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Clinico- pathological study on Rabbits infected experimentally with *Salmonella enteritidis* isolated from human

A Thesis

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In Partial Fulfillment of the Requirements for the Degree of the Master of Science in internal and preventive Veterinary Medicine (Zoonosis)

By

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بسم الله الرحمن الرحيم

فتعالى الله الملك الحق ولا تعجل بالقرآن

من قبل أن يقضى إليك وحيه

وقل رب زدني علما

صدق الله العظيم

سورة طه - الآية (**114**)

Dedication

This work here is dedicated to
My Husband……,
My family……,
My children……..., and to all people who supported me during my education period.

Ayat

Acknowledgement

Initially, I thank Allah for all good that I got and that earn of force and patience and ability to do this modest scientific effort.

I would especially like to thank my supervisors Asst .Prof. Dr.Khalid Mahmoud for providing me with this opportunity, for offering their advice, guidance, encouragement, patience, support throughout my study, and for their assistance and advice on completing this research and thesis writing

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Examination Committee Certification

We, the examining committee, certify that we have read this thesis entitled (**Clinico- pathological study on Rabbits infected experimentally with *Salmonella enteritidis* isolated from human)** and have examined the student in its contents, and that in our opinion it is adequate for awarding the Degree of Master of Science in veterinary internal and preventive medicine/Zoonosis.

Asst. Prof. Dr.

Assist. Prof. Prof.

Dr. Khalid Mahmoud hammadi

Summery

Nowadays the infection with *S.enteritidis*, in human beings has wide range of distribution especially in new children. However, in animal no such systemic studies have been done so far. Therefore this study has been carried out in order to investigate the effect of the experimental infection of *S.enteritidis*, in Rabbits under control conditions and the next parameters have been included (clinical, pathological(macro and microscopic), and test of cell mediated immunity at 21 days post infection. The diagnosis of the bacteria determined by culture, biochemical test(Api-20E system and VITEk 2 system).

Sixty local breed rabbits of both gender were used in current study, the age range was between 8-12 months, and their weight range was between 1500-1900 gm. In the first experiment twenty rabbit were used estimate the infective dose of *S.enteritidis*, and the rest 40 rabbits were divided randomly into two groups (n=10), group control one, and (n=30), infected group. In the infected group the bacteria was given via intraperitoneally route, and the control group rabbits were given phosphate buffers saline.

The results shown that, the infected dose is 4.5×10^8 cells which was associated with clinical sings without mortality, the body temperatures, heart rate and respiratory rate were increased with dullness and diarrhoea, anorexia, and restlessness, also they passed frequent cloudy or milky urine in addition to dyspenea and dehydration.

During the gross lesions, investigation, in the pathological aspects, which include sever swollen and congested with yellowish spots on liver (necrosis). The gall bladder was enlarged combined with enlargement of the kidneys and the heart was flabby. The small intestine characterized with flaccid and filled by clear to watery fluid content and mucus in large amount, the large intestine showed enlargement and congested in a comparison to control group intestine.

In addition to the previous findings, in histological outcomes were found and recorded during (24, 48, 72, 96,120,144,168 hours) second and third weeks. The result in the small intestine showed sever infiltration of inflammatory cells (macrophage and lymphocyte), sluffing of the epithelium, exudate and there is necrosis, liver showed sever infiltration of inflammatory cells, necrosis and there is granuloma, lung showed damaged of the alveoli, and present of exudate. In the heart, there is few inflammatory cells infiltration.

Moreover, we found that the bacteria *S.enteritidis* was widely spreads in internal organ of the infected rabbits with infective dose, and we noticed that *S.enteritidis* has the ability to invade the most internal organs (spleen, liver, kidneys, lung, heart, brain, small and large intestine) but in varying degree. No such changes have been found in the control group, which gives negative bacterial results.

In the immunological aspects, in the infected rabbits right, there was a significant increase within 2 day post immunization which exhibited the higher average thickness then it decreased after 3 days.

In the infected group, there was a significant increase in the phagocytic indices (35.16) as compared with control group (14.5).

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List of abbreviations

Aberrations	Key or full name
μm	Micromole
ADH	Arginine dehydrolase
Ag	Antigen
ANOVA	Analysis of variance
APi 20	Analytical profile index for Enterobacteriaceae test
B cells	B lymphocyte
BPW	Buffer peptone water
C	Capsular antigen
CD4	Cluster differentiation
CD8	Cluster differentiation
CFU	Colony forming unit
CIT	Citrate test
DCs	Dendritic cells
DNA	Deoxy nucleotide
DTH	Delayed type hypersensitivity
EFSA	European Food Safety Authority
EU	European Union
GEL	Gelatinase

GHs	Glycolytic hydrolyses
GIT	Gastrointestinal tract
gm	Gram
H Ag	Flagella antigen
H_2O	Water
H2S	Hydrogen sulphate
HIV	Human immunodeficiency virus
IB_S	Pound
ID	Infective dose
IFN	Interferon
IL	Interleukin
IND	Indol test
IP	Intra-peritoneal
KB	Kilo base
Kh	Kilo hertz
LDC	lysine decarboxylase
LPS	Lipopolysaccharide
LSD	Least significant differences
M cell	Microfold cell
mg	Milligram

ml	Mole
MLNS	Mesenteric lymph node
Nacl	Sodium bicarbonate
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NK	Natural Killer
NOS	Nitic oxidase synthesis
NTS	Non typhoidal Salmonella
O Ag	Somatic antigen
O2	Oxygen
ODC	ornithine decarboxylase
OIE	World Organisation for Animal Health
PBS	Phoshate buffer saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
RES	Reticuloendothelial system
rpm	Round per minutes
SCV	Salmonella-containing vacuole
SEF	Samonlla fimbriae
SEFA	*Salmonella enteritidis* fimbrin gene
SOP	Standard Operating Procedure

SPI2	Salmonella pathogenicity island
SPV	Plasmid virulent gene
SPVRADCD	Plasmid virulent gene
SS	Salmonella Shigella
T cells	T lymphocyte
T3SS	Type 3 secretion system
TAD	(Tryptophan deaminase)
TNF	Tumor necrotic factor
TSI	Tribble sugar iron
URE	Urease test
V	Virulent
VI	Virulent intigen
VITEK2	Automated microbial identification
VP	Voges–Proskauer
VPI	Voges–Proskauer
W	Wat
WHO	World organization for animal health
XLD	Xylose lysine deoxycholate
PI	Post infection

Chapter one
Introduction

1.1 Introduction

Salmonella species are responsible for outbreaks of human gastroenteritis and enteric fever all over world. This bacterial species is widely distributed in different animal's species that are used as food and could reach aquatic animals through fecal contamination of water and represents a serious public health problem. According to annual report of OIE (2016), Salmonellae caused the highest number of foodborne infections of gastrointestinal tract. Furthermore, such cases were reported to be 1.4 million yearly in USA (Barrow and Neto, 2011).

The first strain of *Salmonella* was isolated in 1885 by Dr. Daniel E.Salmon. Today, the genus is known to consist of the two species *S.bongori* and *S.enterica*, which have a high genomic sequence similarity (96-99%). *S. enterica* is responsible for most infections in human and other animals that were reported as warm-blooded whereas many cold blooded animals were reported to be infected with contrary *S.bongori*, but are poorly pathogenic for humans (Kaniuk NA *et al* .2011).More than 2500 *Salmonella* serovar have been identified but most of them are not pathogeneic. The three main serovars being pathogenic to human are *Salmonella enterica* subspecies enterica serovar *Typhimurium, Enteritidis* and *typhi* which are pathogenic to human the other one induce self- limited gastroenteritis and bacteremia. Howevr *S.Typhimurium* and *S. enteritidis* infections in elder, younger and immunocompromised people lead to a typhoid fever (OhlsoMB. 2008).

Many reports mentioned that salmonella food-borne illness was associated with S.Enteritidis and S. Typhimurium, and the source of infections came from contaminated poultry products, egg, and meat (Kramarenko *et al*., 2014:Thung *et al.*,2016*)*.In Europ and United State, most outbreaks of salmonellosis in humans were attributed to serovar *Enteritidis* of *Salmonella enterica* (Gould *et al*., 2013) that might be associated to consumption of

contaminated eggs or other poultry products (Braden, C.R. 2006). Salmonellae was isolated from feather, skin and contents of digestive tracts of living birds, accordingly live poultry can be considered as main reservoir; carcases of infected birds and improper processing, bad packaging and transportation, evisceration, and cooking in abuse temperature might act as best ways of bacterial contamination with salmonellae (Zhang *et al.*, 2013).

Foodstuffs that contaminated with salmonellae made it unsafe for human consumption (Agunose, A. 2007). *S.enteritidis* is zoonotic and is transmitted to human via ingestion of contaminated meat, egg and milk (Radriquez,*et al.*, 2014). It may cause infections associated with systemic infection or several clinical symptoms characterized by diarrhea and septicemia and may lead to death in severe cases. *Salmonella* can be harboured by asymptomatic carriers (Smith -Palme *et al*., 2003). Many salmonellae infections were reported due to direct or indirect contact with infected animals in veterinary clinics, at homes or zoos, but foodborne infection still the main source of human salmonellosis. The M-cells of payer's patches, the columnar epithelial cell lining at lower part of small intestine or the colon epithelial lining of proximal part were the first sites of salmonella entrance to sub epithelial places that then aided in salmonella transportation to mesenteric lymph nodes, liver, and spleen.

Infection with Salmonella depended on three factors, the infective dose, level of immunity and predisposing factor influencing (Venter BJ 1994).

1.2 Aims and objectives of this study:

1. To estimate infective (ID) of *Salmonella enteritidis* in experimental animals (rabbits).

2. To observe changes that might affect such animals clinically or in different organs at post-mortem.

3. To follow the sequence infecting bacteria spread in different organs of infected animals.

4. To determine the cell mediated immune response activity during the experimental infection.

Chapter two

Literatures review

Literatures review

2-1: General features:

The family Enterobacteriaceae included many genera, *Slamonella* genus was one of them. This genus included many bacteria that were gram negative rods, facultative anaerobes, motile with peritrichous flagella, non-spore former, their sizes ranged from the wide of 0.8 to1.5 µm and a length ranged from 2 to 5 µm. two salmonella species were not motile, these are *S.pullorum* and *S. gallinarum* (Abdullahi, 2010).

Humans and animals were susceptible to salmonella infection as foodborne salmonellosis, paratyphoid fever or typhoid fever (Ray and Ray, 2004).

Thy have the ability to grow in low temperature (4-8℃) and low pH (4.4 to 8) (Krulwich *et al.*, 2011), as facultative anaerobes, they grew with low oxygen level but cannot survive hot environmental temperature above 70℃ (Radostits *et al*., 2007).

Sodium deoxycholate, sodium tetrathionate and brilliant green in a medium can inhibit the growth of other enteric bacteria but not Salmonella. Production of gas from glucose, acid and H2S appeared as some of their growth characteristics in addition to utilization of sucrose and lactose in the media (Jawetz *et al* .,2007)

2.2: Classification of Salmonella:

The genus *Salmonella* was grouped with family *Enterobacteriacae* of the Order *Enterobcateriales*. This order was classified within the *Phylum* of *Proteobacteria* of the kingdom *Bacteria* (Brenner *et al.*, 2005)

There are two species in the genus Salmonella which are S.bongori and S. enterica (Grimont and Weill, 2007). An unusual strain was isolated from

environment *S.subterranea* and proposed as a third species (Shelobolin *et al.*, 2004).

There are six subspecies were reported from the main species S.enterica, furthermore, lysis by bacteriophage and biochemical activities were used to differentiate these subspecies (Grimont and Weill, 2007). They were mentioned in table (2.1)

Table (2-1): Classification of Salmonellae species and subspecies

Original subgenera	Current nomenclature
SubspeciesI	Subspecies enterica
Subspecies II	Subspecies salamae
Subspecies IIIa	Subspecies Arizona
Subspecies IIIb	Subspecies diarizonae
Subspecies IV	Subspecies houtenae
Subspecies VI	Subspeceis indica

Moreover, and to misunderstand or confusion with names of serovar *S.enterica* subspecies enterica the V symbol was retained (Grimont and Weill, 2007*).* According to Kauffman- white scheme that depend on O to AG somatic antigens of the bacteria, 2500 serotypes of Salmonella were reported (Popoff *et al.*,2004; Domenech,2007).

Most human and food animal's infections were attributed to serovars of subspecies enterica. Cold-blooded animal infections, some occasional infections in human and/or other environmental contamination were connected to other serovars. Some disease of free-living or captive amhibians or reptiles, sheep and turkey was found to be due to infections with subspecies of diarizonae and Arizona (OIE, 2010).

Identification of the prevalent serotypes of Salmonella in humans should be done for developing a control program for an region which is vary than another, because Salmonella prevalence and their serotype may different considerably between localities (OIE., 2016).

2.3: Characteristics of *Salmonella:*

2.3.1: Bacterial structure:

The cell wall of gram negative bacteria (Quinn *et al.*, 2004), consists of three components, virulent antigen (VI), lipoprotein, outer membrane and lipopolysaccharide (LPS).

The bacterial protein that sticks on peptidoglycan and stabilize the outer membrane of gram-negative bacteria is the lipoprotein. Endotoxin in gram negative bacteria is the molecules of lipopolysaccharide (LPS) that in case of host infection stick on outer cell and caused lysis. Most salmonella infections were conducted through oral routes and this was attributed to the size of LPS antigen in salmonella as it was the biggest and the most effective (Brooks *et al.*, 2001).

2.3.2.1: Flagellar antigen (H-Ag):

Flagella of Salmonella were forming bacterial antigen which is heat-labile and composed of protein known as flagellin, this antigen (H-Ag) is also inactivated by formaldehyde and alcohol (Old, 1996).

The phenomenon of phase variation made Salmonella as a unique organism when compared to other enteric bacteria. This phenomenon is associated with presence of two types of flagellar antigen H1-Ag and H2-Ag which were significantly different from each other, and were associated with

ability of salmonella to escape the antibody attack of immune system (Kotetishvili *et al* ., 2002) .

2.3.2.2: Somatic antigen (O-Ag):

The O-Ag resists formaldehyde and alcohol and heat stable (Old, 1996). This is composed of polysaccharide that forms the most outer portion of lipopolysaccharide (LPS). Each four to six sugars were forming one O subunit, and polysaccharide of O-Ag was composed of several polymers of O subunits. These O subunits are forming the somatic O antigens and different in the subunits linkage, the covalent bonds and the form of sugar (Anonymous, 2004).

Lipopolysaccharide (LPS) is the most potent endotoxin that was responsible in inflammation and injury in the host infected with gram negative bacteria. This glycolipid was also responsible for membrane stability of the bacterium (Williams, 2007).

2.3.2.3: Virulent antigen (VI – Ag):

The most outer part of some salmonella serovars is known as capsule, and this capsular structure is known as VI-Ag. *Salmonella typhi*, *S. dublin* and *S.paratyphi* are the only serovars that contained the VI-Ag (Saha *et al.*, 2002). Immunological detection of Salmonella serotypes was depended on capsular antigen that can be purified by chemical methods (Fluit, 2005).

Flagellar antigen was used for serotypes identification and classification as it was more specific when compared to other salmonella antigens. Somatic antigen was cross reacted with some genera of Enterobacteriacae, but generally most salmonella strains of S.enterica spp enterica caused human infections (Scherer and Miller, 2001).

2.4 Epidemiology:

S.enteritidis is caused sever diseases in humans (number of hospitalization and death associated with infections),(Scallan *et al.*,2011). It is

important pathogen for humans and animals (Eisenstei,1999;Akbarmeher,2011).It is one of the major causative agent of food borne disease outbreaks(Onyango *et al.*,2009) and also a public health concern all over the world (Cardinale *et al.*,2005).

The young children, older adults, and people with weakened immune system are the most to have severe infections (CDC, 2012).

At first *salmonella enteritidis* was described as "fatal septicemia" or "white diarrhoea" (Andino and Hanning, 2015).

Salmonella enteritidis is recognized as an important pathogen to puplic health and notified as a cause of bacterial diarrhoea worldwide (Medeirose *et al.*, 2001).

The contamination of food is the usual source of human infections, and poultry products are considered the major infection route for human (Noda *et al.*, 2010).

S. enteritidis was the most serovar in the mid-1980s to date (Omwandho and Kubota, 2010, Abd El-Ghany *et al.*, 2012).

Dogru *et al.*, (2010) in Turkey, mentioned that the most important common frequently of nontyphoidal Salmonella (NTS) serotypes isolated from 400 chicken carcasses were (*S. enteritidis, S. hadar, S. virchow and S. typhimurium).*

Salmonella enterica serovars were isolated within the last 3 years in Czech Republic. These strains originated from humans, cattle, and chicken and included isolates of S.agona, *S. typhimurium, S. enteritidis, S. agona, S. hadar, and S. infantis* serovars that were detected by using PCR method. (Karasova *et al.*, 2009).

In Poland studied salmonellosis in human, and found that the most frequently isolated serotypes are (*S.enteritidis*, S. *typhimurium,S.infantis, S. hadar and S. Virchow*), children aged 2 years are the most affected age group (Czerwinski *et al*.,2008).

A serotyping study in Spanish hospitals, found that the most important common frequently of 771 non-typhoidal Salmonella NTS serotypes isolated from the stools of patients with diarrhea were *(S. enteritidis, S. derby, S. typhimurium, and S. hadar*). These strains characteristically cause gastroenteritis and they can produce invasive infections with serious complications (Avial *et al*., 2005).

Salmonella enteritidis was isolated from flocks of geese in Denmark, and found to be colonized in from age of two weeks but in intermittent status (Christensen *et al.,* 2011)

Salmonella enterica represents the major cause of bacterial foodborne infection in the United State (Scallan *et al.*,2011).*Salmonella* enterica serovar *Enteriditis* is considered the major cause of humans salmonellosis outbreaks in the United State and Europe(Collard *et al.*,2005). The disease transmitted mainly through foodstuff and via contaminated with the pathogen, and it effects more than 90 million people worldwide yearly, with Varity morbidity and mortality rates (Majowics, 2010).

European Food Safety Authority (EFSA) in 2015 reported that *S.enteritidis* accounted for 575 European Union outbreaks, additionally it was attributed to contaminated foodstuff in 116 of these outbreaks.

Majowicz *et al.*, (2010) reported that 155, 000 deaths yearly among 93.8 million cases were attributed to salmonella gastroenteritis infections.

Salmonella Newport, *S.Typhimurium* and *Salmonella enteritidis* were reported to be responsible for NTS infections (4%, 12%, and 65%) of human cases respectively during the period extended from 2001 to 2005 worldwide according to the report of foodborne disease surveillance network (Galanis *et al.,* 2006).

Latin America, Asia, and Europe recorded *Salmonella Enteriditis* clinical isolates in 87% 38% and 31% respectively. The most common isolated serotypes of Salmonella in Africa were *S.Typhimurium* and *S. Enteritidis* and counted 25% and 26% respectively. Whereas the most reported isolate of salmonella in North America was *S.Typhimurium* and 29% of gastroenteritis infection cases. Furthermore, 21% of such clinical cases were due to *S.Enteriditis* (Galanise *et al.,* 2006). Underdeveloped and industrialized countries were still increasingly facing problems associated with non-typhoid salmonella infections even with application of good sanitation and hygiene (Majowicz *et al.,* 2010) People with HIV and children of 3 years old and below were suffering from invasive NTS infections especially in Sub-Saharan African and in undeveloped countries increased the mortality rate up to 25% (Gordon *et al.,* 2008). Contrary Asia showed less frequency of invasive NTS infections (Khan *et al.*, 2010).

In Iraq *S.Enteritidis* are isolated from buffalo from faecal samples and organs as a percentage of 13.64 %(Yousef, 2014).

In Thi-Qar province during 2015 from Twentey –Four isolates were diagnosed the percentage of *S.Enteriditis* is 12.5% from fecal samples of children (Ezat, 2016).

2.5: Transmission

Human salmonellosis was strongly linked to contaminated food of animal origin. Ingestion of contaminated fruits and vegetables, direct contact with infected people, pet animals like reptile, cats and dogs might be regarded as another mods of salmonella transmission (Freitas Neto *et al*,. 2010). The animal role in transmission of salmonella between herds and flocks in addition to human food contamination were also reported (OIE, 2010).

Salmonella was transmitted either horizontally and vertically (Hameed.,*et al* 2014). *Salmonella enteritidis* was transmitted vertically through transovarian infection and contamination of eggs (Andino and Hanning, 2014).

Human salmonellosis and especially in immune comprised people and children can be also attributed to contact with pet animals as these animals might harbour serovars of salmonella and shed it to the environment. The roof rats (Rattus rattus) play important role in the maintenance and spread of S. enteritidis inside the layer premises (Kabir, 2010). Animals that commonly infected with Salmonella serovars which may pose a risk to human include ,birds, rabbits, amiphibians, dogs, cats fish ,hamestar ,guinea pigs, mice ,horse, lizard, turtles and snakes, although. Furthermore, majority of salmonellosis in humans were connected to direct contact with snakes, lizards, and turtles that have been used as pet animals (Bruins *et al*.,2006; Berrtand *et al*.,2008).

Shedding of salmonella serovars in faecal materials of birds, rats and flies for weeks or months was reported as a source of environmental contamination (OIE,2010).

Chickens, cows and swine that pastured on contaminated food with salmonella acted as reservoirs and transmit the agent with their movement, and they also contaminate the environment that became as a source for human infection either through contaminated water or contaminated food. Some other

salmonellae do not have animal reservoir like Salmonella paratyphi and Salmonella typhi, they were transmitted to healthy individual by ingestion of food handled improperly by infected (Newell *et al.*, 2010).

Infected birds or contaminated bird feeders might transmit salmonella to cats that ingested such infected birds or when became in contact for long time with contaminated feeders (Riyaz-UI-Hassan *et al*., 2004; Oie, 2005). Contamination of environment can also be originated from infected animals and humans as they secret salmonella with their faecal materials (Hoszowski and Wasyl, 2001) and making a closed cycle of circulation for salmonella (Wray and Wray, 2000).

2.6: Pathogenesis:

The main source of Salmonella is the human intestinal tract, farm animals, wild birds, reptiles, and occasionally insects (Andino and Hanning, 2015).

Salmonella serovars can overcome the mammalian defence system as they were important pathogens for human and animals, and developed affected mechanisms to protect themselves from such host defences (Prior, *et al., 2009*).

Virulence plasmids were detected in some but not all isolates of salmonella like *S.typhimurium, S. pullorum, S. enteriditis, and S.dublin* (Rotgar and Casadesus, 1999).

Morbidity and mortality due to gastroenteritis caused by salmonella were reported worldwide. The use of laboratory animals as an experimental models in salmonella infections promoted the understanding of their pathogenesis in intestinal mucosa. Many studies concerned with the effects of bacterial products on susceptible cells of such animals in attempt to understand the immune responses to the

salmonella infection. The studying of main characteristics of intestinal dendritic cells and the presence of novel lymphoid follicle in crypto patches were studies well during infections with salmonella (Grassl *et al.*, 2008).

Serotypes of salmonellae were managed their uptake by the cells of intestinal lining, so that they were invade the cells of the host and survive inside phagocytes (Ohi and Miller, 2001)

Gianella *et al.*, (1976) reported four stages for pathogenesis of infection with salmonella these were included: Bacterial colonization in the intestine of infected host, disruption of the mucosa of intestine that led to invasion of bacteria, exsorption of fluid, and finally the spread of the pathogen to the reticuloendothelial system.

Fecal-oral transmission is the initial route by which Salmonella infect humans and animals, Salmonella have to be survive in the stomach acidic environment (Foley *et al.*, 2013) and be able to compete with intestinal flora to multiply and invade lymphoid tissue (EFSA, 2010), and other gastrointestinal tract (GIT) organs (Foley *et al.*,2013). Salmonella confront a barrier that represented by epithelial and immune cells lining GIT and competes with its microflora then colonize the gut (Sadeyen *et al.*, 2004; Foley *et al.*, 2013). Fimbriae represent a first class of structures involved in the attachment of bacteria to mucosal surface of intestine (Santos, 2013) although flagella have also been reported to function as adhesions (Asten and Dijk, 2005; Haiko and Wikström, 2013) then the bacteria reach into the interstitial space of the lamina propria where phagocytized by the different phagocytic cell (Velge *et al.*, 2012).

Salmonella cells multiply within macrophages, in *Salmonella*, the SPI-2 T3SS harbours genes that able to suppress antigenic presentation in dendritic

cells, and lead to limits the immune response of the host to infected cells (Foley *et al*., 2013).

Following the organisms engulfing by macrophages, they disseminate to organs rich in reticuloendothelial tissues (RES), like spleen and liver, which are the main location of multiplication and a second invasion may occur and be localised in other organs (pericardium, myocardium, and/or lungs) when the body defence mechanism become inadequate (Kabir, 2010).

The SPI-2 T3SS is known to block fusion of vesicles harbouring active NADPH phagocyte oxidase and the SCV membrane thereby enhancing the survival of *Salmonella* in macrophages (Torres and Fang, 2001). It is required for the replication of microorganism in the macrophage by translocate bacterial effector proteins across the vacuolar membrane, where the bacteria reside (Hensel, 2000).

Salmonella can survive inside the host cells vacuole, they were replicate and survive that facilitate their ability to invade bone marrow, liver and spleen (Christenson, 2013).

Bacterial attachment to the mucosa of intestinal tract is the first important and required stage in pathogenesis of salmonella. This attachment is facilitated by the surface stricture of bacterium that included some matrix proteins like collagens and laminin (Hsu and Lin, 2005).

The first element that early monitors defense mechanism to salmonella infection in the intestine of infected host is Payer's patches. Activation, redistribution, and recruitment of phagocytes were achieved as a first step of defense response against salmonella accession of host tissues. Anti-microbial molecules that produced by recruited monocyte control the replication of bacterium, but these cells were weak for presentation of bacterial antigens.

Contrary, presence of bacterium as a direct factor for stimulation and the indirect effect of cytokines increased DCs maturation the capacity in antigen presentation. Furthermore T lymphocyte stimulation was enhanced by the secretions of mature DCs (Tam *et al.,* 2008).

There are many factors involved in the severity of salmonella infections to human, the serotype of salmonella, the health of the host and the age of individual. Most salmonella serotypes were pathogenic and might cause fatal diseases, immune compromised patient suffered from immunosuppression were susceptible to infection more that healthy one furthermore aged individuals and children below 5 years of age more susceptible than others(OIE,2010).

In order to access the cells of susceptible host, salmonella was able to perform its own phagocytic activity. This was under genetic control induced by Salmonella pathogenicity island (SPIs). This island was involved in in the process of salmonella invasion of host cell. It was detected as genetic cluster in chromosomal DNA of salmonella (Grassl and Finlay 2008). Thus it was regarded as important character of salmonella in invasion of non-phagocytic cells of human tissues (Hansen-Wester *et al.,* 2002). Human got salmonella infection through the ingestion of contaminated food, then salmonella attached to intestinal lining and invaded the host cell by the action of SPIs that allowed the secretion of multi-channel proteins of type III secretion system. This protein facilitates the invasion of salmonella to cytoplasm of host cells. The signal transduction pathway of salmonella effectors was activated and the actin cytoskeleton of host cell is stimulated resulting in the formation of cytoplasmic extensions to surround the salmonella similar to that in phagocytosis (Takaya *et al.,* 2003). Virulent salmonella strains are those can persist in host cells whereas un-persisted salmonella strains regarded un-virulent (Bakowski *et al.,* 2008). This was followed by formation a vacuole in host cell cytoplasm formed from normal membrane of host cells and surrounded the engulfed salmonella. The engulfed salmonella secreted proteins mediated by type III secretion system to

alter the lysosomes enzymatic secretion that joined the formed vacuole. Accordingly salmonella protects its self from the action of proteolytic enzyme of lysosome and can survive and replicate inside host cells and carried by reticuloendothelial system (Monack *et al.,* 2004).

2-7 Virulent factors

The virulence of *Salmonellae* depends upon an array of factors, encoded by genes that lead to their colonization, invasiveness, intracellular survival and damage to host tissues (Mir *et al*., 2010). It is suggested that a combination of these factors that specific to each serovar including the presence of plasmid virulence genes (spv), surface cell structure, flagellin, and pathogenicity islands (SPIs) is involved in severity of salmonellosis (Andino and Hanning, 2015).

