However, In group four (4^{th}) that served as control positive group incubation with effective dose ($1x10^7$cfu/ml) of virulence *L.monocytogenes* the current finding revealed that the most cases(aborted fetus, healthy offspring, stillbirth, and fetal resorption) occur in this group and at the third period of gestation (15days), while the pregnancy failure placed in first gestation period (5days).

This observation was an agreement with (Doyle, 2001), who investigated that the pregnant host characterized by suppression acquired immune response particularly cell-mediated immune response associated with reduced T helper (Th)1 response, due to influence of progesterone, also (Allerberger & Wagner, (2010), showed that the *Listeria monocytogenes* can cause meningitis, septic gastroenteritis, spontaneous abortion, premature labor and stillbirth in humans and animals, especially in ruminants such as cattle, goat and sheep, Doganay, (2003), demonstrated that during pregnancy, listeriosis can be asymptomatic or can give rise to subclinical symptoms like a nonspecific fever despite the insidious development of fetoplacental infection resulting in abortion, stillbirth.

Also, placenta stage development can influence on entry efficiency of *L. monocytogenes* during pregnancy these results agreement with (Robbins *et al*., 2010) reported the stage of the placental development can influence on entry efficiency of *L. monocytogenes*, the maternal blood can fill the intervillous space when the placenta is fully developed at the end of the first trimester of pregnancy in which maternal blood starts to flow from spiral arteries to these space, in addition to the *L.monocytogens* can enter the placenta through invading extravillous cytotrophoblasts from maternal deciduas through the cell to cell spread or through maternal leukocytes (Robbins & Bakardjiev, 2012), and Baud, & Greub, (2011) showed that the life cycle pathogens such as *L.monocytogenes* which successfully targets and multiplies within the cells of the placenta and fetus that lead to the death of the fetus and abortion.

In addition to, results revealed that most abnormal condition was occurrence in third stage of gestation more than other stage and these result compatible with (Robbins *et al*., 2010), that investigated The stage of the placental development can influence on entry efficiency of *L. monocytogenes*, the maternal blood can fill the inter villous space when placenta is fully development at the end of the first trimester of pregnancy in which maternal blood starts to flow from spiral arteries to these space the Madjunkov *et al*. (2017), reported that the *L.monocytogenes* can mostly infected the fetus third trimester, due to increase fetal grow and metabolic demands associated with continuous grow of the villous blood vessels at the beginning of the third trimester of pregnancy who explained by (Gude *et al*., 2004), moreover (Schwab & M, 2003), demonstrated that the occurrence exceptionally in first trimester and rarely in second trimester of pregnancy, but Listeriosis can mostly occur in the third trimester of pregnancy, also Lamont *et al*. (2011), reported Listeriosis during pregnancy is generally associated with a spectrum of adverse outcomes during the third trimester, including miscarriage, preterm labor, stillbirth, and neonatal infection. The spontaneous abortion and failure of pregnancy in the trimester of gestation in the current study, these result may support information of Irvin *et al*. (2008) who found the *L. monocytogens* cause preterm labour, stillbirth, spontaneous abortion with fetal and neonatal death in about 20–60% of infected cases. although L.M was identified to cause animal spontaneous abortion and pregnancy disorder, it has been shown that pregnancy failure is largely associated with immune disorders caused by pathogen infection or tissue inflammation, support for above results *L.monocytogens* can infect the trophoblasts and induce recruitment and differentiation of monocytes/ macrophages and the latter induce proinflammatory cytokine and chemokine production to support trophoblast growth and survival who reported by (Fest *et al*., 2007) and cytokine productions, including pro-IL-1β (Machata *et al*., 2007), so Increased IL-1β production has been considered an important contributor for animal spontaneous abortion, Therefore speculated that the inflammasome activation might play a role in *L. monocytogens* -induced pregnancy failure (Wu *et al*., 2010)

Immunohistochemistry detection of *Listeria monocytogenes* Ag in the tissue section

