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IMMUNOLOGICAL AND HISTOPATHOLOGICAL STUDY OF THREE TYPES OF ANTIGENS FROM *S. PSEUDINTERMEDIUS*

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ABSTRACT

This study aimed to aims to prepare three types of antigens from *S. pseudintermedius*, *Staphylococcus* was isolated and identify the *Staphylococcus* species from otitis externa samples of dogs as well as from the tonsillitis, rhinosinusitis and skin lesion so human. Including. The collection of 100 samples involved 50 from humans and 50 from dogs from different location in Baghdad governorate during November (2021)to March (2022).the samples were cultured on Mannitol salt agar, purified, and identified according to colony morphology, Gram stain, and biochemical tests. The bacterial isolates were confirmed by VITEK technique. The results showed that 35out of 50 dog samples were positive for *Staphylococcus* while 27 out of 50 human sample were positive. Prepare three types of antigens from *S. pseudintermedius* which considered the most prominent isolate in this study ,and their immunogenic activity was evaluated by cellular immunity study. For this purpose, twenty rabbits randomly divided into four groups (five animals in each group) the first group was immunized with (WSAg) 32/mg subcutaneously, the second group was immunized with (peptidoglycan)25mg/ml subcutaneously, the third group was immunized with (cell wall)35mg/ml subcutaneously, the fourth group was injected by PBS (pH7,2) as a control group. The cellular skin reaction was observed by using WSAg in delayed hypersensitivity (DTH) skin test at 21days post first immunization. the result of skin test shows appearance of erythematic and skin thickness among all the three immunized groups while no positive skin reaction occur in the control group after 24 and 48 hours post examination. The challenge dose was given for all groups at day 28 post first immunization. after Challenge dose animals wereobservedclinicaly.one animal of control group was died 48 hrs after challenge dose. The other control group animals show signs of illness. All animal groups were scarified after 7 days of challenge dose and the Histopathological study show a pathological changes in control group that indicate a severe infection with Listeriosis, this supported by appositive isolation of *L. monocytogenes* .all immunized groups show different cellular immune response characterized generally by aggregation of mononuclear cells and other inflammatory cells in internal organs with negative bacterial isolation.

Keywords: Wsag, Listeriosis, Antigens, Staphylococcus, VITEK

1 INTRODUCTION

Staphylococcus are tiny gram-positive cocci they got their name because they looked like bunches of grapes, currently, more than40 known species of *Staphylococcus* exist **(Al-Hasnawi, 2017; Jasim, 2012 and Krute and Bose, 2015).** Due to their ubiquity and adaptability, it inhabits the skin, skin glands and mucous membranes of people, other mammals and bird, as well as the environment **(Vrbovská *et al*., 2020).**Mammals can develop localized and systemic infections from *staphylococci* ranging from small wounds infections to potentially fatal illness including endocarditis and osteomyelitis **(AL-Osmonov *et al*., 2013; Hussein, 2017 and Ahmed, 2017**). *Staphylococcuse* spp. can cause many sever disease in animals for example in a cattle, sheep and goat the main infection is mastitis .in dog and cat may cause otitis and skin infections. In laboratory animals specially rabbits and gineapigs *Staphylococci* may cause severe pneumonia **(Radostits *et al*.,2007; Ali, 2007 and Kanaan and AL-Shammary, 2013).** Many researchers had used different subunit antigens of bacteria. This protocol was initially conducted in2002 in annual meeting preceeding that conclude that twenty-one centuries must be the century of using subunite particles of bacteria in all bacterial vaccines becauseusing of sub unite vaccine is safer for human and animals and most all bacterial parts can given immune response even with killed bacteria **(Wykoff, 2002; Hayyawi, 2012 and Yassin *et al*., 2022**).

2 MATERIAL AND METHODS

Isolation of *S.pseudintermedius* was done according to (Kasimand Al-Zubaidy, 2022)

2.1 Prepration of Whole *S. pseudintermedius* Sonicated Antigen (WSAg)

The whole sonicated *S. pseudintermedius* antigen was done according to **(Tahi*etal*.,2021 and Ibrahim, 2012).**

2.2 Preparation of cell wall and peptidoglycan

The cell wall and peptidoglycan antigen of *S.speudintermedius* were extracted according to **(Ziamko and Okulich, 2014)**

2.3 Experimental design

To identify Staphylococcus samples collected from 50 human samples with tonsillitis, rhinosinositis and skin lesions and 50 dog samples with otitis. Samples were inoculated into brain heart infusion broth(BHIB), then cultured on mannitol salt agar .Bacterial identification was done depending on colony morphology, gram stain, biochemical test (catalase, coagulase) and VITEK. Extraction of antigens was done using the most important and most prominent isolate of *Staphylococci t*hese antigens were whole sonicated antigen, peptidoglycan and cell wall antigen. After extraction, the concentration of protein in each extracted antigen was estimated using Biuret method, Doses of immunization of laboratory rabbits were estimated according to (**Al-dafai, 2013)**. *Listeria monocytogenes* Isolate used in this study obtained from the unit of zoonotic Diseases Baghdad University College of veterinary medicine, and was confirmed by vitek system. the Challenge dose of *Listeria monocytogenes* 1×10^7 CFU/ml according to (**Calderon-González *et al*.,2014).** Culture *L. monocytogenes* was prepared on brain heart infusion broth at 37°C for 24 hrs and centrifuge at 3000 rpm for 20, these diment was hed three times with phosphate buffer solution PBS (pH 7.2) and re-suspended with 1 ml of PBS, in tube. The concentrations of bacteria in diluents tubes had compared to stander Macfarlane tube,

2.4 Experimental laboratory animals

Twenty healthy rabbit's local breed, and all rabbits from male sexes with 1500-2000 gm weight. The rabbits were housed in laboratory animal house of college of veterinary medicine they were housed in cages as stainless-steel wire with fully ventilated room at temperature 25. the animals were provided with food and water and adapted for two weeks before initiating of experiment. The animals were divided into four groups each consist of five animals and treated as follow: First group: immunized with whole *S. pseudintermedius* sonicated antigen S/C with (protein 32mg/ml) concentration as primary dose followed by booster dose after 14 days. Second group: immunized with peptidoglycan of *S. pseudintermedius* S/C (protein 25 mg/ml) concentration as primary dose followed by booster dose after 14 days. Third group: immunized with cell wall of *S. pseudintermedius* S/C (protein 35 mg/ml) concentration as primary dose followed by booster dose after 14 days. Fourth group: served as control was injected S/C with1ml of Phosphate buffer solution as primary dose followed by booster dose after 14 days. At 21-day post first immunization, Delayed Type Hypersensitivity Skin Test (DTH) was done to all groups. And at 28 days post first dose of immunization the rabbits of all groups were challenged orally with (1ml) of bacterial suspension containing 1×10^7 CFU/ml of viable virulent *Listeria Monocytogens* (**Calderon-González *et al*., 2014).** At 35 day post primary dose of immunization the rabbits of immunized groups were scarified then post-mortem examination was done to all scarified rabbits.