2.7.1. Salmonella pathogenicity islands (SPIs):

SPIs were grouped as SPI-1, SPI-2, SPI-3, SPI-4, SPI-5 and SPI-6. SPI-1 was reported in mediation of bacterial invasion in non-phagocytic cells of the intestinal tissues, whereas, SPI-2 was mediated the replication and survival of salmonella in phagocytic cells (Dieye *et al.,* 2009) and induced under low PH intracellularly (Barrow, 2000), Salmonella need SPI-3 to survive in macrophages in environment with low magnesium. SPI-4 stimulate bacterial genes to secret toxins. Encoding and production of T3SS effector proteins was mediated by SPI-5. Proteins transferring to the host cells or the environment as to reflect to external stimulation were mediated by SPI-6 (Foley *et al.,* 2013).

2.7.2. Virulence plasmids:

Plasmids of Salmonella enterica different in size (from 2 kb to more than 200 kb), the virulence plasmids in size (50–100 kb) are the best group that described and present in serovars (*dublin, enteritidis, cholerae-suis, typhimurium, gallinarum,* and *pullorum*), they all encode

spvRABCD genes contributed in Salmonella intra-macrophage survival (Rychlik *et al.*, 2006). Salmonella plasmid virulence locus (spv) is present on each of the plasmids and it important for Salmonella multiplication in the reticuloendothelial system (Asten and Dijk, 2005; Foley *et al*., 2013).

2.7.3. Toxins:

Salmonella have many toxins including exotoxin and, endotoxin; exotoxin is divided into enterotoxin and cytotoxins (Asten and Dijk, 2005). Development of the diarrhea in infected animal or human was attributed to the effect of cytotoxin that damaged the epithelia cells of the intestine either by cation chelating on membrane of mucosal cells or by cytokines released due to the infection and induced inflammation as indirect cause (Murray, 1986). Serovars of *typhi*, *typhimurium*, and *enteritidis* were contained salmolysin as an exotoxin (Asten and Dijk, 2005; Foley *et al.*, 2013).

Septic shock or syndrome was mediated by endotoxin that caused tissue injury in the host as a result to severe infection of the host with Gram negative bacteria. The resulting disease was attributed to the response of the host to endotoxins of bacteria (Williams, 2007).

2.7.4. Fimbriae:

These structures mediated the attachment of bacteria to mucosal surface during the process of colonization and pathogenesis of infection. They were filament structure on the surface of bacteria and sometimes called pili and known to be composed of protein of low molecular weight (Mirmomeni *et al.*, 2008). Salmonella species have genes coding for at least (12) different types of fimbriae, including SEF14, SEF17, SEF18 and SEF21, the majority of these structures are located in many members of the genus (Cogan and Humphrey, 2003). *S. enteritidis* is expressed fimbriae SEF14 that composed of sefA protein

mediated by a gene carrying the same name; they are play important role for adhesion of these Salmonella serovars to the reproductive tract tissues (Mirmomeni *et al*., 2008; Foley *et al*., 2013).

2.7.5. Flagella

Flagellar filament has the flagellin (protein) which alerts the host of early infection (Barrow and Neto, 2011). Flagella help to increase immune response like *S. typhimurium* and *S.enteritidis* eliciting a stronger inflammatory response (Chappell *et al*., 2009; Foley *et al*., 2013).

2.7.6. Lipopolysaccharide

A lipid complex and polysaccharide that associated with the outer membrane of bacteria Gram-negative is called Lipopolysaccharide (Cogan and Humphrey, 2003). Surface polysaccharides may have role in *Salmonella* colonization in the host tissues (Coward *et al*., 2013) its structure different among bacterial genera (Cogan and Humphrey, 2003).

Salmonella O-antigen is a part of lipopolysaccharide (LPS) (Addwebi *et al*., 2014) which mask the determinants on the surface of bacterial cell to prevent interaction with humoral immune system and phagocytic cells (Quinn *et al*, 2004) also LPS protects *Salmonella enteritidis* from certain antimicrobial peptides produced by the host (Coward *et al*., 2013).

Payre's patches were the site of salmonella colonization, and during the systemic infection bacteria can use M-cells to penetrate the intestine barriers, reach the mesenteric lymph nodes and the through the lymphatic circulation salmonella reach liver and spleen with aid of phagocytes. This was followed by invasion of blood circulation that helped the salmonella tp participate in different organs and tissues, their multiplication and replication might induces severe systemic infections (Rodriguez *et al*., 2006).

The most important causes of morbidity and mortality in humans were sepsis and bacteremia of NTS like *S. enterica* and *S.typhimurium* as were reported to be the main causes of human infections (Brent, 2006).

2.7: Clinical signs:

2.7.1: Clinical signs in human:

In human cases salmonella infection was grouped according to causative agents as typhoid salmonellosis and non-typhoid salmonellosis (NTS). Gastroenteritis in human due to salmonella might be appeared after 6 hrs post exposure to bacteria up to 3 days. Long incubation period up to 14 days was also reported. For example in incubation period of 10-14 days were reported with cases of eneric fever.

Different clinical forms or manifestations due to salmonella infections were reported like:

- ❖ Gastroenteritis
- ❖ Enteric fever
- ❖ Chronic carrier state.
- ❖ Bacteraemia and other extraintestinal complications.

(Sheorey and Darby, 2008).

2.7.1.1: Gastroenteritis:

Non-typhoid salmonella, were Salmonella strains other than *S.paratyphi* and *S.typhi* which. Infections with NTS were reported in animal reservoirs and caused what known as stomach flu. Myalgia's, abdominal cramps, nausea, headache, vomiting, and non-bloody diarrhoea were the main manifested clinical signs due to the infection with NTS with less frequent of splenomegaly and hepatomegaly (Hohmann, 2001). The infection was also characterised by short incubation period of 6 to 12 hrs but the disease might last for 10 days as it was self-limited (Crump *et al.*, 2008). Appendicitis, cholecystitis, and

pancreatitis might be included in NTS gastroenteritis but were regarded as complications (Hohmann, 2001). Immunocompromised individuals, aged people, infants and young children were highly susceptible to NTS infections and might be appeared with severe or acute symptoms (Scallan *et al* .,2011).

2.7.1.2: Enteric fever:

Enteric fever was caused by S.typhi, whereas S. paratyphi, A, B and C caused paratyphoid fever. The both bacterial agent were known as typhoid salmonella. Enteric fever term was used for both infections, as their clinical symptoms were not distinguishable (Connor and Schwartz 2005).

More than one week might be required for enteric fever to be established in the susceptible human host. The main symptoms were appeared as constipation, diarrhoea, abdominal pain and headache. The this was followed by fever (Bhan *et al.,* 2005). Constipation was noticed in immunosuppressed patients, while children were commonly showed diarrhoea (Thielman and Guerrant, 2004). Fever was noticed to develop slowly from above 37 ℃ (37.5 to 38.2℃) to high fever up to 41.5 ℃ especially during the second week of illness. Moreover, such fever can persist more than one month if the patient was not subjected to a course of treatment (Patel *et al*, 2010).

Appearance of rose spots on the chest and abdomen of the patient was noticed in addition to hepatomegaly, splenomegaly bradycardia and myalgia (Kuvandik *et al.,* 2009).

2.7.1.3: Chronic carrier state:

Patients passing the acute stage of salmonella infection and continued to shed the bacteria for more than a month in their stool were defined as chronic carrier. In typhoid salmonella, humans were the only typhoid salmonella reservoir. Their infections were passed through

ingestion of contaminated food and water with stool of chronic carriers (Bhan *et al.*, 2005).

Elderly people, women and infants were the predominant patients infected with enteric fever, but they might become chronic carriers and form 5% of such patients (Gonzallez-Escobedo *et al.*, 2011) when compared to 0.1% of patients with NTS infections, whereas animals were the reservoirs in case of NTS (Hohmann 2001).

2.7.1.4: Bacteraemia and other extraintestinal complications:

S.Cholearaesuis, and *S.Dublin* were reported to be highly associated with bacteraemia in comparison to other salmonellae that also do bacterima, a condition associated with bacterial invading to blood stream of the host flowed the intestinal invasion by the same causative pathogen (Woods *et al.*, 2008).

Bacteraemia was associated with high fever, septic shock and may be ended with death and mostly associated with NTS. No rose spots were noticed with such bacteraemia as was noticed in other salmonellae that caused enteric fever. Salmonella plasmid virulence factor was attributed to bacteraemia of NTS and connected to genetic factors in these bacteria (Guiney and Fierer 2011).

Persistence of NTS in host cells was reported to be associated with bacterial gene that causing virulence increase in unknown mechanism (Gulig *et al.*,1993). It was noticed that extra complications were occurred in patients with NTS bacteraemia like meningitis, pneumonia, endocarditis, cellulitis and urinary tract infections; such bacteraemia was reported in 5% NTS infected patients (Shimoni *et al.*, 1999; Arii *et al* .,2002).

2.7.2: Clinical signs in animals:

The mortality and morbidity may reach 100% especially in septicaemic cases. Salmonella infections might be associated with high morbidity and mortality in with high mortality in pigs, poultry and young ruminants. The disease was common in lactating animals, pregnant and young animals fed with commercial raw foodstuff, but it was common to find asymptomatic salmonella infections among animals. According to the severity of the disease clinical signs can be grouped into three categories chronic enteritis, acute enteritis and septicemic (Radostitis *et al.*, 2007)

2.7.2.1: Chronic enteritis:

Chronic enteritis due to salmonella infections was mostly associated with fatigue, body weight loss, bloody diarrhea and anorexia. The disease occurred in cattle and horses occasionally, but common in pigs.

High morbidity and mortality associated with gastrointestinal infections with salmonella were reported worldwide. The great goals beneficially noticed and helped in understanding of such infections was the use of laboratory animal in experimental infections, that also helped in understanding of changes induced in salmonella infected animal from both clinical signs and pathogenesis (Grassl *et al.*, 2008).

Many clinical manifestations were observed in animals infected with salmonella like necrosis of extremities, respiratory signs, abortion, arthritis and septicaemia, but enteric infections and signs were the most predominant. Some other animals did not show clinical signs like poultry and pigs (OEL, 2010).The most susceptible domestic animals to the infection were those young and pregnant (Wary and Wary, 2000).

2.7.2.2: Septicemic form:

Calves and foals infected with salmonella showed high fever and the body temperature may range from 40.5 to 42 ℃. ataxia, pneumonia, prostration depression and dullness.

2.7.2.3: Acute enteritis:

Abortion was common in animals with acute enteritis due to salmonella. These infected animals showed complete anorexia, severe diarrhea and sometimes dysentery with bad smell feces that containing blood and mucus. The body temperature ranged from 40 to 41C. Furthermore, the disease was common in all animal species and particularly in adults.

In rabbits, the clinical signs can range from asymptomatic carriers to diarrhoea, (Harcourt and Nigel, 2002) who classified the clinical signs into three forms:

- Chronic enteritis: characterized by the rabbit becoming carriers and may shed bacteria in their feces intermittently for a long time.
- Acute enteritis: characterized by fever, diarrhoea, emaciation, loss of appetite, anaroxia and the disease can progress rapidly to death.
- Septicemic form: characterized by diarrhoea, fever, septicemia and rapid death.

AL-Mansory (2009) in Iraq observed normal faeces, normal appetite, slight depression, and slight increase in body temperature in rabbits injected with S.enteritidis experimentally

2.8: Gross pathological findings:

Salmonellosis often produces an enterocolitis, lesions occur in the villi of the small intestine, lymphoid tissues and colonic mucosa. The invasive *Salmonella* has a cytotoxic effect on epithelial cells later diphtheritic

pseudomemmbrane form at the mucosal surface. Mesenteric lymph node are enlarged, swelling, and oedematous and may have foci of necrosis, the hepatic lesions are focal necrosis and /or mycrogranulomas, splenomegaly. In the septicemic form, *Salmonella* disseminate to other tissue to produce in same animal focal meningoencephalitis (Carlton and McGavin, 1995; Percy and Barthold, 2007). Al-Naqeeb, (2009) noticed that there was a varying degree of intestinal congestion with a presence of mucus of the infected mice with *S. hadar*, liver and spleen were sever swollen and congested together with petechial hemorrhages on its surface, the gall bladder was enlarged, the kidneys were swollen and the heart appeared flabby. In a study by Al-Mansory, (2009) recorded that the gross pathological changes in rabbits infected experimentally with *S. enteritidis*, are characterized by variable degree of congestion and petechiation of multiple organs, congestion in large intestine. Small intestine were flaccid and filled by yellow watery contents with high amount of mucous, enlargement of payer's patches, engorgement and necrotic foci in liver, enlargement and darkness of gall bladder, spleen was enlarged, lung congested and oedema, with focal haemorrhages**,** kidneys were enlarged. Al-Khafaji, (2008) reported that there was congestion of all internal organs of the infected mice with *S. typhimurium* which died during the first 24 hours post inoculation Muhsen, (2007) noticed that there was severe congestion and haemorrhage in most parts of the gastro intestinal tract of the infected dogs with *S. typhimurium*. Sharma *et al*., (2001) found that the major necropsy findings in goats infected experimentally with *S. typhimurium* were thicken of small intestine with congested mucosa, enlargement oedematous inflamed mesenteric lymph nodes (MLNs) and necrotic foci in liver. There was a varying degree of intestinal congestion, enlargement of gall bladder, spleen and kidneys of the new-borns guinea pigs, which challenged with *S. typhimurium* (Yousef, 2000).

Another study by Yass, (1990) found a varying degree of hyperaemia and/or the presence of mucus, fibrinous or haemorrhagic exudates in the small and large intestine of calves infected experimentally with *S. typhimurium*; the liver is swollen, congested, acute cellular swelling and focal necrosis of individual hepatocytes; the spleen is often swollen together with petechial haemorrhages on its surface; also there is swelling of the mesenteric lymph nodes; the heart is flabby with subepicardial and sub endocardial petechial haemorrhages and the kidneys are swollen and severely congested.

2.9: The immune response to infection with *Salmonella:*

There are more than 2500 serovars of enteric *Samonellae* which were gram negative bacterial rods. Few of them were associated with poultry infection; those were *S.Kentucky*, *S. Heidelberg*, *S.Typhimurium*, and *S. Enteritidis*. These bacteria were able to infect humans and animal hosts; accordingly they were regarded as non-host adapted (Desin *et al*., 2013).

It was reported that two of above mentioned salmonella of poultry *S.Typhimurium* and *S.Enteritidis* were associated to gastro-intestinal infection, accordingly it was suggested that foodborne illness associated with contamination of poultry products with such bacteria (Cheng *et al.*, 2013).

Oral ingestion of salmonella contaminated food or water was the main route of host infection with salmonella, these bacteria resist the effect of saliva lysozyme due the outer membrane structure that shield their wall peptidoglycan (Masschalck and Michiels,2003). When these bacteria move to the stomach of the host they faced the low pH of stomach acid HCl (Ramose-Morales, 2012). The stomach acidic pH in humans ranged from 1.3 during fasting to 4.9 during food ingestion (Russell *et al.,* 1993). Most bacteria can be killed by such acidic environment but some others had developed their mechanism to resist stomach low pH as what happened by *E.coli* that can resist for hours the acidic stomach

media of pH2, while some other bacteria appeared of less resistance like S. enterica (Koutsoumanis and Sofose, 2004).

There are two glycan layers that were incorporated with mucin and oligopolysaccharides. Such complex formed can protect the human gut from local acidic environment and also from the infection, but some bacteria had the ability to degrade this protective complex by the use of certain enzymes like glycosylic hydrolase that have the activity to degrade glycan as that present with salmonella (Arabyan *et al.,* 2016).

The body defence mechanisms included innate immunity in which the body offered defence mechanisms against the invaded microbes and supressed it (Vazquez-Torres *et al.,* 2001). Macrophages and other phagocytic cells played important roles in such innate defences without the needs for T and B lymphocytes, but salmonella was reported to infect B lymphocytes in vitro and in vivo (Yrlid *et al.,* 2001).

Specific defence mechanisms against the invaded microbes can be categorized into two responses humoral and cell mediated immune responses. In humoral response B lymphocytes were differentiated into plasma cells that produce specific glycoprotein antibi against the particular microorganism, bind to the surface of microbes and enhance its destruction by the action of phagocytic cells (Mastroeni *et al.,* 1998). In cell mediated immune response active T lymphocytes were developed and kill the invaded microbes of enhance its destruction by activated phagocytes; generally both immune responses can play roles of protection against the infection with Salmonella (Mastroeni *et al.,* 1993).

2.9.1: Antibody response to Salmonella infection

The ability of salmonella of non-typhoidal type to grow in reticuloendothelial system and especially in macrophages can be regarded as indication of its virulence (Fields *et al.,* 1986). These bacteria also can grow extracellularly, but specific antibodies that be produced in humoral immune response against such bacteria with presence of complement can mediate the killing Salmonella (Maclennan *et al.,* 2008).

The humoral response can be affected by many factors:

There are many factors that affected the humoral immune response to bacteria, and included the followings:

- How much the microorganism is virulent (Gray *et al.,* 1995).
- The number of particular microorganism that initiate the infection (Gray *et al.,* 1996).
- The susceptibility of the host to the infection with particular microorganism that related to host genetic build up (Barrow, 1992).
- The host age (Thorns *et al*., 1996).
- The route of infection (Gray *et al.,* 1995).

2.9.2: Cell mediated immunity:

Dendritic cells (DCs) in the host body prime the specific defense immune response that play an important role in antigen processing, but it was reported that such cells were susceptible to the infection with salmonella in vitro and in vivo, furthermore, cytokines produced by these cells can be activated the infection (Svensson *et al.*, 2000).

These cells were tolerated to commensal and food organisms when they in immature stages in mucosa of the intestine, they were later play significant

roles in the development of body inflammatory responses against the pathogens in the intestine lumen (Steinman and Banchereau, 2007).

Tam *et al.*, (2008) mentioned that neutrophils, macrophages and DCs were derived from myeloid tissues, acting as phagocytes that control the infection caused by bacteria. Their functions and activities might ensure the survival of the host (Tam *et al.*, 2008). The expression and secretion of interferon gamma IFN-γ, tumor necrosis factor TNF-a, IL17, and IL-1 was resulted from severe damage of host intestinal epithelium due to salmonella infection during the clinically active infection (Grassl *et al.*,2008).

Adaptive immune response essentially required the action of different cytokines like IL2, IL15, IL18, tumor necrosis factor (TNF-a) and interferon gamma (IFN-γ). Persistence of granulomas and killing of salmonella by macrophages were mediated by TNF-a (Vazquez-Torres *et al.*, 2001). Macrophages antibacterial activity that mediated and regulated by nitric oxide synthase (SON) was found to be under the control of IFN-γ that was secreted by NK cells under the influence of IL-12 and IL-18 (Mastroeni *et al.*, 1998).

It was reported that more than 600 genes were elicited by the action of IFN-γ that was served as receptor ligand. These genes were important in cellular defences mechanisms that defeated pathogens of intracellular activity (Ehrt *et al.*, 2001).

It was suggested that the main immune responses to salmonella infection is due to T helper cell (CD4), whereas CD 8 (cytotoxic lymphocytes) and antibodies were appeared of less values (Hughes and Galan, 2002).

Cell mediated immune response to a particular antigen can be measured by the use of DTH skin test. Experimentally the antigen can be injected intradermally in the skin and the site of injection should be observed for any changes included swollen of the skin, redness and thickening after 48 to 72 hrs post injection. The development of above-mentioned signs was associated to the stimulation of TH-

1 cells to particular pathogen, and accumulation of phagocytic cells in the site of injection particularly the macrophages (Kindt *et al.*, 2007).

Skin test had been used by many researchers to evaluate the delayed type of hyper sensitivity as had been mentioned in Iraq by AL-Mansory (2009), when he infected rabbits experimentally by *S. enteretidis* and used the same bacteria to determine the cell mediated immune response of type DTH by intradermal inoculation of the skin by the same antigen. The same test was used by AL-Naqeep (2009) but in mice infected with *S.hadar*. Increase in the thickness of mice foot pad was observed when it was inoculated with antigen of the study.

Chapter three

Materials and methods

3.1: Material

3.1.1: Bacterial isolation:

Salmonella enteritidis was obtained from college of veterinary medicine from Baghdad University. The bacteria was characterized and identified by PCR and it is well documented at the veterinary college, Baghdad University under supervision of Professor Emad Jwad Khamas for the degree of master science in poultry disease by Lujain Dhiaa Nasser Al-Khayat.

3.1.2: Laboratory animals:

Domestic rabbits were used in present study; they were obtained from local market. They were 60 in number and their age ranged from eight to twelve months with an average weight of 1700 gm. They were apparently healthy and without any indication for skin diseases. Additionally, clean metal cages were used for their housing in experimental house of the College of Veterinary Medicine/ University of Diyala. In this house the temperature was manged to be 22±°C. Alpha alpha (green food) and commercial pellet were used for their feeding with free access of water and 12 hours artificial light. Furthermore, they were adapted to the new environment for two weeks to be ready for two experiments.

Experiment Number I: In this experiment, the infectious dose (ID) of *S.enteritidis* was determined using twenty healthy rabbits.

Experiment Number II: In this experiment another forty healthy rabbits were used to be infected by the determined ID of the same bacterium of the experiment number I.

3.1.3: Instruments and equipment:

Table (3-1) Equipment, Tool, and their sources that utilized in work

NO.	*Equipment*	*Company (source)*
1	Autoclave	Express (England)
2	Biohazard hood	Vision(Korea)
3	Cool box	India
4	Cooling centrifuge	MSE (England)
5	Distillator	Daihan lab tech.(Korea)
6	Freezer	Concord (Lebanon)
7	Incubator	BINDER(USA)
8	Laboratory centrifuge	MSE (England)
9	Light microscope	Olympuse (Japan)
10	Microtiter pipette	SLAMID (Denmark)
11	Millipore filter (0.22μm)	Difco (USA)
12	Pasteur pipette	China
13	PH Meter	Radiometer(DENEMARk)
14	Refrigerator	LG (Korea)
15	Scissors ,Forceps and Scalpel with Blade	China
16	Sensitive balance	Sartorius (Germany)
17	Vernier Caliper	China
18	vortex	
19	Water bath	MEMERT(Germany)
20	Water-cooled sonicator oscillator	MSE (England)

3-1-4 - Cultural media and biochemical media

Table (3-2):Culture and biochemical media

NO.	Media	Origin
1.	Brain Heart Infusion Agar (BHIA)	HIMEDIA
2.	Brain Heart Infusion Broth (BHIB)	HIMEDIA
3.	Blood media base	HIMEDIA
4.	Nutrient agar	Fluka (Germany)
5.	*Salmonella-shegella* agar (s.s agar)	HIMEDIA
6.	Xylose-Lysine Deoxychoclate agar(XLD)	HIMEDIA
7.	Triple Sugar Iron (TSI)	HIMEDIA
8.	Chrom agar	Paris/France
9.	Urea agar	HIMEDIA
10.	Nutrient broth	HIMEDIA
11.	Buffer peptone water (BPW)	HIMEDIA

3.1.5: Chemicals and reagents:

Table (3-3): Chemicals and reagents

No.	Chemical , Reagent	Origin
1	CaCl	India
2	covac*'s* reagent	Arcomex-(Jordan)
3	Ethanol (70%)	BDH(England)
4	Formalin	BDH(England)
5	Gimsa Stain powder	India
6	Glucose	Merck(Germany)
7	Gram stain reagent	Crescent – (KSA).

8	Hematoxylin and Eosin stain (H and E)	BDH(England)
9	Hydrogen peroxide	local
10	KCl	BDH (England)
11	KH_2PO_4	BDH (England)
12	$MgSO_47H_2O$	India
13	Na_2HP0_4	BDH(Engand)
14	NaCl	(BDH)(England)
15	Oxidase reagents	HIMEDIA(India)
16	Paraffin Wax	Germany
17	Phenol red	India
18	Sterile Urea	HIMEDIA (India)

3-1-6 solutions:

1-Physiological normal saline

2- Phosphate Buffer saline (PBS)

3- Hank's solution

3-1-7: API- 20E Biochemical kits. BIOMERIEX (France)

3.2: Methods

3-2-1: Preparation of cultural media:

(1)- Salmonella chromogenic agar

According to manufacturer instructions, (37.1g) of dehydrated media was dissolved in 1000 ml D.W. at 80℃. The mixture shacked and boiled for one minute to dissolve the medium completely then cooled, to about 50°C and poured into sterile petri dishes.

(2)-Buffer Peptone Water (BPW):

It was prepare by dissolving of (15) mg of media in1000 ml of distilled water, heated for boiling with agitation to dissolve all the media then poured into sterile test tubes and sterilized by autoclaving at 15Ibs/inch2(121℃) for 15minutes then incubated at 37 ℃ for 24 hours (the porpoise of preparing this media for making suspension of the suspected organs to isolate the causative agent).

(3)- Nutrient agar:

It was prepared by dissolving of (28) gm of nutrient agar in glass flask of 1.5 litre capacity. The volume was completed to one litter by adding of distilled water. Heat was used to dissolve the medium by boiling ant continuous agitation. This was followed by autoclaving the content at 121℃, 15 Lb for 15 minutes for sterilization, then the sterilized medium was put in water bath in (55℃) for cooling and then poured in Petri-dishes and incubated at 37℃ for 24 hours for removing the contaminated medium.

(4)- Brain heart infusion agar:

It was prepared like nutrient agar but the amount of medium is (53mg per 1000 ml of distilled water) (using as enrichment media for *salmonellae)*.

(5)- Brain heart infusion broth:

Prepared by dissolving 0f (37mg) of medium in 1000 ml of distilled water, then and 20% glycerine and heated to boil with agitation to dissolve the media and poured in sterile test tube then after sterilized by autoclave incubate at 37℃ for 24 hour.

(6)- *Salmonella Shigella* agar (S.S) agar

The medium was prepared by boiling when one litre of distilled water was added to 63 mg of the S.S agar, dissolved well by heating and boiled with

agitation. After complete dissolving, the medium in the flask was moved to water bath at 55℃ for a while and the poured in petri-dishes.

(7)- Xylose – lysin Deoxycholate agar (XLD).

The same as above-mentioned in (6) was followed in preparation of XLD medium, but 53 mg was dissolved in one litre of distilled water.

(8)- Triple Sugar Iron (TSI).

Triple Sugar Iron is a biochemical medium and gives a positive indicator for Salmonella spp. It was prepared as in preparing trypticase soya agar but the amount of the medium was (63.6 gm/L) and after autoclaving, the test tubes were put in a slant position yet solidifying the medium.

3-2-2 Preparation of solution:

1- Phosphate buffer saline

The method used by Luna and Lee (1968) was followed for the preparation of phosphate buffered saline (PBS) when 100 mls of distilled water was used to dissolve the following chemicals.

Potassium dihydrogen phosphate (KH_2PO_4)	0.2gm
Potassium Chloride (KCL)	0.2gm
Disodium hydrogen phosphate (Na_2HPO_4)	1.15gm
Sodium Chloride (NaCl)	8.5gm

The pH was adjusted to 7.2 and the mixture was autoclaved 121 ˚C 15 Lb for 15 minutes in screw capped glass bottles. After such sterilization, PBS was cooled and stored at 4 ˚C.

(2) Physiological normal saline

The method that mentioned by Collee *et al*., (1996) was used for the preparation of physiological normal saline. It was prepared by mixing 8.5 gm of NaCl with 1000 ml of distilled water. The mixture was then autoclaved at 121 ˚C, and 15Lbs, for 15 minutes for sterilization; cooled and stored at 4 ˚C until use.

3) Formalin working solution (10%):

Formalin in concentration of 40% as stock was used for the preparation of 10% formalin solution when 90 ml of distilled water was added to 10 ml of of stock solution in screw capped 100 ml glass bottle.

