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Experimental Infection Of *Salmonella pullorum* To Study Immunological AndHistopathological Changes Of Broiler

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Abstract: *Salmonella pullorum were isolated from the broiler chicken samples Rose. 12 isolates were identified from a total of 69 samples and identified by conventional cultural, motility and morphological characteristics. And was confirmed by PCR and then prepare a vaccine using the method of freezing and thawing repeated and this method has a clear effect on the growth of bacteria. The second step was to give the bacteria as a vaccine for a total of 30 chicks at the age of 7 days. The second dose was repeated at 14 days, the histological changes of the cecum and liver were observed at 20 days, and the immunological tests DTH, PHA were performed after collecting serum samples at the age of 24 days. Both groups were given a 0.5 ml dose of weakened bacteria and observed gross and histological changes of the internal organs of the chickens.Gross and histological changes were recorded in the chicks before and after the vaccine was administered. It has been concluded that the method of freezing and thawing has a clear effect as a vaccine.*

Keywords: *Salmonella pullorum, Immunological , Histopathologicaland Broiler*

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I. Introduction

Salmonella entericaS. enterica include more than 2500 Serovars source the genus *Salmonella*, which is the most common from a species *Salmonella*, and is responsible for various diseases, many of these species have significant roles in the medical and veterinary fields , approximately 99.5% of the isolated species belong to *S.enterica*, which causes acute intestinal inflammation and rather than systemic diseases Grammato *et al*., 2013.Pullorum diseasePD and Fowl typhoid FT, are two diseases specific for avian, these diseases cause significant economic losses around the world, *S. Pullorum* causes PD infect young birds is an acute systemic. *S. gallinarum* causes FT effects at adult birds , and all ages may be susceptible to infection is an acute or chronicBarrow and Freitas , 2011.*Salmonella* transmission vertical from the mother to the chicks via egg, but remain the big problem is the horizontal transmission through carriage, feed, and vectors including humans, rodents, and insects Foley *et al*,.2008 ; Wales *et al.,* 2013.*Salmonella* like many pathogens transmission by direct ingestion ,also possible transmission through the inhalationLopez *et al*., 2012.For the prevention and control of *Salmonella* can be used a vaccine, where he was important in the production of meat and eggs free of salmonella, both Live attenuated and killed vaccines with broad impact to reduce mortality and shedding pathogen to environment Priyantha 2009 ; Hossain 2011.

During the infection of *Salmonella* and after the arrival of these pathogens to the intestinebegins the body with the innate immunity, which is considered the first line of defense, including epithelial layers, macrophages, complement, dendritic cells, coagulation cascade , cells act as natural killer and coagulation cascade, another function to these cells retain for intestinal homeostasis through integral pathogen signals Peterson and Artis ,2014.

II. Material And Methods

Field Study: Of the total 69 samples collected from broiler chickens, which had signs of respiratory and diarrhea, in the province of Salah al-Din, and was sent to the laboratory of the microbiology of Veterinary Medicine, the University of Tikrit for the purpose of examination and isolation of *Salmonella pullorum*..

Isolation and Identification of Salmonellaspp. Procedure :The study was conducted using traditional methods of detection and diagnosis of *Salmonella*, following the standard guidelines from**OIE, 2012** sample culture on Selenite F broth and incubated for 24 hours at 37°C. Then transfer a drop of broth using the Loop and subculture on *Salmonella-Shigella* agar and XLD for the purpose of testing their fermentation or non-fermentation of lactose sugar, and production of hydrogen sulfide gas **H2S**, incubated at 37 °C for 24 hours **Menghistu*et al.,* 2011**. Further confirmations have been made on colonies suspected of *Salmonella* by conventional biochemical test.

Biochemical test: Biochemical diagnosis was based on the methods developed by**Quinn *et al.,* 2004,**Where one or more pure colonies of isolated bacteria were identified, culture the bacterial colonywere subjected tothe slant

Triple Sugar Iron **TSI** agar, sugar **Carbohydrate** fermentation test, citrate utilization, urease reaction, indole reactionandsulfide- indole- motility**SIM** Test.

Half Lethal Dose LD50: The bacteria was growth on nutrient broth at 37 °C for 24 h, and in the centrifuge with 3000 cycles / min for 15 minutes were concentrated. By PBS three times, for 5 minutes each time were washed and discard the liquid.according to the following Reed and Mueneh.

Freezing And Thawing: This method was used according to Calcott and MacLeod, through freezing the bacteria, then frozen slowly 1 -2 °C / min to at least -70 °C before melting at a slow rate.

