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PATHOLOGICAL CHANGES AND ANTIGEN LOCALISATION IN THE SMALL INTESTINES OF RABBITS INFECTED WITH *EIMERIA MAGNA*

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Abstract: Coccidiosis is a major disease caused by various *Eimeria* species in rabbits. The aim of the present study was to investigate the haematological and pathological changes in rabbits infected with *E. magna.* Moreover, the localisation of coccidial antigens was examined in the intestines of rabbits with two kinds of serum as primary antibodies. In the present study, forty-five 28-day-old weaned rabbits were randomly divided into three groups and reared in three separate places. Group A was infected with 20×10^3 sporulated oocysts of *E. magna*, group B was only used to produce anti-*E. intestinalis* serum by infecting them with 3×10^3 sporulated oocysts of *E. intestinalis*, and group C was designated as the control group. According to histopathological evaluation of group A, the epithelial cells of the jejunum and ileum were parasitised with a large number of oocysts and other stages of *E. magna*. The haematological results showed that red blood cell counts, haemoglobin counts, haematocrit levels and the percentage of lymphocytes were significantly decreased in group A compared with group C ($P<0.01$), but white blood cell counts and the percentage of neutrophils were significantly increased ($P<0.01$). The weight of group A began to decrease on the 5th day after infection, and this decrease continued until the 9th day. Immunohistochemistry staining revealed that two kinds of coccidial antigens were basically located at the same sites of the intestine when anti-*E. intestinalis* serum and anti-*E. magna* serum were used as primary antibodies. Most likely, *E. magna* and *E. intestinalis* antigens have some similar antigenic determinants; this finding provides a theoretical basis for screening for common antigens of these two coccidian species.

Key Words: *E. magna*, *E. intestinalis*, *Oryctolagus cuniculus*, antigen detection, intestinal histopathology.

INTRODUCTION

Rabbit coccidiosis is a parasitic protozoal disease caused by a variety of *Eimeria* species that live in the intestinal and hepatobiliary epithelia of rabbits (Akpo *et al.*, 2012). At present, there are 11-15 internationally recognised *Eimeria* species; *Eimeria stiedai* parasitises the liver, whereas the other species of *Eimeria* parasitise different parts of the intestine (Eckert *et al.*, 1995, Li *et al.*, 2009). All rabbit species are susceptible to *Eimeria* and it mostly affects weaned kits to 4-month-old rabbits (Pakandl *et al.*, 2008).

Eimeria magna is recognised as one of the most common pathogenic species in rabbits, and it parasitises the ileum and jejunum (Licois *et al.*, 1995). Rabbits infected with *E. magna* can present anorexia, acute emaciation, diarrhoea and other clinical symptoms (Sadek Bachene *et al.*, 2018). *E. magna* is mainly pathogenic due to its ability to destroy the intestinal villi, thereby leading to malabsorption, generating toxins and promoting intestinal bacterial invasions. Intestinal infections are caused by the invasion of sporozoites, which are released from sporocysts into the lumen of the upper intestine and then migrate to enterocytes (Shirley *et al.*, 2005).

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Rabbit coccidiosis is generally a mixed infection of multiple *Eimeria* species in the clinical picture (Ming-Hsien *et al*., 2010). Therefore, it is expected that a kind of vaccine that can effectively resist mixed infection with multiple *Eimeria* species will be developed. A mixed immune challenge test of *E. magna* and *E. media* was proven to establish effective immune protection without interference after rabbits were immunised at the same time for both species (Akpo *et al*., 2012). This result illustrated the feasibility of developing a combination vaccine for rabbit coccidiosis. Furthermore, little information is available on the detection of pathogenesis of rabbit coccidiosis by immunohistochemistry (IHC), as most of the reports are on *Eimeria intestinalis* and *Eimeria coecicola* (Renaux *et al*., 2001; Pakandl *et al*., 2006). To the best of our knowledge, there are no reports on *E. magna*.