In our present study evidence, of *L. monocytogenes* antigens based on the avidin-biotin complex (ABC), the immunoperoxidase technique was performed on formalin-fixed tissue organs (Rat) in research groups. Listeriosis characterized by focal necrosis, microabscesses, perivascular cuffing, and gliosis with the presence of macrophages and/or neutrophils and other inflammatory cells was observed at histological examination. Positive *L. monocytogenes* antigens were successfully identified by immunohistochemistry (IHC) in the tissues of all infected animals. Because the ability of bacteria to survive in the phagocytic and many non phagocytic cells, which can invade the tissue and induce many pathological lesion associated with nature of tissue and ability of the immune system in the body to eliminate this pathogen, this evidence was inconsistent with (Rolhion & Cossart, 2017) reported that the *Listeria monocytogenes* is a facultative intracellular pathogen whose expression of virulence factors is controlled by multiple mechanisms regulated by RNA, also (Allerberger & Wagner, 2010), showed the clinical manifestations of infection with *L.monocytogenes* include sepsis, meningitis/encephalitis, abortion, and gastroenteritis. Severely congested blood vessels, thrombosis, and haemorrhage with necrosis and apoptosis cells recorded in examined organs (liver) post-infection may be indicated that these pathogen cause bacteremia and sepses, these results were similar to these described by Sile and Norwood, (2002), who recorded pyknotic and necrosis of hepatic cells infection, the lesions in the spleen of infected animals may due to toxic effects of

L.monocytogenes in lymphoid tissues, this result was an agreement with Zainab *et al*. (2017), who investigated that the histopathology of liver tissues post-infection revealed degenerative changes and severe necrosis in hepatic lobules exclusively at 6 and 24 hours.

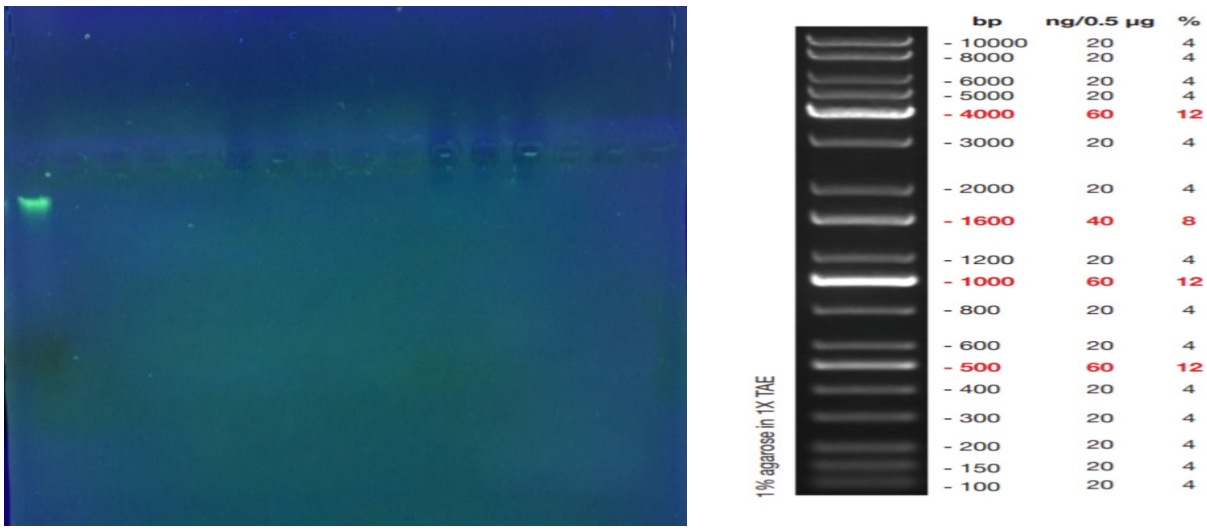


Fig. 1 : Gel electrophoresis of genomic DNA extraction from bacteria, 1% agarose gel at 5 vol /cm for 1:15 hour.