Experimental chicks*:*

The chicks that were used in the experiment were divided into two groups, the first of which consisted of 30 chick - a one - day Rose type called the experimental group and the second called control group, under similar conditions, fed on pellets and drinking water free of additives.

First dose: All chicks (30 chicks) were given 0.1 ml (2 x 10^{-7}cfu / mL) of the live attenuated bacteria at the age of 7 days.

Second dose: At the age of 14 days, all chicks received 0.1 ml 2 x 10^{-7}cfu / mL of live attenuated *Salmonella*.

Challenge dose*:*At 28 days of age, all birds in two groups **n = 15** were orally challenge with 0.5 ml2 × 10^{-7}cfu / ml of suspension containing *Salmonella* strain.

Gross lesion:Gross lesion was determined for each of the internalorgansin two group vaccinated and control group, and then show the change lesions in 28 day.

Immunological tests:

Delayed-type Hypersensitivity test DTH: On day 20 post vaccination, 0.1 ml of *S.pullorum* were injected in the right wing, the left wing as control injected with 0.1 ml sterile PBS, at 24 and 72 hours right after injection the expansion diameter was measured, The results were expressed as the mean increase in the sensitivity area at periodic intervals after injection.

Passive haemagglutinationPHA test: PHA test was used to determine the antibody titers in chickens vaccination by *Salmonella pullorum*. In 17 days after post vaccination on age 24 day,sera collected from the vaccinated group and control group stored at freezing until use .This test was conducted according to**Herbert, 1987** methods.

Sample collected:

After *S. Pullorum* vaccination on day 7day 0 of vaccine, and 2nd vaccine on 14 day day 7 of vaccine, 2 birds from two group were euthanatized at 20 days from age 13 day post vaccination. By cervical dislocation for gross andhistopathologicalstudies,the tissue samples of the liver and intestines cecum, were sent to the laboratory and are subject to formalin 10% for tissue samples. On day 28 21 post vaccinationchallenge dose 0.5ml /bird and after two day, histopathological studies.

III. Result

In this study, a total of 12 **57.1%***S. Gallinarum / pullorum*,of 69 samples were identified as *Salmonella* sp. This was done through the use of cultural techniques and biochemical tests. Results obtained from the cultural, morphological and motility test of *Salmonella* isolates sp.

PCR Result: *Salmonella* isolates have been confirmed using the target genes specific to the A gene, using self-styling primers, 150bp for *S. pullorum*and 285bp for *Salmonella* sp.

Determination of LD_{50}: Dilution 10^{-5} showed the death of half the number of chicks when experimenting with *Salmonella pullorum*, which contains 200 cells / ml of a bacterial number. Thus, the LD50 lethal dose of 2 x10^{-7} cells / ml, as shown in table no

Result Freezing and Thawing:*Salmonellapullorum* was culture before freezing, with 250 cells at 1 mL after culture on stored agar at 37 °C / 24 h and 250 cells /ml. After thawing was 200 cells per 1 ml.

Table 1: Results of cultural, motility and morphological characteristics of the isolates of *Salmonella* spp.
N.B: SS= Salmonella-Shigella , XLD= Xylose Lysine Deoxycholate , TSI = Triple Sugar Iron.

SS agar	XLD agar	Staining characteristics	TSI agar	Motality
small, smooth ,transparent round shapes and blackness at their center	Colonies appear smooth with black center	Pink short rod, gram negative arranged in single or pairs	Black color colonies against a yellow background	-Ve *S. pullorum, S. gallinarum*

Table2: shows the number of death and alive for each dilution and bacterial acount of this dilution.

No.	Total sample sent to lab.	Broiler	Layer	positive	Negative	Positive%
1	13	13	0	5	8	23.8%
2	22	18		2	16	9.6%
			4	0	4	--
3	14	11		6	5	28.6%
			3	2	1	9.5%
4	4	4	0	0	4	--
5	6	6	0	4	2	19%
6	10	8		2	6	9.5%
			2	0	2	--
Total	69	60	9	21	48	100

Gross Lesion results: Gross lesions were observed, in the vaccinated group post challenge, after challenge 0.5 ml with *S. pullorum* recorded and photographed during necropsy, all the internal organs were normal**liver , intestine, heart, bile and kidney**.The other organs no clear lesion observed. in control group gross changes were observed enlarged in the liver , with green-yellowish color, and friable, not in all cases figure 1. Kidney was enlarged, mild congested in intestine figure 2.