In the present study, the pathogenicity of rabbits infected with *E. magna* was investigated by histopathological evaluation, routine blood analysis and assessment of weight gain. Moreover, antigens were found in the intestines of the rabbits infected with *E. magna* using either anti-*E. magna* serum or anti-*E. intestinalis* serum as primary antibodies. This finding provides a theoretical basis for further screening for common antigens of these two coccidian species.

MATERIALS AND METHODS

Experimental animals

Forty-five weaned rabbits (age=28 d, weight=1108.95±50.45 g) were purchased from Nanchang Longping Rabbit Co., Ltd. (the farm meets local hygiene standards). The faeces were inspected for the presence of *Eimeria* by saturated brine flotation every day (Eckert *et al*., 1995; Balicka-Ramisz *et al*., 2020). The rabbits were qualified for inclusion in this experiment after oocysts of coccidia could not be detected for two weeks. Subsequently, the rabbits were randomly divided into three groups and reared in three separate sterilised rooms. Group A (n=15) was infected with 2×10^4 *E. magna* oocysts as in previous research (Tao *et al*., 2017), to assess the effect of such infection; group B (n=15) was infected with 3×10^3 *E. intestinalis* oocysts as in previous research (Coudert *et al*., 1993), for the sole purpose of obtaining anti-*E. intestinalis* serum, and group C (n=15) was designated as the control group and was given the same volume of phosphate-buffered saline as group A.

After the first infection, groups A and B were re-infected by gavage three times every two weeks to obtain anti-*E. magna* and anti-*E. intestinalis* serum. The infection dose of group A was 5000 oocysts, and that of group B was 1000 oocysts. Between 0-14 d after the first infection, rabbits in group A (n=9) and group C (n=9) were weighed on an empty stomach every morning. All procedures were performed in accordance with the Ethical Guidelines of Animal Experiments throughout the experiment.

Oocysts

E. magna and *E. intestinalis* oocysts were donated by the College of Veterinary Medicine, South China Agricultural University. After multiplication, collection and sporulation, the oocysts were preserved in 2.5% potassium dichromate solution at 4°C. Before inoculation, the oocysts were washed with normal saline solution to remove the potassium dichromate.

Haematoxylin and eosin (HE) staining

Six rabbits from group A and group C were randomly chosen and anaesthetised, and the duodenum, jejunum and ileum were collected on the 7th day of infection. The tissues were immediately fixed in 4% paraformaldehyde for histopathological examination and IHC staining (procedure detailed below). Forty-eight hours later, the three segments of the small intestine were cut into approximately 1 cm^3 tissue blocks, which were successively washed with tap water, dehydrated using graded ethanol solutions, transparentised with xylene, embedded in paraffin, and then made into 5 µm-thick slices. After HE staining, the slices were observed under a light microscope and subjected to histopathological evaluation.

Blood index analysis

Blood was taken once from each anaesthetised rabbit in group A and group C before infection and again on the 7th day after rabbits were first infected. Two millilitres of cardiac blood were collected and placed in an ethylenediaminetetraacetic acid EDTA K2 anticoagulation tube, and routine blood tests were performed on an MB860 Automatic Hematology Analyzer. The following routine blood indices were estimated: red blood cell counts (RBCs), white blood cell counts (WBCs), haemoglobin (Hb) counts, percentage of neutrophils (NE%), percentage of lymphocytes (LY%) and haematocrit (HCT) levels (i.e., the proportion of red blood cells). On the 14th day after the last infection, 5 mL of cardiac blood were collected from anaesthetised rabbits from group A, group B and group C, kept at room temperature for 15 min, and then centrifuged (3000 r/min, 20 min) to separate the serum. The serum was preserved at –80°C until further processing to prepare the anti-*E. magna* serum, anti-*E. intestinalis* serum and a negative serum control as primary antibodies for subsequent IHC staining.

