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### Βιβλιογραφική αναφορά:

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## Nervous coccidiosis in a calf

Ş. Değirmençay<sup>1\*</sup> , S. Çomaklı<sup>2</sup> , S. Özdemir<sup>3</sup> , B. Hanedan<sup>1</sup> 

<sup>1</sup>Department of Internal Medicine, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey

<sup>2</sup>Department of Pathology, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey

<sup>3</sup>Department of Genetics, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey

**ABSTRACT:** In this case report, a 9-month crossbred Swiss brown calf has been reported with neurological symptoms including seizures, opisthotonus, hyperesthesia, nystagmus, convulsions of the head and extremity muscles, watery brown faeces and mild blood in faeces. *Eimeria* spp. oocyst was found in the parasitological examination of the faeces, and it was suspected of nervous coccidiosis. When the brain tissue was examined, the numerous different forms of *Eimeria* spp were histopathologically detected in the brain parenchyma and brain capillaries. *Eimeria* spp which were found in the brain and large intestinal tissues were confirmed as *Eimeria zuernii* using specific primers with qRT-PCR. This case report emphasizes that *Eimeria zuernii* is located in the brain tissue of cattle for the first time in the world. We suspect that these agents may travel to the brain through the enteric blood vessels, and all the neurological symptoms detected in this calf may be due to the presence of these agents in the brain tissue. Therefore, this could be crucial knowledge on the pathogenesis of nervous coccidiosis.

**Keywords:** *Eimeria zuernii*; calf; nervous coccidiosis; neurological findings; brain.

*Corresponding Author:*

Şükrü Değirmençay, Department of Internal Medicine, Faculty of Veterinary Medicine, Atatürk University, 25240, Erzurum, Turkey  
E-mail address: s.degirmencay@atauni.edu.tr

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## INTRODUCTION

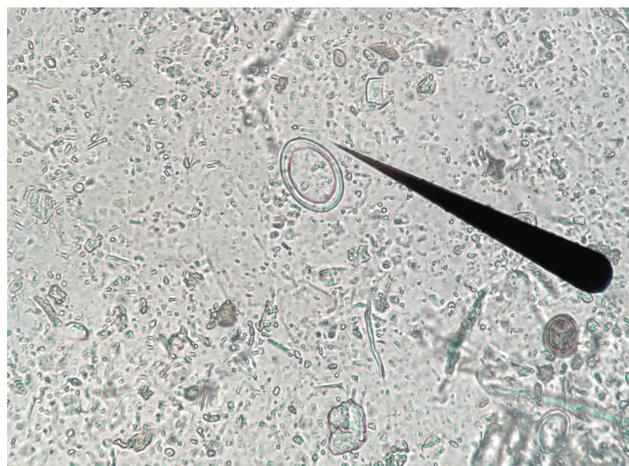
Nervous coccidiosis is a neurological syndrome associated with enteric coccidiosis in calves and a year-old cattle (Mackay and Van Metre, 2015). It is noteworthy that the nervous coccidiosis occurs mainly in the winter months (Jubb, 1988; Mackay and Van Metre, 2015), just as in this case report. The fatality rate of cattle developing nervous coccidiosis is much higher than that of cattle with only enteric coccidiosis (Hill and Ebbett, 2000). Fortunately, the syndrome is seen in a very small percentage of those cattle manifesting clinical cases of enteric coccidiosis possibly 4 per cent or lower (Reppert and Kemp, 1972). *Eimeria zuernii* (*E. zuernii*) has been detected in more than 90% of nervous coccidiosis cases (Arslan et al., 2015; Hill and Ebbett, 2000; Jubb, 1988). In this disease, acute diarrhoea, muscular tremors, convulsions, opisthotonus, nystagmus, and blindness occur (Bowman, 2014). The patients seem normal between seizures (Reppert and Kemp, 1972). The pathogenesis of the disease is still unknown. Several contradictory hypotheses have been proposed as a possible causative factor of the nervous signs such as vitamin A deficiency, thiamine deficiency, lead poisoning, bacterial meningoencephalitis, anemia, serum Na, K, Mg, Ca and P imbalances and hypoglycaemia. Nonetheless, none of them has been found directly associated with this syndrome (Isler, 1986; Isler et al., 1987b). Isler et al. (1987a) detected a labile neurotoxin only in the serum of coccidiosis cattle with neurological findings and speculated that neurotoxin may cause neurological signs. However, the importance of this neurotoxin on the pathogenesis of this disease was unknown. As can be seen, many hypotheses have been proposed on the pathogenesis of nervous coccidiosis, but the pathophysiological mechanisms of the disease have not been fully resolved yet.