Histopathological changes : The results of the experimental infection in the chicken showed the following pathological changes in the organs **cecum and liver**, which were sampled at the age of 20 days, and two days after the challenge dose was given as follows:
In the cecum at 20 day found, the surface of the intestinal mucosa was covered by a simple columnar epithelium, and there was a degeneration of epithelial cells and a dissociation of the mass in the intestinal lumen, the goblet cells also hyperplasia. The inflammatory cells were invaded the epithelial cells at the mucosa. The lamina propria was highly engorged with inflammatory cells. Figure 3 show these effect.In between the bundles of smooth muscles fibers of tunica muscular showing lymphocytic infiltration .The blood vessels in the tunica serosa were congested with blood. with infiltration by inflammatory cells in the serosa fig.4.

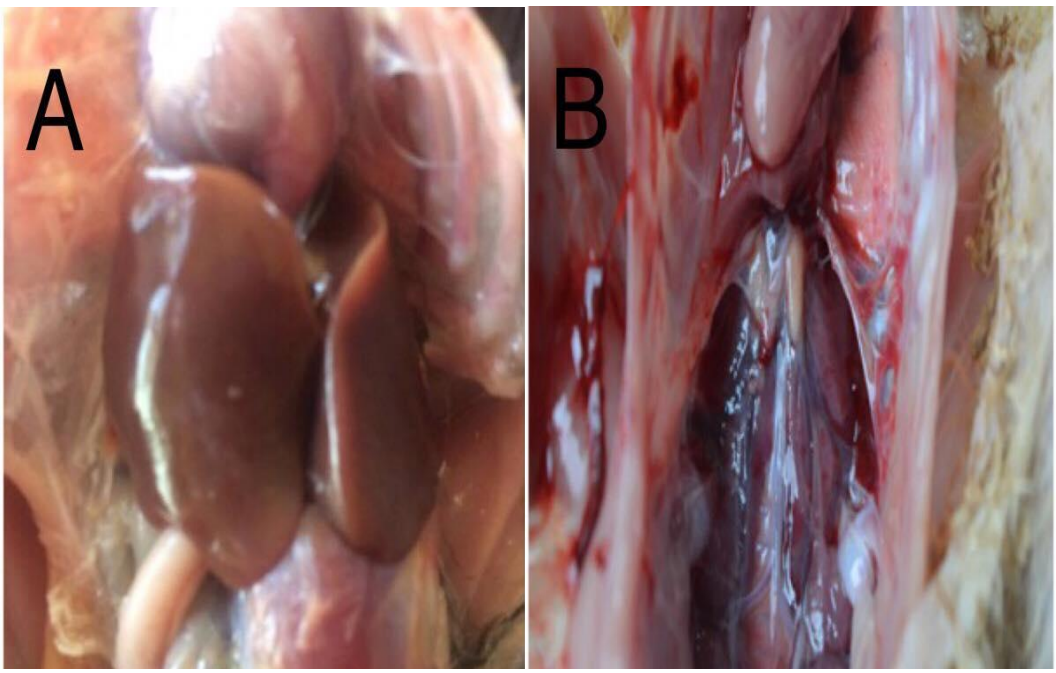

Figure1 : A; Normal liver and intestine of vaccinated chicks at 28 day age. B; Normal internal organ kidney, heart and lung.