Immunohistochemistry (IHC) staining

After being warmed in a 60°C incubator for 20 min, the tissue sections were dewaxed with xylene, hydrated in gradient alcohol solutions, incubated in citrate buffer solution at 98°C for 15 min, and then cooled to room temperature. Peroxidase activity was blocked using H_2O_2 (3%) for 10 min, and the slices were then incubated with normal goat serum as a blocking reagent for 30 min at 37°C. The anti-*E. magna* serum, anti-*E. intestinalis* serum and negative serum were used as primary antibodies, which were diluted to a concentration of 1:200 µL. The slices were incubated with ready polymer adjuvant for 20 min and then incubated overnight with polyperoxidase-anti-rabbit IgG as a secondary antibody. The slices were stained with 3, 3'-Diaminobenzine (DAB) solution for 5 min, the reaction was stopped with distilled water, and the slices were counterstained with haematoxylin stain. Finally, the slices were dehydrated in gradient alcohol solutions, transparentised with xylene and sealed with neutral gum. The staining results were analysed under a light microscope, and photographs were taken.

Statistical analysis

The data were coded and entered into SPSS software (version 25) (Frey, 2017). Rabbit body weight and routine blood indices were analysed using analysis of variance (ANOVA) to determine the differences between means. The results are expressed as the means±SE (standard errors). $P<0.05$ was considered as significant.

RESULTS

Histopathological evaluation of the small intestine

Histopathological changes in the duodenum

In the control group, the morphology and structure of the intestinal wall were normal, exhibiting rich and complete intestinal villi. Epithelial cells of the intestinal villi were arranged in an orderly manner and stained brightly. The villi of the duodenum in group A were relatively intact and exhibited a normal shape that was similar to those observed in the control group, and no parasites were found (Figure 1A-D).

Histopathological changes in the jejunum

In the control group, the structure of the jejunum mucosa was complete, the outline of the intestinal epithelial cells was clear, and epithelial cell arrangement was regular. All layers of the intestinal tissue were intact. There were severe histopathological changes in the microstructure in group A. The intestinal absorptive epithelium was occupied by a large number of *E. magna* at different developmental stages. The intestinal villi were shed, and their structures were disrupted. At 400× magnification, we observed that the intestinal absorptive epithelium was parasitised by a large number of gametophytes and unsporulated oocysts. In addition, a few bleeding spots with inflammatory cell infiltration were observed in the submucosa (Figure 2A-D).

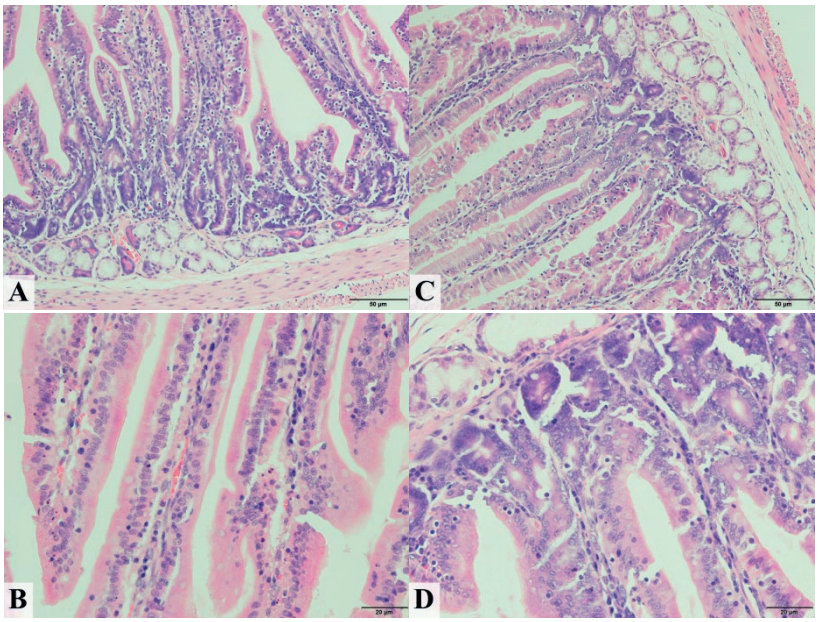

Figure 1: Histopathological changes in the duodenum. (A), (B): Rabbit duodenum in the *E. magna*-infected group at 200× and 400×, respectively. (C), (D): Rabbit duodenum in the control group at 200× and 400×, respectively.