The key objective of this case report emphasizes that, for the first time, *E. zuernii* can be located in the brain parenchyma and capillaries of cattle with nervous coccidiosis. This presence of *E. zuernii* in brain tissue was demonstrated by histopathological and qRT-PCR analysis. We speculate that this agent may travel to the brain through blood vessels and be responsible for the development of neurological signs. This stands for an important finding on the pathogenesis of nervous coccidiosis.

## CASE HISTORY

A 9 months old, 100 kilograms of crossbred Swiss brown calf with neurological symptoms, a watery

brown color of faeces containing mild blood was brought to the Animal Hospital of the Veterinary Faculty of Atatürk University in February. In the anamnesis, the owner said that the calf had been sick for 3 days, lost weight, and fallen on the ground, trembles on his head and feet, mild diarrhea in the morning of the presentation to the hospital. In addition, it was found out that the patient had never been ill before, but tablets containing oxfendazole-oxyclozanide (Ceva Animal Health LLC, Turkey) were administered for antiparasitic treatment as a prophylactic manner a month ago. In the clinical examination of the calf; nervous symptoms were dominant. In this context; opisthotonus, hyperesthesia, nystagmus, foaming in the mouth, lateral recumbency, periodically convulsions of the head and extremity muscles were detected. Between the seizures, it was observed that the patient was normal, lying in the lateral position, but the seizures started again (after 1 min) spontaneously or following stimulations. Rectal temperature was 38.2 °C, respiratory rate was 24 per minute and heart rate was 88 per minute. Parasitological examination of the faeces taken from the rectum revealed only *Eimeria* spp. oocyst (Figure 1) and the nervous coccidiosis was suspected.



**Figure 1.** *Eimeria* spp. oocyst from the faeces of the calf with nervous coccidiosis

Sulphadoxine-trimethoprim (15 mg/kg/day for 3 days; Topkim-Topkapi Pharmaceutical Premix Industry and Trade Inc, Turkey), flunixin meglumine (2.2 mg/kg/day for 2 days; Fulimed, Alke Pharmaceuticals, Turkey), vitamin B12 (20 mcg/kg/day for 3 days; Deva Holding Joint Stock Company, Turkey), Vitamin A, D and E (1 ml/50 kg, single dose; Ceva Animal Health LLC, Turkey), and fluid treatment (Lactated Ringer and 5% Dextrose) were administered for treatment, which alleviated the neurological

**Table 1.** Primer sequences of four types of *Eimeria*

Type of <i>Eimeria</i>	Forward	Reverse	bp	References
<i>E. bovis</i>	tcataaaacatcacctccaa	ataattgcgataagggagaca	238	(Dyková et al., 1983)
<i>E. cylindrica</i>	gacatttaaaaaaccgattggt	ggctgcaataagatagacata	304	
<i>E. ellipsoidalis</i>	caacgttttctttctatca	actgcatgagagagagcg	148	
<i>E. zuernii</i>	aacatgtttctaccactac	cgataaggaggagacaac	244	

symptoms. Despite the decrease in the frequency of occurrence of neurological symptoms, the calf died on the 4th day of treatment.

As for the histopathological examination, large intestine (cecum area) and brain tissue (areas close to the cerebral cortex) samples were obtained from the necropsied calf with separate tools in order to eliminate the risk of contamination and were fixed in a 10% buffered formaldehyde solution. Then, the routine alcohol-xylol process was applied and blocked in paraffin. 5 µm sections from the blocks were placed on the microscope slide. Hematoxylin-eosin (HE) and Periodic Acid Schiff (PAS) were performed to the sections in accordance with the manufacturer's recommendations (CB6125.0500 PAS Stain Set - McManus).

qRT-PCR was performed with specific primers to detect if the agents, histopathologically observed in the large intestine tissue (cecum), brain parenchyma and capillary, were *Eimeria spp.* Three FFPE biopsies from the intestine and brain were applied to deparaffinization and total RNA isolation process. Excess paraffin was cut off from the tissue block with a scalpel. Paraffin sections (10- 50 mg) were put into a 1.5 RNase free microtube and incubated at 60°C for 10 min for melting the paraffin. The sections were transferred into liquid nitrogen. Then, the frozen tissue was ground into powder using a pestle. The powdered sections were transferred into a new microtube and 1 mL of xylene was added and mixed thoroughly by vortexing 10 min at room temperature to extract the remaining paraffin. After centrifugation at 14,000 ×g for 2 min, the supernatant was removed and 1 mL of ethanol 100% was added to the pellet and vortexed for 10 min. Then, the samples were centrifuged at 14,000 ×g for 2 min and the supernatant was cautiously poured off. This step was repeated three times by adding 1 mL of each 96%, 80%, and 70% ethanol. The centrifuged pellet was air-dried at room temperature for 10 min to remove the ethanol (Zununi Vahed et al., 2016).