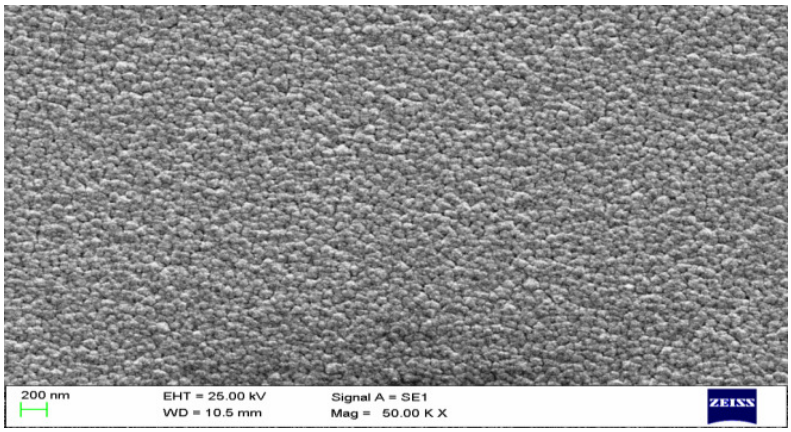


Fig. 2 : show SEM pictures, it can be observed that the morphology of the product is particle-like and nearly uniform in diameter (20 ± 10 nm). The software used in the calculation of this work was MBF_Image J. program. It is a public domain Java image processing program inspired by NIH Image for the Macintosh. It runs, either as an online applet or as a downloadable application, on any computer with a Java 1.1 or later virtual machine.

Table 1 : The mean and standard Error of skin thickness of immunized animal with killed whole Sonicated *Listeria monocytogenes* Ag and non-immunized animal after 24- 48hr.

Group	Foot pad thickness (mean ± SE)	
	24 hr.	**48 hr.**
G1	A 2.10±0.19b	B 1.38±0.03b
G2	A 2.22±0.07a	B 1.89±0.02a
G4	A 1.18±0.008c	B 0.86±0.19cXX
LSD	0.2945	

Means with a different letter in the same column significantly different (P<0.05) , the different small letter horizontally refer to present significantly different(P<0.05)

Table 2 : The mean and standard Error of serum level of (IgG, IL2, TLR2) in immunized and non-immunized animal at 28 days post immunization

Group	IgG	IL-2	TLR2
G1	12.78±0.17b	165.99±1.85b	14.82±0.46b
G2	15.61±0.37a	187.50±2.20a	18.27±0.42a
G4	9.72±0.26d	102.88±3.49d	8.25±0.79d
LSD	0.8421	7.3592	1.5398

Means with a different letter in the same column

Table 3 : Number of aborted fetus, abnormal and healthy offspring in animal Challenge with $1x10^7$ CFU/ml of virulence *L.monocytoges* after A (5) days and B (15) days from gestation.
significantly different (P<0.05)

Group A	P	A	S	F	O	N	Failure of pregnant
G1	6	2	1	1	-	32	1
G2	6	1	1	-	-	37	1
G3	6	10	8	5	3	15	7
G4	6	-	-	-	-	41	0
Group B	**P**	**A**	**S**	**F**	**O**	**N**	**Failure of pregnant**
G1	6	1	1	1	1	31	0
G2	6	1	1	1	-	37	0
G3	6	13	12	11	4	2	1
G4	6	-	-	-	-	40	0