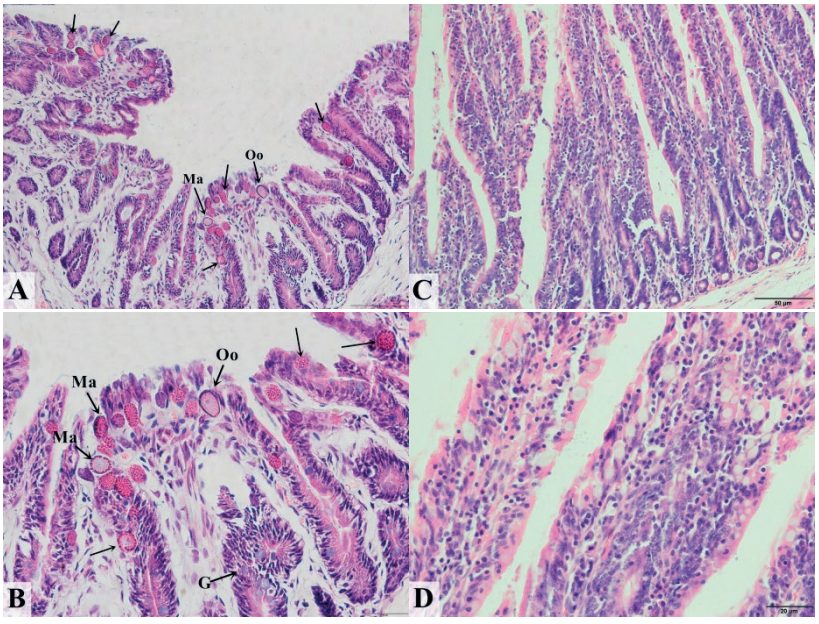

Figure 2: Histopathological changes in the jejunum. (A), (B): Rabbit jejunum in the *E. magna*-infected group at 200× and 400×, respectively. Ma: macrogametocyte; Oo: oocyst. (C), (D): Rabbit jejunum in the control group at 200× and 400×, respectively.

Histopathological changes in the ileum

In the control group, the ileum exhibited a complete structure and distinct tissue layers. The epithelial cells of the intestinal villi were orderly arranged and had clear boundaries. At 200× magnification, we observed that in the ileum in group A, there were a large number of *E. magna* oocysts and coccidia at other life-cycle stages in the villi and intestinal glands. The intestinal villi were blunt and fused together, wider than in the control group, slightly atrophied, and partially exfoliated. At 400× magnification, a large number of *E. magna* in the villi was observable, which were gradually more numerous towards the lower part of the villi. Even in the neck of the intestinal glands, there were *E. magna* at many different life stages, most of which were gametophytes and unsporulated oocysts. Moreover, massive infiltration of inflammatory cells was observed in the lamina propria (Figure 3A-D).

Haematological changes in rabbits infected with E. magna

In the control group, all of the blood indices were within the normal range (Cam *et al.*, 2008). RBCs, Hb counts, HCT levels and the LY% were significantly decreased by 30.70, 32.17, 35.83 and 44.92%, respectively, in group A compared with the control group on the 7th day after infection (P<0.01). In contrast, WBCs and the NE% were significantly higher in group A than in the control group on the 7th day after infection (P<0.01), increasing by 54.60 and 35.40%, respectively (Figure 4).

Antigen localisation in the small intestine

In slices subjected to IHC staining, the immunoreactive products of the antigen were expected to be stained yellow to brown. In the control group, negative staining was observed in all three intestinal segments. Additionally, the duodenum of group A did not show significant positive staining (Figure 5A-F). However, there were obvious brownish-yellow (positively stained) regions in the intestinal villous epithelial cells of the jejunum when anti-*E. magna* serum and anti-*E. intestinalis* serum were used as primary antibodies, indicating that there were *E. magna* oocysts or gametophyte antigens in these areas (Figure 6A-F). In the ileum, obvious brown (positive) areas were observed in the villous epithelial cells and the neck of the glands in group A. The positive area in the ileum was more extensive than