2.5 Delayed Type Hypersensitivity Skin Test (DTH)

This test was done at 21day post primary dose of immunization and carried out according to (**Hudson and Hay, 1980**). One side flank region of all animals of immunized and control groups was clipping and shaving and intradermal injection in the center of this region was done with 0.1 ml of whole sonicated antigen of S*. pseudintermedius* using insulin syringe.

The results of the test were recorded by measure erythema diameter and thickness of skin at region of injection after 24 and 48 hours after injection and using vernia for this purpose.

2.6 Macroscopic examination

After 7 days post challenge dose the Animals were euthanized using closed ether jar. All surface body was accurately observed for the presence of external lesions. Then the carcasses were opened by longitudinal abdominal incision all internal organs were accurately observed to indicate any lesions or congestion. Smears were taken from internal organs for culturing to indicate the presence of *L. monocytogens*.

2.7 Histopathological examination

At 35 day post primary immunization, the sample was collected from vital organs of experiment animals were collected which included; lung, liver, intestine, spleen and kidneys. The tissues were fixed in %10 buffer formaldehy desolution immediately after removal, after72hrs of fixation the specimen were washed with tap water and then processing was routinely done with assest of routinely with set of upgrading alcoholic concentration from%70 to absolute 100% for 2 hrs in each concentration to remove water from the tissues, then the clearance was done by xylol, the specimens were soaked with semi-liquid paraffin wax two stages. Then blocks of the specimens were made with paraffin wax and sectioned by rotary microtome at 5mm for each tissue sample. All tissues were stained with Hematoxlin and Eosin (H&E) stain and the histopathological changes were observed under light microscope **(luna, 1968**).

3 RESULTS AND DISCUSSION

Table 1: Antigens, method and protein concentration

Antigen	Method of Extraction	Protein concentration
wsAg	Tahi*etal*.,2021	43mg/ml
peptidoglycan	ZiamkoandOkulich, 2014)	7.1mg/ml
Cell wall	ZiamkoandOkulich,2014)	7.8mg/ml

These antigens were used to immunize three groups of rabbits as was designed in experimental design. The three groups showed cellular immune response. this was detected by skin testing (delayed type hyper sensitivity) and Histopathological changes after post mortem.

3.1 Delayed Type Hypersensetivity–skintest

The results of this test in the three immunized groups in addition to control group were represented by mean of skin erythema and induration and showed in table (4.7), (4.8) and figure (4.9). The skin test results indicate the presence of cellular immune response to the antigen **(kindt *et al.*, 2007**). The reaction in this test depends on the presence of memory T cell for CD4 and CD8 (**John and MacArther, 1997).** also the Delayed Type hyper sensitivity test depends on both T helper cells (TH1) drive responses and cell recruitment and chemotaxis to the site of injection. (**Dietert *et al.*, 2010).** After antigen injected intradermally it is taken up by Langerhans cells and these migrate to the draining lymp he node in which the antigen present in gto memory T cells that respond by generating Th1effector cells. The Th1cells recognize antigen when the yen counter it in the skin and accumulate around the antigen. (**Tizard, 2017).** the Th1 cells are responsible in secreting a variety of cytokines that recruite and activate macrophage and other nonspecific inflammatory cells **(Subhash, 2012)**. The indurations occur due to accumulation of activated macrophages and another inflammatory cells in the area of injection and the erythema or the red appearance of skin occur due to increase the permeability of blood vessels due to infiltration of the inflammatory cells (**TiZard, 2009).** The variation in the diameter of erythema indicates different degree of immune response (**Bercovich, 1995)** the higher response may related to higher specify that lead to high skin reaction **(Davis *et al.,*1990).**

Table 2: Mean±SE of erythema in all groups against WSAgat 24-48 hours post skin test.

hours	Erythema mean cm.			
	G1	G2	G3	G4
24	2.26±**0.927**	2.86±**0.12**	2.66±**0.18**	0.300±**0.0316**
48	1.46±**0.812**	2.04±**0.12**	1.98±**0.12**	0.120±0.0200

Table 3: mean ±SE of skin thickness in all groups against WSAg at 24-48 hours post skin test.

hours	Skin Thickness mean mm.			
	G1	G2	G3	G4
24	2.66±**0.92**	2.77±**0.8**	2.86±**0.9**	0.58±0.22
48	3.26±**0.84**	3.80±**0.15**	3.76±**0.9**	0.60±0.19

- G1: immunized with whole *S. pseudintermedius* Sonicated Antigen
- G2: immunized with Peptidoglycan
- G3: immunized with Cell wall
- G4: control group (not immunized with Antigen)