(4) Hank's balanced salt Solution HBSS:

Prepared as in following:

Solution A:

Potassium Chloride (KCl)	0.8 g.
Sodium Chloride (NaCl)	16 g
Calcium Chloride ($CaCl_2$)	0.28 g.
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.4 g.
Distilled water	100 ml

Solution B:

Poassium Dihydrogen Phosphate (KH2PO4)	0.12 g
Disodium phosphate (Na_2HPO_4)	0.3 g.
Glucose	2.0g

Indicator (Phenol red)	0.2 g
Distilled Water	100ml

The working solution was prepared by mixing one volume from solution A with one volume of solution B and 18 volumes of distilled water. The mixture was well mixed, the pH was adjusted to 7.2 and the mixture was sterilized by filtration through 0.22 μmMillipore filter and stored at 4℃ unitl used (Metcalf *et al.,* 1986).

5) Killed Yeast suspension:

The method that described by Metacalf *et al.,* (1986) was followed. Sterile normal saline in amount of 150 ml was used to dissolve 50 gm of dried *Saccharomyces cerevisiae*, the mixture was the boiled using water bath, filtered by sterile cotton gauze in two layers, and frozen until used.

3-2-3: Identification of isolates:

Isolates were identified according to different tests, including gram stain, biochemical tests and VITEK 2 COMPACTS.

3-2-3-1: Gram stain:

A small colony of *Salmonella* organisms was picked up by a bacteriological loop and a thin smear of bacteria made on glass slide, the smear was fixed by gentle heat (Muktruzzaman *et al.,* 2010)

Gram stain determines the staining characteristic of bacteria as well as the purification (Dutta *et al.,* 2015). *Salmonella* is gram-negative bacteria and appear red colour (Quinne *et al.,* 2004).

3-2-3-2: Biochemical testes:

Biochemical tests conducted according to (were Standard Operating Procedure "SOP", 2007).

3.2.3.2.1: The test of catalase:

Pure bacterial culture on nutrient agar was used in this test; single bacterial colony was picked up by the use of sterile loop needle. The colony was placed on clean microscopic glass slide. This was followed by addition of few drops of hydrogen peroxide. The mixture was observed to notice arising of gas bubbles that indicate the positive reaction due to Oxygen (O_2) production (Hemraj *et al.*, 2013; Quinne *et al.*, 2004).

3.2.3.2.2: The test of oxidase:

White filter paper was used in this test; the paper immersed in freshly prepared oxidase reagent and removed. This was followed by spreading of pure bacterial colony that picked up from pure bacterial culture on nutrient agar. Positive reaction can be recognized by the development of colony colour to dark purple within few seconds. The fresh oxidase reagent can be prepared mixing 0.1g of tetramethyl p-phenylenediamine dihydrochloride with 10 ml of distal water (Hemraj *et al.*, 2013).

3.2.3.2.3: Lactose fermentation:

The isolates were cultured on MacConky agar at 37 ˚C for 24 hours. Pale colony is an indicator for lactose non-fermented bacteria.

3.2.3.2.4 - Triple sugar iron (TSI) agar slant reaction:

Platinum bacterial loop needle was used to spread the picked up pure bacterium culture on the surface of TSI slant agar and to be also stabbed till the butt of TSI tube agar. The spread and stabbed tube culture was incubated at 37°C for 24 hrs. Color changes in both surface and butt TSI slant into pinkish and yellow respectively with production of hydrogen sulfide (H2S) were recorded as the positive reaction for Salmonella (Hossain *et al.*, 2006;Abdullahi, 2010).

3.2.3.2.5. - Urase test

Two to three colonies of bacteria were inoculated to the urea broth tubes then for 24hrs. At 37 ℃.they were incubated, urease-positive culture become pinkish-red color is indication to an alkaline reaction while when the medium remain pale yellow (no change in the colour) indication to urease –negative reaction; *Salmonella* is usually gave a negative reaction (Abdullahi, 2010).

3.2.3.2.6 -: Api-20E system (Analytical profile index for Enterobacteriaceae test):

This system was offered an essential of 20 small tubes for biochemical identification of a bacterium belonged to *Enterobacteriaceae*. It was used according to Atlas, (1995) and the instruction manual of the manufacturer.

3.2.3.2.7 - Indole test:

Inoculated the bacterial culture within 5ml of nutrient broth then incubated for 18 hrs.at 37℃ then added 0.5ml of kovac' s reagent, shaken well and after 1 minute examined; positive result appear as a red color in the reagent layer while in negative result there is no red colour appear (Mukhtaruzzaman *et al.*, 2010).

3-2-3-3 :Slide agglutination test:

The test was done by using standard antisera for *Salmonellae* according to method of (Collins and Lyne, 1987).

1-Two drops of normal saline were put on clean slide each drop was separate from another then a colony was taken from the bacterial culture by sterile loop and mixed well with each two drops .

2-One drop of standard antisera was added to one of the two drops with well mixing and the other drop is considered as control

3-The clear agglutination after one minute is considered as a positive result.

3.2. 4 - VITEK 2 COMPACT System

Complete identification of bacterial isolate can be performed by the use of Vitek® compact system. By the use of colourimetry on biochemical test, this system can identified the required bacterium within two hours. Colonies of suspected bacterium can be suspended in 0.45% sterile saline. The formed turbidity can be subjected to spectrophotometer to obtain its optical density with use of Vitek GN cards included in Biomérieux Vitek 2 System. Accordingly many bacterial species and yeasts can be identified using this system.

3-2-5: Preparation of bacterial stock

Preparation of bacterial stock from the completely diagnosed and identified bacterial isolated can be done. For this purpose brain heart infusion agar was prepared and poured in test tubes as slant. The slant agar was inoculated with required bacterium and incubated at 37°C for 24 hrs kept at 4°C and monthly re-cultured to insure their viability. Moreover, and to keep the bacterial stock for long period, the required bacterium can be cultured in brain heart infusion broth mixed with glycerol as 20%, cultured with required bacterium, incubated at 37°C for 24 hrs and then the cultured tubes moved to deep freezer.

3-2-6: Preparation of soluble antigen:

The method that described by Rehman *et al*., (2005) was used for preparation of soluble bacterial antigen. *S.enteritidis* was grown on brain heart infusion broth for overnight, and the bacterial suspension was sediment by centrifugation for 30 minutes at 2000 rpm and 4°C. The sediment was washed

with sterile PBS (pH 7.2) for three times. Sonication of the resulted and washed bacterial suspension was done for 5 minutes at 20 KHz and 105 W. The sonicated bacterial suspension was tested by Gram stain and cultured on blood agar to insure the sterility and complete sonication of bacteria. This was followed by another centrifugation run of sonicated bacteria at 2000 rpm for 30 minutes at 4°C. The supernatant was collected, its protein contents were adjusted to 200 µg/ml by the use of refractometer, and frozen at -20°C until be used.

3-2-7: Estimating the Infectious dose (ID):

3-2-7-1: Rabbits groups

Twenty of healthy rabbits of both gender were selected, the body weight range from (1500-1900) gm and age range (8-12) months; they were divided into five groups, each group contained four rabbits.

3-2-7-2: The bacteria

Brain heart infusion broth was used in amount of 10 per sterile test tube. This broth was inoculated with 5 colonies of *Salmonella entritidise,* incubated at 37 C for 18 hrs and followed by centrifugation of bacterial growth in refrigerated centrifuge at 8000 rpm for 15 minutes. The sediment was collected and washed three times by sterile PBS (pH7.2). After the last centrifugation, the pelleted bacteria was re-suspended in one ml of sterile PBS and ten-folds serial dilutions were made from re-0suspended bacteria starting from 10^{-1} to 10^{-10} using the same pH7.2 sterile PBS as a diluent. Counting of bacteria in each dilution was performed using the viable plate count method that described by Miles and Misra, (1938). The following bacteria counts (4.5 x 10^{7}cells), (4.5 x 10^{8} cells) and (4.5 x 10^{9} cells) were used as inoculums in experimental infection.

Twenty domestic rabbits were used and grouped into five groups, four rabbits each. Faecal samples were collected from all experimental rabbits and tested for Salmonellae. The first three rabbit groups were inoculated intraperitoneally (IP) each group (4 rabbits) with particular bacterial cell count (C.F.U/ml) dose above-mentioned. The forth group of rabbits was used as control and the rabbits were inoculated each with 1ml sterile PBS (pH 7.2).

Experimentally *S.enteritidis* inoculated and control rabbits were observed daily for 30 days. Any death among rabbits was recorded and connected to the bacterial dose used. Group of rabbits that did not reported any death but showed clinical signs were choose; two rabbits from such groups were randomly selected, killed and subjected to necropsy finding for *S.enteritidis* isolation from internal organs.

3.2.8: Experimental infection of the rabbits with *Salmonella enteritidis* intraperitoneally (IP):

Experimental infection of rabbits with *S.eneritidis* was conducted with some modification according to Freter (1956).

In this experiment:

1-Adaptation of all rabbits to new environment in animal house was performed for two weeks prior the starting of bacterial inoculation.

2- Drinking water for experimental rabbits was prepared, boiled and cooled.

This was followed by grouping rabbits into two main groups (A and B):

A: Included 30 healthy rabbits that were prepared to be inoculated with the identified bacterium intraperitoneally (IP).

B: Included 10 healthy rabbits used as control and injected by with sterile PBS using the same dose size and route.

All rabbits were examined as follows:

3.2.9: Clinical signs:

Experimentally infected rabbits were observed daily to record clinical signs that was associated with infection and appeared as, increase body temperature, diarrhoea, increased respiratory and heart rates, dehydration, dehydration, septicaemia and death.

3.2.10 Body weight measuring:-

Body weight of infected and control rabbits was measured daily from day 0 till the end of experiment and recorded and connected to animal status due to infection.

3.2.11 Fecal examination:

All experimentally infected animals and control were subjected to fecal swabs collection that were inoculated on primary medium and then on S.S. and XLD agars for the purpose of *S.enteritidis* re-isolation.

3.2.12Post-mortem examination:

Post-mortem findings were included:

A: Gross examination of viscera (macroscopic examination):

This was included the visual observation and recording any gross changes in visceral organs of infected animals in comparison to control rabbits; these changes might be the colour, size, consistency, shape, location and cut section appearance.

B: Histopathological examination:

In five successful days starting from 24 hrs PI to 144 hrs PI in 24 hrs interval, PM samples were collected from different organs of infected rabbits and control. These samples were included heart, lung, spleen, bladder, kidney, liver, and intestine. These tissue specimens were fixed by buffered formalin of

10% for 72 hrs, washed by tape water and processed with a set of alcohol of gradient concentration starting from 70% to 100% in interval of 10 for two hrs for dehydration. Xylol was used for clearance of processed tissue, which was then embedded in paraffin wax in its semi-liquid status at 58°C for two stages. The resulted processed tissue in paraffin blocks was subjected to sectioning of 5µm thickness using a microtome. Cut tissues on glass slides were subjected to staining with hematoxylin & eosin (H&E) and examined under light microscope for histopathological changes in comparison to control tissues Luna and Lee, 1968).

3.2.13: immunological testes:-

3.2.13.1 Cellular immunity:

Delayed type hypersensitivity test DTH (skin test):

Method that was described by Hudson and Hay, (1980) for testing the DTH by skin test was followed, in which soluble antigen of *Salmonella enteritidis* have protein about 250μg/ml was used to be inoculate intradermally of infected rabbit skin of right abdomen and in amount of 0.1 ml. The skin of left abdomen was used as control and injected with sterile PBS (pH 7.2) of same inoculum size. The thickness of the skin at the site of inoculation was measured before and after injection and for two rabbits after 24, 48 and 72 hrs post injection by the use of Vernier and calliper.

3.2.13.2 Phagocytic index:

The phagocytic index in the blood of infected animals and control was detected according to method described by Weber et al., (1982). The engulfing activity of phagocytes was measured when 250 µl of Hanks solution was mixed with same volume of rabbit blood with heparin. This mixture was received 50 µl of killed yeast, then the final suspension was incubated at 37°C for 30 minutes. Using of clean microscopic glass slide, thick smear from the mixture was made.

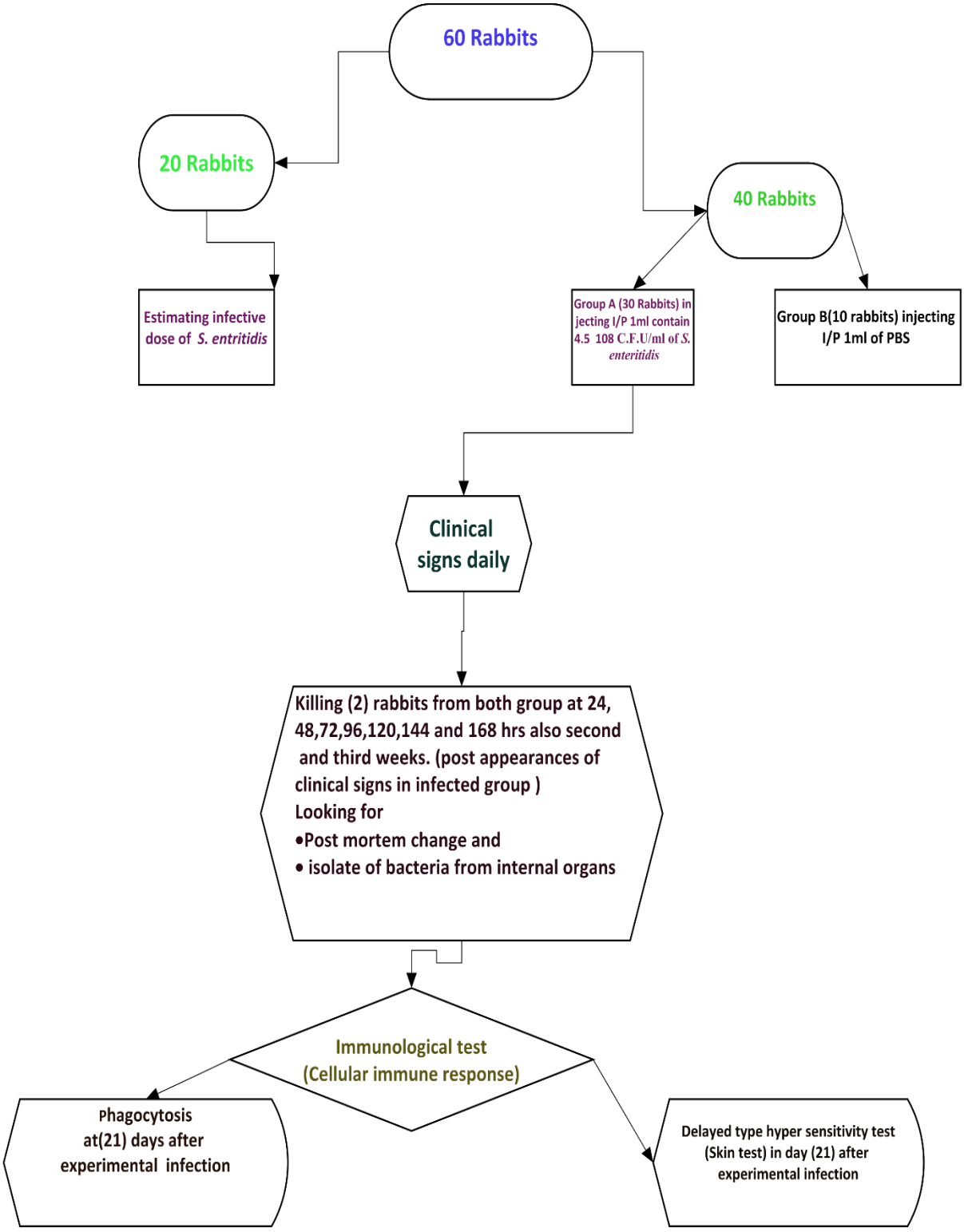
This smear was fixed in methanol and stained with for 10 minutes with 10% Gimesa. The extra stain was removed by washing with tape water, air dried and subjected to microscopic oil immersion lens testing. The phagycytic index can be calculated using the following equation when 200 cells were at least be calculated.

$$\frac{\text{No. of engulfing cells}}{\text{All no. of phagocytic cells}} = \text{ratio of Phagocytic index\%}$$

3.2.14: Statistical analysis:

According to Steel and Terris (1980) Graph and Prison of version 7 statistical analysis and ANOVA variance analysis were used. Furthermore, and to point the statistical differences in treatment means, least significant detecting was used. Significant differences were indicated by ($P \leq 0.05$) and marked by letters.

Diagram (1): Study design of the experimental infection of the rabbits with *S. enteritidis*



Chapter four

Results and discussion

4- Results

4.1: **Bacterial Isolate Identification:**

According to Quinn *et al* (2004) the below mentioned steps were followed to identify the bacteria isolate:

4.1.1: The Characteristic of Bacterial Culture on Classic Media

The initial diagnosis of suspected bacterial isolate must be examined on different culture media. *Salmonella* spp. Showed different characteristic when grown on different media. First, turbidity in the pre-enrichment and enrichment broth were seen. On the solid media, Salmonella colonies appeared small, smooth, round and transparent on S.S agar with black centre due to hydrogen sulphide production. On plates of BHI agar, Salmonella colonies have rounded and pale appearance. On XLD agar, the colonies appeared smooth with black centre. When growing on the chromogenic agar that specific for *Salmonella*, colonies were variable in size, convex and mauve in colour.

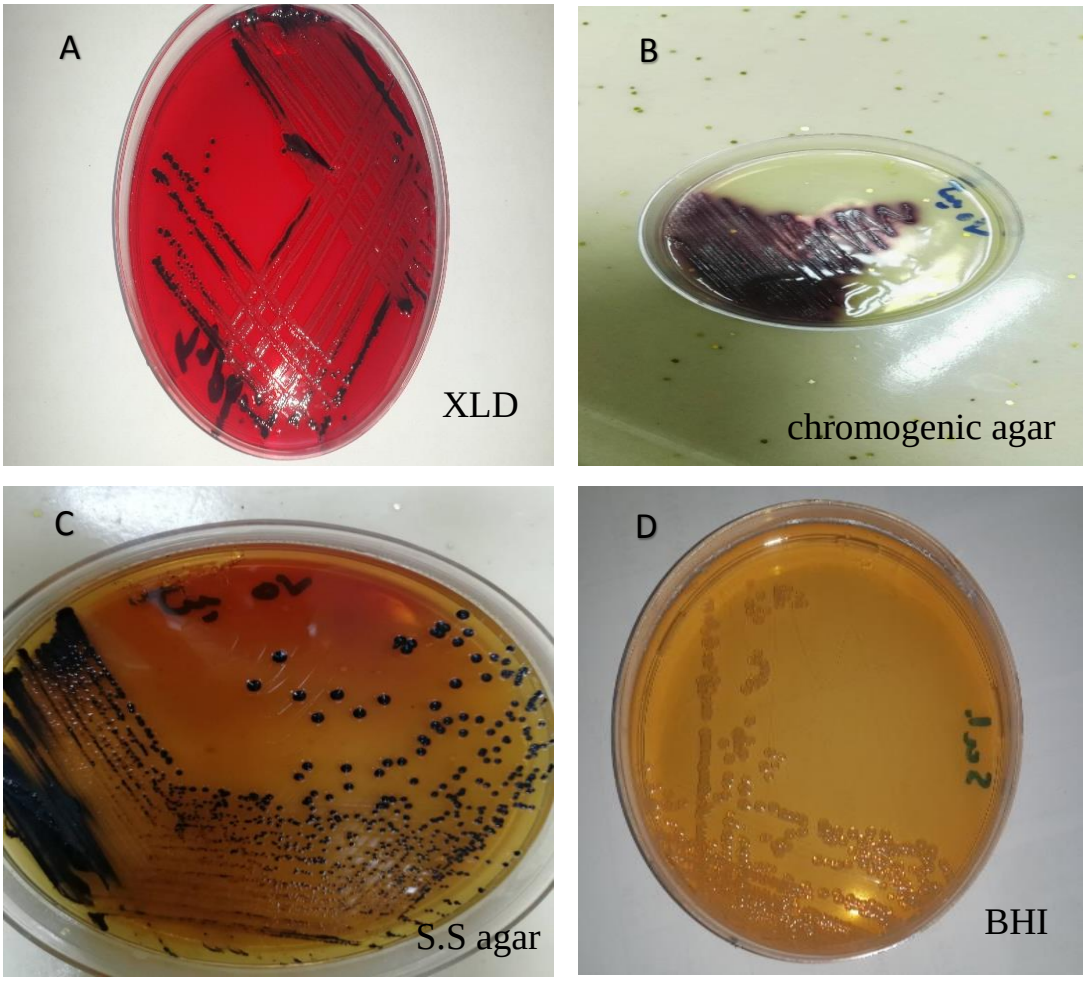


Figure :**(4-1)** Colonies of *S.enteritidis* on XLD and chromogenic agar,S.S agar and BHI agar.

4-1-2: Microscopic examination:

Under the light microscope, the bacteria seen as gram- negative, small rod in shape and arranged in single or pairs.

4-1-3: Biochemical identification:

For detection of *Salmonella*, specific biochemical tests were used. All species of bacteria were catalase positive and oxidase negative. On TSI slants, Salmonella were glucose fermented but did not ferment lactose and sucrose with H_2S and gas production in majority of slants. The slants were appeared red (alkaline reaction) and bottom were yellow (acid reaction). *Salmonella* spp were indole and urease negative (Table (4-1).

Table (4-1) Biochemical testes of Salmonella (positive +/ negative -)

Test	Results
Catalase	+ve
Indol	-ve
Oxidase	-ve
Triple sugar iron (TSI)	Pink/yellow+H_2S+gas
Urease	-ve

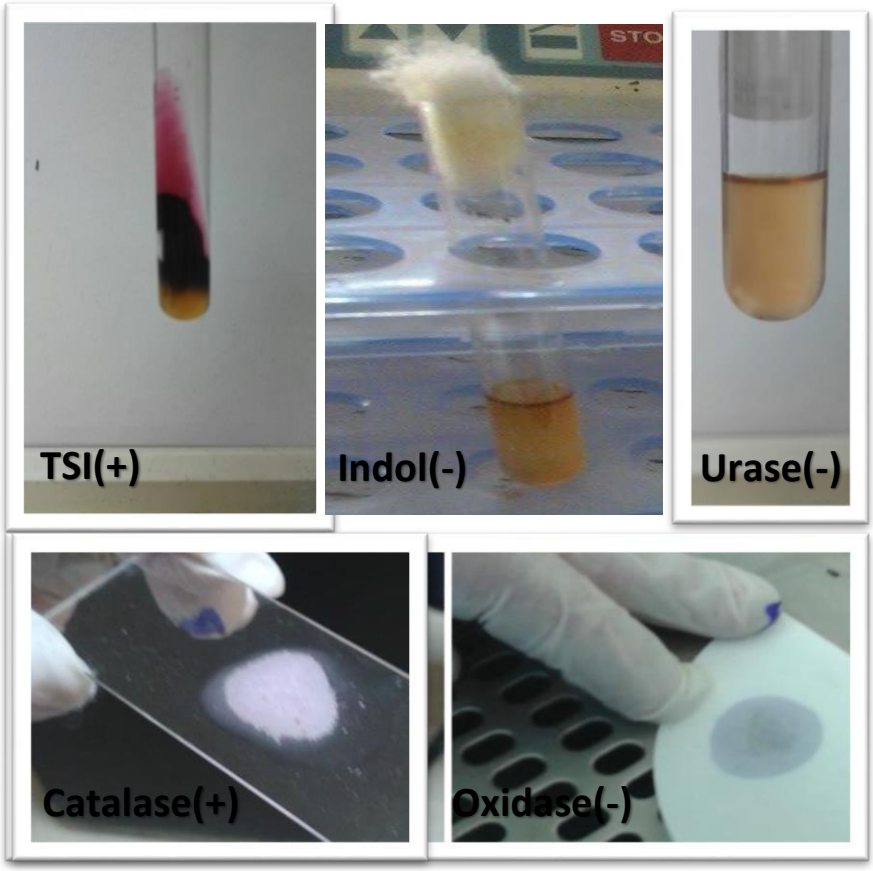


Figure: (4-2) results of biochemical test of *S.enteritidis*.

4-1-4: Slide agglutination test

After using standard antisera specific for *Salmonella enteritidis* the agglutination occurred obviously, after one minute (using O and H polyvalent antisera) they were positive for both that indicated this strain is *S. enteritidis*.

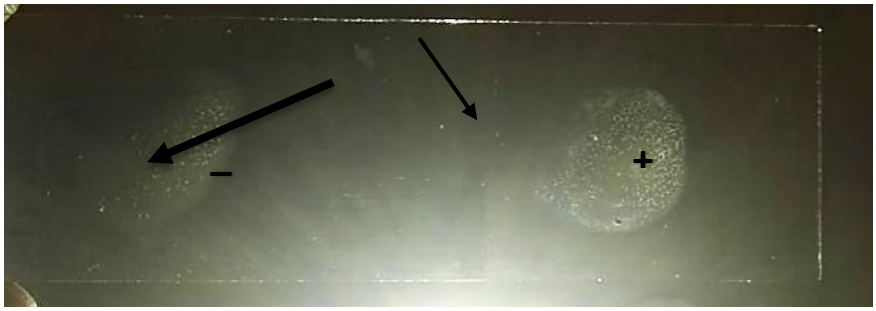


Figure: (4-3) slide agglutination reaction for serological test

4-1-5: VITEC® 2 – Compact microbial identification:

It have ability to identify a large range of microorganisms, after the culture were, incubate at 37℃ for 24 hours and the result of the biochemical reaction of this test demonstrate the our isolation was *S.enteritidis* as in appendix (1)

4-1-6: Api- 20 test

Api-20E system shoed the following results: The inoculated bacterium was positive for citrate utilization, ornithine decarboxylase, lysine decarboxylase, H_2S production, and also positive for utilization of the following sugar either for oxidation or fermentation which are, glucose, mannitol, inositol, sorbitol, rhaminose, melibiose and arabinose.

The bacterium was negative for the following: β-galactosidase, arginine dehydrolase, acrtoin production, tryptophane deaminase, urease, gelatinase, and indol production.

The bacterium was also negative for the utilization of following sugars for oxidation or fermentation: sucrose, cytrochrome-oxidase and amygdaline.

The numerical number of processed bacterium in Api-20E was 6704752 that gave an indication of Salmonella isolation in (99.9%) as presented in figure (4-4).

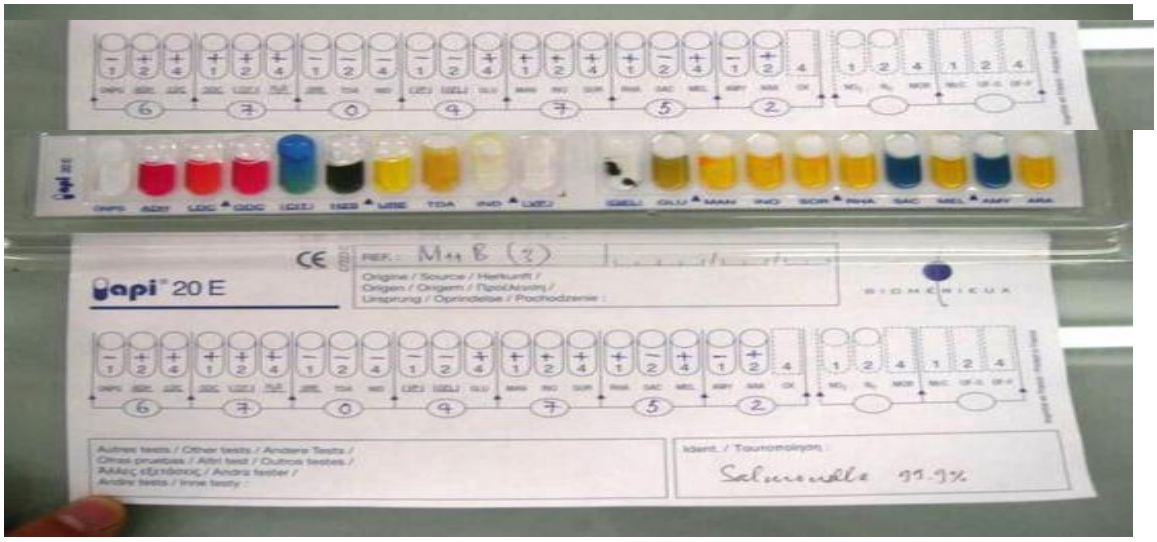


Figure :(4- 4) APi -20 system

(+) positive

(-) Negative

4-2: Results of experiment number I:

Determination of Infective Dose (ID)

The number live and dead rabbits in each group was recorded from week one PI to week 6 PI (table 4-2). The ID was selected as that dose used in rabbits and caused clinical signs like loss of appetite, decrease body weight, dullness, and fever but all inoculated animals were live. This ID found to be $4.5x10^8$ CFU/ml.