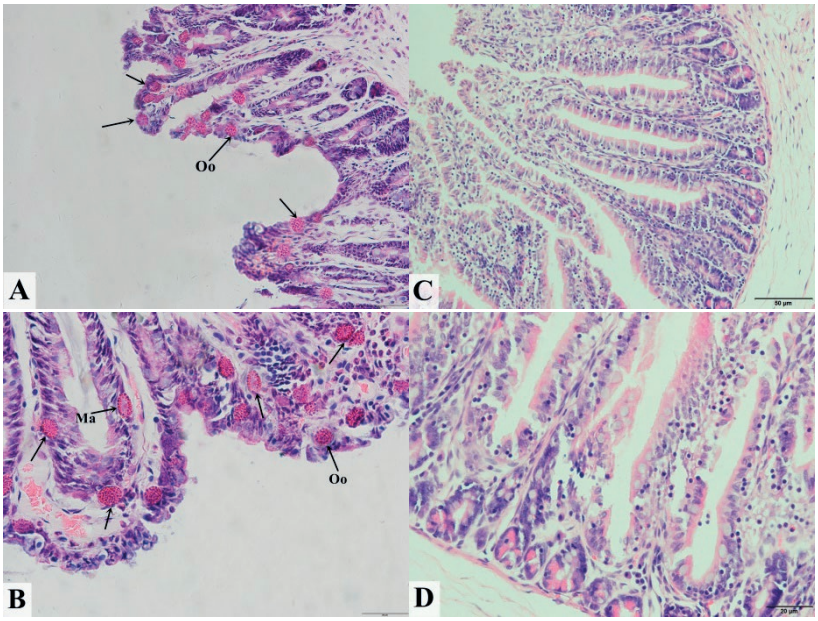

Figure 3: Histopathological changes in the ileum. (A), (B): Rabbit ileum in the *E. magna*-infected group at 200× and 400×, respectively. (C), (D): Rabbit ileum in the control group at 200× and 400×, respectively.

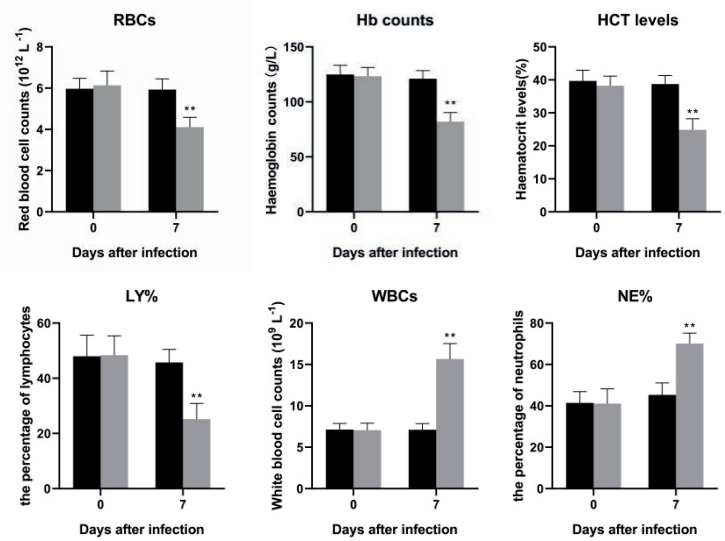

Figure 4: Red blood cell counts (RBCs), haemoglobin (Hb) counts, haematocrit levels (HCT) and the percentage of lymphocytes (LY%) in group A, which was infected by *E. magna*, were significantly decreased compared with those in group C, the control group. In contrast, white blood cell counts (WBCs) and the percentage of neutrophils (NE%) were significantly increased (** $P<0.01$). ■ Control; ■ *E. magna* infection.

that in the jejunum. Furthermore, the parasite antigen had similar locations in intestinal tissues when anti-*E. magna* serum and anti-*E. intestinalis* serum were used as primary antibodies. In contrast, there was no brown-yellow (positive) area when negative serum was used as the primary antibody (Figure 7A-F).