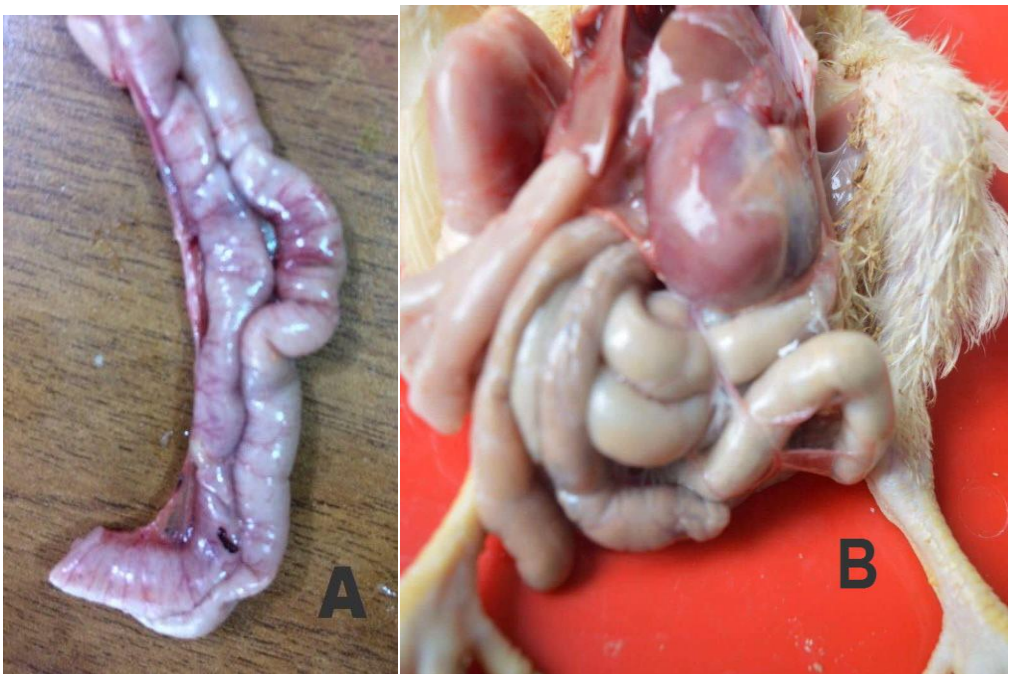

Figure2; A: Mild hemorrhagic enteritis in control group at age 28 day after challenagwith 0.5ml virulant SP. **B**: Normal intestine in vaccinated chicks reseved 0.5ml virulant SP.

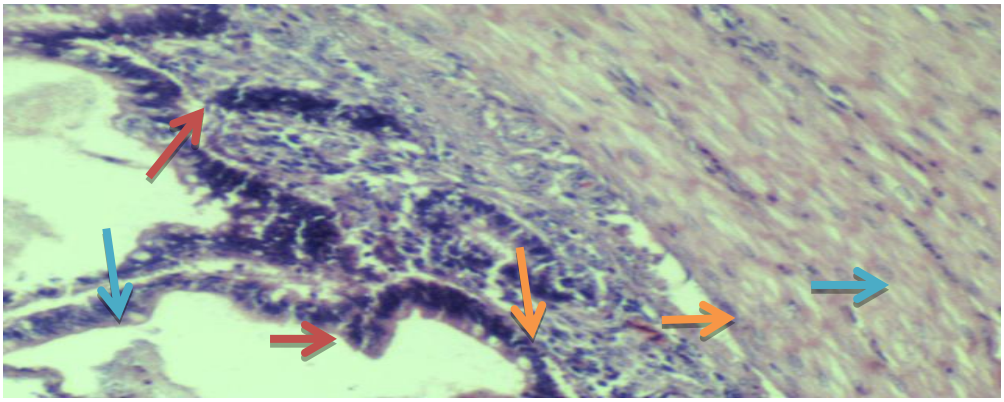

Figure3 :cecum of chicks at20 day, show degenerated epithelial cellappearsgoblet cell hyperplasia , extensive infilammatory cell ,H & EX 400.

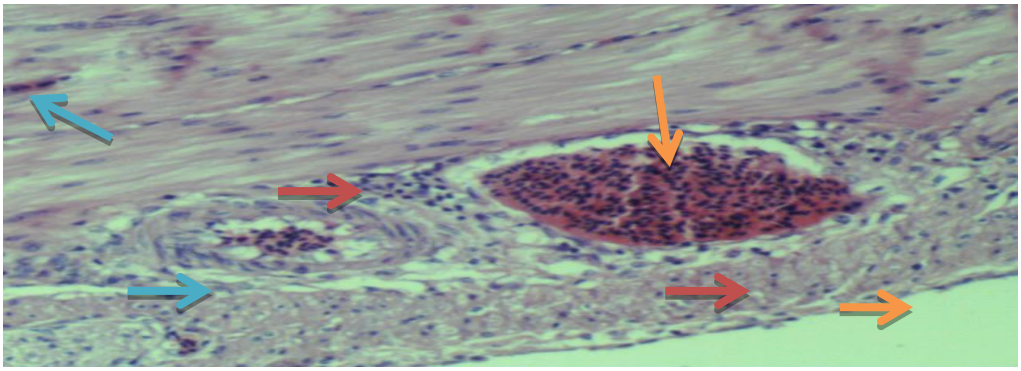

Fig. 4: of the cecum of chicks20 dayvaccinated blood vessels congested, lymphocytic infiltration , in serosa infiltration of inflammatory cells H & E X 400.

After 28 days of age and two days after the dose challenge the following changes are observed: -
The mucosal villa were numerous, covered by simple columnar epithelium certain number of these cells were degenerated and invaded by inflammatory cells ,these was masses of epithelial cells and mucus located in the lumen of intestine. As the figure 5.

