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# Serological and Histopathological evaluation for *Encephalatozoon Cuniculi* infection in Laboratory Rabbits: A Guide in selection of Rabbits for research and Toxicology studies

**Avinash V J*, Kalaiselvan P, Senthil R, Parthiban N, Rajesh K, Vishal M M, Nataraju G J, Madhusudan P G, Prahalada S R**

*Safety Assessment Department, Syngene International Limited,
Biocon Park SEZ, Bommasandra IV Phase, Jigani Link Road, Bangalore 560099, India

Corresponding author:
Dr. Avinash Jadhav MVSc, DIBTP, DABT. Safety Assessment Department,
Syngene International Limited,Biocon Park SEZ, Bommasandra IV Phase, Jigani Link Road,Bangalore 560099, India.
Phone: +91 9620 3811 77, Email: avinash.vitthalrao@syngeneintl.com

## Abstract

The objective of this study was to determine the prevalence of *Encephalitozoon cuniculi* infection through serologic and histopathologic examination of laboratory rabbits from suppliers in India. One hundred and thirty-one New Zealand White rabbits procured from two different Indian suppliers were used for this study. All rabbits were clinically normal at receipt and during the period of experiment. Serological examination was carried out for the detection of *E. cuniculi* antibodies using ELISA tests. The select tissues were collected and processed for light microscopic evaluation. Among 131 rabbits evaluated 43 (33%) were seropositive for *E. cuniculi.* The microscopic changes in the brain and spinal cord (granulomatous inflammation and/or perivascular infiltrates) and kidneys (interstitial nephritis) were consistent with *E. cuniculi* infection. A good correlation (84%) between serological and microscopic findings was observed. Other background microscopic findings were minimal and consistent with hepatic (3%) and intestinal (5%) *Eimeriasis* (Coccidiosis). The microscopic changes consistent with otitis externa and otitis media, possibly related to external parasitic infection were observed in a few (11%) rabbits. Based on these findings, it is recommended that, rabbits should be serologically screened for *E. cuniculi* at supplier's breeding colony to remove carriers. Furthermore, before supplying rabbits to research facility, serology should be performed by the supplier to exclude seropositive animals. In addition, the research facilities also should consider performing serological testing to ensure that only seronegative animals are selected for experiments. This will minimize the variability in test results, avoid spurious observations and aid in scientific data interpretation

**Key words:** Rabbit, serology, histopathology, *E. cuniculi*.

## Introduction

In India, rabbits have been extensively used both in academic and industrial research facilities. The New Zealand White is the preferred rabbit strain, which has been used in pharmaceutical industry, especially for testing of large molecules. In India, rabbit has also been used in various toxicity studies as well as in drug potency studies.

Infectious micro-organisms may affect laboratory animal population in various ways. A few are pathogenic and may induce clinical signs with variable degree of severity. However, most microorganisms induce no clinical sign or produce only mild disease. Dormant infections often become activated by experimental procedures (e.g. stress and immunosuppression) or environmental influences like transportation, suboptimal humidity or temperature [28] and may pose difficulties in interpreting experimental results.

Encephalitozoonosis is a common infectious disease in domestic rabbits. Its prevalence in rabbits has been reported in various regions of the world [31, 34, 16, 2, 26, 12]; however, in India the disease has not been studied extensively. Its causative agent, *Encephalatozoon Cuniculi* (*E. cuniculi*) is an intracellular parasite belonging to genus microsporidia. *E. cuniculi* can infect various mammals, including rabbits, rodents, horses, carnivores and humans. The main host for *E. cuniculi* is the rabbit and infections are usually seen in sub-clinical form. The common target organs of *E. cuniculi* infection are brain, kidney and eye, but liver, lungs and heart can also be affected. The predominant microscopic alterations with *E. cuniculi* infection include granulomatous meningoencephalitis and interstitial nephritis [11]. The spore is the infective form of the parasite. The spores are shed in the urine of infected rabbits and infection usually occurs via ingestion of food and water contaminated with urine. The transplacental infection from dam to offspring has been

reported [4]; however, this route of infection appears less frequent and play a role in intraocular development of the parasite [33, 3]. In many rabbits, the disease can persist for months without any clinical sign. In experimental animal breeding facilities, the parasite may be prevalent but with good hygiene standards, infections can be controlled [11, 22]. There is a potential for spreading of infection from infected rabbit to rodents especially in long term experiments [25].