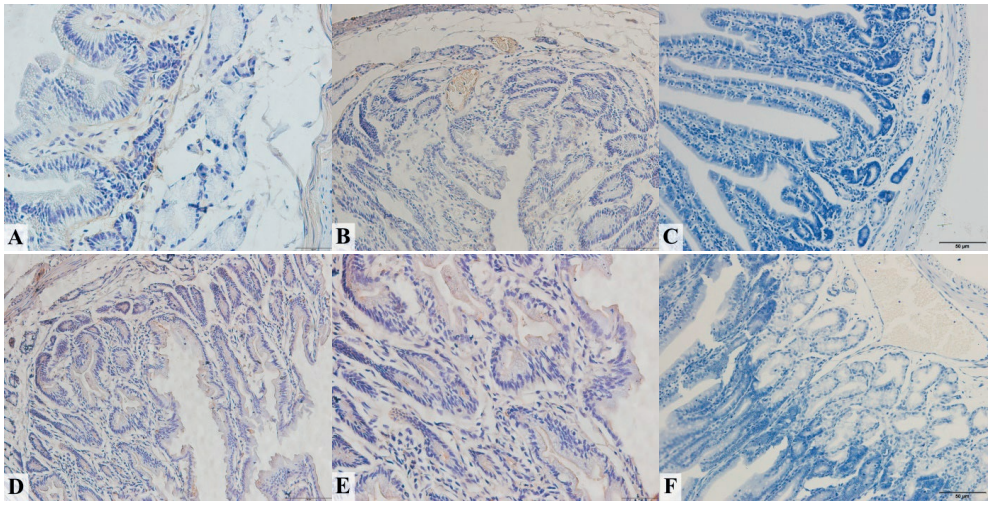

Figure 5: Antigen localisation in the duodenum. (A), (B), (C): IHC results for the group infected with *E. magna* when anti-*E. magna* serum, anti-*E. intestinalis* serum and negative serum were used as primary antibodies at 400×, 200×, and 200×, respectively. (D), (E), (F): IHC results for the control group at 200×, 400×, and 200×, respectively.

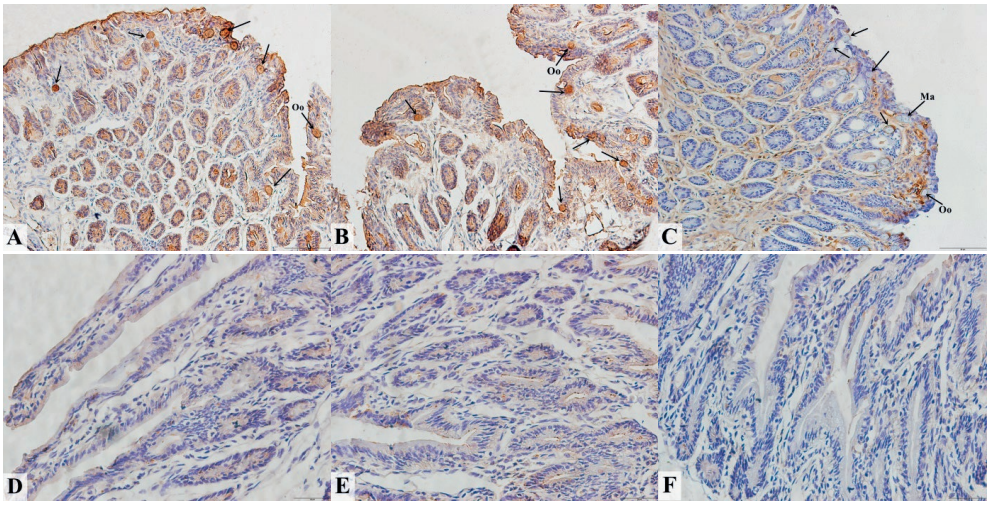

Figure 6: Antigen localisation in the jejunum. (A), (B), (C): IHC results for the group infected with *E. magna* when anti-*E. magna* serum, anti-*E. intestinalis* serum and negative serum were used as primary antibodies at 200×, 200×, and 200×, respectively. (D), (E), (F): IHC results for the control group at 400×, 400×, and 400×, respectively.

Effect of E. magna infection on weight

From the 1st to 14th day after infection, the weight of the control group continually increased. The weight of group A began to decrease on the 5th day after infection, continued to decrease until the 9th day, and then began to increase slowly. There was no significant difference in body weight between the groups before the 5th day after infection ($P>0.05$). From the 5th day to the 14th day, there was a significant difference in body weight between the groups ($P<0.01$) (Figure 8).