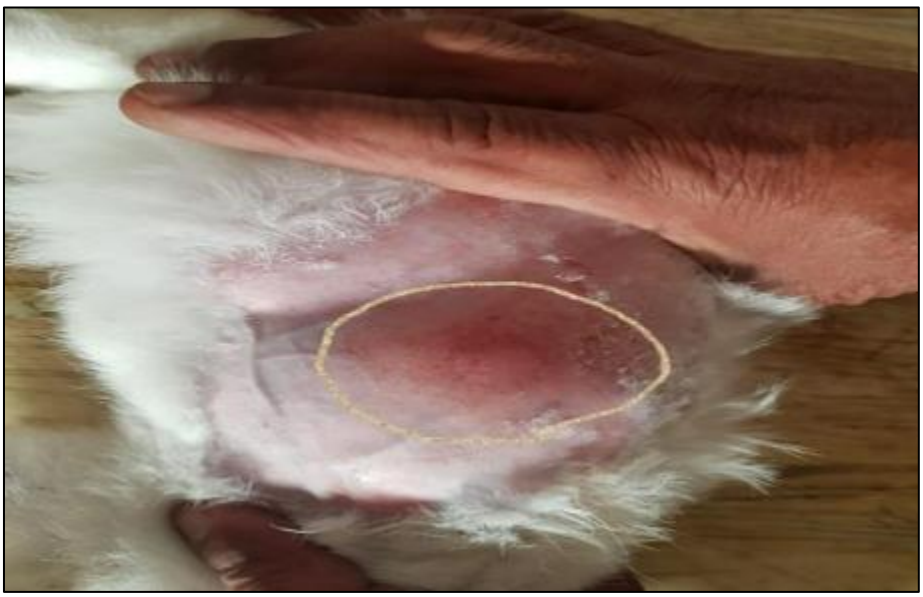

Figure 1: Positive reaction of skin test in one animal of one immunized group

3.2 Clinical signs observation and bacterial isolation

Signeslikedecreaseorlossapetitewithclearlostofconditionanddepressionappearedon all the animals of control group after giving the challenge dose. after 48 of challenge dose one animal of control group was died and after 5 days another animal in the same group was died. Pathological investigation to these 2 animals was done immediately after death. Bacterial isolation was done firstly from dead animals directly after death. the other animals were euthanized after 7 days of challenge and the bacterial isolation was also done. *L. monocytogenes* were isolated from all animals of control group that died and authenized and no bacterial isolation was shown among the animals of immunized groups

3.3 Pathological examination Gross Examination

The gross examination was done after 7 days of challenge with *Listeria monocytogens*. grossly control positive group show sever and generalized congestion throughout the carcass of animals of this group. Also enlarged liver with white spots were seen, with engorged gallbladder. Mucous inside intestine also seen with engorgement of blood vessels of small intestine wall. These pathological gross lesions were illustrated in **Figure (2, 3).**

Figure 2: Gross appearance in control positive group shows enlarged liver with congestion and presence of white spots and engorged gall bladder

Figure 3: Gross appearance in control positive group show engorgement of mesenteric blood vessels in small intestine

Animals of the three groups that immunized show almost normal carcass with apparently low or no congestion with normal internal organs appearance. No significant gross lesions were seen. These were illustrated in **Figures (4, 5)**.

Figure 4: Gross appearance in the lung of one animal of immunized group, shows no gross pathological changes.

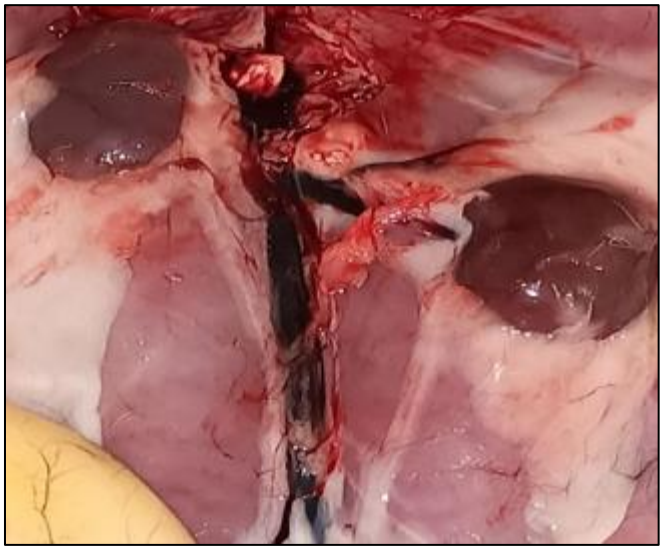

Figure 5: Gross appearance in kidneys of one animal of immunized group, shows no gross pathological changes.

The gross pathological lesions in control group that reported in this study were also recorded by (**Moshtaghi *et al*., 2006)** in mice inoculated experimentally with *Listeria Monocytogens*.

3.4 HistopathologicalExamination

Histopathological picture of control group show severs pathological changes throughout the organs examined. Congestion was the main picture especially in liver **Figure (6, 7**) due to proliferation of inflammatory cells especially neutrophil and mononuclear cells with dilatation of blood vessels and accumulation of mononuclear cells around blood vessels **figure (8)**. In intestinal wall section there is focal destruction in the epithelial cells of crypts **figure (9).** In lung there is thickening of internal alveolar septa due to accumulation inflammatory cells. **figure (10)** and congested capillary blood vessels. In spleen sever depletion of white pulp due to necrosis of lymphocytic cells **figure (11).** Kidney section show congestion of blood vessels with sever vacuolar degeneration of epithelial lining cells of renal tubules and excessive inflammatory cells **figure (12)** and hyaline cast in the lumen of renal tubules that suffering from vacuolar degeneration. All the above histological lesion indicates generalized septicemic infection and this was described by (**Rouquette and Berche, 1996)** as a feature of listeria infection. Infiltration of mononuclear cells in liver and depletion of white pulp of spleen were confirmed an important histopathological picture of listeria infection by (**Moshtaghi *et al*., 2006**). Alterations of kidney section that reported by our study such as hyaline cast and signs of nephritis with the signs enteritis in intestinal wall section were noticed in mice infected with virulent *Listeria monocytogens* by **(Mainou-flower 1988)**. The immunized groups showed histological changes in internal organs included generally congestion, infiltration of mononuclear cells and phagocytic or other inflammatory cells. The haveir mononuclear cell infiltration was in the lamina propria of small intestine of immunized groups this may related to the infection with *Listeria monocytogenes* orally and that immunized groups had the ability to induce cellular immune response that produce these cells to migrate and localized in lamina propria to eliminate infection **Figure (13, 14).** The highest response was in group one and two and this may indicate the ability of whole sonicated antigen and peptidoglycan to induce the higher immunity (**Tizard, 2009).** Liver of group one have also higher infiltration of mononuclear cells in and fibrosis of wall of hyperplastic epithelial lining bileduct **figure (15).** The aggregation of inflammatory cells developed formation of granulomatous inflammation which consist of number of macrophages (Histiocytes) this was obvious in group two and three **figure (16, 17)**. This changes in liver indicate the presence of cellular immunity and the large number of these cells in liver may be due to the nature of the function of liver in nutritional metabolic balance and elimination of toxins, body west product, metabolic substances and foreign materials including pathogen (**Kumar *et al*., 2009**)**.** these lesions are the characteristics feature of granulomatous inflammation that generated by a fusion of activated macrophages **(Hawryluk *et al.,* 2010).** In lung the thickening interalveolar septa due to mononuclear cells infiltration in group one **figure (19)** and hyperplasia of bronchial associated lymphoid tissue in wall of airways **figure (18)** indicate the high immune response and the ability of whole sonicted antigen as a defending mechanism against pathogen **(luna, 2019)** while in lung of group two there is proliferation of alveolar macrophages in alveolar space **figure (20)** this also indicate the immunogenic response in this group. In kidney of group one there is aggregation of neutrophil and in dilated congested blood vessels **figure (21**) while in group two moderate vacular degeneration in epithelial lining cells was shown **figure (22)** the overall picture of presence of mononu clear cells in other organs indicate the production of pro inflammatory mediators such asIL12 **(cassatella *et al*., 1995).** The infiltration of inflammatory cells in three immunized groups may related to the active production of tumor necrotic alfa (TNF-α) which is proximal mediator of neutrophil chemotactic factor (**Netea *et al*., 1999)** and also related to that neutrophils infact is the first cell for defence. In spleen