Coccidiosis is a protozoan infection of rabbits seriously impairing their growth. It is one of the important diseases of rabbit and major cause of morbidity and mortality [21]. Different species of coccidial (*Eimeria*) parasite are causative agents of the disease. Prevalence of various coccidial species in domestic rabbit has been reported earlier [17, 14, 19, 29, 7]. Rabbit coccidiosis is transmitted by oral ingestion of the sporulated oocysts by the susceptible host and the infection develops into disease in young rabbits primarily, while adults are mostly carriers [5]. In commercially reared rabbits, coccidiosis occurs in subclinical form. This disease occurs in two forms, hepatic and intestinal, the latter being more common than the former [5]. Hepatic coccidiosis is caused by *Eimeria stiedae* and intestinal coccidiosis by various other *Eimeria* species. In India, hepatic coccidiosis in rabbits has not been studied extensively unlike intestinal coccidiosis.

External parasitic infestation of ear is common in rabbits. External ear examination is one of the routine clinical examinations in rabbit colonies. The gross morphological changes on external ear can be observed during routine clinical examination; however, changes in middle ear generally go undetected due to its anatomic location. A few histological studies on external ear in rabbits have been carried out [1]; however, information on the histological changes in the middle ear of rabbit is limited.

This study was conducted with the primary objective to evaluate for the prevalence of *E. cuniculi* in rabbits through serological and histopathological evaluations in laboratory rabbits. Additional common background microscopic findings in the study related to Eimeria infection and inflammation of the ear (otitis) are also presented in this article

# Materials and Methods

## Animals

One hundred and thirty-one male New Zealand White rabbits were used in this study. These rabbits were procured from two different rabbit suppliers (will be referred in future as Supplier A and Supplier B) in India between April 2017 and August 2017. The age of the rabbits ranged from 12 to 24 weeks at necropsy. Rabbits were maintained in a controlled environment with a temperature range of 17 to 23°C, a relative humidity range of 30 - 70 %, a light and dark cycles of 12 hours each and at least 15 fresh air changes per hour. Rabbits were individually housed in a designated experimental room of the facility in clean and sterilized stainless- steel rabbit cages. Rabbits were fed *ad libitum* with maintenance diet except during overnight fasting prior to blood collection. Potable water filtered through reverse osmosis was provided to all rabbits. The approval for use of animals was obtained from Institutional Animal Ethics Committee. Blood collection and euthanasia procedures were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The rabbits were part of different experiments however, the rabbits were allowed a washout period of minimum of two weeks before the start of this study and were apparently normal clinically till necropsy.

## Blood Collection and Serological Examination

The rabbits were fasted overnight (12 to 16 hours) prior to blood collection. Just before sacrifice, blood was collected from each rabbit from the central auricular artery and used for serological examination. Blood was centrifuged at 5000 rpm for 10 min, serum was collected and stored at -80°C until use. Serological examination for *E. cuniculi* IgG antibody was carried out using ELISA kit (Rabbit ECUN ELISA Kit, XpressBio Life Science Products, MD, USA) according to the manufacturer's instructions.

## Necropsy and Organ collection

Rabbits were sacrificed by exsanguination under deep anaesthesia with intravenous injection of thiopentone sodium and gross examination was performed. Samples of selected tissues (brain, spinal cord, kidneys, lungs, heart, liver, external and middle ear, epididymides, stomach and intestines) were fixed in 10% neutral buffered formalin. Eyes were preserved in Davidson's fixative and testes were preserved in modified Davidson's fixative.