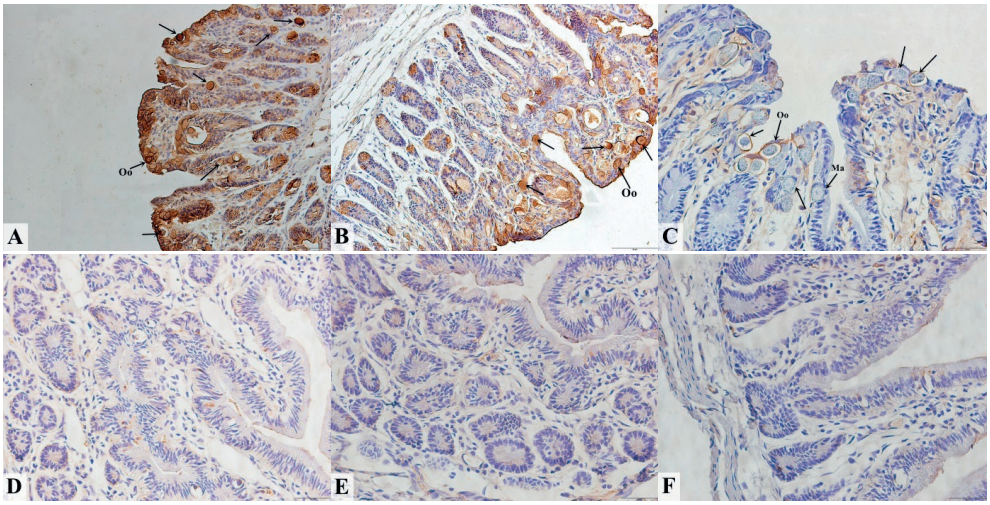

Figure 7: Antigen localisation in the ileum. (A), (B), (C): IHC results for the group infected with *E. magna* when anti-*E. magna* serum, anti-*E. intestinalis* serum and negative serum were used as primary antibodies at 200×, 200×, and 400×, respectively. (D), (E), (F): IHC results for the control group at 200×, 200×, and 400×, respectively.

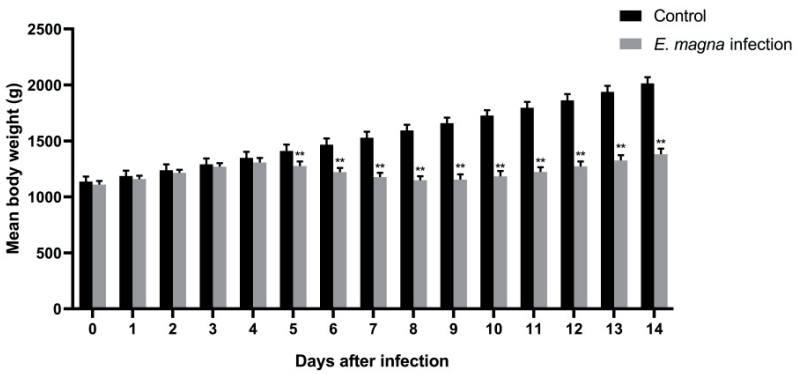

Figure 8: Mean body weight in group A, which included rabbits infected with *E. magna* (n=9), and group C, the control group (n=9). The weight of group A decreased from the 5th day to the 9th day. The weight of group C continually increased (** $P<0.01$).

DISCUSSION AND CONCLUSIONS

Eimeria magna is a widespread species found in rabbit breeding facilities that causes considerable economic losses in the rabbit industry due to weight gain decreases, diarrhoea and even mortality (Sadek Bachene *et al.*, 2018). After experimental infection with *E. magna*, Sadek Bachene *et al.* (2018) observed that the intestinal villi were destroyed and that there was diffuse infiltration of plasmocytes and lymphocytes in jejunal and ileal tissues. These results are similar to what we have observed. In the present study, a large number of *E. magna* of different stages parasitised the epithelial cells of the jejunum and ileum, and villi shedding and structural disruption occurred. In addition, a few bleeding spots with inflammatory cell infiltration were observed in the submucosa of the intestine.