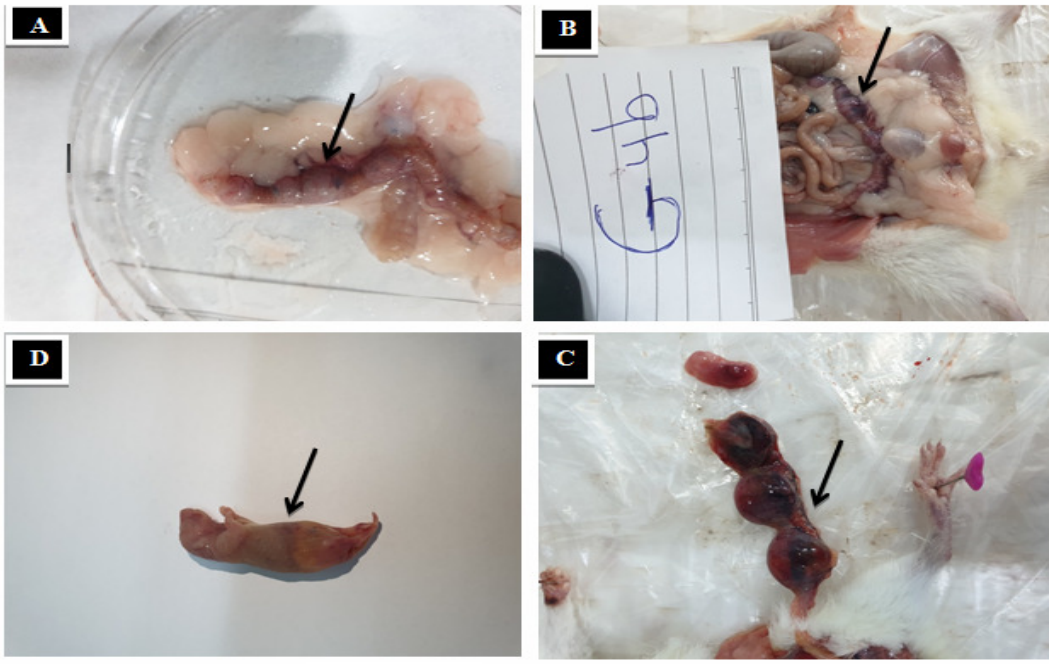
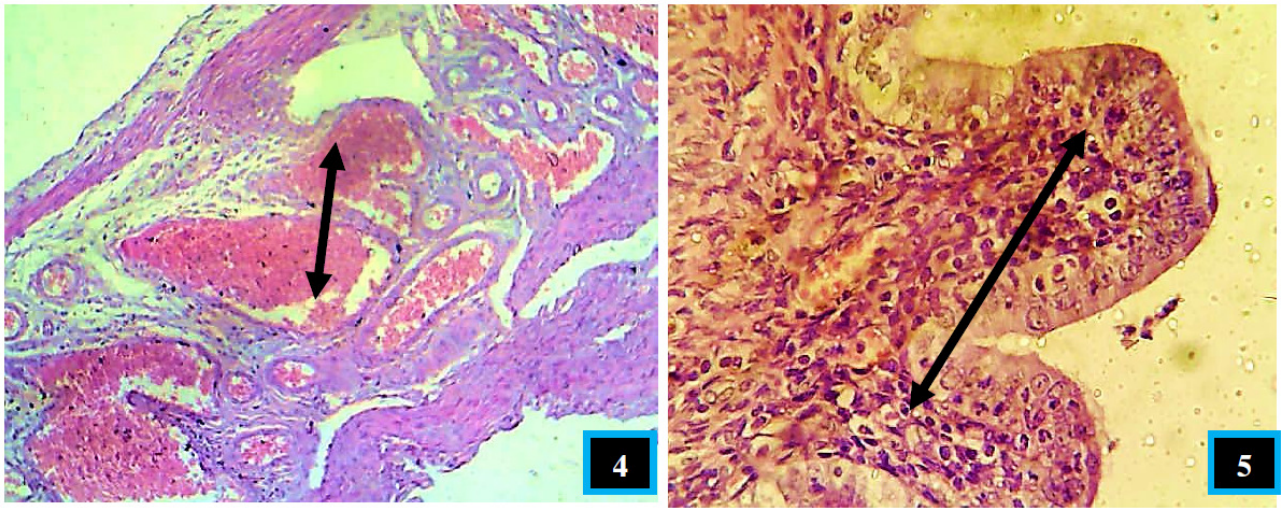
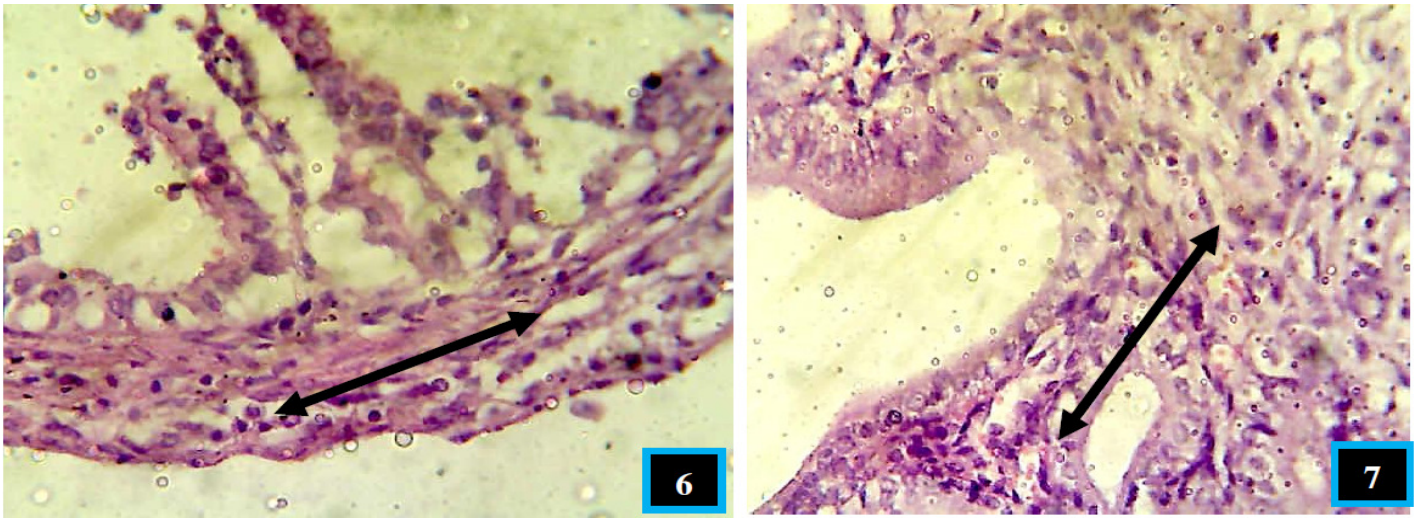