## Histopathology

The tissues were trimmed, processed, embedded in paraffin, sectioned at 5-micron thickness and stained with haematoxylin and eosin (H&E) and subjected to light microscopic evaluation.

# Results

## Serology

Out of 131 rabbit's sera tested, 88 (67%) samples were negative and 43 (33%) samples were positive for *E. cuniculi*. None of these rabbits showed any clinical signs. Out of 43 seropositive rabbits, 36 (84%) had microscopic changes in brain and kidneys consistent with *E. cuniculi* infection and 7 seropositive rabbits did not had microscopic changes. Among the 88 rabbits which were seronegative, 8 (9%) rabbits had microscopic changes in the brain suggestive of *E. cuniculi* infection. There were no major differences in the seroprevalence of *E. cuniculi* between the rabbits of two suppliers (refer Table 1).

## Histopathology

### *Changes in the kidney and brain consistent with E. cuniculi infection:*

The characteristic microscopic changes consistent with *E. cuniculi* infection were observed in brain and kidneys. The changes in the brain were characterized by focal to multifocal, non-suppurative granulomatous inflammation and/or perivascular infiltration of mononuclear cells. Foci of granulomatous inflammation contained lymphocytes and glial cells often surrounding the central area of necrosis (Fig 1). Occasionally, inflammation of the meninges was also observed in a few rabbits. Granulomatous inflammation was predominantly observed in cerebrum; however, cerebellum was also occasionally involved. In a few rabbits, perivascular infiltration of mononuclear cells was observed in spinal cord. All rabbits having spinal cord change also had changes in the brain. The changes in the kidneys included minimal to mild interstitial infiltration of mononuclear cells, tubular dilation, tubular basophilia and interstitial fibrosis (Fig 2). At necropsy, most of the rabbits did not show gross changes in kidneys; however, in two rabbits, kidneys had multiple irregular and depressed- subcapsular foci (Fig 3) that correlated microscopically with interstitial fibrosis and inflammatory changes.

There was excellent correlation (84 %) between serological results and histopathology findings. There were a few seronegative rabbits (20%) with microscopic changes consistent with *E. cuniculi* infection and a few seropositive rabbits (16 %) without microscopic changes in brain or kidney. These findings are consistent with published literature [8]. Results of serology and microscopic changes in the brain and kidneys are summarized in Table 1.

### *Changes in liver consistent with hepatic coccidiosis*

Out of total 131 rabbits, 7 rabbits were observed with microscopic changes in the liver suggestive of hepatic coccidiosis caused by *Eimeria stiedae*. At necropsy, irregular yellowish white raised foci were observed on the surface of liver in a few rabbits (Fig 4). The microscopic changes were observed both in hepatic parenchyma and bile duct. The bile ducts changes were observed in two forms. In first form, bile duct was enlarged and lined by hyperplastic columnar epithelial with papillary projections extending into the ductal lumen, resembling adenomatous hyperplasia with presence of parasitic oocysts (Fig 5). In second form, the dilated bile duct was lined by flattened epithelium with minimum to no papillary projections and the lumen was filled with oocysts (Fig 6). The hepatic parenchyma showed granulomatous inflammation with central area of necrosis and infiltration by lymphocytes and giant cells (Fig 7). These microscopic findings are consistent with hepatic coccidiosis [27, 30].

### *Changes in jejunum and ileum consistent with intestinal coccidiosis*

The changes in jejunum and ileum were characterized by presence of various stages of the Eimeria parasite, predominantly in the epithelial cells lining the villi, and lymphocytic infiltrates in mucosa (Fig 8), suggestive of intestinal coccidiosis. Out of 131 rabbits, 4 rabbits had this finding in both jejunum and ileum and only in the ileum of 2 rabbits.