Table (4.2): infective dose of *S.enteritidis* in rabbits

Groups	Dose cells	Alive	Dead	Percent mortality
1	4.5×10^{11}	0	4	100%
2	4.5×10^{10}	1	3	75%
3	4.5×10^{9}	2	2	50%
4	4.5×10^{8}	4	0	0%
5	PBS	4	4	0%

No. of rabbits in each group =4 Total no. of rabbits =20

The ID of *Salmonella enteritidis* in this experiment was similar to the ID of other non-typhoid salmonella, that mentioned by AL- Talib,(2010) who found that the infective dose of *S.Newport* in rabbits 2×10^{8} C.F.U./ml. Other studies also mentioned that the ID might be ranged from 10^{5} to 10^{10} (Blaser and Newmann, 1982). An another study the ID of *S.enteritidis* in experimental infection of rabbits was determined as 2x 10^{8} C.F.U./ml (AL-Mansory, 2009).

Experimental infection of rabbits of present study was performed by intraperitoneal injection, and the findings were in agreement with those of AL-Qaisi (2004) who reported that the inducing infection by intraperitoneal rout was better than oral infection. This is might be attributed to the fact that gastrointestinal tract offered large number of barriers like IgA, acidity and the completion of normal intestinal flora when compared to the peritoneal cavitythat lacked such barriers.

4-3: Experimental infection of rabbits with infective dose of S.enteritidis.

4-3-1: Clinical signs:

The rabbits of group which infected by intraperitoneal route with ($4.5x10^8$CFU/ cells)show dullness, loss of appetite , fever, decrease activity, very mild diarrehea, dehydration, and lethargy. Also unresponsive to stimuli. Body temperature were more than 39.5℃ after one day post infection and disappeared gradually, recovery was complete within (7-9) days, other clinical signs were restlessness and dullness, which appeared after (24) hours post infection followed by diarrhea. Which continued for three days, the color of feces was dark black to light green and paste in consistency with or without mucus when compared with feces of the rabbits in the control group. These clinical signs were compatible with study of harab (2010) in rabbits which infected experimentally with *S.hadar* showed the same clinical signs . in our study then these clinical signs disappeared gradually and recovery was complete within (7-9) days

Figure :(4-5) Infected rabbits showing diarrhea during the first week of *S.entritidis* infection

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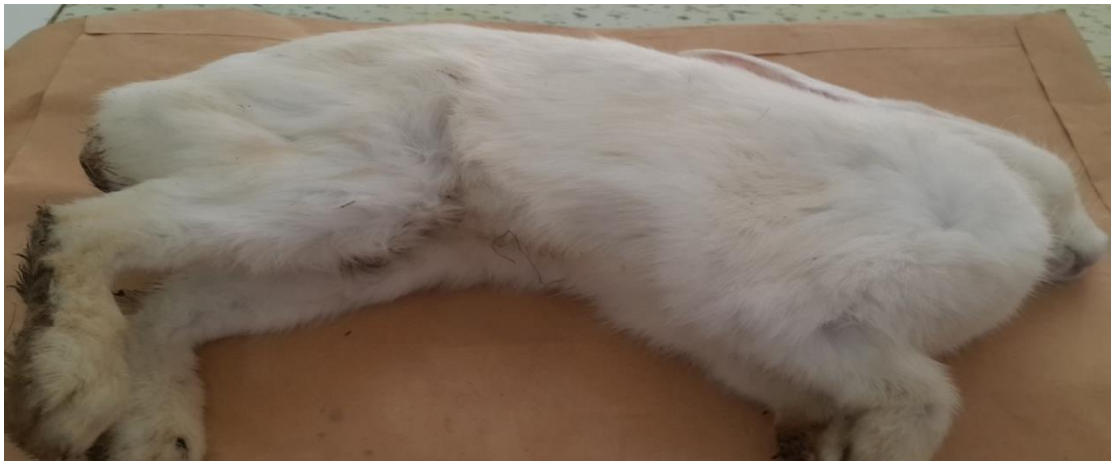

Figure :(4-6) Infected rabbits showing emaciation during the experimental time.

The control group did not show any change in behavior or other signs.

The results of the present study was agreement with AL-Mansory, (2009) which recorded the same clinical signs on rabbits infected with *S.enteriditis* .

Rabbits have been chose as a model for diarrheal disease and its sequelae associated with salmonellosis (Brutzki *et al*., 2001).

S.enteritidis invade the mucosa of small and large intestine and produced toxins, then stimulates the release of pro-inflammatory cytokines and acute inflammatory reaction occurs. *Salmonella* can be disseminated from intestine to other organs and cause systemic infection (Monack and Falkow. 2004).

When daily examination of rectal temperate was carried out before infection (at zero time), the two groups (control and infected have shown normal range (38.51) and (38.4) respectively. After that the body temperature increased significantly ($P \leq 0.05$) in the infected group at first day post infection(41.01) then it gradually subsided and the animals appear normal at day (14 and 45) when compared with control group which remained with the normal value in all time of experiment. Table (4-3).

Table (4-3): Mean of rectal temperature (℃) in the infected group with *S. enteritidis* and the control group.

Body temperatures changes C°				
Groups	**Time post infection(days)**			
	Zero	**1**	**2**	**3**
Infected	38.51±0.06Aa	41.01±0.35Ba	39.51±0.23Ba	38.68±0.22Ba
Control	38.4±0.09a	38.4±0.09b	38.67±0.20b	38.82±0.25a

Different small letters vertically (between groups) and capital letters horizontally (between periods) denote significant differences ($P \leq 0.05$), among groups during the period of experiment, data represents as M±SE.

The increase of the body temperature is due to endogenous pyrogen in the cytokine IL- 1 which produced by macrophage as a result to response to endotoxins. These endogenous pyrogens circulating in the blood stream and act on stimulating the thermoregulatory centre in the hypothalamus to cause fever (Eales 2003; Coico *et al.*, 2003) this in agreement with mentioned by (Harab 2010).

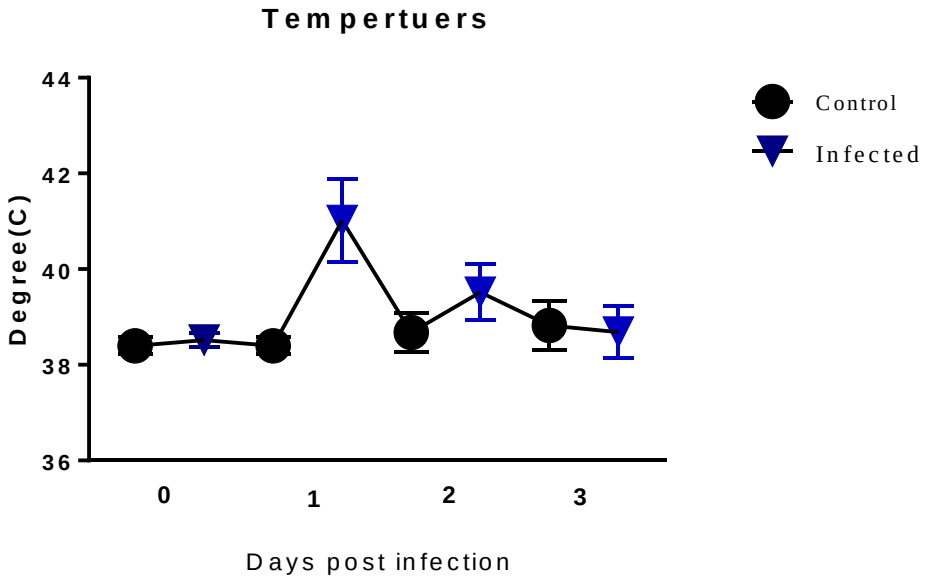

Figure :(4-7) Body temperature in infected and control group

When daily examination of Respiratory rates before infection (at zero time) the two group (control and infected) showed normal range (52) (37) respectively after that respiratory rate increased a significant ($P \leq 0.05$) in the infected group at first day post infection (81.33∓2.45) then rabbits appeared normal at day (14 and 45) as compared with control which remain in normal value all time of experiment as in table (4-4).

Table (4-4): Means respiratory rate (breath/minute) in the infected group with *S.enteritidis* and the control group:

Respiratory rate changes in minutes				
Groups	**Time post infection(days)**			
	Zero	**1**	**2**	**3**
Infected	52±1.78Aa	81.33±2.45Ba	64±1.78Ca	57.33±1.33Aa
Control	37±2.51b	43±4.72b	34±1.15b	38±2b

Different small letters vertically (between groups) and capital letters horizontally (between periods) denote significant differences (P≤ 0.05), among groups during the period of experiment, data represents as M±SE

In the infected group there is increase in respiratory rate and thirst may be contributed with to fever and diarrhea, to reparation the excessive losses of fluids and electrolytes and maintain the temperature of the body, this in agreement with study of (Frizzell *et al.*, 2001).

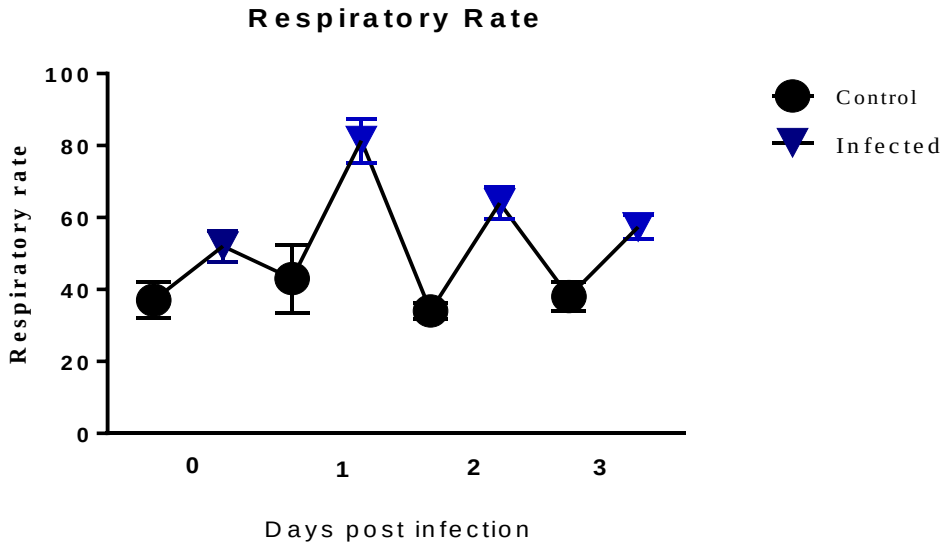

Figure (4-8): Respiratory rate in infected and control group

The results of heart rate showed gradually increased during 1 day post infection and the peak noticed a significant (P≤ 0.05) increased in the infected group (175.33±1.90) at first day of experiment, while the animals of control group remain in normal value all time.

Table (4-5): Mean heart rate (rate/minute) in infected group with *S.enteritidis* and control group.

Heart rate changes in beat/minutes				
Groups	**Time post infection(days)**			
	Zero	**1**	**2**	**3**
Infected	134±3.38Aa	175.33±1.90Ba	144.66±4.05Ca	133.33±3.21Aa
Control	134.66±1.15a	137.33±3.05b	133.33±1.15b	129.33±4.16a

Different small letters vertically (between groups) and capital letters horizontally (between periods) denote significant differences ($P \leq 0.05$), among groups during the period of experiment, data represents as M±SE

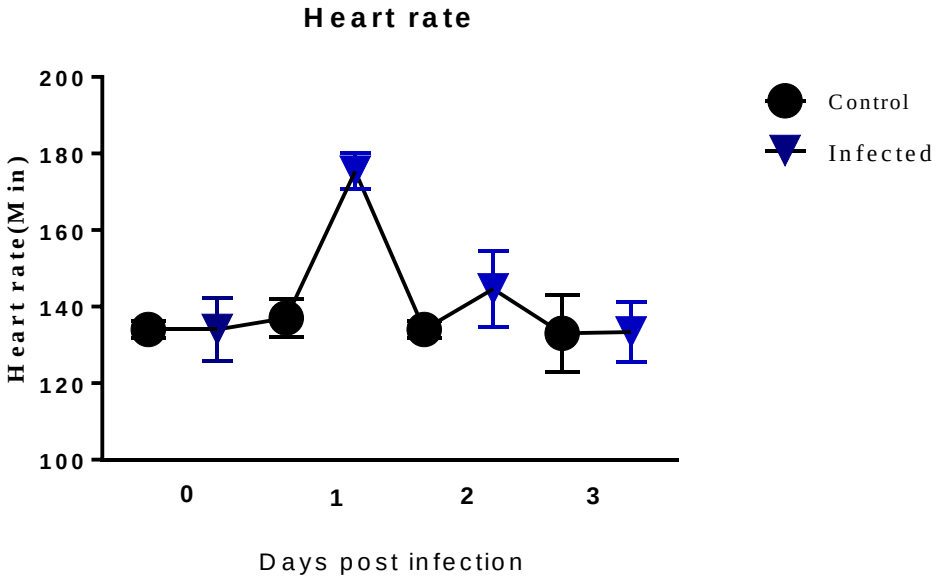

Figure (4.9): Heart rate of infected and control group

The results of body weight(rabbits-gm/week) there is a significant decrease in body weight after infection at (4 and 6) weeks PI when compared to control rabbits that showed increase in body weight (Table 4-6).

Table (4-6): The rabbit body weight mean in grams/week of the animal group infected with *S.enteritidis* and the control group.

Body weights changes in grams				
Groups	**Time post infection(weeks)**			
	Zero	**2**	**4**	**6**
Infected	1861±45.51Aa	1760.16±46.53Ba	1675±38.18C	1631.66±34.46Ca
Control	1865±41.28a	1912.5±42.54b	1936.25±38.20b	1954.25±36.19b

Different small letters vertically (between groups) and capital letters horizontally (between periods) denote significant differences (P≤ 0.05), among groups during the period of experiment, data represents as M±SE

Decreased of body weight after infection of *S.enteritids* due to strong anorexia was in agreement with findings of Fabienne *et al.*, (2002), while animals of control group showed increased in body weight due to increase food intake and normal activity of body metabolism.

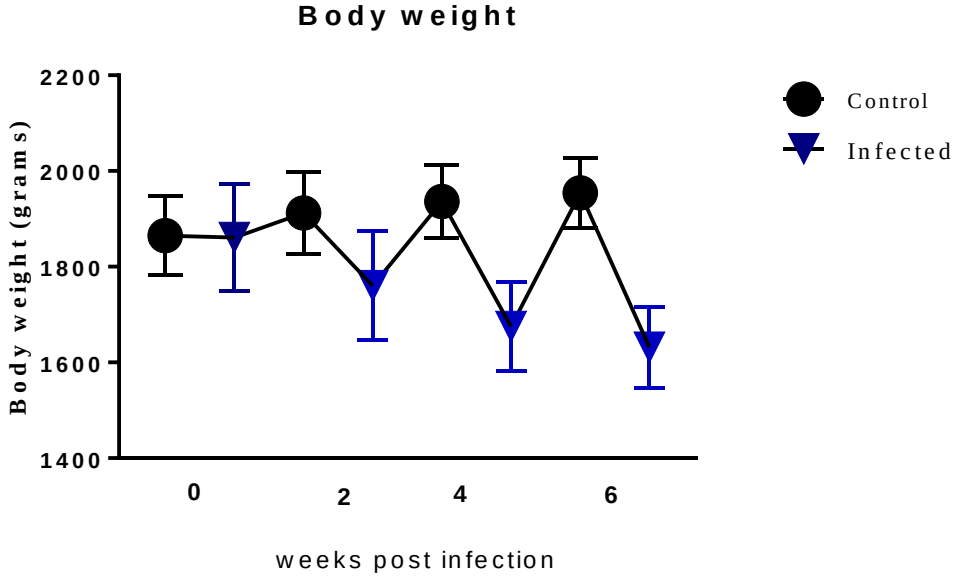

Figure (4-10): Body weight of infected and control group

4-4: Post mortems change in organs of rabbits:

A- Control group:

There are no pathological changes in internal organs of control group used in the experiment.

B- Infected group with infectious dose of *S. enteritidis*:

The internal organs of sacrificed rabbits, which injected intraperitoneal with infectious dose (4.5×10^8 C.F.U/ml),were examined in these time (24, 48, 72, 96, 120, 144, 168) hours, second and third week post infection. Two rabbits sacrified in each time and were found as fallow:

First week after infection

Abdominal viscera show flaccid of small intestine and filled with clear to yellow watery contents and there is sever congestion of large intestine.

Liver enlarge in size (hepatomegaly) and dark- red in color .necrotic foci areas (white –yellow) in color.

Lung: enlarge in size, congested, with pale –white in color and focal hemorrhagic.

Kidney: enlargement of both kidney and there is congestion.

Heart: enlarged and congested with flappy and there is pericarditis.

Small intestine: dilated and filled with watery clear to yellow, flaccid, thin wall.

Large intestine: congestion and filled with feces.

Spleen: enlarge in size and congestion.

Gall bladder: enlarge in size and dark in color.

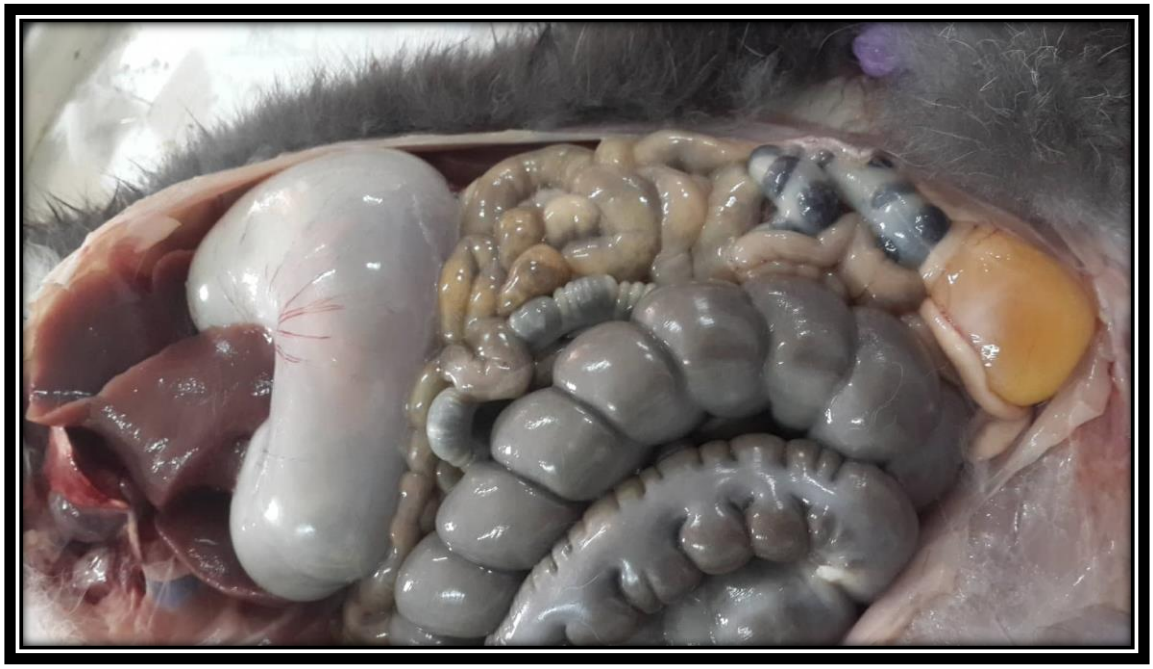

Figure (4-11) Normal internal viscera which shown the normal structure without any abnormalities

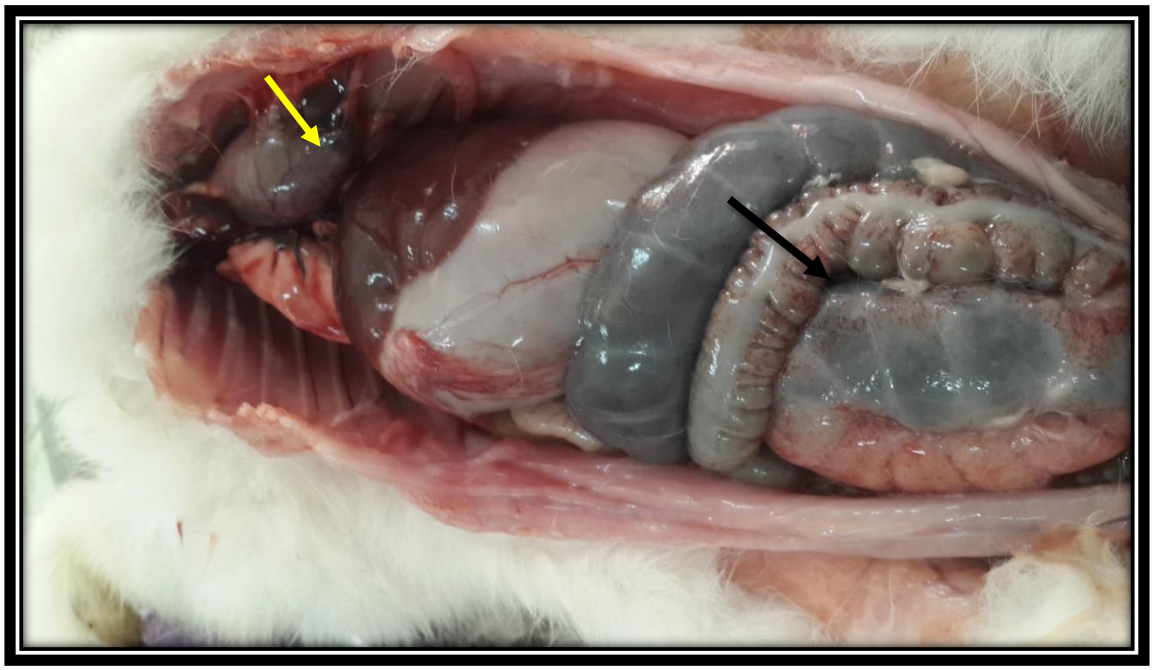

Figure (4-12): Abnormal abdominal viscera of infected rabbit showed sever congestion of large intestine, congestion of heart and pericarditis

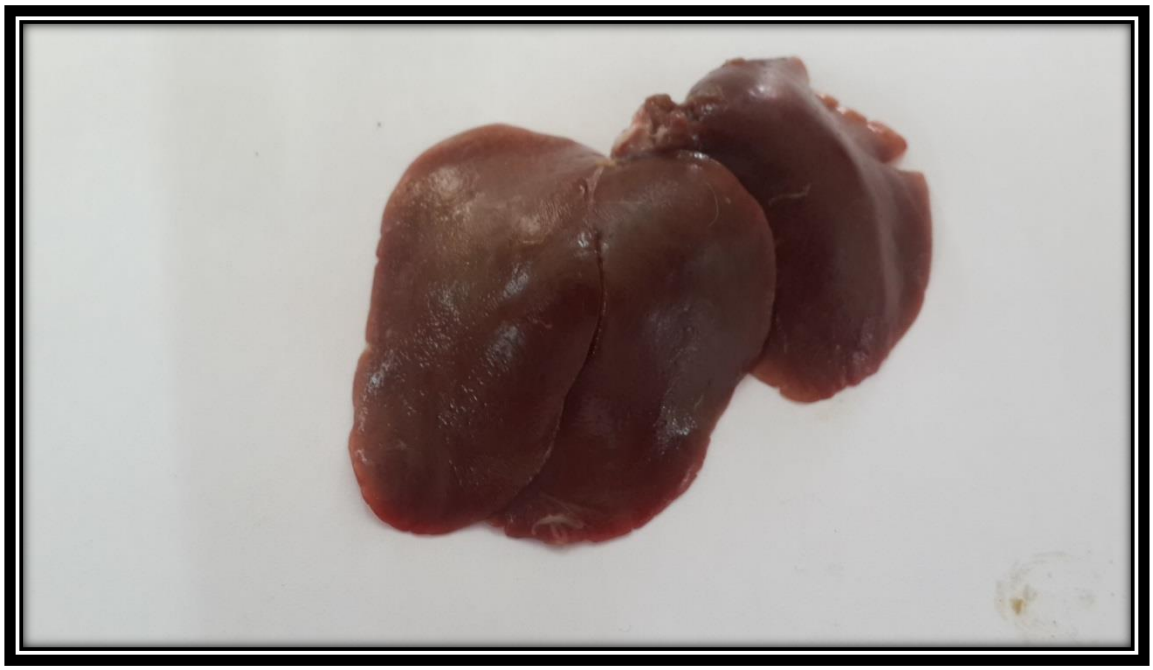

Figure (4-13): Normal liver no pathological changes

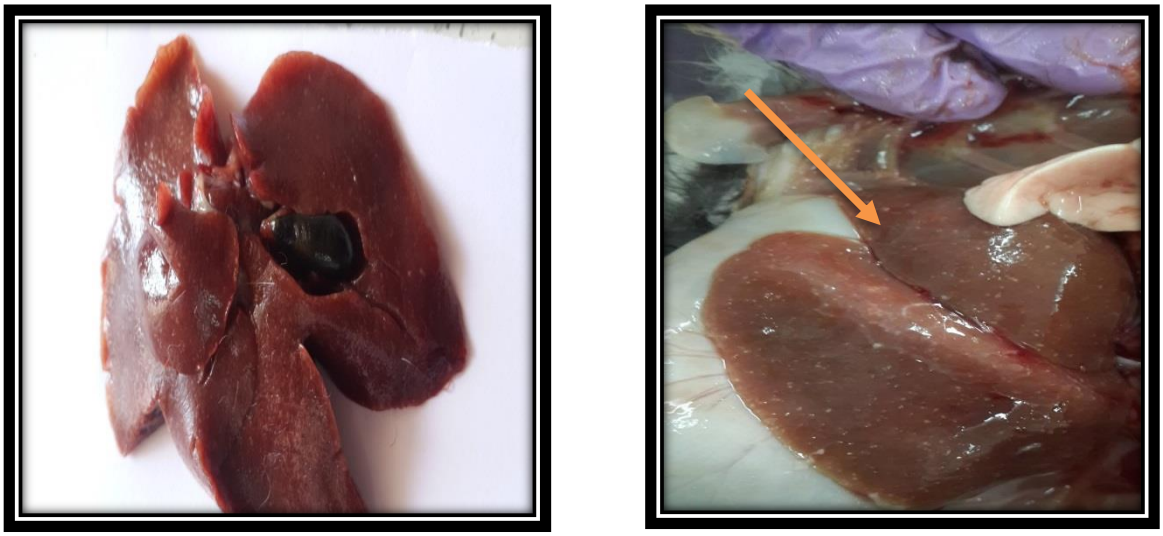

Figure (4-14) Liver of infected rabbit showed enlargement, congested and necrotic foci, and fibrosis