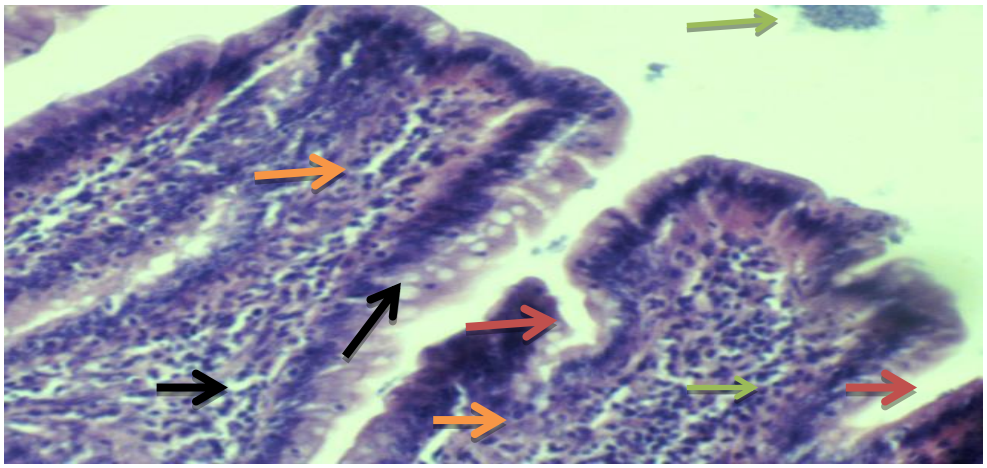

Fig5: of the cecum after 28 day and two day challenge dose(*pullurum* inoculation degenerated villi , masses ofepithelialcells in the lumen ,infilteratedinflammatory cells ,separation in epithelial cells . H & EX 400.

In the liver at 20 day found. The parenchyma of the liver was containing a masses of the liver cells some of these cells are lost its nuclei , there was an atrophy of certain number of liver cell .The sinusoid were wide and highly extensive in between the masses of liver cells , these sinusoid appeared occupied by inflammatory cells with Kupffer cells. As the figure 6.Lymphocytic aggregation was noted around the central vein, which had congestion of blood figure 7.

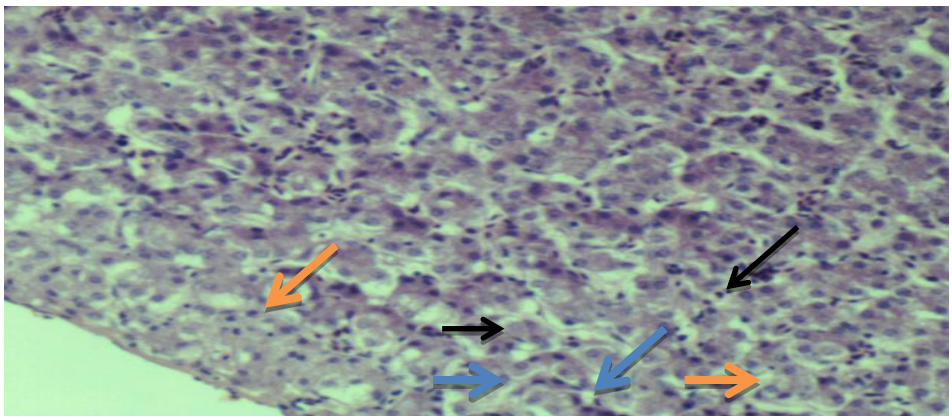

Figure 6: liver at 20 day : appear liver cells atrophy nucleated inflammatory cells with kupffer cells , sinusoid wide and extensive between liver cell H & EX 200.