### *Changes in external and middle ear*

The microscopic changes on external ear skin ranged from minimal lymphocytic infiltration in dermis to moderate inflammation involving both epidermis and dermis. The changes were characterized by mononuclear cell (macrophages, lymphocytes and plasma cells) to mixed cell (macrophages, lymphocytes, plasma cells and heterophils) infiltration in the epidermis and dermis, hyperplasia of hair follicles, hyperkeratosis, epidermal basal cell hyperplasia and/or cellular exudate on the surface of epidermis. The microscopic changes in middle ear included mononuclear cell to mixed cell infiltration including inflammatory cell exudate in the tympanic cavity (Fig 9). Out of total 131 rabbits, 16 had changes in external and middle ear. The exact cause of these findings was undetermined but may have been related to external parasitic infestation. Results of microscopic changes related to coccidiosis and otitis are summarized in Table 2.

## Discussion

Seropositive result is a strong indication of *E. cuniculi* prevalence in the rabbit colony, but conventional titres do not necessarily correlate with the degree of parasitism. Rabbits show a considerable individual variation in their immune response [20]. Similarly, in this study, IgG titer ranged from 0.00 to 2.35 supporting considerable individual variation in immune response. Titer in seropositive animals ranged from 0.31 to 2.35 with generally good correlation between the titer and severity of microscopic findings in the brain and kidney. However, a few seronegative rabbits also had the microscopic findings consistent with E.cuniculi infection, consistent with published literature [8] (refer Table 3). Some rabbits persistently show higher antibody levels for years without any clinical signs while others become seronegative soon after initial infection [18]. Antibody titres remain high over several months after exposure to *E. cuniculi*, then decrease slightly and persist for years with fluctuating levels [32]. Antibodies are also transmitted from an infected dam to its offspring, which can persist for approximately 4 weeks. After decrease in maternal antibodies, offsprings become susceptible to natural infection. If infected at this point, after an initial seronegative period, seroconversion occurs at 8 to 10 weeks [13].

There are different methods for detection of circulating antibodies to *E. cuniculi*. ELISA, indirect fluorescent antibody technique and carbon immunoassay methods have been used

in the diagnosis of encephalitozoonosis [9]. However, the measurement of serum IgG to *E. cuniculi* cannot distinguish between active, early, reactivated or chronic infection [9, 6]. Therefore, presence of IgG in the serum is just considered as an indication of exposure or infection status. IgM is more indicative of active infection. Hence, measurement of IgM in combination with IgG, gives a better indication of the infective status of the affected rabbit [15].

The definitive diagnosis of encephalitozoonosis *in vivo* is difficult because neurological or renal disease do not preclude other diseases. Serological detection of antibodies is the most sensitive diagnostic method during the early stage of infection [9, 8]. Accordingly, in current study, IgG measured did not distinguish between active and latent infection, however, there was good correlation between seropositivity and presence of microscopic changes, suggesting IgG may serve as good technique to screen for *E. cuniculi* exposure in laboratory rabbits.

When a rabbit is infected for the first time, antibodies start to rise after three to four weeks. From this time, it takes at least four weeks for microscopic changes to become apparent in the kidney or the parasite to be excreted in the urine. Microscopic changes in the brain are generally seen at much later stage, usually more than eight weeks after antibodies are detectable [8]. Traditionally, the coexistence of granulomatous encephalitis and interstitial nephritis has been considered diagnostic for *E. cuniculi* infection because of its consistent and characteristic pattern [24]. The brain has been shown to be the most promising organ for the detection of *E. cuniculi* using morphological and immunohistochemical methods [24]. Although extensive literature review reveals the lesions caused by *E cuniculi* are commonly found histologically in the CNS, these lesions are not consistent and do not correlate with the reported clinical signs [20].

Rabbit coccidia parasitize in distinct parts of the intestine and in different depths of the mucosa [23]. Though intestinal coccidiosis is most common form of coccidiosis in rabbits, this study had similar incidences for hepatic and intestinal coccidiosis. Mixed cases of hepatic and intestinal coccidiosis have been reported [10]; however, this study did not have any mixed case of hepatic and intestinal coccidiosis.