Total RNA isolation was observed from the collected tissue pellets through the utilization of Trizol (Invitrogen, USA) and in line with the kit's procedure.

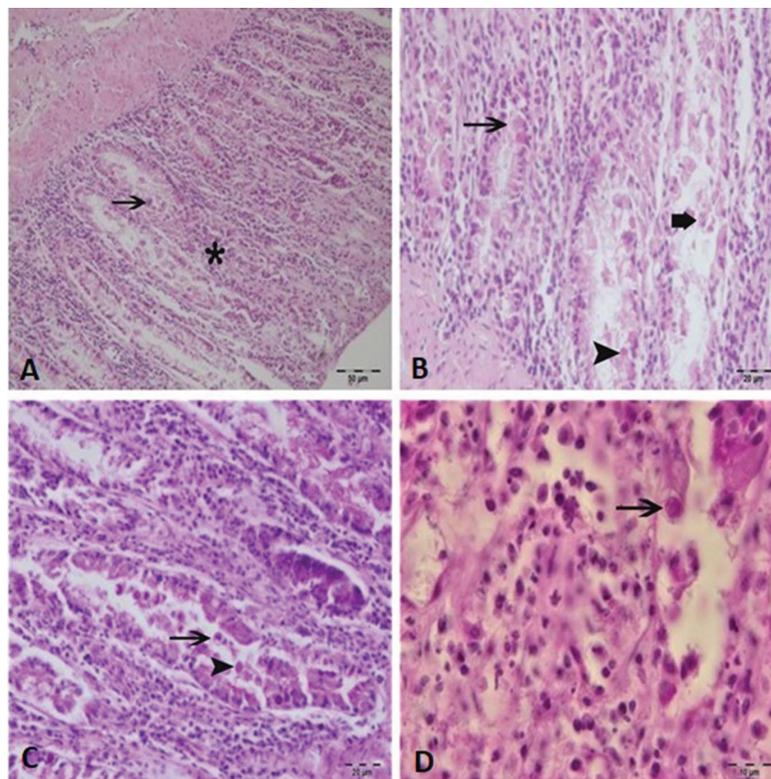
Following that, the RNA concentration was measured by virtue of NanoDrop (Epoch Microplate Spectrophotometer, USA). RNAs were run in a 1.5% agarose gel in 1XTBE solution for one hour at 80 volts with a view to controlling total RNA quality and visualized by gel imaging system and the RNA quality was determined.

DNase I (Thermo Scientific, USA) was employed against DNA contamination in isolated RNA samples. DNase I treatment was performed in line with the protocol provided in the kit. Subsequently, 1 µg was taken from these RNAs and cDNAs were synthesized using the miScript Reverse Transcription Kit (Qiagen, Germany) in line with the protocol provided. The purity and quantity of the obtained cDNAs were measured by virtue of spectrophotometer (Epoch Microplate Spectrophotometer, USA), and the cDNAs were diluted at the same ratios. Subsequently, the cDNA samples were stored at -20°C for utilization in Real-Time PCR studies.