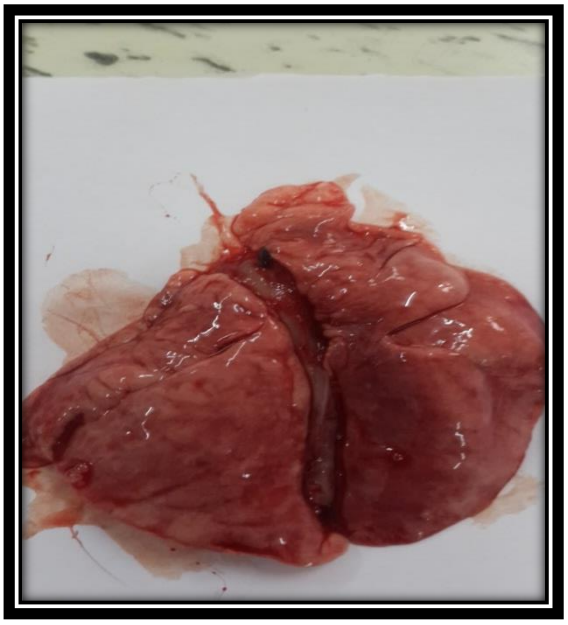

Figure (4-15): Normal lung

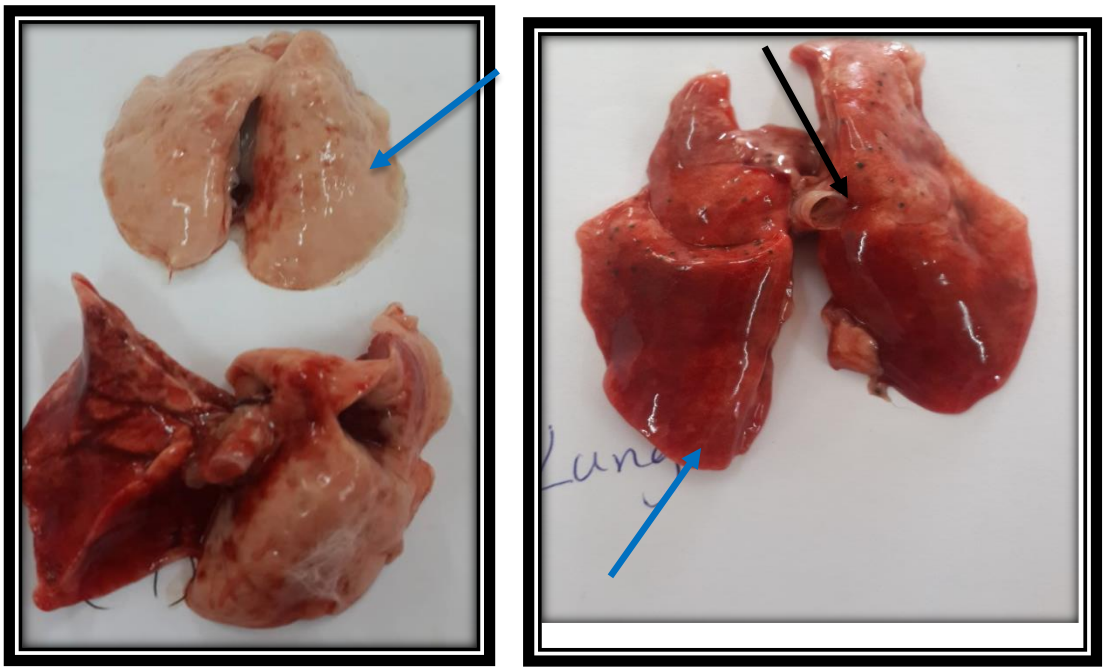

Figure (4-16): Lungs showed enlargement in size and there is

Congestion and focal haemorrhages, pale –white in colour

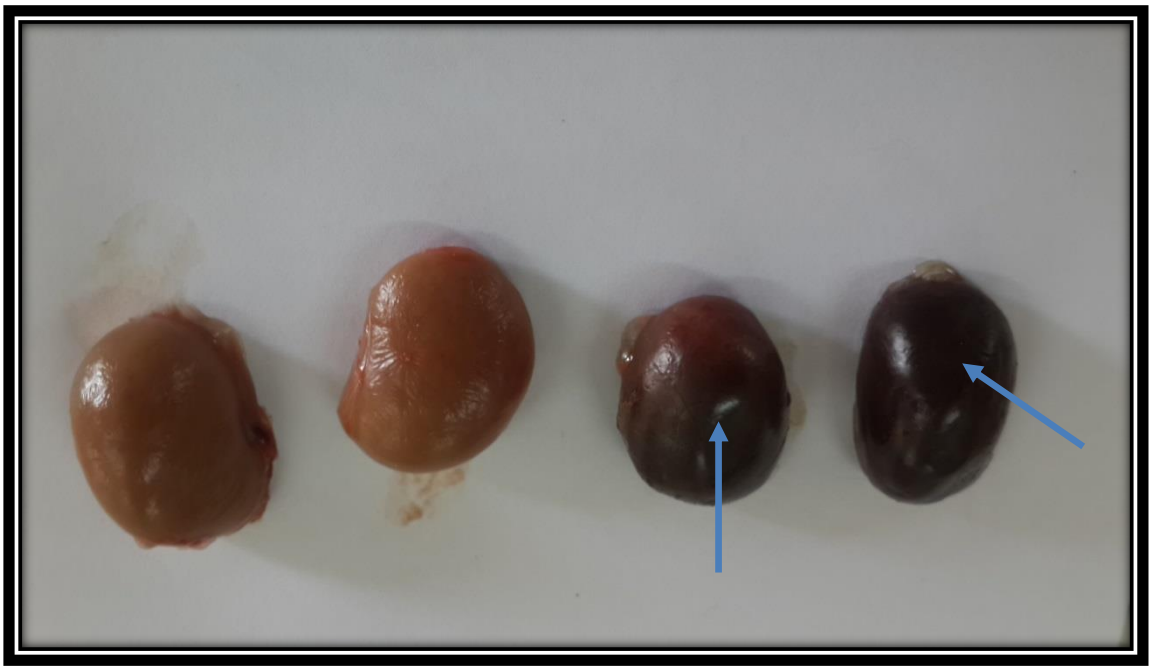

Figure (4-17): Kidney in the right side is normal while in left side there is congested and dark in colour

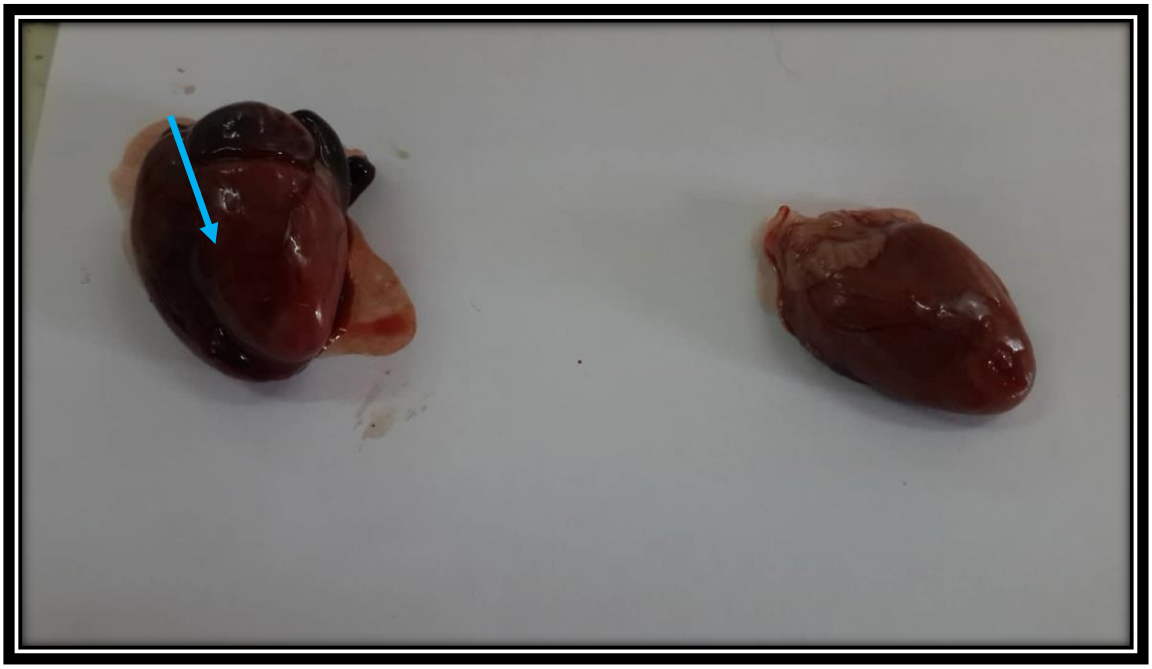

Figure (4-18): - Heart in the right side is normal while in left side there is enlargement , congestion .

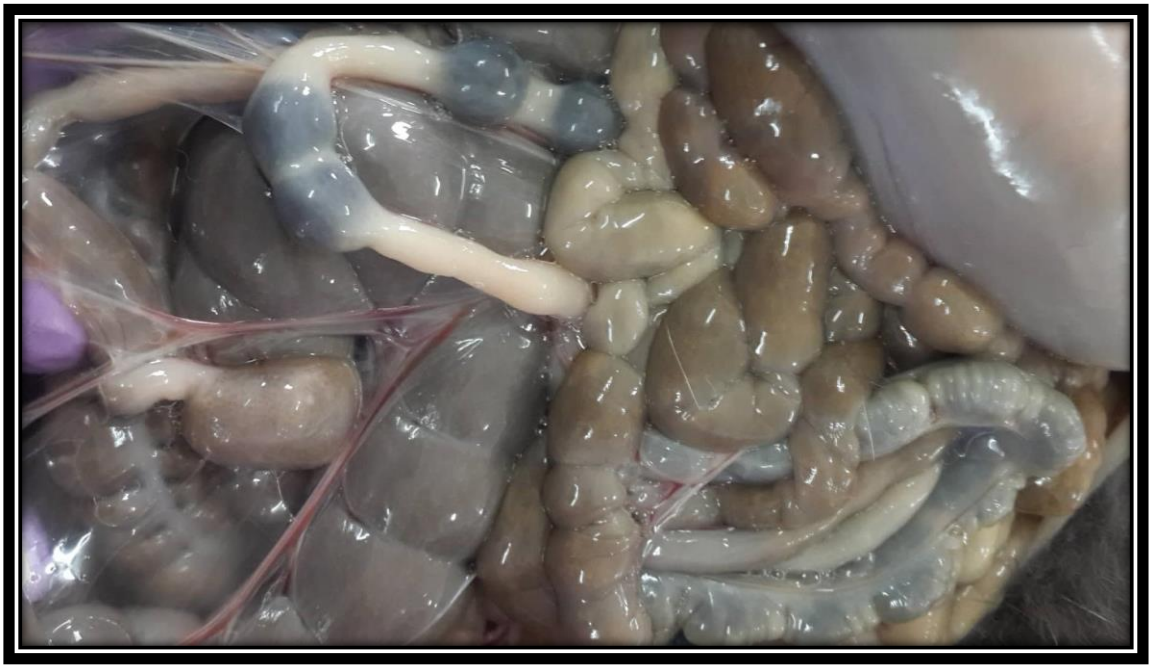

Figure (**4-19):** Normal small intestine

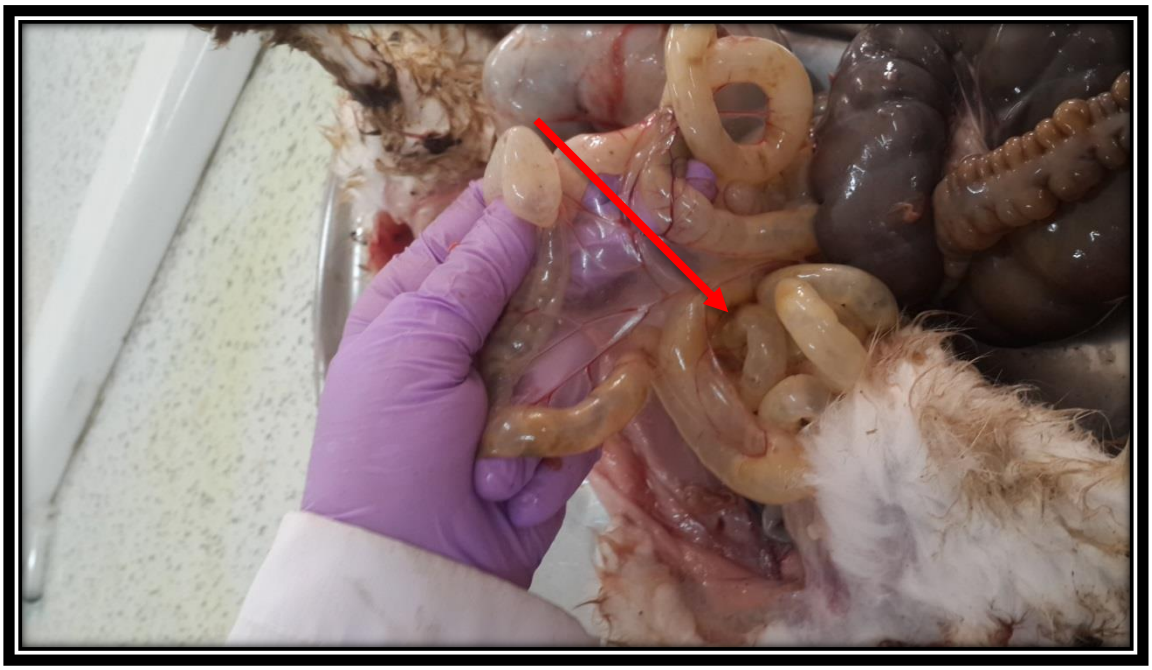

Figure (4-20): - Small intestine of infected group showed flaccid and filled by clear to yellow watery content and mucus in large amount.

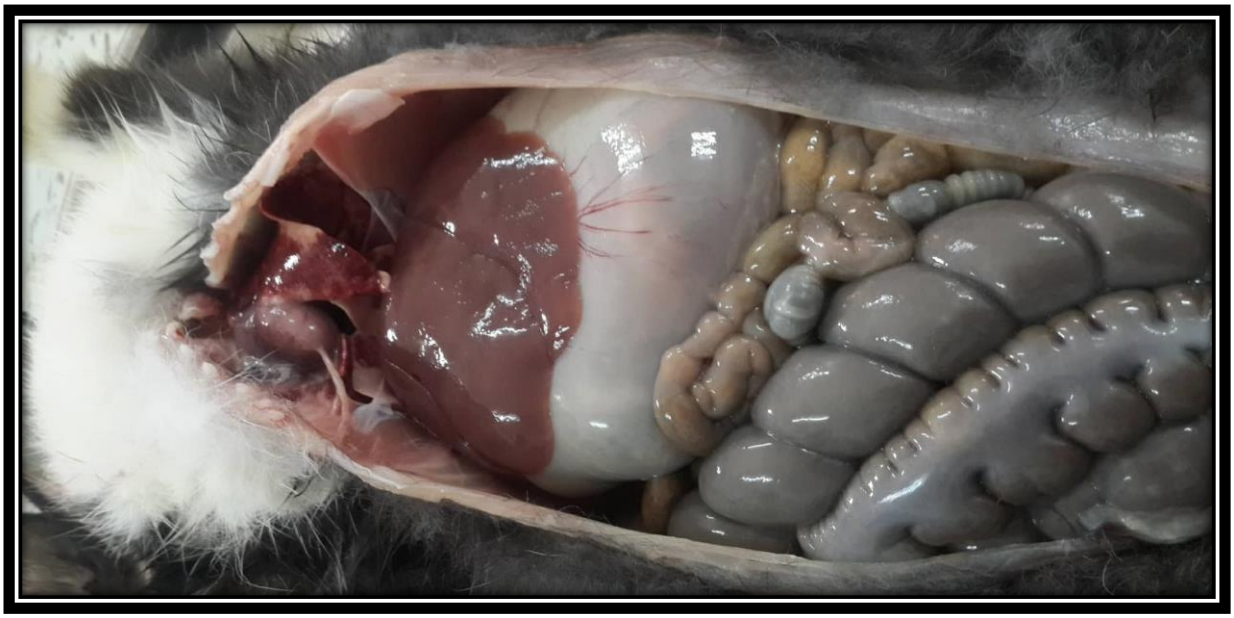

Figure (4-21): Normal large intestine

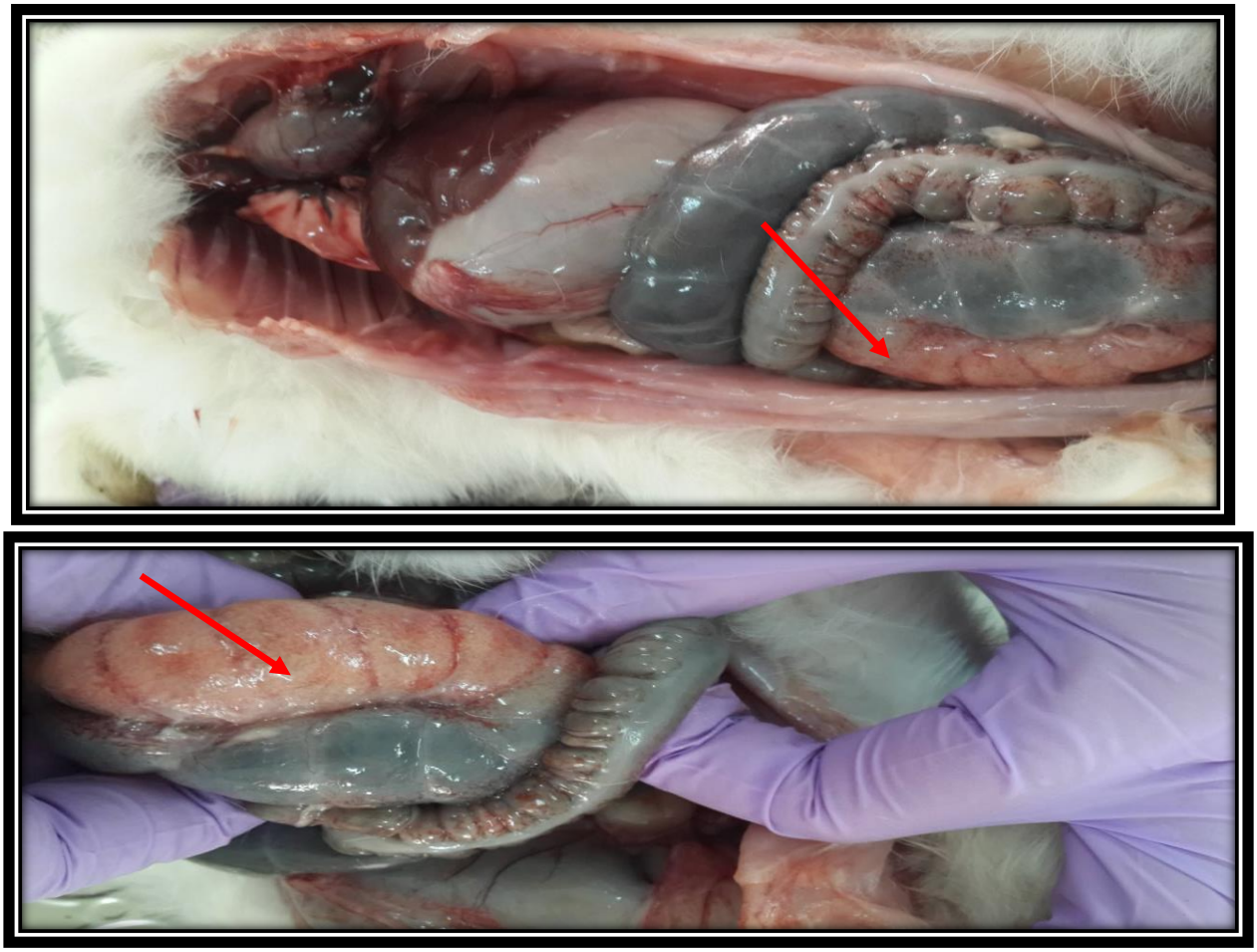

Figure (4-22): Large intestine of infected group showed congested and enlargement

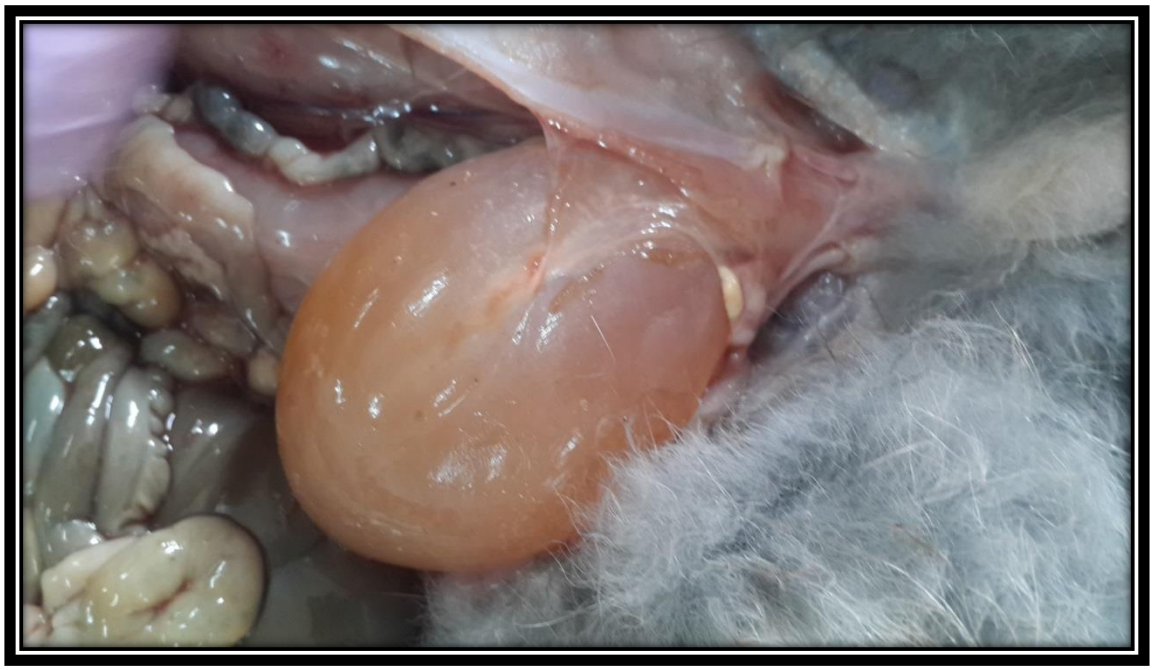

Figure (4-23): - Bladder of infected rabbit showed enlargement and filled with urine

Microscopically: in the intestine of control rabbits the payer patch is normal and no pathological lesions (figure 4-24) but the infected intestine there is infiltration of inflammatory cells (macrophage and lymphocyte) also there is exudate, sluffing of epithelium and necrosis figure(4-26).

The liver showed infiltration of inflammatory cells, necrosis, fibrosis and there is exudate, figure(4-34) in severe cases there is granuloma and amyloid degeneration(infiltration of homogenous protein material)figure(4-36) .lung, there is sever infiltration of inflammatory cells(macrophage and lymphocyte) ,damaged of the alveoli , and there is exudate and necrosis figure (4-42). In the heart, there is mild infiltration of inflammatory cells in striated muscle (mononuclear cell). Figure (4-49).

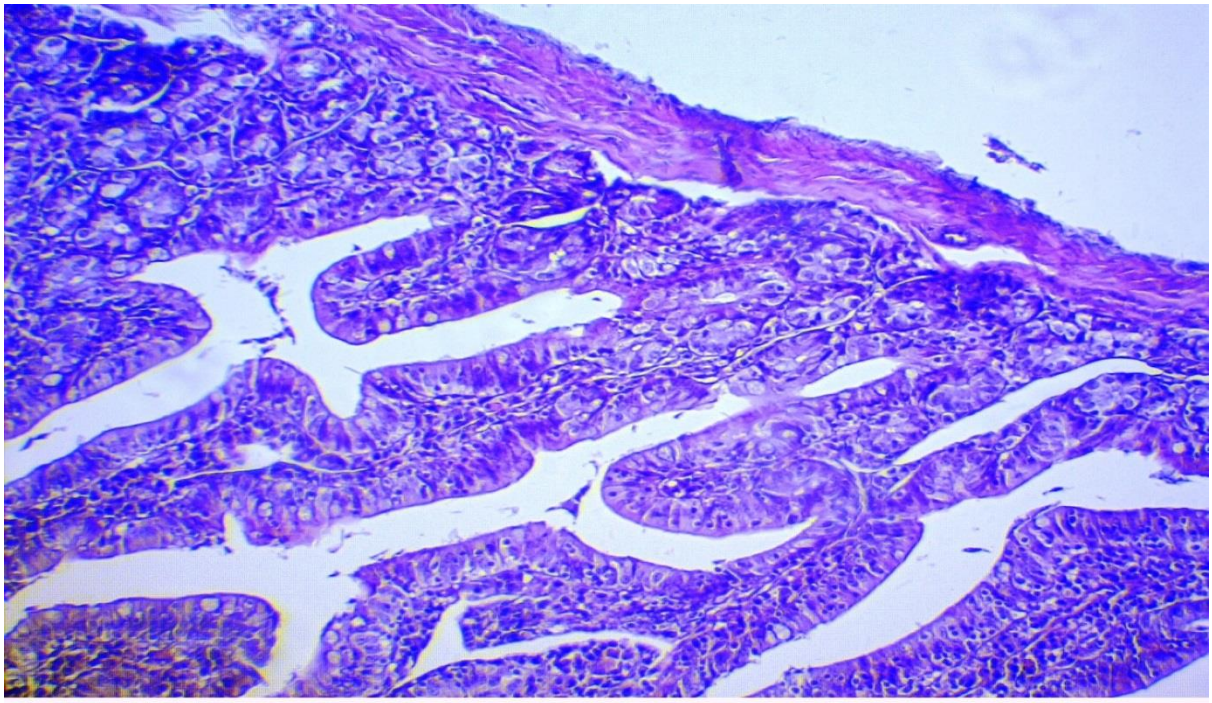

Figure **(4-24):**Normal small intestine there is no histopathological changes (H&E 20X)

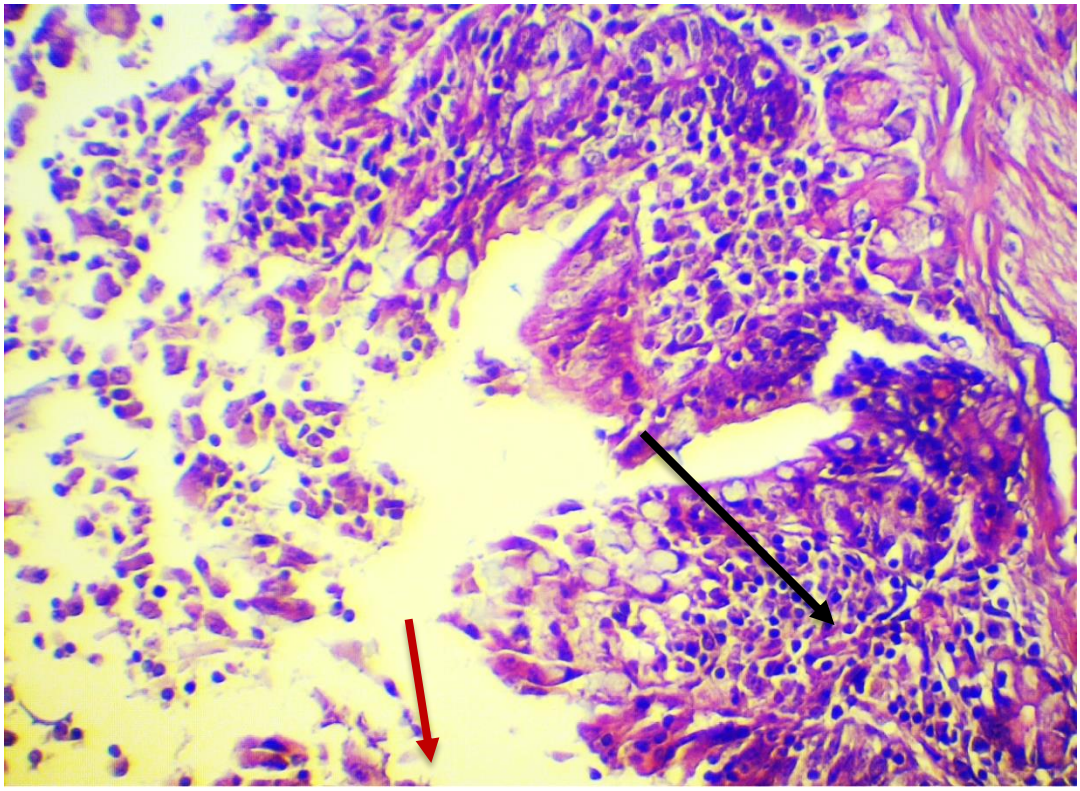

Figure (4-25): Infected intestine after 72 hour showed infiltration of inflammatory cells ⟶ sluffing of epithelium ⟶ (H&E stain20X).