Although, there are effective treatments available for coccidiosis and ear canal infections, there are no effective treatment available for *E. cuniculi* infection. Periodic serological monitoring, good hygiene practices, isolation of positive cases and/or culling of breeding stock is an effective method to eliminate *E. cuniculi* from the colony.

Based on the results of serologic and histopathology data in rabbits from two suppliers in India, it is evident that Encephalitozoonosis is highly prevalent subclinical infection in the laboratory rabbits. It is recommended that, rabbits should be serologically screened for *E. cuniculi* at supplier's breeding colony to remove carriers. Further, before supplying rabbits to research facility by the supplier, serology should be performed to exclude seropositive animals. In addition, the research facilities should also consider performing serological testing to ensure that only seronegative animals are selected for experiments. This will minimize the variability in test results, avoid spurious observations and aid in scientific data interpretation

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**Table 1: Serological and Microscopic Observations in Rabbits consistent with *E. cuniculi* Infection**

| | Total | Supplier A | Supplier B |
|---|---|---|---|
| Number of rabbits | 131 | 103 | 28 |
| Seropositive for *E. cuniculi* | 43 (33 %) | 32 (31 %) | 11 (39 %) |
| Seronegative for *E. cuniculi* | 88 (67 %) | 71 (69 %) | 17 (61 %) |
| Seropositive with microscopic changes in brain and/or kidneys | 36/43 (84 %) | 27/32 (84 %) | 9/11 (82 %) |

**Table 2: Incidence of Microscopic Changes consistent with Coccidiosis and Otitis**

| Microscopic changes | Total (N=131) | Supplier A (N=103) | Supplier B (N=28) |
|---|---|---|---|
| Hepatic coccidiosis | 7 (5 %) | 0 | 7 (25 %) |
| Intestinal coccidiosis | 6 (5 %) | 6 (6 %) | 0 |
| Otitis | 16 (12 %) | 15 (15 %) | 1 (4 %) |

**Table 3: *E. cuniculi*: Microscopic changes with corresponding serology titers**

| Microscopic changes with severity | IgG Titer for *E. cuniculi* | |
|---|---|---|
| | Seropositive | Seronegative |
| | 0.31 to 2.35 (43) | 0.00 to 0.18 (88) |
| Brain: Granulomatous Inflammation/Perivascular infiltration- Minimal | 0.31 to 2.35 (31) | 0.00 to 0.17 (7) |
| Brain: Granulomatous Inflammation/Perivascular infiltration- Mild | 1.15 to 2.23 (3) | 0.00 (1) |
| Kidney: Interstitial infiltration/inflammation- Minimal | 0.31 to 2.35 (24) | 0.00 to 0.18 (13) |
| Kidney: Interstitial infiltration/inflammation- Mild | 0.91 to 2.17 (9) | Nil |

**Note:** Number of animals are indicated in parenthesis. As per recommendation of ELISA kit manufacturer, animals with serum IgG Titre ≥ 0.3 are considered seropositive for *E. cuniculi*.

**Fig 1:** Brain- Granulomatous inflammation with central area of necrosis (arrow) in cerebrum. H&E stain, 10X.

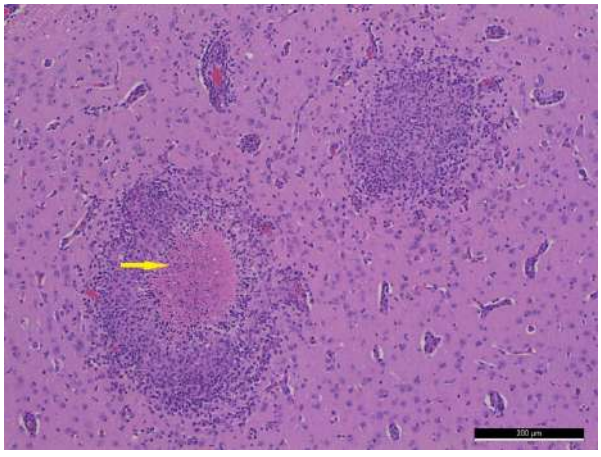

**Fig 2:** Kidney- Interstitial fibrosis (arrow) associated with mononuclear cell infiltration, tubular basophilia and dilation. H&E stain, 10X.