apoptosis in the white pulp resulting in the formation of spaces with cellular depris in group one **figure (23)** and hyper plasia of white pulp in group two **figure (24)**. May related to the massive inflammation of phagocyted due to immune responses **(McGaha and Karlsson, 2016).** In group three marked proliferation of lymphocyte in periarteria sheath **figure (25)** indicate the presense of immune proliferative micro environment which related to obvious immune response (**Matsuno *et al*., 1989)**.

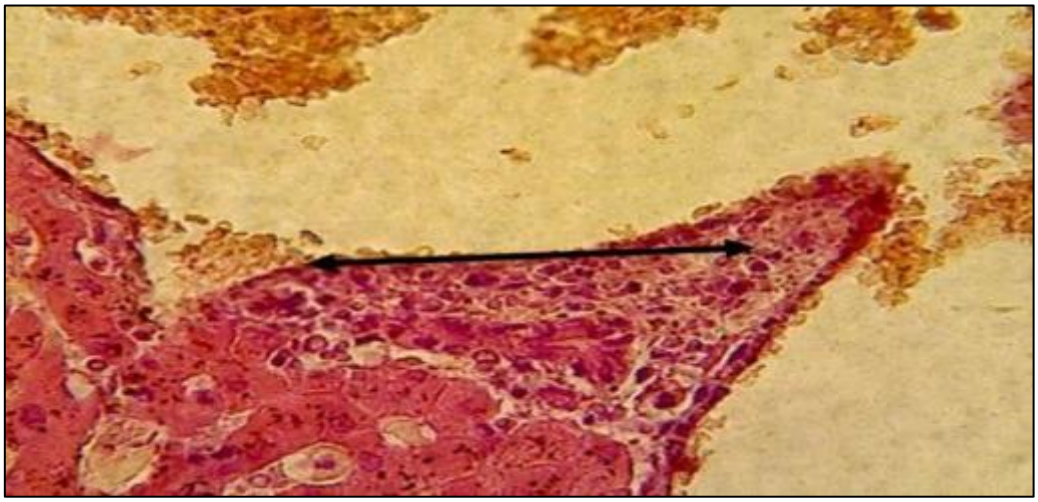

Figure 6: Histopathological section in the liver of animal in control positive group; shows congested blood vessels and inflammatory cells particularly neutrophila and mononuclear cells infiltration in liver parenchyma (H & E stain 400X).

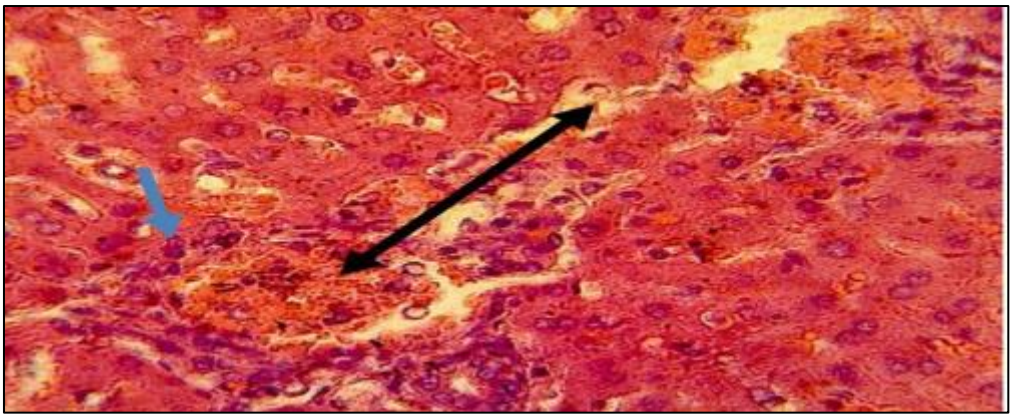

Figure 7: Histopathological Section in the liver of control positive group; shows neutrophils in dilated congested blood vessels and sinusoids in addition to mononuclear cells around bile duct in portal area (H & E stain 400X).