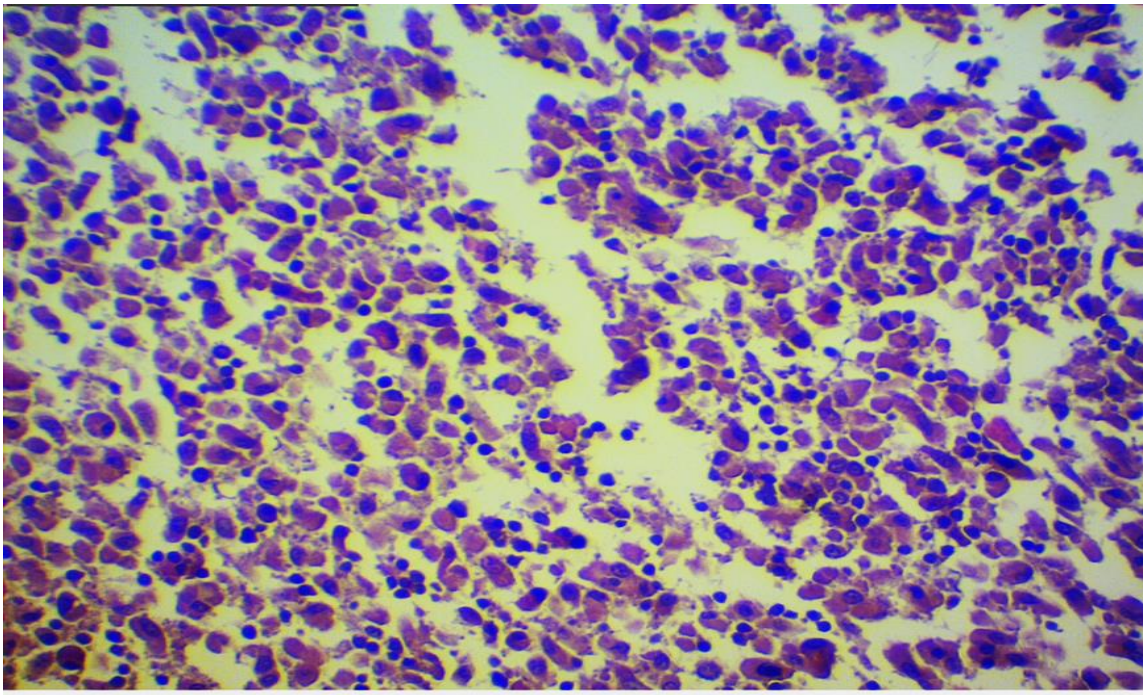

Figure (4-26): Infected intestine after 96hour showed infiltration of inflammatory → cells, necrosis → and exudate → (H&E stain 20X).

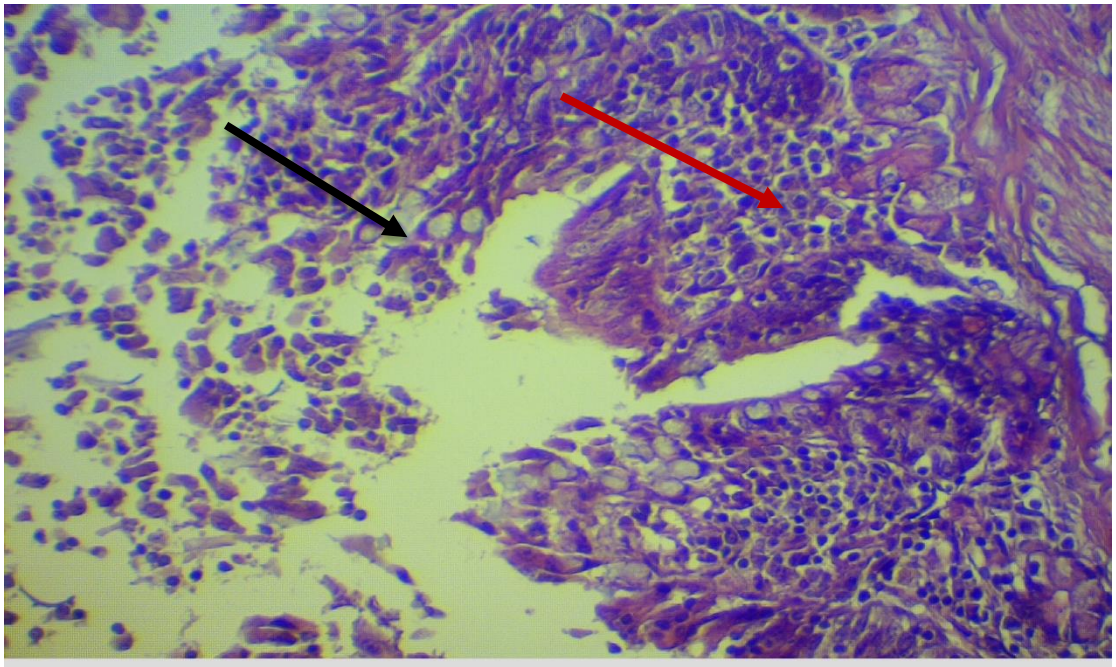

Figure (4-27): Infected intestine after 120 hour showed sluffing →, necrosis and infiltration of inflammatory cells → (H&E stain 20X).

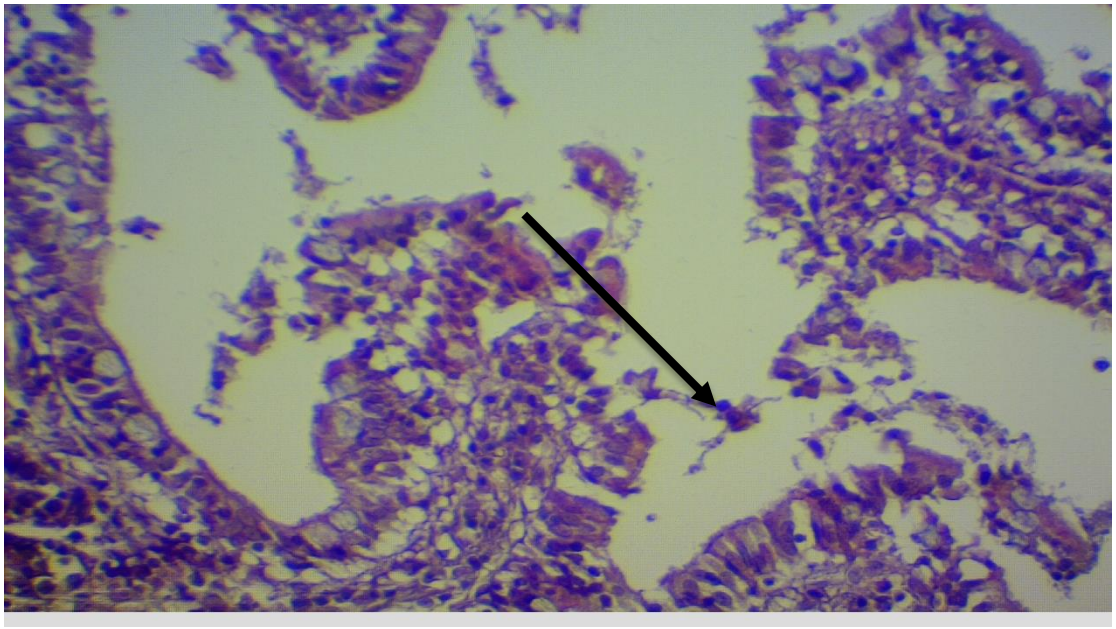

Figure (4-28): Infected intestine after 1 week showed sloughing of mucosa (H&E stain 20X).

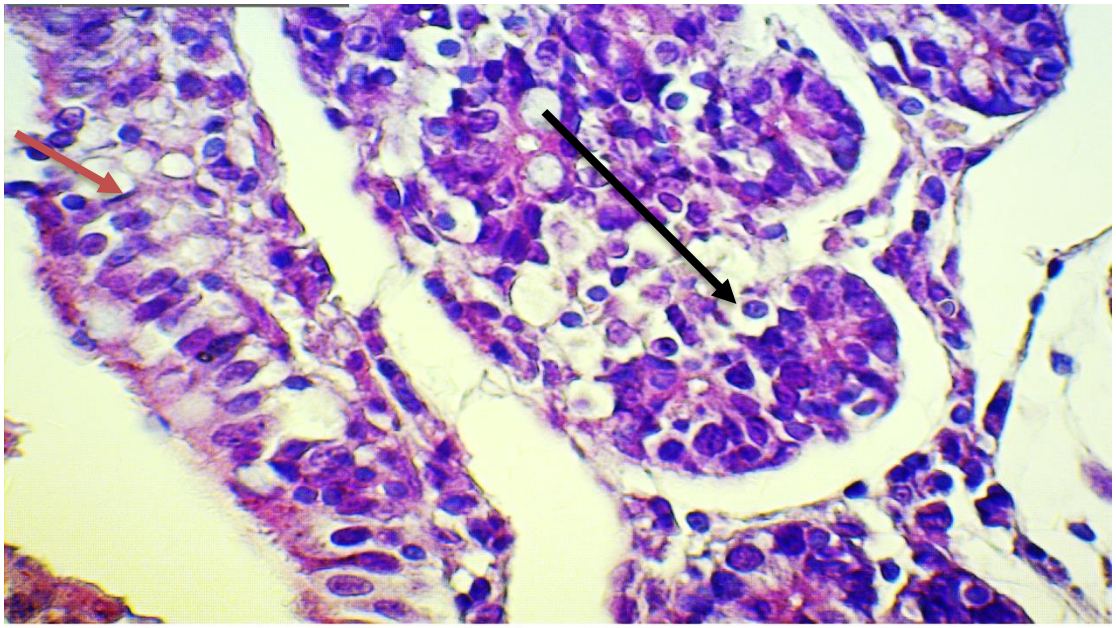

Figure (4-29): Infected intestine after 2 weeks, there is present of infiltration of inflammatory cells ⟶, exudate and necrosis ⟶ (H&E stain 20X).

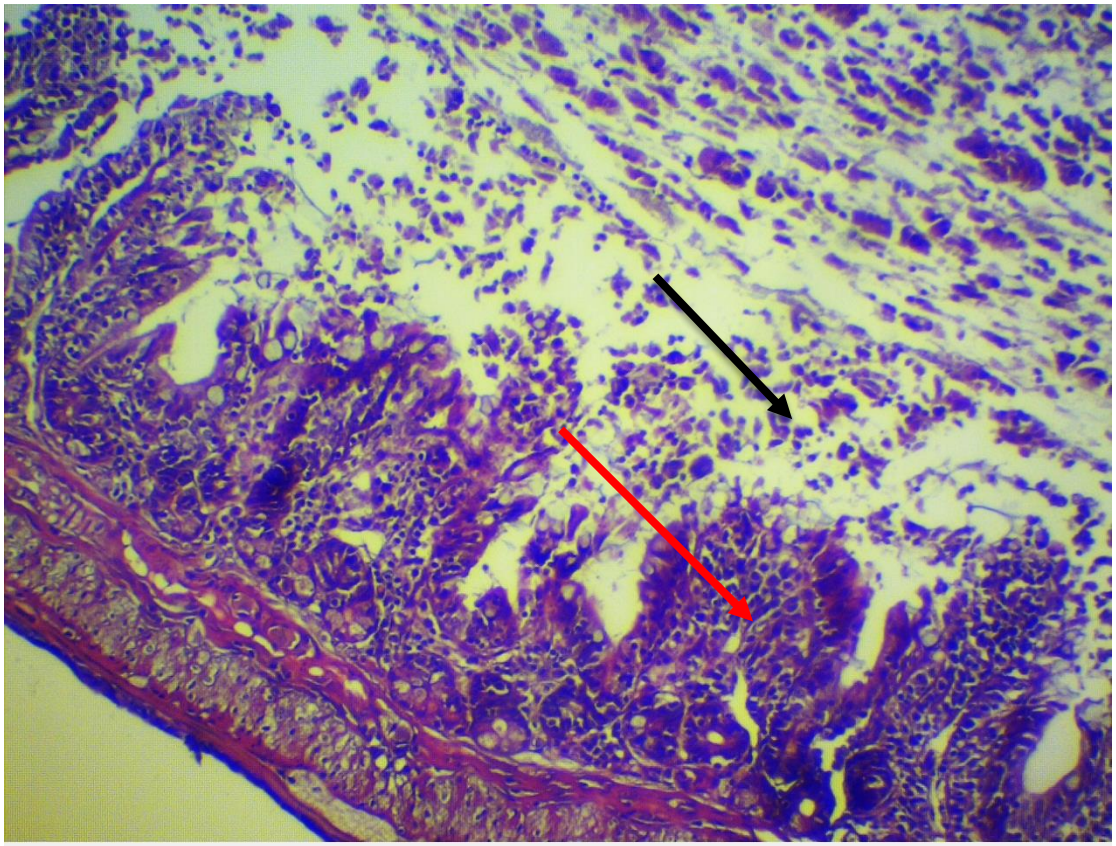

Figure (4-30): Infected intestine after 3 weeks of infection showed sloughing of epithelium → and infiltration of inflammatory cells → (H&E stain 10X).

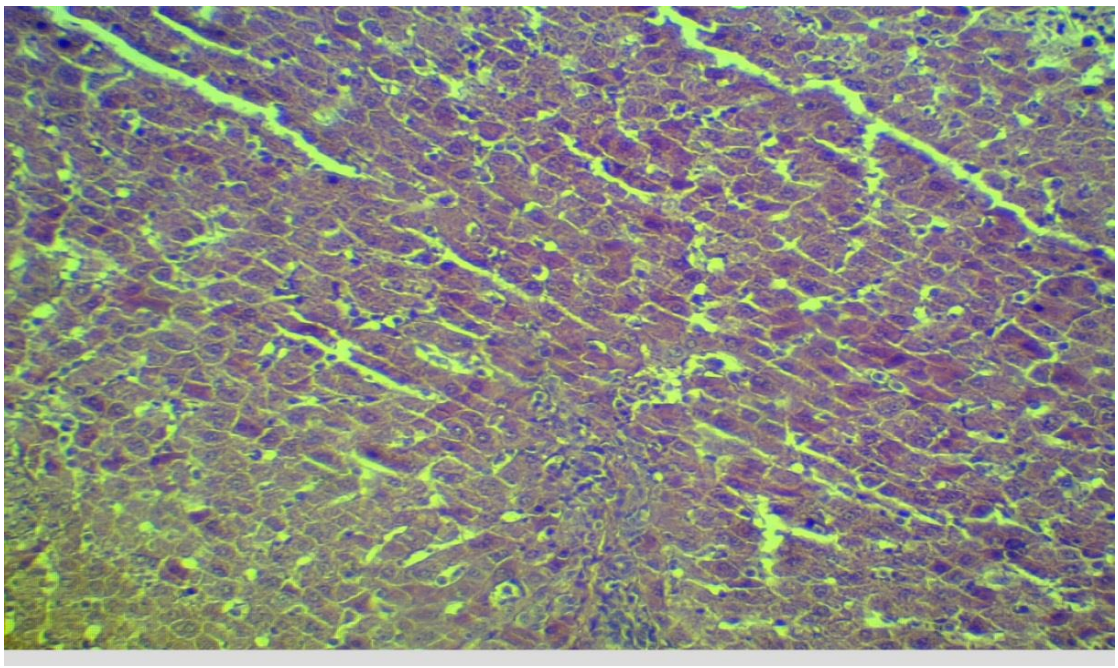

Figure (4-31): Normal liver no histological changes (H&E stain 20X)

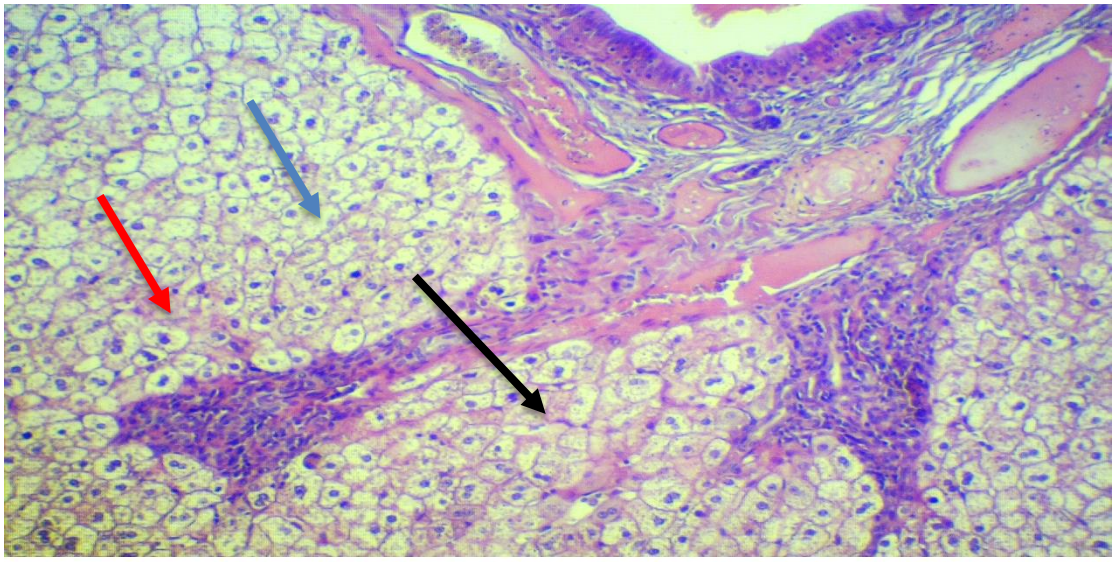

Figure (4-32): Liver of infected animal after 24 hour of infection, there is infiltration of inflammatory cells ⟶, few exudate and congestion ⟶ and hydropic degeneration ⟶(H&E stain 20X).

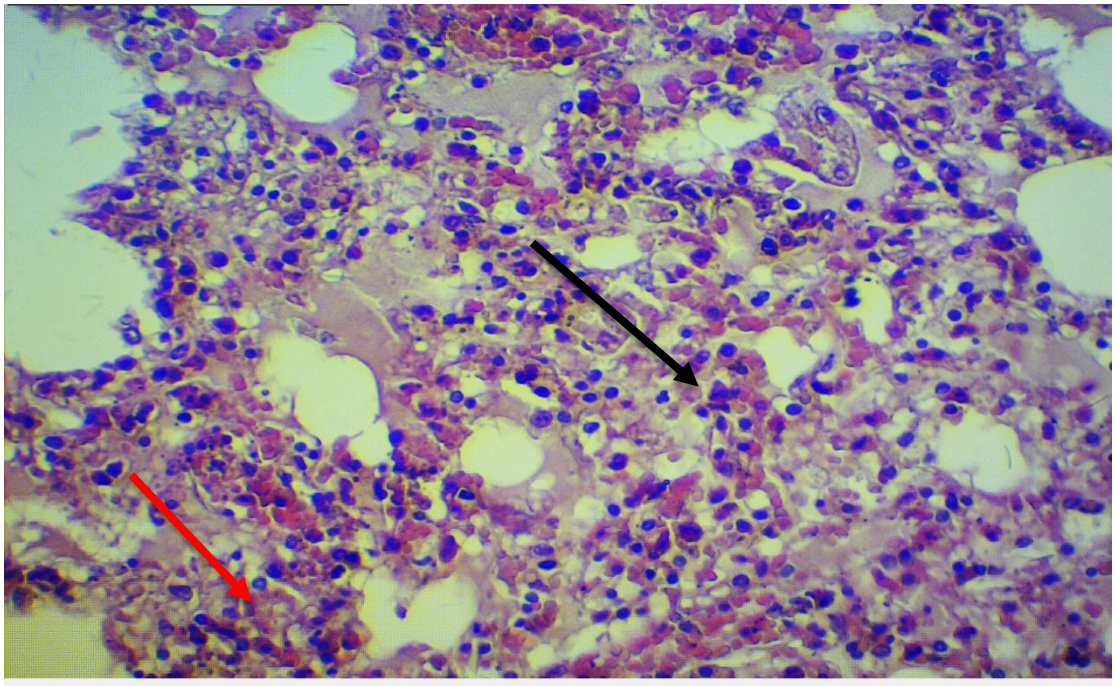

Figure (4-33): Liver of infected animal after48 hour there is infiltration of inflammatory cells ⟶ , congestion and few exudate ⟶(H&E stain 20X)

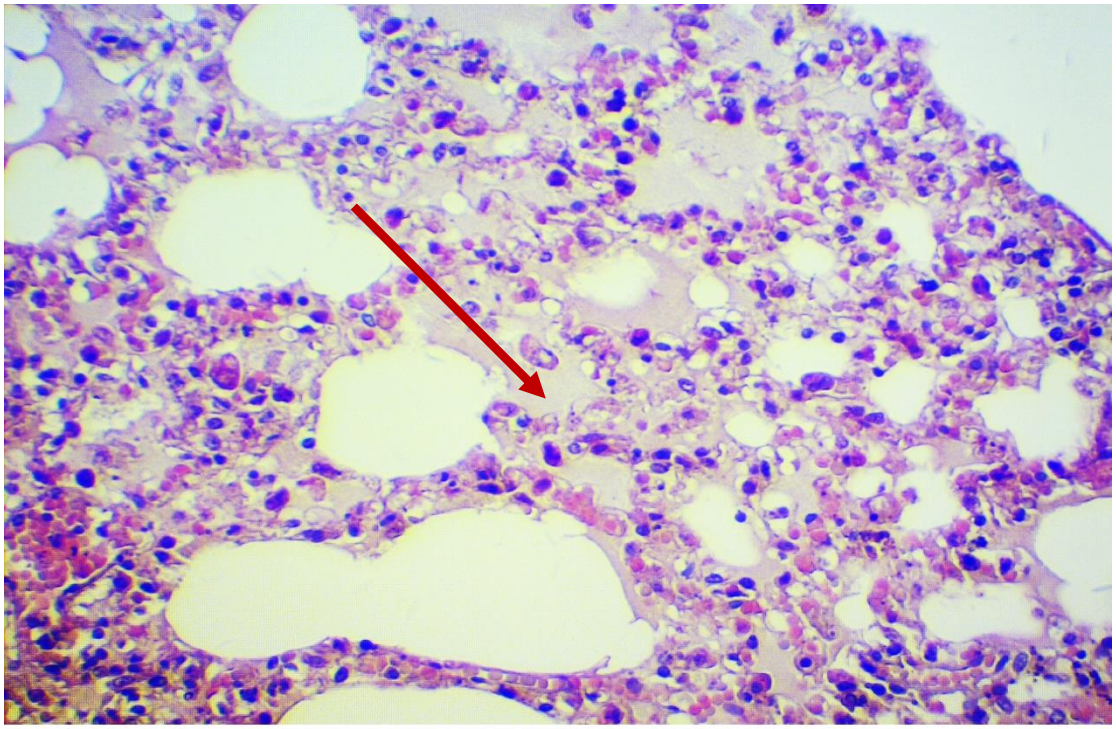

Figure (4-34): Infected liver after 72 hour showed few exudate and necrosis (H&E stain20X)

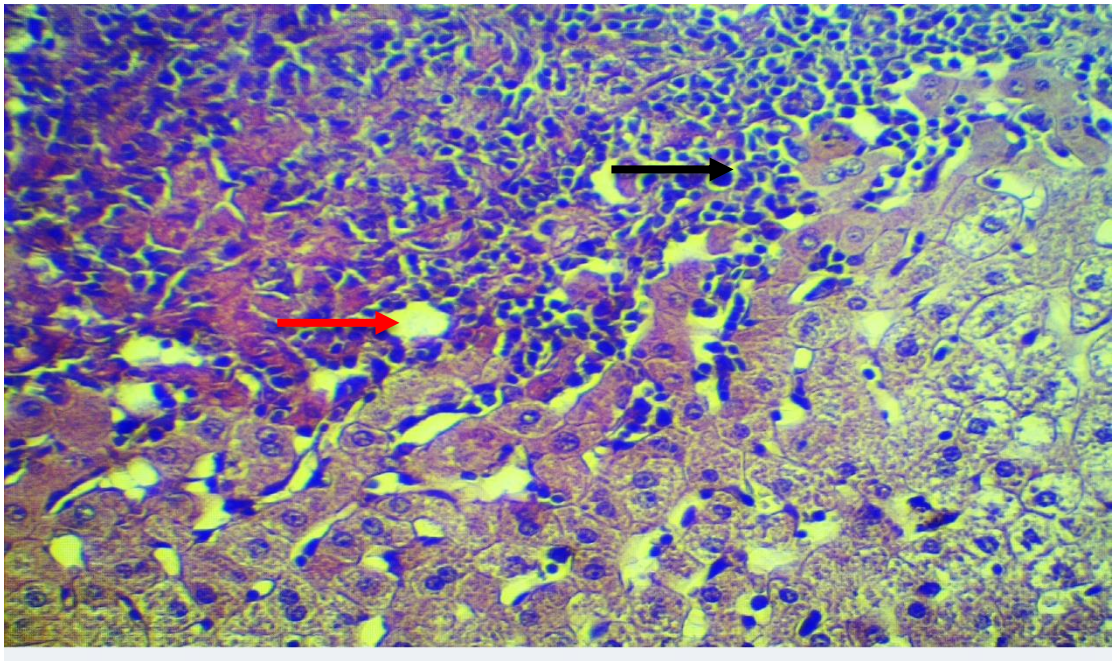

Figure (4-35): Infected liver after 96 hour there is sever inflammation, ⟶exudate and necrosis⟶ (H&E stain 20X)

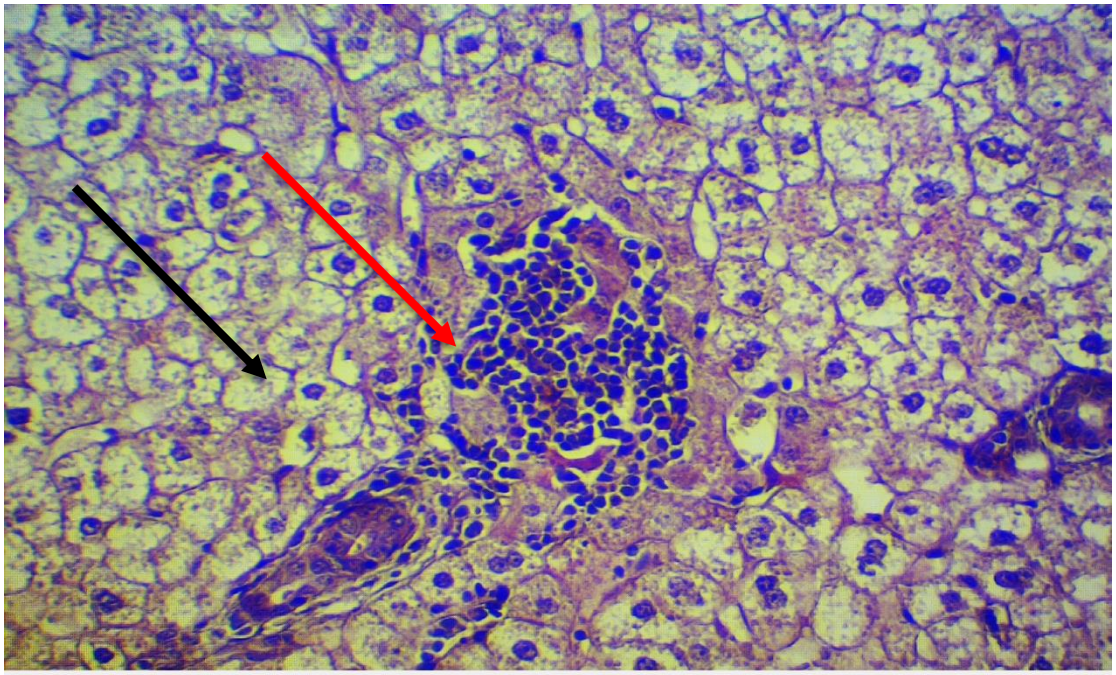

Figure (4-36): Infected liver after 120 hour, in the center of slide there is early stage of granuloma, → hydropic degeneration and exudate → (H&E stain 20X).