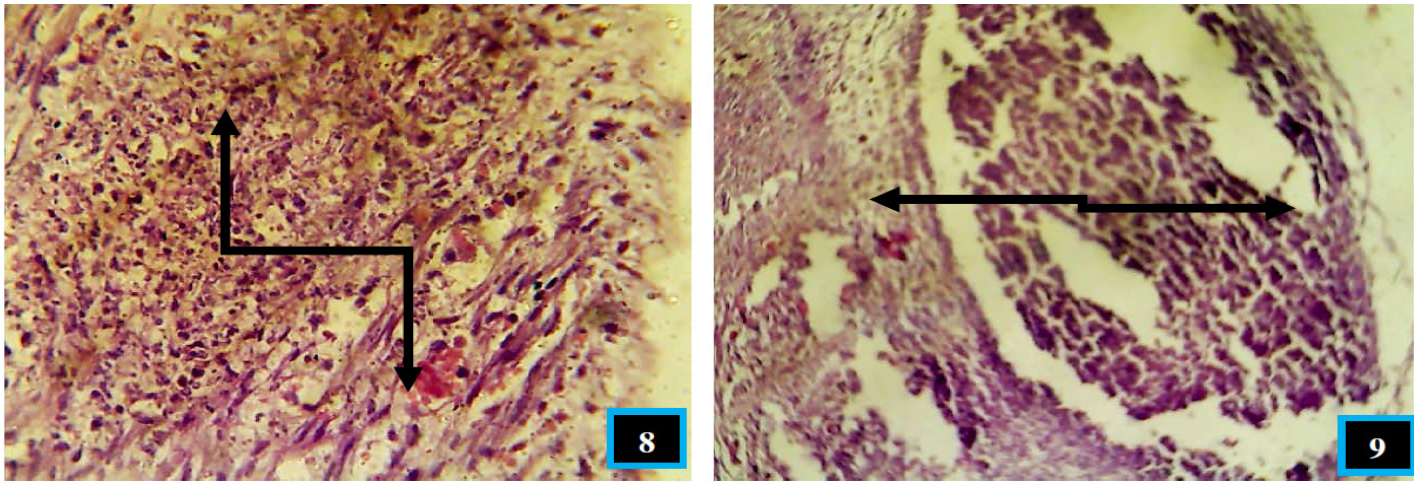
Fig. 3 : Multi pathological condition of fetal resorption in A, B and C, the aborted fetus appeared in D Challenge with $1x10^7$CFU/ml of virulence *L.monocytoges.*



Figs. 4,5 : Section in the uterus of immunized animal at 20 days post-infection shows congested blood vessels with few mononuclear cells infiltration in the muscular layer with normal n serosal layer (H & E stain 400X).