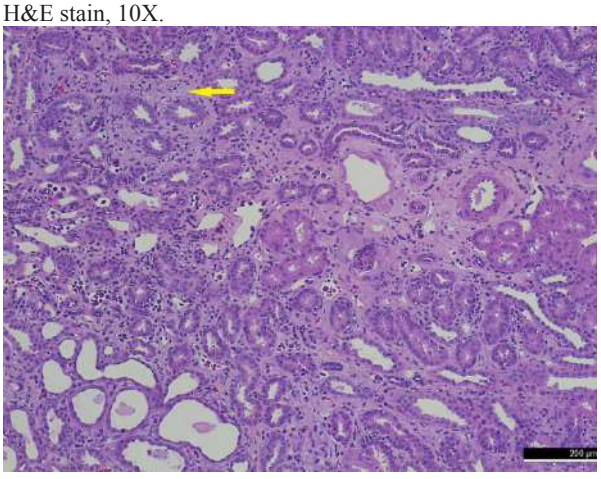

**Fig 3:** Kidney- Grossly observed multiple irregular, depressed, sub-capsular foci suggestive of E. cuniculi infection.

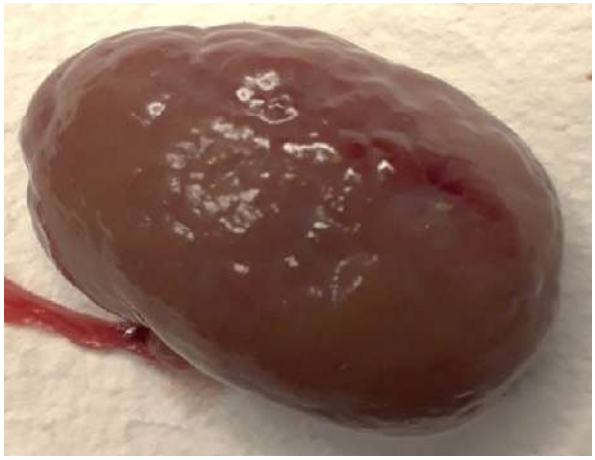

**Fig 4:** Liver- Grossly observed irregular yellowish white raised foci on liver suggestive of hepatic coccidiosis.

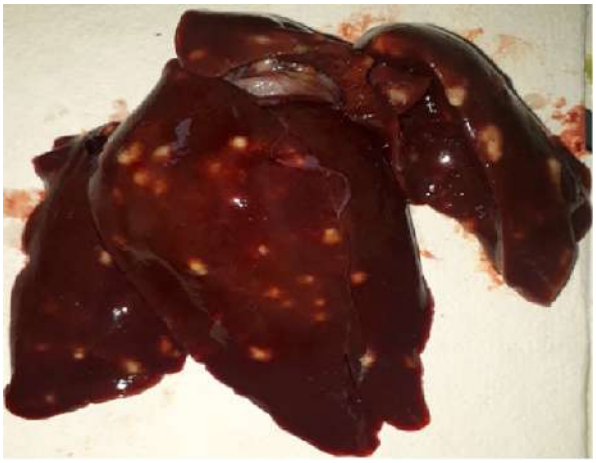

**Fig 5:** Liver- Enlarged bile duct lined by hyperplastic epithelium with projections (green arrow) and presence of Eimeria stiedae oocysts (yellow arrow) in the lumen. H&E stain, 4X.