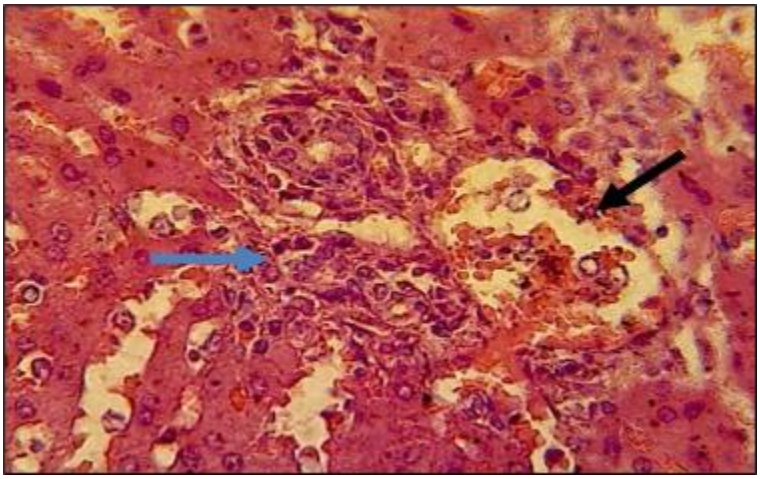

Figure 8: HistopathologicalSectionintheliverofcontrolpositivegroup;showsneutrophils in dilated congested blood vessels and mononuclear cells around bile duct in portal area (H & E stain 400X)

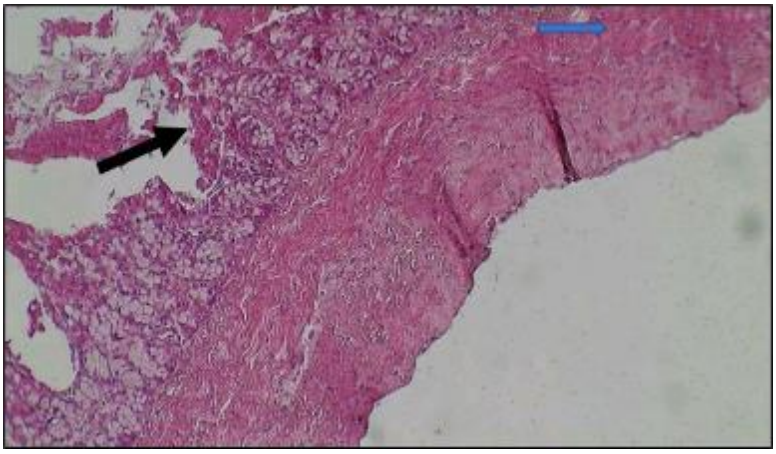

Figure 9: Histopathological section in the intestine of control positive group; shows focal destruction of epithelial cells in crypts (H & E stain 100X).

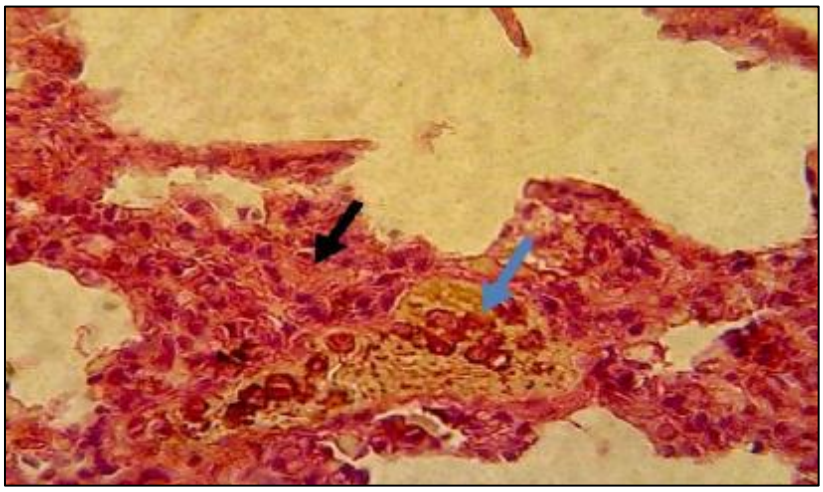

Figure 10: Histopathological Section in the lung of control positive group; shows increase thickness of interalveolar septa due inflammator cells infiltration and congested capillary blood veseeles (H&E stain 400X).

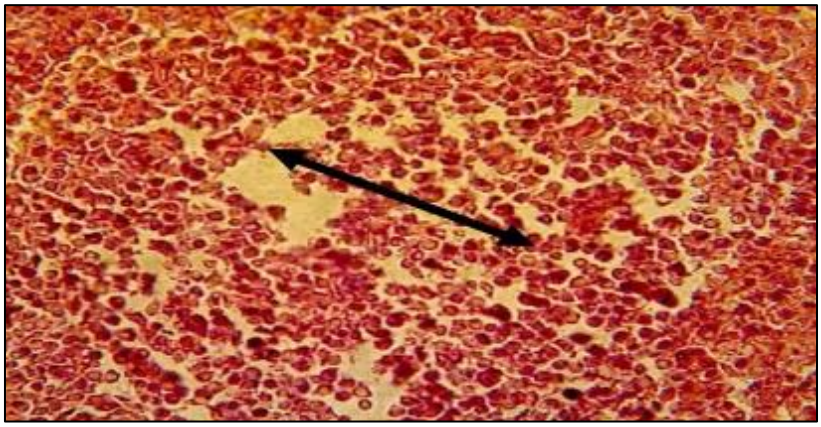

Figure 11: Histopathological Section in the spleen of animal of control positive group; shows severe depletion of white pulp due to necrosis of lymphocytic cells (H & E stain 400X).

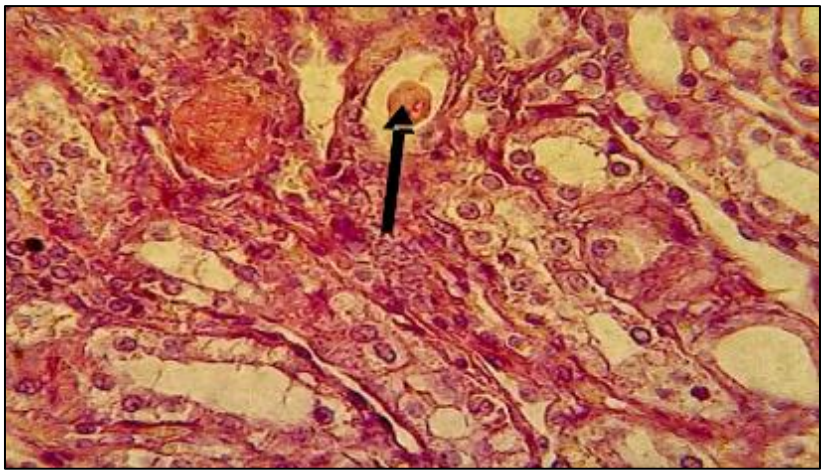

Figure 12: Histopathological Section in the kidney of control positive group shows hyaline cast in the lumen of renal tubules suffering from marked vacuolar degeneration (H & E stain 400X).