Some physiological indices in the blood of rabbits infected with coccidia change considerably, including erythropoenia, leucocytosis and lymphocytosis (Hana *et al.*, 2011), which can be used to evaluate the infection status of coccidia by discriminant analysis (Adenaike *et al.*, 2018). RBCs, Hb counts, HCT levels, the LY%, WBCs and the NE% changed more significantly in infected than uninfected status in the present study. In line with our observations, Srinivasu *et al.* (2019) reported lower levels of total erythrocyte and haemoglobin content in the infected groups than in the control group. The decrease might be the result of rupture of the villous epithelium, as we observed in several bleeding spots in the infected animals. The loss of blood might lead to osmotic imbalance in blood vessels and dilution of the blood, leading the haematocrit level to decrease (Srinivasu *et al.*, 2019). Coccidial schizonts developed in the intestinal villi and damaged a large number of intestinal epithelial cells (Pakandl *et al.*, 2009), and this is likely what caused the haemorrhagic enteritis and led to an increase of WBCs in group A in the present study.

Neutrophils, a type of leukocyte, play a central role in innate immunity (Chu *et al.*, 2018). The leukocytes that are mainly recruited during inflammation are neutrophils and monocytes (Moro-García *et al.*, 2018). Hence, this explains why the number of neutrophils increased when intestinal inflammation occurred in rabbits infected by *E. magna*. Moreover, the body's resistance and immunity decreased after the rabbits were infected with *E. magna*, resulting in a decrease in the lymphocyte percentage.

Weight gain is the most reliable criterion for evaluating the health status of farmed animals and measuring the intensity of infection in growing rabbits (Pakandl *et al.*, 2009). Here, rabbits infected with *E. magna* lost weight from the 5th day until the 9th day after the first infection, after which their weight began to slowly recover. Similarly, Ryley and Robinson (1976) reported that the growth of rabbits infected with *E. magna* was normal for the first three days, but from day 3 or 4, the growth rate was reduced or weight was lost. In this study, the rabbits lost weight due to the massive reproduction and development of coccidia *in vivo*, resulting in diarrhoea and a reduction in food intake.

As expected, no IHC positive staining was observed in the intestinal tissues of the control group when any of the three kinds of serum were used, as rabbits in the control group had no antigens to react with *E. magna* and *E. intestinalis* antibodies. However, the localisation of coccidial antigen in the intestinal tissue was roughly the same for rabbits infected with *E. magna* when anti-*E. intestinalis* serum or anti-*E. magna* serum was used as the primary antibody. This reveals that schizosomes, gametophytes and other stages of *E. magna* can be simultaneously recognised by the two kinds of serum. Because *E. magna* antigen could be detected by *E. intestinalis* antibodies, *E. magna* and *E. intestinalis* antigens probably have some similar antigenic determinants. The sporozoites of *E. magna* first pass through the duodenum and then enter epithelial cells in upper parts of the villi of both jejunum and ileum, which is similar to what occurs for *E. intestinalis* (Pakandl *et al.,* 2006).

Studies on coccidial antigens have mostly identified a variety of common antigens and proteins, but they have been mostly limited to chicken coccidiosis; only *E. stiedai* has been previously studied in rabbits (Song et al*.,* 2017). Liu *et al.* (2020) identified twenty-one *Eimeria acervulina* sporozoite antigens that can be recognised by antisera from chickens infected with *E. acervulina*, *E. tenella* or *E. necatrix*. Studies in chickens and our results support the need for further comparisons of coccidial antigens parasitising rabbits.

In conclusion, the epithelial cells of both jejunum and ileum were parasitised with a large number of oocysts. Sloughing of the villous epithelium and massive inflammatory cell infiltration were observable. The present results indicate that *E. magna* and *E. intestinalis* have common antigens, and this finding provides basic evidence for further identifying common immunodominant antigens and developing multivalent anticoccidial vaccines.

Conflict of interest: The authors declare that they have no conflicts of interest.

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