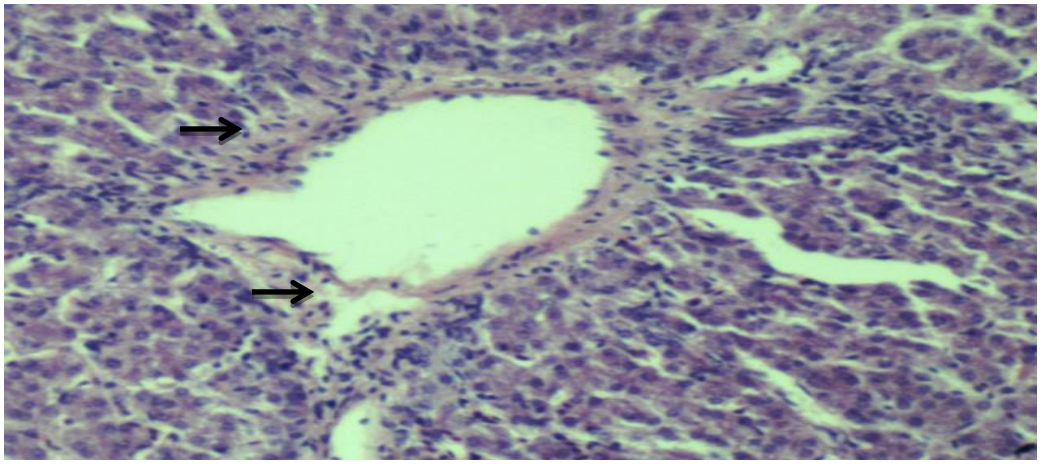

Fig.7: liver at 20day of age, showing Lymphocytic aggregation around centrial vein . . H & E X 200.

After 28 days of age and two days after the dose challenge the following changes in liver are observed: -The interstitial C-T was containing inflammatory cells , Kupffer cells loss nuclei. The capsular of the liver was containing foci of inflammatory cells **fig. 8**.

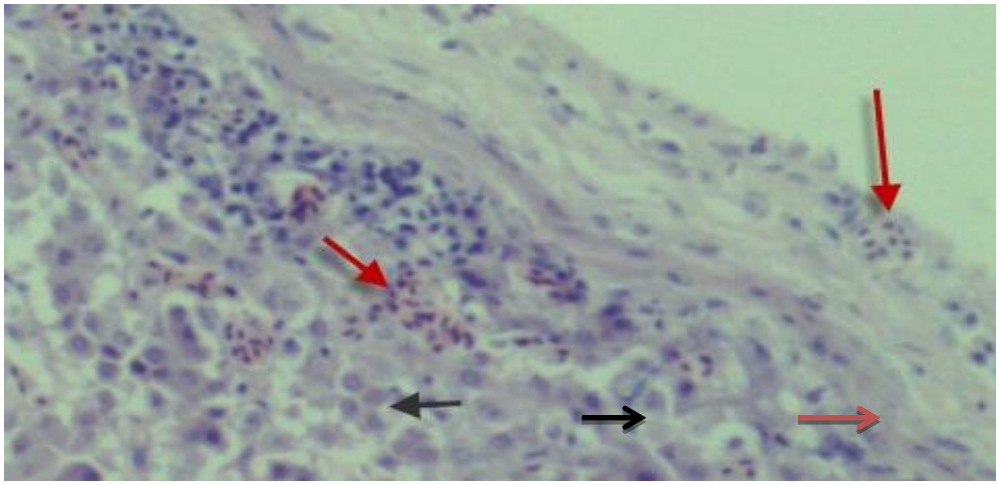

Figure 8: liver of vaccinated chicks at 28 day, observedKupffer cells lossnucleiinflammatory cells in capsular and parenchyma H & E X 200.

The control groups after challenge with 0.5 ml *S. pullorum* at **2X10^{7}cfu /ml** in age 28 day, the cecum show degeneration the goblet cell, and sloughing in the lumen intestine inflammatory cell in epithelial cell of the mucosa**fig.9**.

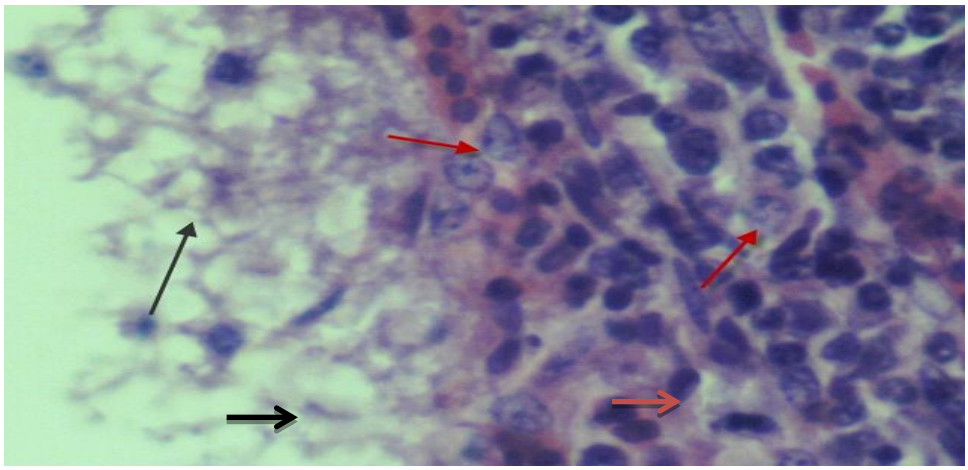

Figure 9: cecum of control chicks after challenge 0.5ml of *SP*observed degeneration the goblet cell sloughing gobletcell in the lumen . H & E X 200.