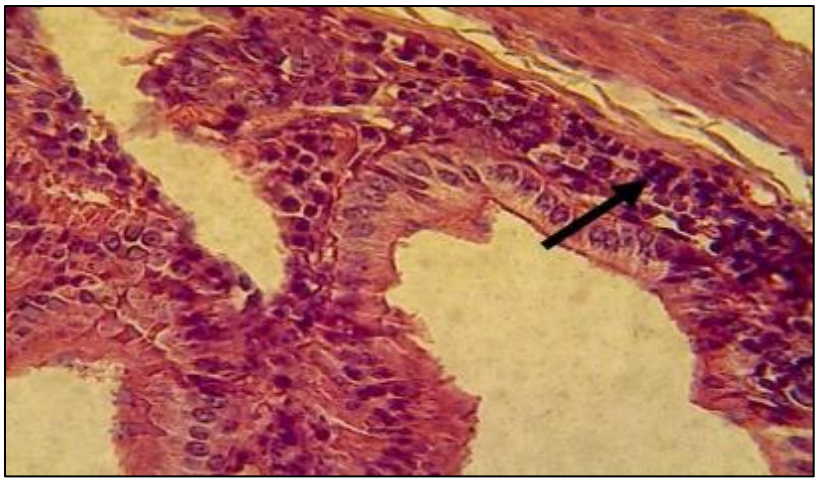

Figure 13: Histopathological Section in the intestine of animal in G1 (immunized with WSAg); Shows mononuclear cells infiltration in the lamina properia (H & E stain 400).

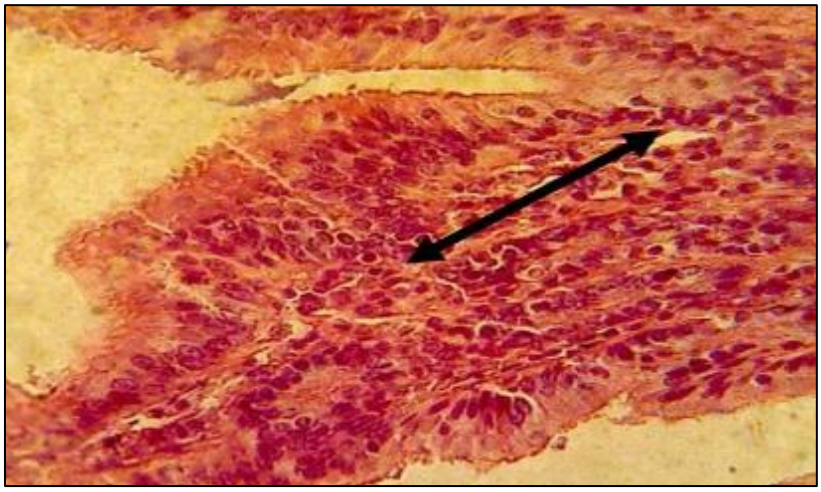

Figure 14: Histopathological changes in the intestine of G2(immunized with PGN); shows marked mononuclear cells infiltration in the dilated lamina propria (H&E stain 400X).

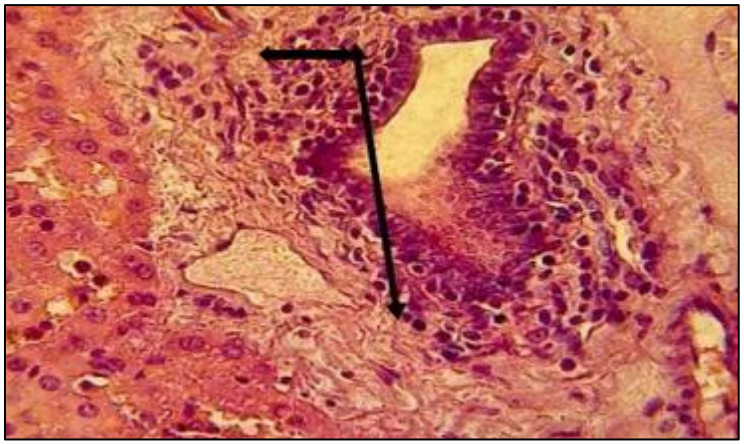

Figure 15: Histological Section in the liver of G1(immunized with WSAg); shows mononuclear cells infiltration in fibrosis in wall of hyperplastic epithelial lining of bile duct (H & E stain 400X).

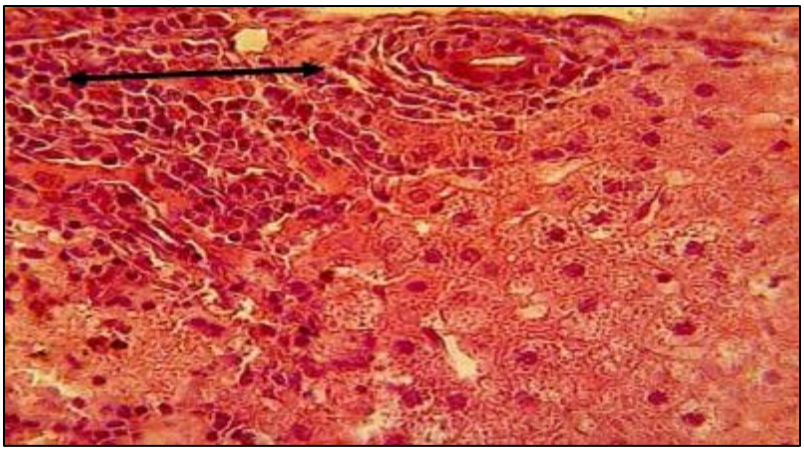

Figure 16: Histopathological Section in the liver of animal in G2 (immunized with PGN); shows granulomatous inflammation in the liver parenchyma (H & E stain 400X).