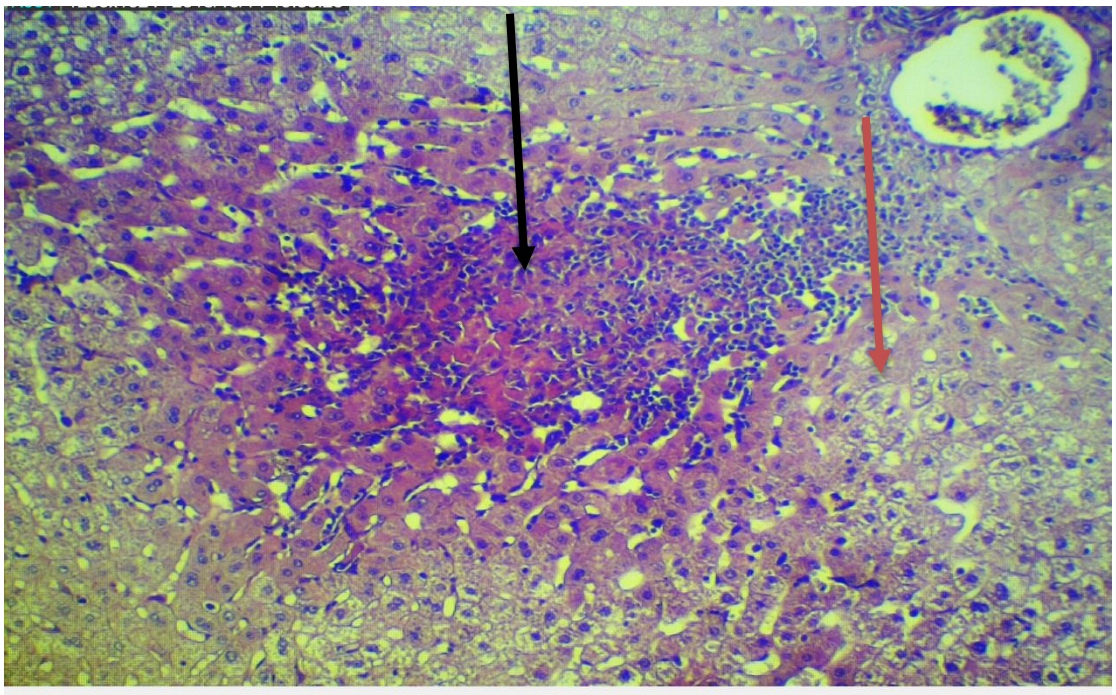

Figure (4-37): Infected liver after 144 hour there is presence of granuloma (macrophage and lymphocyte), → amyloid infiltration → and few necrosis (H&E stain 20X)

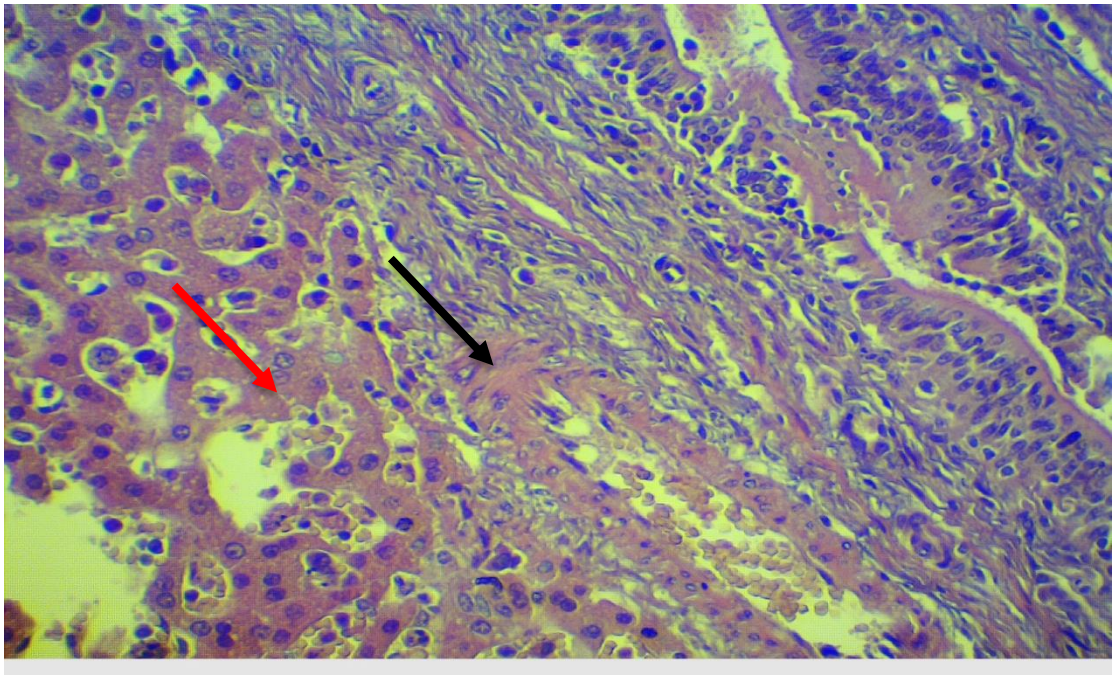

Figure (4-38): Infected liver after 168 hour after infection, there is exudate, → few fibrosis → (H&E stain 20X)

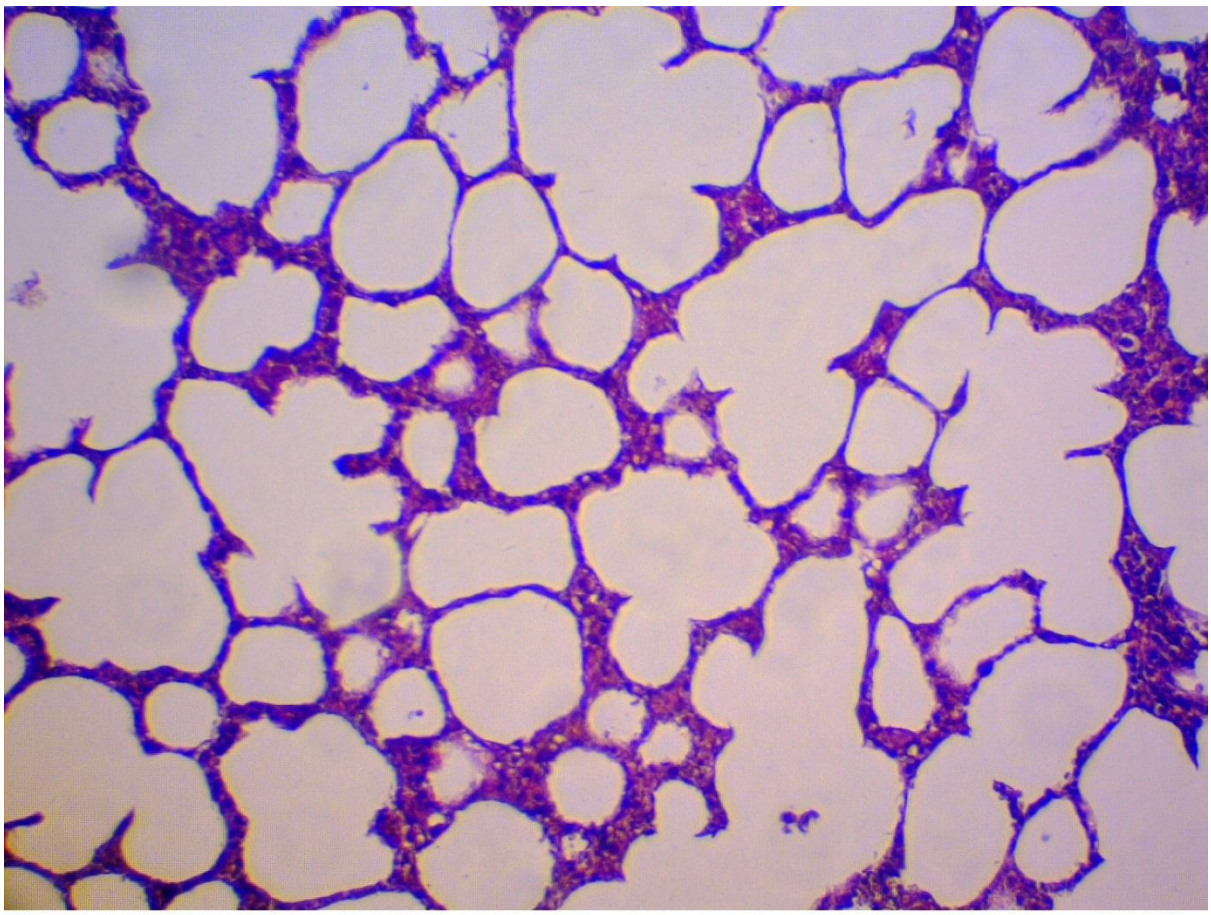

Figure (4-39): Normal lung there is no histological changes (H&E stain 20X)

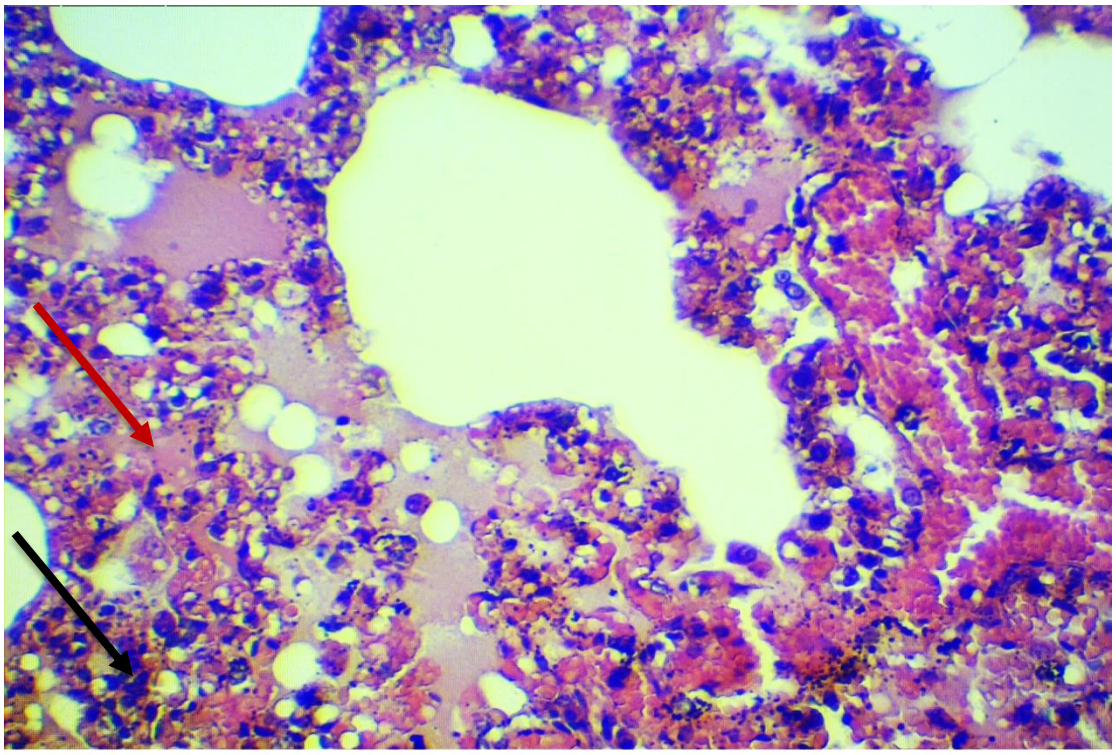

Figure (4-40): Infected lung after 24 hour of infection, there is infiltration of inflammatory cells ⟶ and exudate ⟶ (H&E stain 20X).

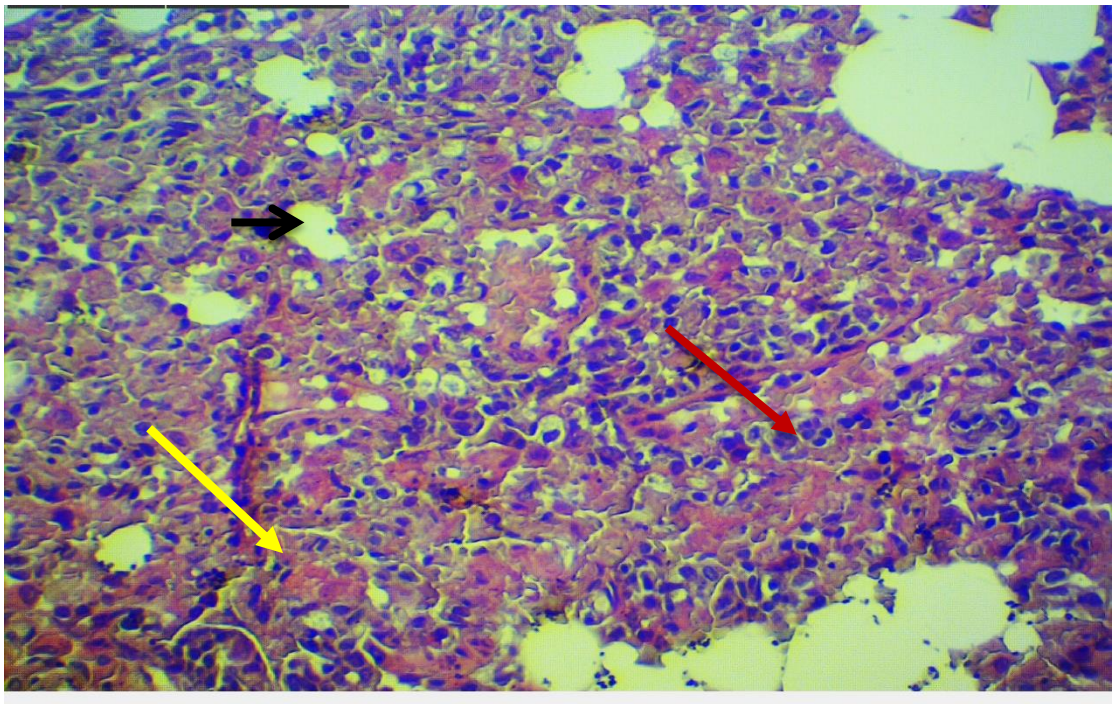

Figure (4-41): Infected lung after 48 hour after infection there is infiltration ⟶ of inflammatory cells ⟶, necrosis and exudate ⟶ (H&E stain 20X) . .

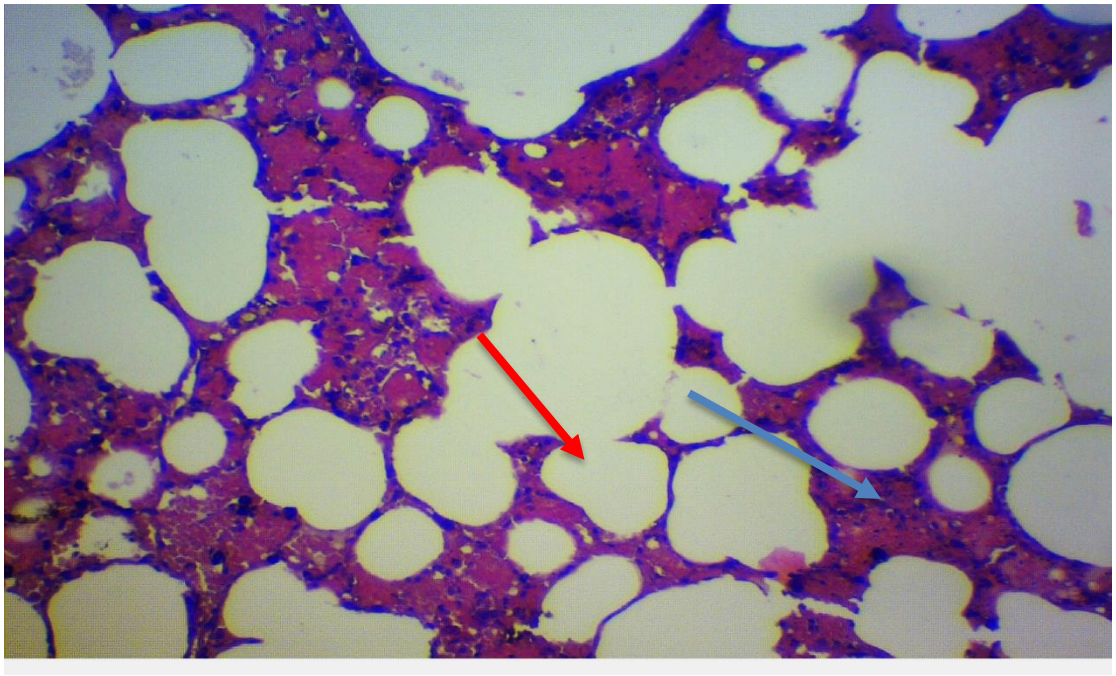

Figure (4- 42): Infected lung after 72 hour after infection, there is few infiltration of inflammatory cell, exudate and damaged of alveoli (H&E stain 20X).

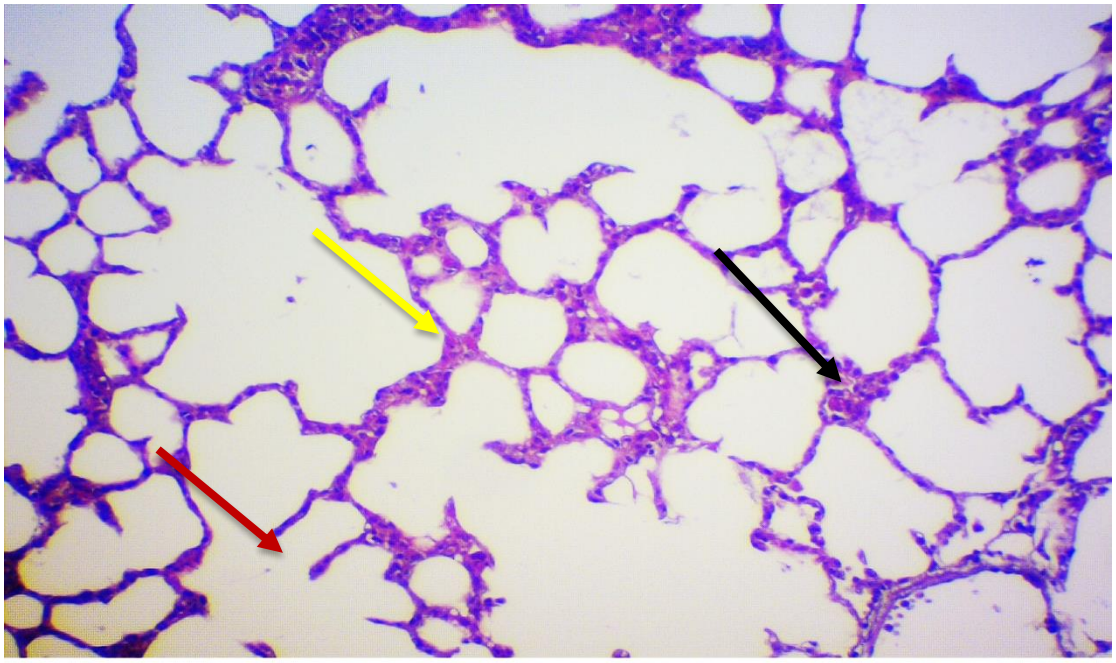

Figure (4-43): Lung of infected animal after 96 hour, sever infiltration of inflammatory cells, damaged of alveoli , presence of exudate and congestion (H&E stain20X).

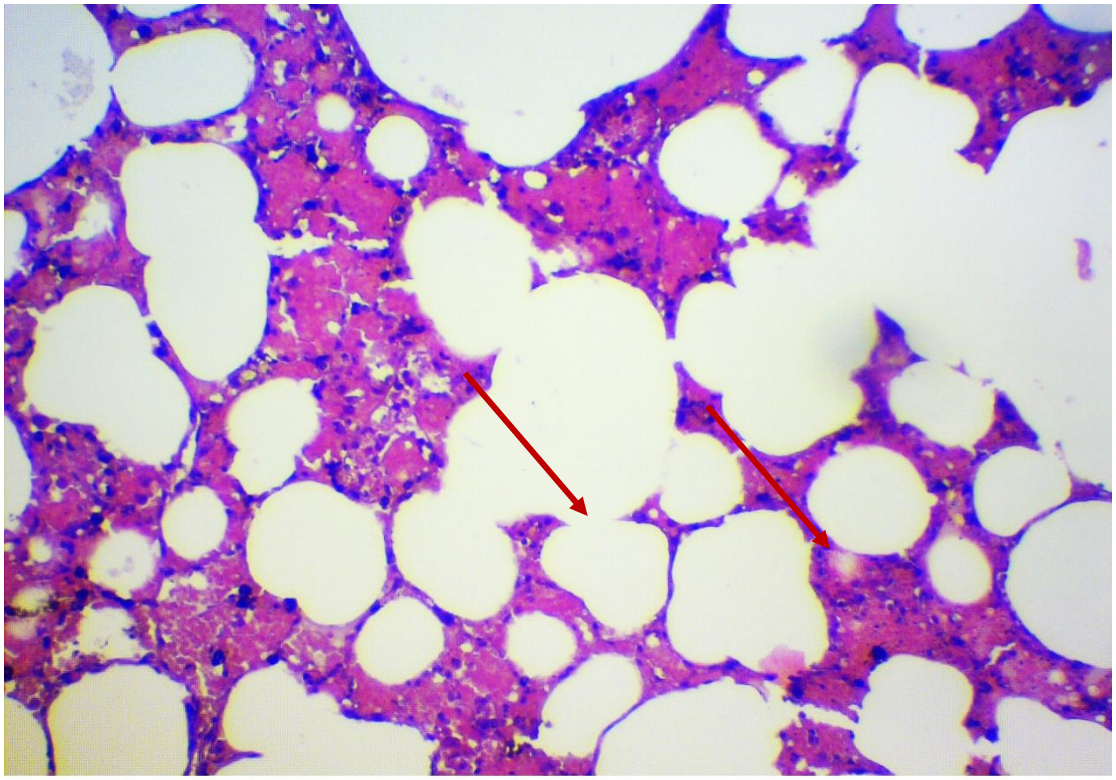

Figure (4-44): Infected lung after 120 hour there is exudate in septal alveoli, and damage (H&E stain 20X).

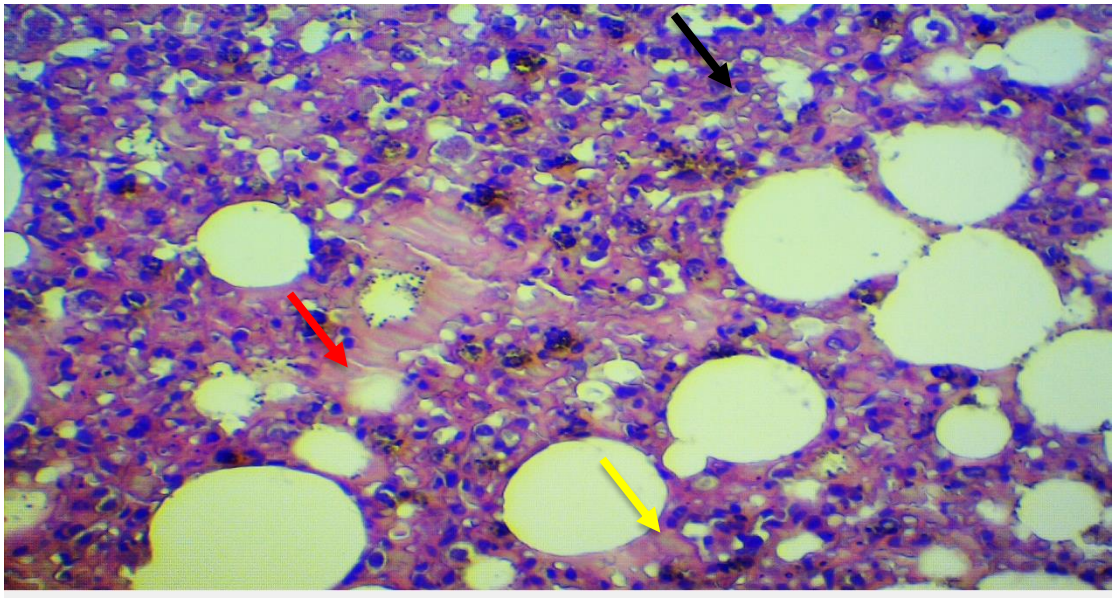

Figure (4-45): Infected lung after144 hour there is infiltration of inflammatory cells ⟶, necrosis ⟶ and exudate ⟶ (H&E stain 20X).

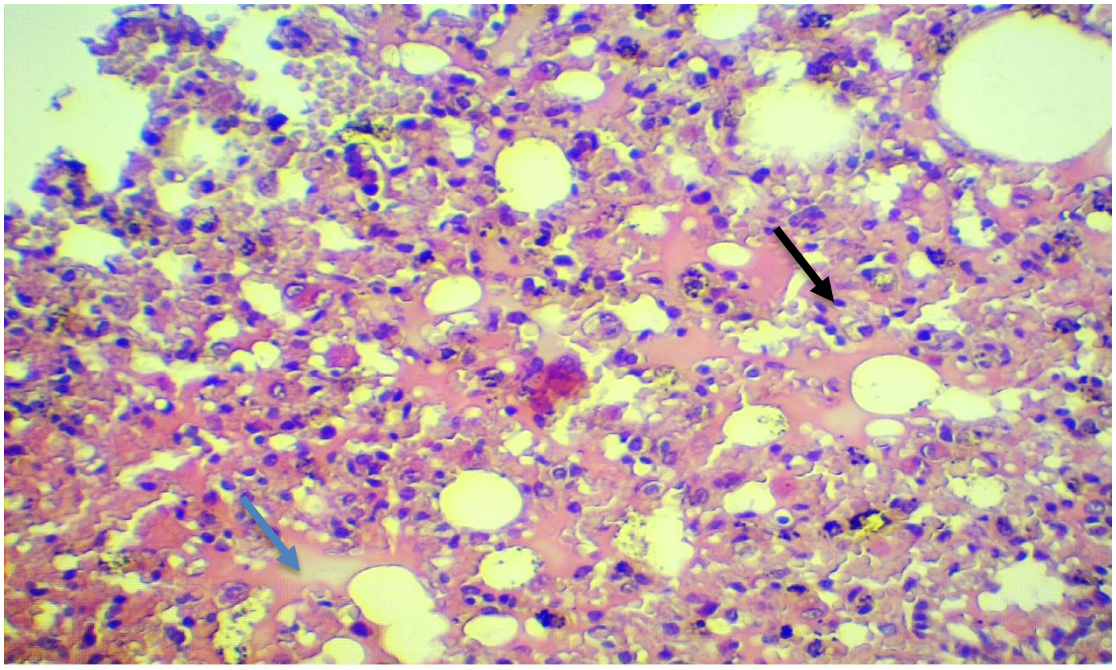

Figure (4-46): Infected lung after 168 hour of infection, there is infiltration of inflammatory cells, ⟶ necrosis and exudate ⟶ (H&E stain 20X).

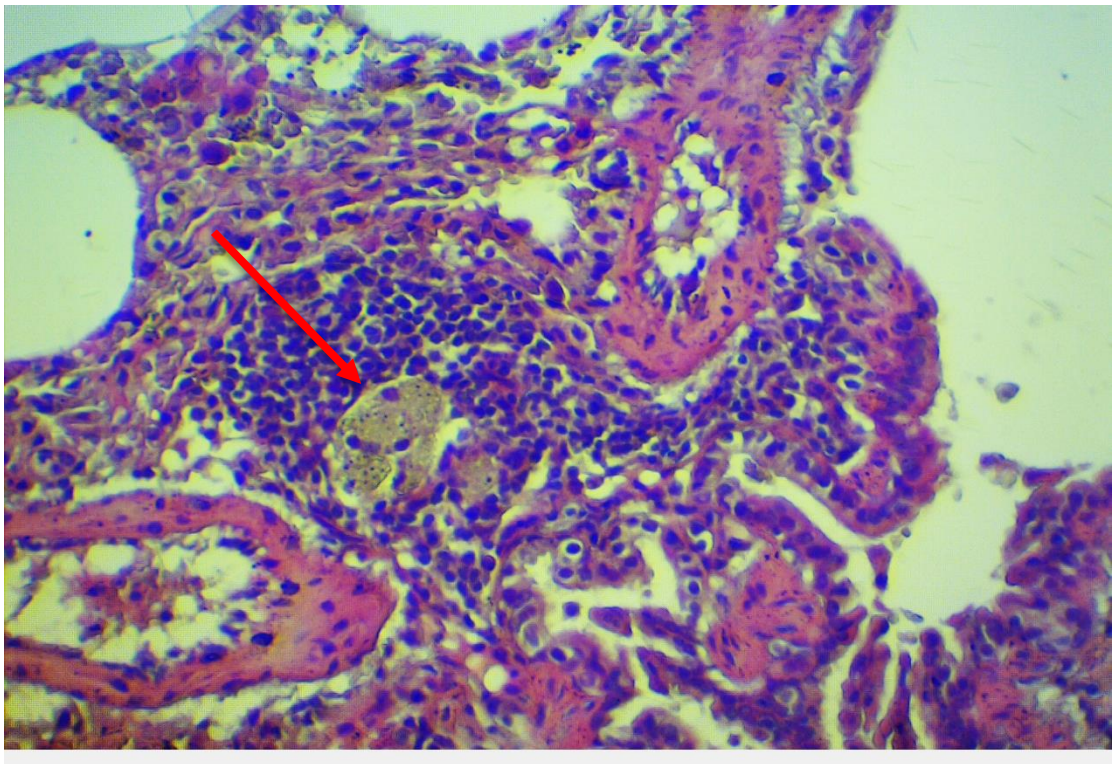

Figure (4-47): Infected lung after 168 hour in other slide there is granuloma in the center of slide and started of cassation necrosis (H&E stain 20X).