The parenchyma of the liver was containing a masses of many individual the liver cells some of these cells are lost its nuclei, the sinuosids were severely congested with blood .With presence of Kupffer cells in side these sinusoids **fig.10** .

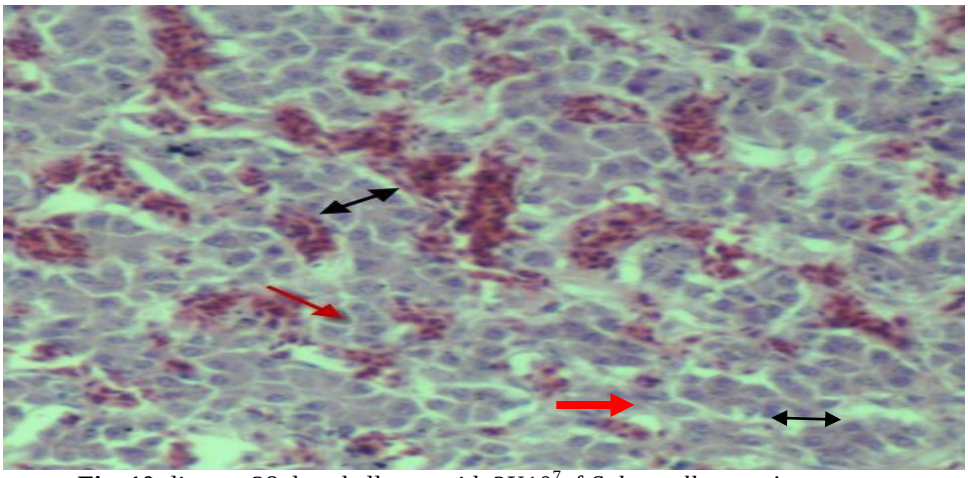

Fig. 10: liver at 28 day challenge with $2X10^7$of *Salmonella entericaserovar pullorum* ,show Kupffer cells inside congested sinusoids , characterized by the dissolution of their nuclei H & E X 200.

Passive haemagglutination test Results: After 20 days of giving the first dosevaccine dose the serum was collected from 10 chicks used to detect the indirect hemagglutination, in a 10-fold test plate.

Table 3: Result of Passive haemagglutination test . + = result positive agglutinateserum and RBCs is diffused . _ = result negative agglutinate serum and RBCs is spot.

#	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Titer
A	_	_	+	+	+	+	_	_	_	_	**8**
B	_	_	- /+	_	_	_	_	_	_	_	**8**
C	_	_	_	+	+	+	+	+	_	_	**16**
D	_	_	+	+	+	_	_	_	_	_	**8**
E	_	_	+	+	+	+	_	_	_	_	**8**
F	_	_	_	- /+	_	_	_	_	_	_	**16**
G	_	_	+	+	+	+	_	_	_	_	**8**
H	_	_	_	+	+	+	+	_	_	_	**16**
I	_	_	_	_	_	_	_	_	_	_	**<2**
J	_	_	+	+	_	_	_	_	_	_	**8**
Con -	+	+	+	+	-	-	-	-	-	**4**	

Dilution 10^{-3}**8** is the first dilution in which red blood cells agglutinate with the antigen in the hole plate test.

Delayed-type Hypersensitivity testDTH:A total of 10 chicks were used in this test to determine the effect of the vaccine dose on the induction of skin reaction, the test, age at 20 days 14 days after the first vaccination dose, was injected under the skin in a few feathers in the wing of about 0.1 ml S. P. were chosen right lateral side of abdomen of chicks, another side contain same amount of PBS, result was described in table 4. The wing thickness diameter was measured in the injection area after 24 and 48 hours of injection by vernier caliper **micron**Figure 11 shows that effect.

Table4 : Diameter of swollen after 24 , 48 hours from bacterial injection.