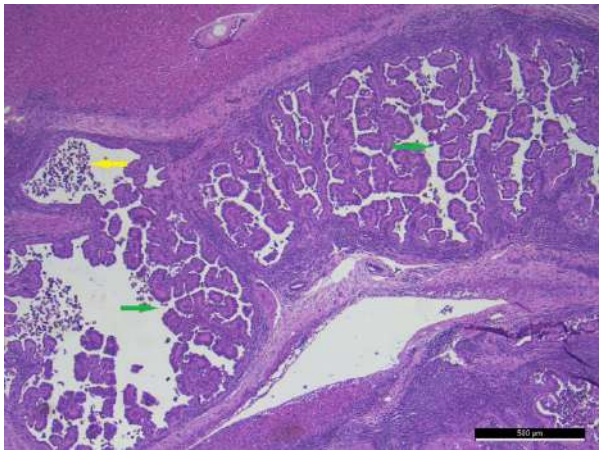

**Fig 6:** Liver- Dilated bile duct with flattened epithelium with lumen having Eimeria stiedae oocysts (arrow). H&E stain, 4X.

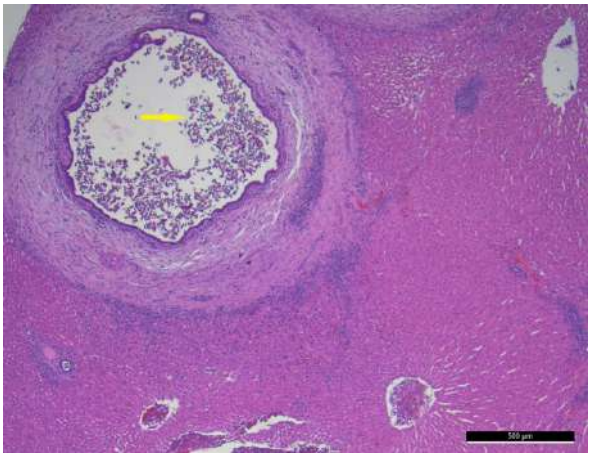

**Fig 7:** Liver- Granulomatous inflammation of hepatocytes with central area of necrosis (arrow). H&E stain, 10X.

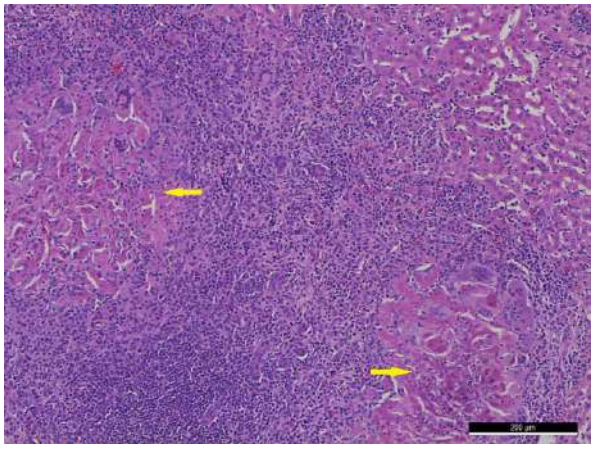

**Fig 8:** Ileum- Developmental stages of coccidial parasite (arrow) in epithelial cells with mononuclear cell infiltration in mucosa. H&E stain, 10X.

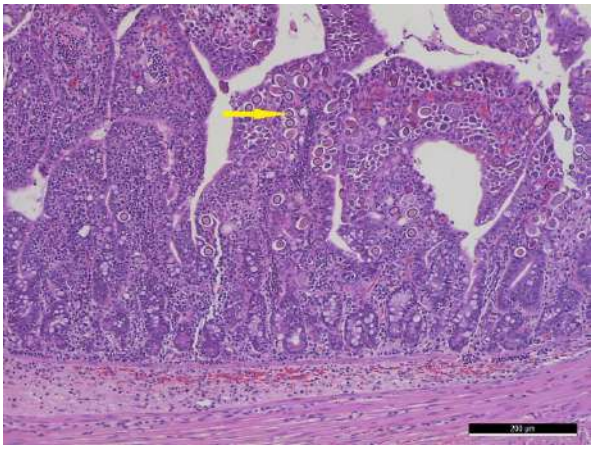

**Fig 9:** Middle Ear- Sub-epithelial inflammation with inflammatory cell exudate in tympanic cavity. H&E stain, 10X.