Figs. (6,7) : The section in the oviduct and uterus of immunized animal treated with Nanoparticles at 20 days post-infection shows mononuclear cells infiltration in the endometrium (H & E stain 400X).



Figs. 8,9 : The section in the uterus of the non-immunized animal at 20 days post-infection shows severe necrotic neutrophils infiltration between muscle fibre and abscess in the serosal layer (H & E stain 400X).

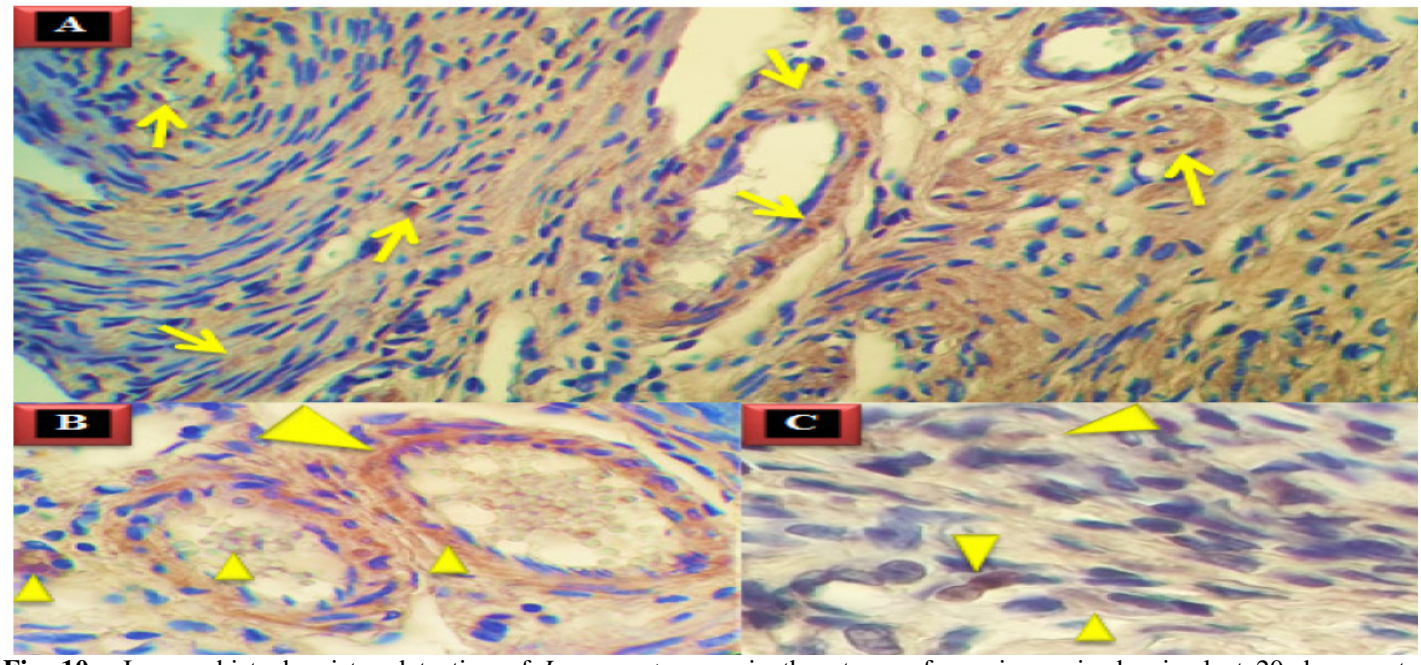


Fig. 10 : Immunohistochemistry detection of *L.monocytogenes* in the uterus of non immunized animal at 20 days post-infection showed sever immune positive cells, in (Fig.A) positive reactions are indicated the presence of bacteria in the endothelial layer of Bvs with inflammatory cells infiltration with yellow arrows, yellow arrows indicate severe inflammatory cells infiltration particularly neutrophils between muscle fibre and present these cells in congested blood vessels with higher magnification showing a detail with positive cells (Fig. B, C)(400X, 100X).

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