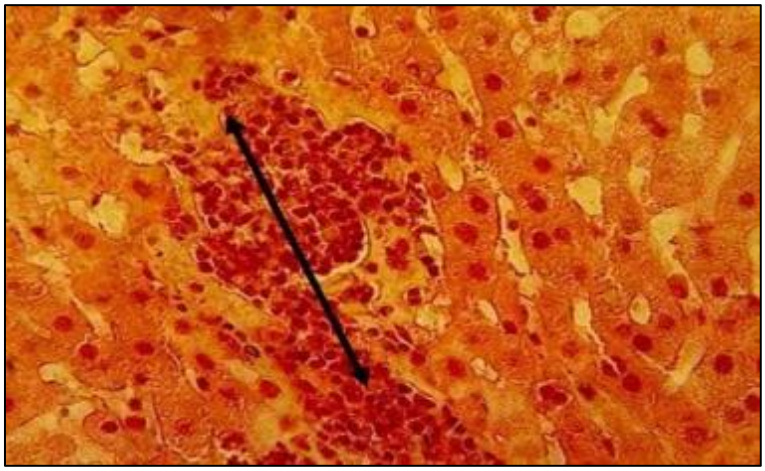

Figure 17: Histopathological Section in the liver of G3 (immunized with cell wall Ag.) shows multiple granulomatous lesions (H & E stain 400X).

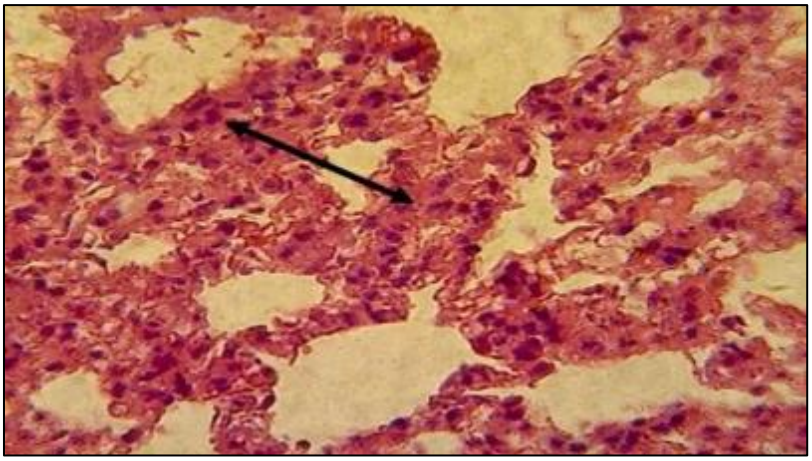

Figure 18: Histopathological Section in the lung of animal in G1(immunized with WSAg); shows increase thickness of inter alveolar septa due mononuclear cells infiltration (H & E stain 400X).

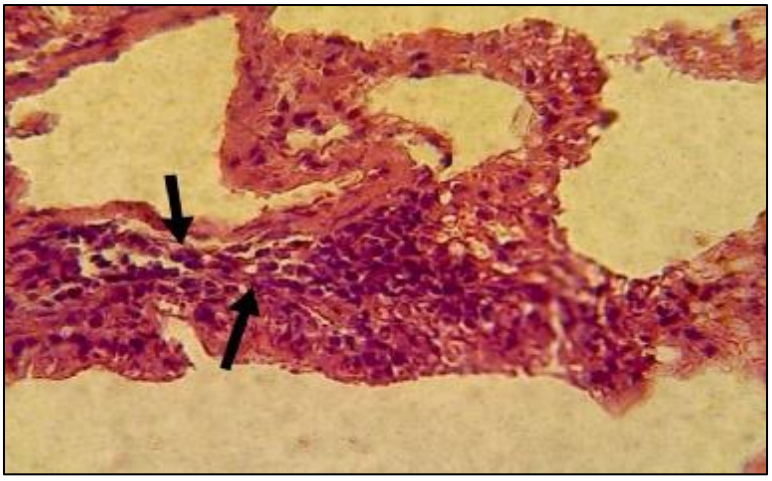

Figure 19: Histopathological Section in the lung ofG1(immunized with WSAg); shows moderate hyperplasia of bronchial associated lymphoid tissues in wall of airways (H & E stain 400X).

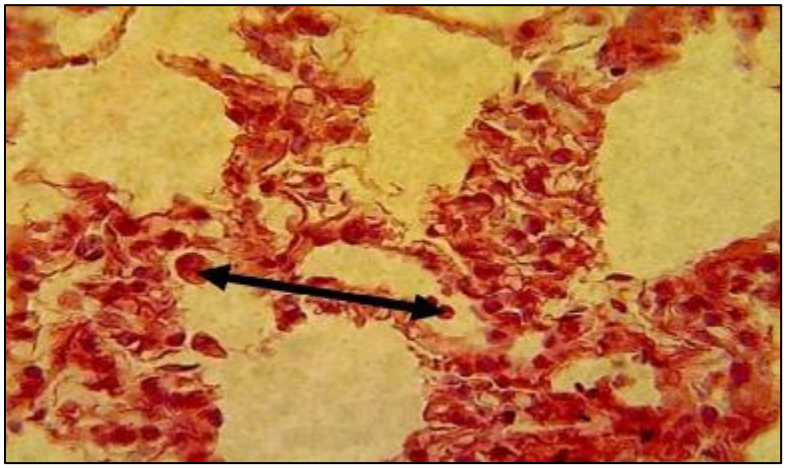

Figure 20: Histopathological Section in the lung of animal in G2 (immunized with PGN); shows proliferation of alveolar macrophages in alveolar spaces (H& E stain 400X).

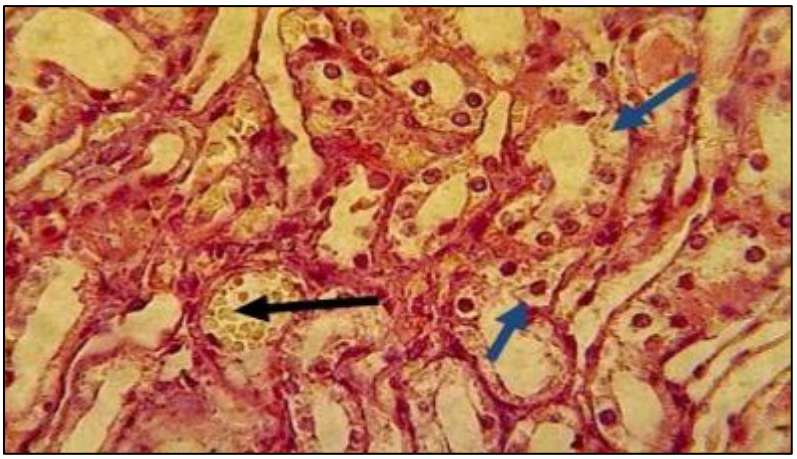

Figure 21: Histopathological Section in the kidney of G1(immunized with WSAg); shows neutrophils in dilated congested blood vessels with acute cellular degeneration of epithelial cells lining renal tubules (H&E stain 400X).