#	Mic. / 0 h	24 /h	48 /h	Control
1	9	9.5	11	9
2	9	9.5	10.5	9
3	9	10	11	9
4	9	9.5	10	9
5	9	11	12.5	9
6	9	9	9.5	9
7	9	10.5	10.5	9
8	9	10	11	9
9	9	9.5	10	9
10	9	9.5	10.5	9

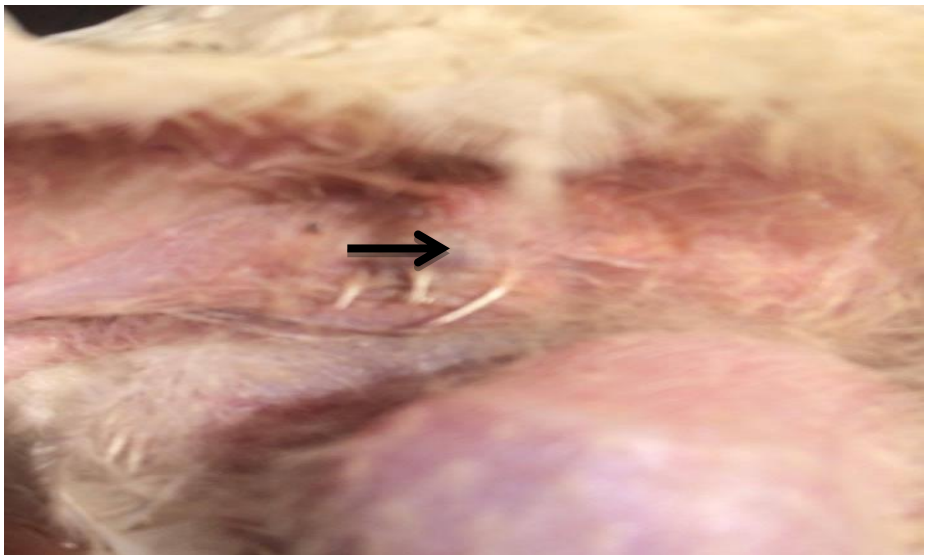

Fig. 11; DTH reaction of skin increased thickness and scab formation
after 48 h injection s. c. 0.1 ml from *S. pullorum*.

IV. Disscusion

Jacirol Islam et al.2016, and others found that the rate of *Salmonella pullorum* was 47.73**% n = 21** which is more than what was achieved in our study which was 57.1% **n= 12 a** total of 69 samples.In Iran **Jamshidi*et al*.2009**, was diagnosed 27 *Salmonella* isolates out of a total of 1125, which is less than what was found in this study.In slow freezing formation large amount from crystal in extracellular , while rapid freezing lead to small intracellular ice crystals formation, Ice crystals large in size and lead to cell damage by deactivate internal structures, cell walls and membranes **Jay, 2005). Obafemi and Davi ,1986**found that*Salmonellatyphimurum* affected by double freeze-thawing with viability reduced by 99.0% and 95.6% respectively.

Gross Lesion :

Haider*et al*.,2012after experimentally infected orally with 2 x10^7**CFU** dose of *S. Pullorum*, the gross findings haemorrhagic and congested liver and observed necrotic foci,also found lung congested, edematous and brown coloured, in the ceca lumen found caseous materials semi-solid, cheesy material and button like ulcer and swelling, congestion in spleen.In this study, most birds with virulent *S. pullorum* challenge did not show prominent gross lesion of pullorum disease. These results are in agreement with Rahul *et al*. 2015 Other changes in one case the liver included enlarged a bronze discoloration, this similar to result of **Nazir*et al.* 2014**

Histopathological changes:

In this study observed in the cecum hyperplasia goblet cells also lymphocytic infiltration and degeneration and desquamation of the epithelium**,** these observations are similar to those from**Nazir*et al*., 2014** .Degenerated villi of intestine Similar microscopic lesions were reported by **Haider*et al*.,2012**Lourenço*et al*.,2016.

Degenerative necrosis and infiltration of mononuclear cells in liver in present results corresponding with that of **Hossain*et al*.2006cells** are lost its nuclei, inflammatory cells with kupffer cells, extensive necrosis of the hepatocytes similar lesions also reported by **Garcia *et al.* 2010**

Passive haemagglutination: PHA test was conducted for determination of antibody titre of the sera of vaccinated and unvaccinated chickens. Control chicks PHA titre of sera samples were recorded as table 3,which was closely related to the findings of **Ferdous*et al.* 2008**.In this study the titre of antibodies vaccinated chicks observed the lowest antibody titre was8 and the highest antibody titre was 128, this result is similar to that found by Bhattacharya *et al*., 2004 and others, where they noted that the titre of the antibodies caused by the vaccination reached the peak.

DTH Results: The results in this study are in agreement with reports in which induction of the DTH reaction in response to *Salmonella* immunization was shown to be responsible for protective immunity in *Salmonella***infections Gupta *et al.* 1996** *al*. 2005.

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