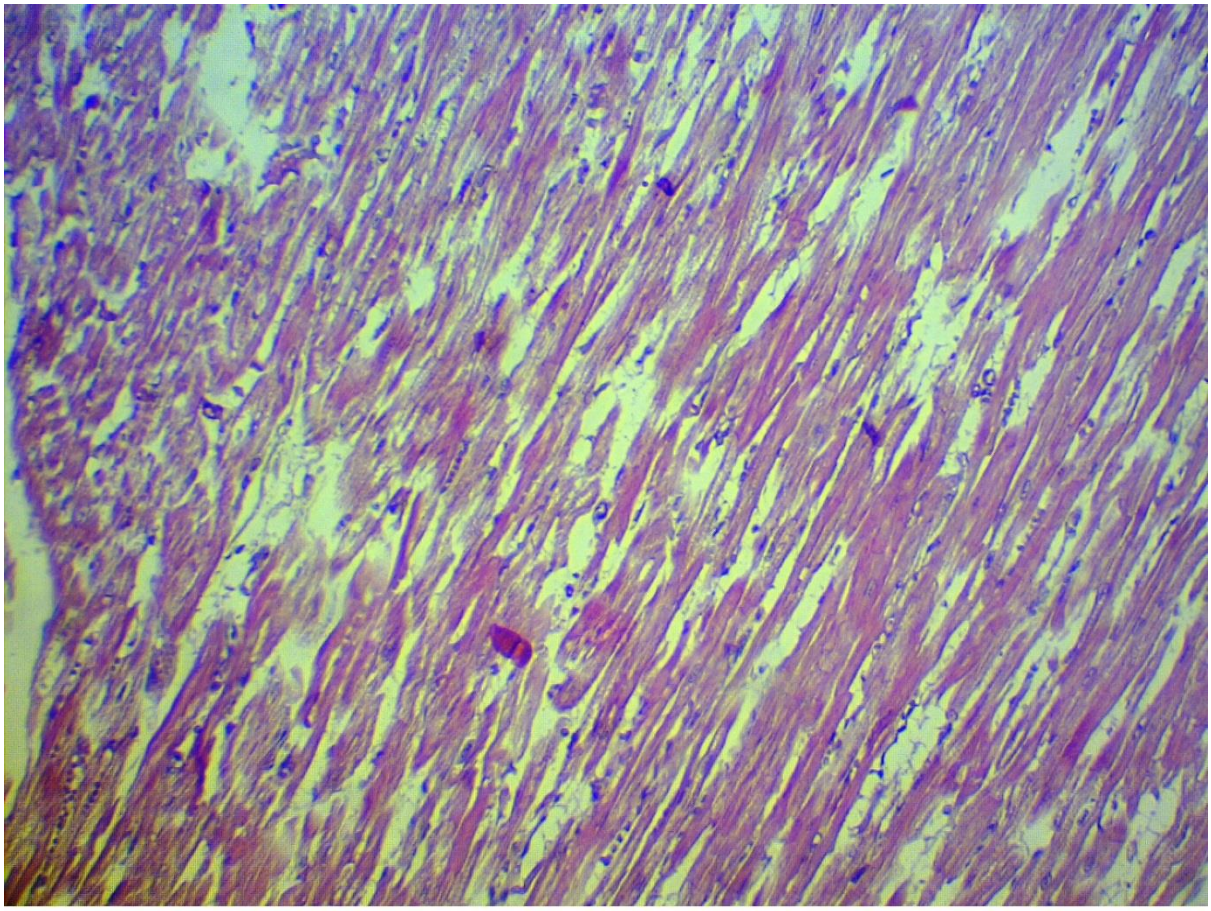

Figure (4-48): Normal heart no histopathological changes (H&E stain 20x)

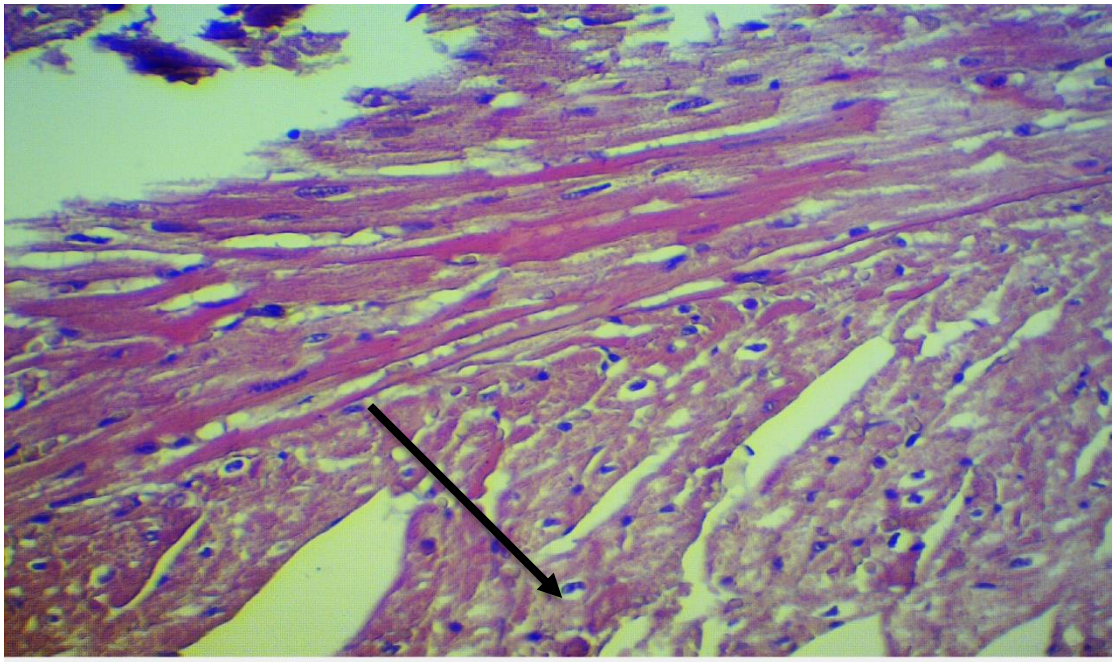

Figure (4-49): Infected heart after 72 hour of infection there is a few inflammatory cells (H&E stain 20X).

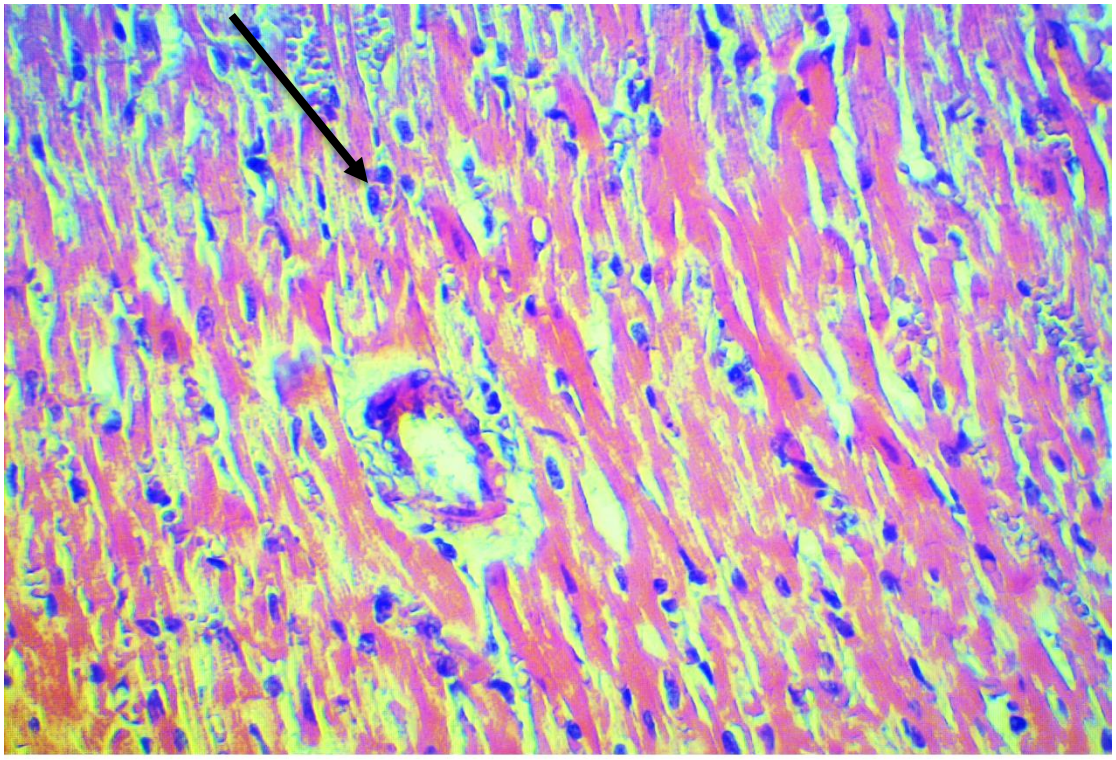

Figure (4-50): Infected heart after 120 hour from infection see few inflammatory cells (H&E stain 20X).

4-5:Re-isolation of *S.enteritidis*

The results of re-isolation of *S.enteritidis* showed that bacteria were spread in different internal organs in different degree for 7 days from starting the experiment.

❖ **Small intestine**

S. enteritidis was highly spread in the small intestine after (24, 48, 72, 96, 120, 144,and 168) hours and second and third weeks after induce infection from most rabbit which infected intraperitoneally with infective dose only 2 rabbits gave a negative result in 6 and 7 days.

❖ **liver**

Liver tissue samples that were collected from postmortem findings of infected rabbits with *S.enteritidis* 144 and 168 hrs PI did not showed any bacteria when attempted the re-isolation of S.enteritidis. Contrary all liver

tissue samples that collected from such animals after 24 hrs PI to 120 hrs PI were positive to such re-isolation.

❖ **Spleen**

S. enteritidis is moderate isolated from spleen of infected rabbits(only in 3 and 4 day after infection).

❖ **lung**

S. enteritidis was isolated from lung at (24, 48, 72, 96, 120, and 144 hours)

❖ **Kidney**

S. enteritidis was isolated from kidney at (24, 48, 72 and 120 hours)

❖ **heart**

S. enteritidis was recorded and isolation from blood heart of infected rabbits at (24, 48, 72, 96,120, and 144 hours)

❖ **large intestine**

The bacterial isolation from large intestine of infected rabbits showed highly at time (24, 48, 72, 96, 120, 144, and 168 hours)

❖ **Brain**

Only one isolate was recorded at time 72 hours from infected rabbits.

Table (4-7): Bacterial re-isolation from internal organs of infected rabbits

Period	No.of rabbits	Small intestine	liver	spleen	lung	kidney	Heart blood	Large intestine	brain
24 hrs	1	+	+	_	+	+	+	+	_
	2	+	+	_	+	+	_	+	_
48 hrs	1	+	+	_	+	+	+	+	_
	2	+	+	_	+	+	+	+	_
72 hrs	1	+	+	+	+	+	+	+	+
	2	+	+	_	+	+	_	+	_
96 hrs	1	+	+	+	+	_	+	+	_
	2	+	+	_	+	_	+	+	_
120 hrs	1	+	+	_	+	+	_	+	_
	2	+	+	_	+	_	+	+	_
144 hrs	1	+	+	_	+	_	+	+	_
	2	+	_	_	+	_	+	+	_
168 hrs	1	+	+	_	_	_	_	+	_
	2	+	_	_	_	_	_	+	_
2ed week	1	_	_	_	_	_	_	_	_
	2	_	_	_	_	_	_	_	_
3rd week	1	_	_	_	_	_	_	_	_
	2	_	_	_	_	_	_	_	_

(+): There is bacterial isolation

(−): No bacterial isolate

4.6: DTH (Delayed type hypersensitivity test) response:

Cell mediated immunity against particular fungal, bacteria or viral antigens can be assessed by DHT when each of such antigens was intradermally inoculated (Sadaoka *et al.*, 2008) .Turk, (1980) mentioned that some inflammatory responses can be seen at the site of inoculation like increase in skin thickness, redness of skin, vesicle formation that might be followed by ulceration.

DTH test depended on ability of and recognition activity of T-helper to particular antigen. This was followed by secretion of certain lymphokines like IL-1 that leads to proliferation and differentiation of T lymphocyte to be T-helper cells. In turn, these cells will secret chemotactic factor like IL-2 and cytolytic factor like INF-Y. The accumulation of macrophages and other inflammatory cells at the site of infection lead to increase in the thickness of skin (Roy *et al.*, 2011). In the present study, skin thickness at the site (right abdomen) of infection was observed and increased significantly when compared to control site. It was clearly noticed at 48 hrs PI and declined in the following 24 hrs.

Table (4-8) DTH (Thickness of skin) ($M \mp SE$)

DTH (skin dimeter test) in mm				
Groups	**Time post infection(days)**			
	Zero	**1**	**2**	**3**
Infected	0±0 A	13.99±2.16B	22.78±1.74 C	20.56±1.08 C
Control	0	0	0	0

Different small letters vertically (between groups) and capital letters horizontally (between periods) denote significant differences (P≤ 0.05), among groups during the period of experiment, data represents as M±SE

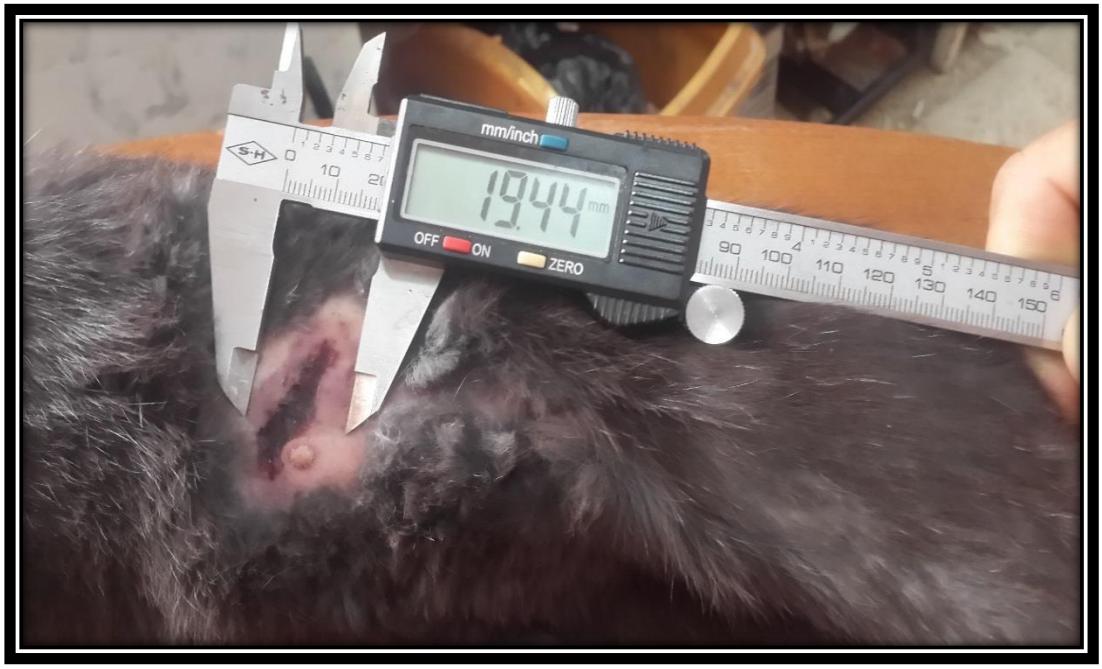

Figure (4-51): Showed thickness of skin of abdomen after injection with soluble antigen *of S. enteritidis*

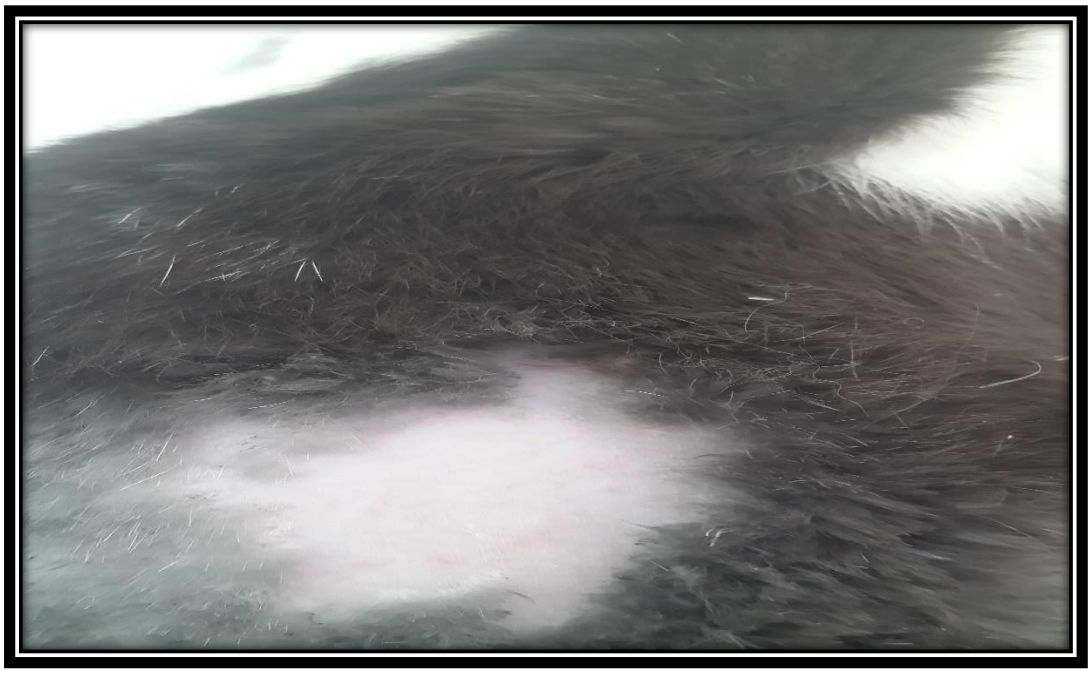

Figure (4-52): Control rabbit with no changes in the skin thickness during the experiment after Phosphate buffer saline injection.

It is obvious that cell mediated immunity was induced during the infection by *S.enteritidis*. The result was in agreement with Strindelius *et al.*, (2002) who used this test as the measure of cellular immune

responses in mice immunized with *S. enteritidis antigen*. Also, resemble to many researcher who found the same results of cellular immune response induced by other *Salmonella* serovars such as *S.typhimurium* (AL-Khafaji .2008) and *S.hadar* (AL-Naqeeb .2009).

DTH test is used as diagnostic methods to detect the cellular immune response. Skin thickness was resulted due to aggregation of very large number of monocyte and microphages at the site where the antigen there. IFN-γ and IL were released from previously sensitized Th1. These chemokines are chemotactic factors for inflammatory and phagocytic cells to site of infection (Mastroeni *et al.*, 1988).

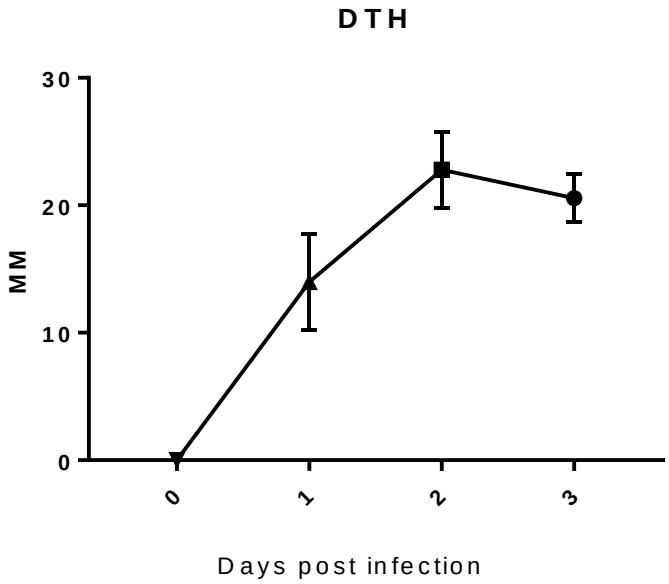

Figure (4.53): Thickness of the abdomen skin after injection with soluble antigen *of S.enteritidis*

4.7: Phagocytic indices:

Phagocytic indices were increase significantly ($p \leq 0.05$)in rabbits of infected group gives (35.16±4.24) in comparison to control group of rabbits that showed phagocytic index of (14.5±1.65)

Table (4-9): The Indices of Phagocytic activity of Salmonella infected rabbit group.

Phagocytic index	
Infected	35.16±4.24 A
Control	14.5±1.65 B

Different letters vertically (between groups) denote significant differences (P≤ 0.05), among groups during the period of experiment, data represents as M±SE

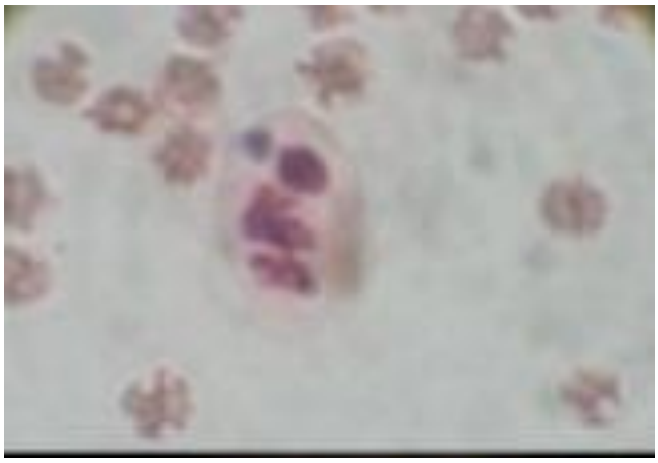

Figure (4.54): Shows the phagocytic cell engulphing the Yeast cell (Saccharomyces cervices) (Geimsa stain X100)

Killed yeast was used in the test of phagocyte, as it was well known that antimicrobial activity can be enhanced against forgin antigens. Phagocytosis can be stimulated by *S.enteritidis*, accordingly destruction of bacteria can be resulted due to the action of activated phagocyte that released their enzymes like proteases or due the opsonizing activity (Magnusson, 2009).

Chapter Five

Conclusions and recommendations

Conclusions

1. Our results are clearly establish that this bacteria is pathogenic in rabbits and associated with pathological, immunological and histological alteration.

2. More changes (histological and pathological) were seen in the liver, intestine and lung.

3. This bacteria is highly infectable and spreadable to all organs

4. The effective dose used here is optimal to do such infection in rabbits

5. *Salmonella enteritidis* is associated with cell mediate response represented by DTH in the skin that changed significantly at 21 days post infection.

Recommendations

1. Future work could be included more parameters like Real time PCR beside the investigated one here in this study in order to make more data regarding this recent widely distrusted pathogen.
2. Study the humeral immune response ,microbial antibiotic and sensitivity test
3. Ultra-structural study on *S.enteritidis* in different field animals.
4. Trial vaccine could be prepared and evaluated from these bacteria in animals.
5. Watching the hematological and biochemical changes caused by *S. enteritidis.*

Chapter Six

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الخلاصة

تعد الإصابة بجرثومة *S.enteritidis* واسعة الانتشار في الانسان خصوصا في الأطفال حديثي الولادة على العكس في الحيوان لا توجد دراسة نظامية لدراسة تأثير هذه البكتريا، لذلك أجريت الدراسة الحالية من اجل التحقق والتأكد من تأثير *S.enteritidis* بعد الإصابة التجريبية في الارانب تحت ظروف مسيطر عليها والتي تضمنت المعايير التالية: التغيرات السريرية والمرضية (مجهريا وعيانيا)و دراسة الاستجابة المناعية الخلوية في اليوم 21 بعد الإصابة.

اما الدراسة التشخيصية للبكتريا اعتمدت على زراعة البكتريا وفحص الكيموحيوي مع فحص ال Api 20ونظام الفايتك.

في هذه الدراسة استخدمت 60 ارنبا محليا من كلا الجنسين والتي كانت أعمارهم تتراوح بين 8-12 شهر واوزانهم تتراوح بين 1500-1900 غرام.

في التجربة الأولى استخدمت 20 ارنبا من اجل تقدير الجرعة المصيبة من جرثومة السالمونيلا وال 40 الباقية قسمت عشوائيا الى مجموعتين ن=10 مجموعة السيطرة و ن=30 ارنبا المجموعة المصابة. أعطيت البكتريا من خلال التجويف البريتوني بينما في مجموعة السيطرة أعطيت فقط محلول الملح الفسيولوجي. أظهرت النتائج انه الجرعة المصيبة هي 4.5×10^{8} خلية والتي اظهرت علامات سريرية بدون هلاكات،والتي شملت ارتفاع في درجة الحرارة وزيادة في معدل ضربات القلب والتنفس مع خمول واسهال وفقدان شهية وعم راحة وأيضا رافقها تبول مضبب وحليبي مع ضيق تنفس وجفاف.

خلال الفحص العياني وجد تضخم واحتقان مع بقع صفراء في الكبد (تنخر)، توسع في المرارة يصاحبه تضخم في الكلى والقلب كان مرتخي والامعاء الدقيقة تميزت بالرخاوة وممتلئة بمادة شفافة سائلة والتي تضمنت على كمية كبيرة من المخاط على العكس الأمعاء الغليظة والتي وجدت متضخمة ومحتقنه عند المقارنة مع امعاء مجموعة السيطرة.

بالإضافة الى الاعراض السابقة التغيرات النسيجية سجلت بعد 24,48,72,96,120,144,168 ساعة والاسبوع الثاني والثالث، التغيرات النسيجية في الأمعاء الدقيقة تميزت بارتشاح شديد للخلايا الالتهابية (لمفية وخلايا بلعميه) انسلاخ للظهارة مع نضحة وتنخر. الكبد أيضا اظهر ارتشاح خلايا التهابية وتنخر وورم عقيدي والرئة أظهرت ضرر في الاسناخ مع وجود نضحة في القلب وأيضا وجود ارتشاح للخلايا الالتهابية.

أيضا وجدنا بان جرثومة *S.enteritidis* واسعة الانتشار في الأعضاء الداخلية للأرانب المعاملة تجريبيا ووجد بان لها القابلية لاجتياح اغلب الأعضاء الداخلية (الطحال، الكبد، الكلى، الرئة، القلب، الدماغ والامعاء الدقيقة والغليظة ولكن بدرجات متفاوتة. بينما في مجموعة السيطرة لا توجد مثل هذه التغيرات والتي اعطتنا نتائج سالبة للجرثومة.

من خلال الجانب المناعي وجدنا تغيرات معنوية في الجهة اليمنى للخاصرة للأرانب في المجموعة الممنعة وكان اعلى معدل للسمك بعد 48 ساعة بعد التمنيع والذي تناقص بعد 72 ساعة.

معامل البلعمة زاد بصورة معنوية في المجموعة المصابة(35.16)عند مقارنته مع مجموعة السيطرة (14.5)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ديالى
كلية الطب البيطري

دراسة سريرية، مرضية للأرانب المصابة تجريبيا بجرثومة *Salmonella enteritidis* والمعزولة من الأنسان

رسالة مقدمة إلى
مجلس كلية الطب البيطري/ جامعة ديالى وهي جزء من متطلبات نيل درجة الماجستير في الطب البيطري/ الطب الباطني والوقائي البيطري/ الامراض المشركة

من قبل

آيات جاسم محمد

بأشراف

ا.م.د
خالد محمود حمادي

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