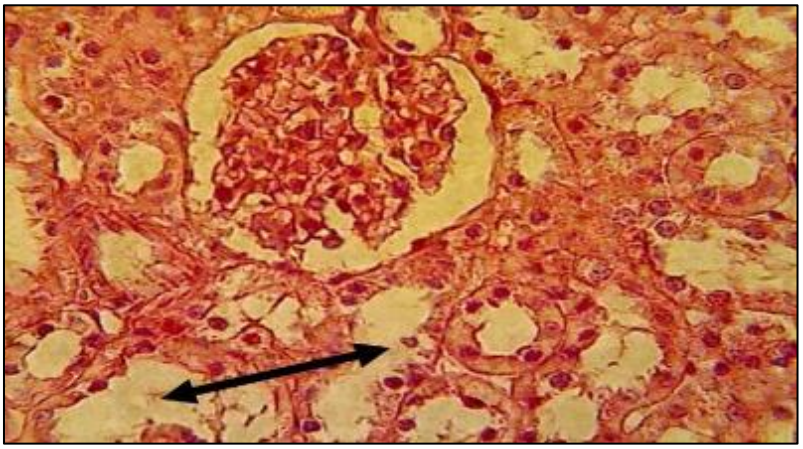

Figure 22: Histopathological Section in the kidney of in G2 (immunized with PGN); shows moderate vacuolar degeneration of epithelial lining cells of renal tubules (H&E stain 400X).

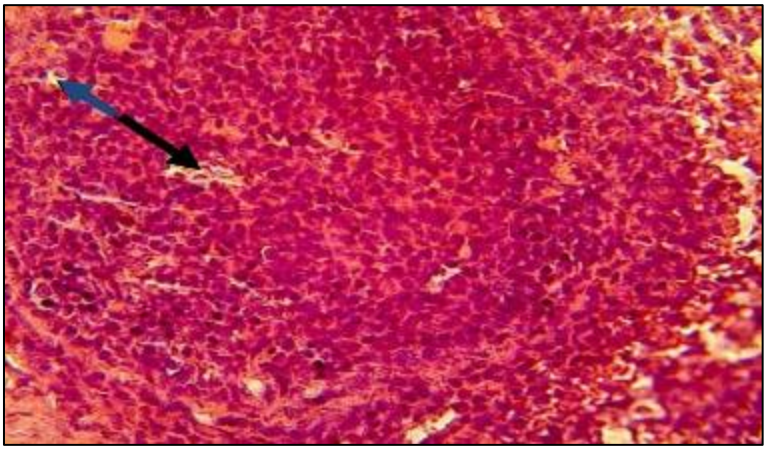

Figure 23: Histopathological Section in the spleen of G1 (immunized with WSAg); shows focal apoptosis in white pulp left spaces contain cellular debris (H & E stain 400X)

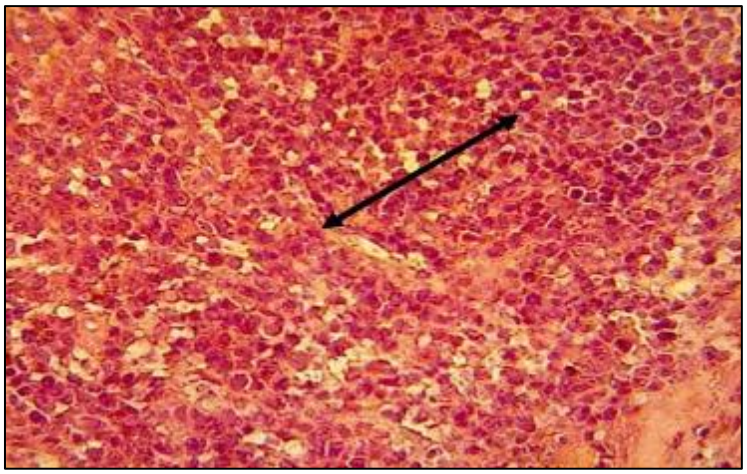

Figure 24: Histopathological Section in the spleen of G2 (immunized with PGN); shows moderate hyperplasia white pulp (H & E stain 400X).

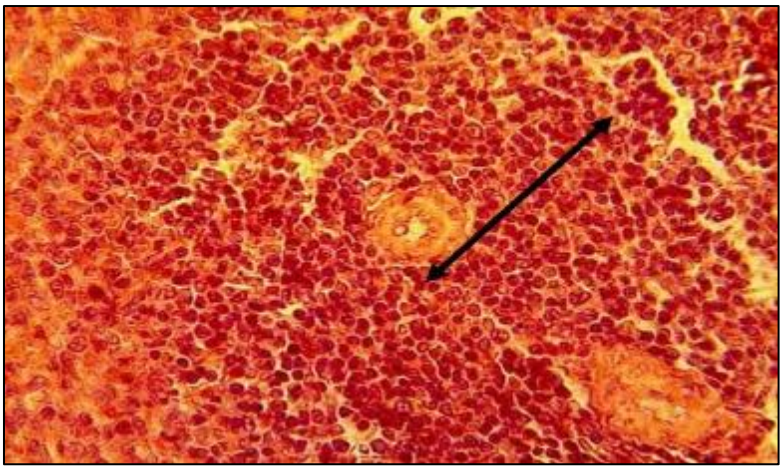

Figure 25: Histopathological Section in the spleen of animal inG3(immunized with cell wall Ag.); shows marked proliferation of lymphocytes in periarterial sheath (H & E stain 400X).

4 CONCLUSION

The three antigens that extracted from *Staphylococcus P. seudintermedius* contains good concentrations of protein. The three antigens gave an acceptable immune response in immunized rabbits against *Listeria mionocytogenes* experimental infection.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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