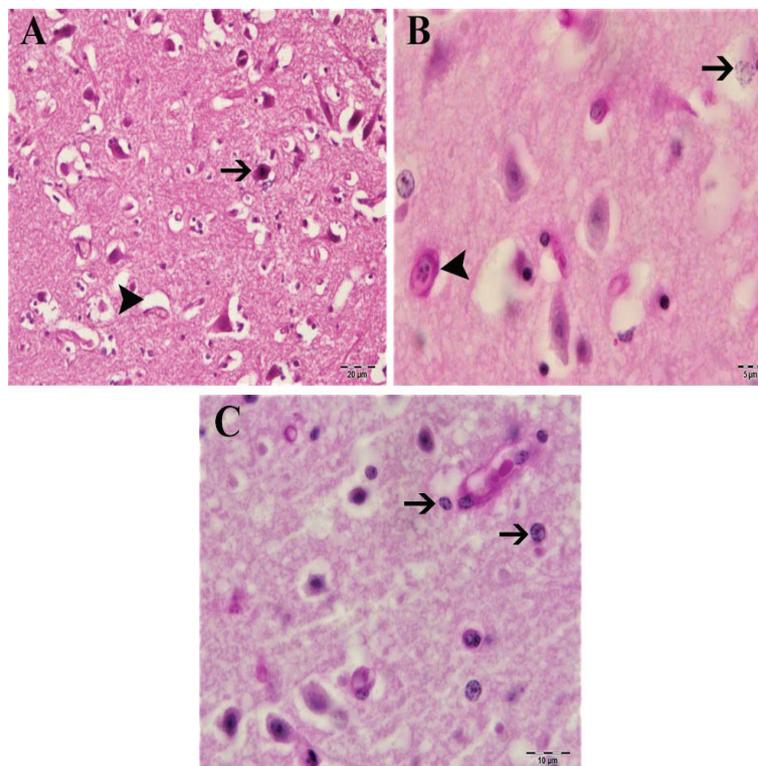
qRT-PCR was performed utilizing the CFX96 BioRad device to determine ribosomal RNA genes of six bovine *Eimeria* species; *E. bovis*, *E. cylindrica*, *E. ellipsoidalis* and *E. zuernii*. Master mix content created in Real-Time PCR experiments was as follows: Syber Green 2X Rox Dye Master mix (Qiagen Germany), forward and reverse primers designed for genes, cDNAs as a template and nuclease-free water. The samples were analysed in a Real-Time device following the preparation of master mixes. Reaction conditions and primer sequences of the genes are shown in Table 1. The primer sequences were received from a previously conducted study (Kawahara et al., 2010).

## RESULTS

Enteritis was observed in the large intestines in the histopathological examination using HE staining. Parasitic structures and intense inflammatory cell infiltration were observed in intestinal crypt epithelial cells (Figure 2A). The developmental stages of the *Eimeria* agents, macrogametes and microgametes forms, (Figure 2B-D) and necrotic epithelial cells were detected in the intestinal tissue (Figure 2B).

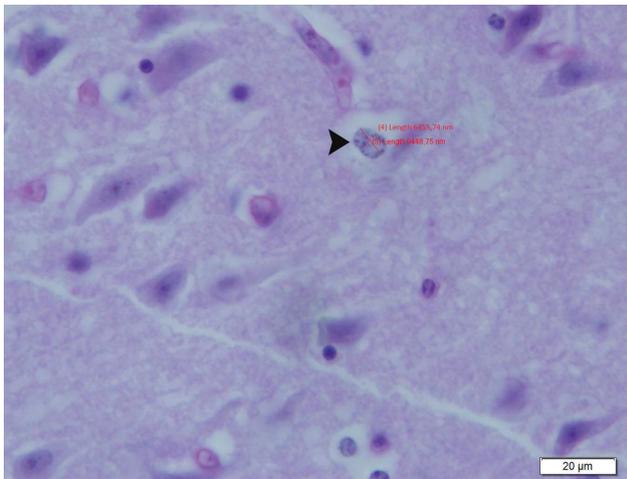


**Figure 2.** Enteric coccidiosis. A) Parasitic structures (arrow) and intense inflammatory reaction (asterisks) in the intestinal crypt epithelial cells, HE&50µm. B) The forms of macrogamete (thin arrow), microgamete (thick arrow), and the necrotic structures (arrowhead) in epithelial cells, HE&20µm C) The forms of macrogamete (arrow) and microgamete (arrowhead) in epithelial cells, PAS&20µm. D) The high magnification of macrogamete appearance in the intestinal crypt epithelial cell. (arrow), PAS&10µm



**Figure 3.** Nervous coccidiosis. A) The neuronal necrosis (arrow) and oedema (arrowhead) in the brain parenchyma, HE&20 µm. B) Eimeria agents-like structures in the brain parenchyma (arrow, arrowhead) PAS & 5 µm. C) Eimeria agents-like structures in and around the capillary (arrows) PAS & 10 µm

Neuronal necrosis and oedema were observed in the brain parenchyma (Figure 3A). Furthermore, we observed Eimeria agents-like structures by PAS staining in the parenchyma (Figure 3B). Eimeria agents-like structures in and around the brain capillary were determined (Figure 3C). Microscopic examination revealed possible schizont forms of an average of 2-4 ellipsoidal coccidia agents at each microscope site. Schizonts were measured as  $5-7 \times 7-9 \mu\text{m}$  in diameter (Figure 4).



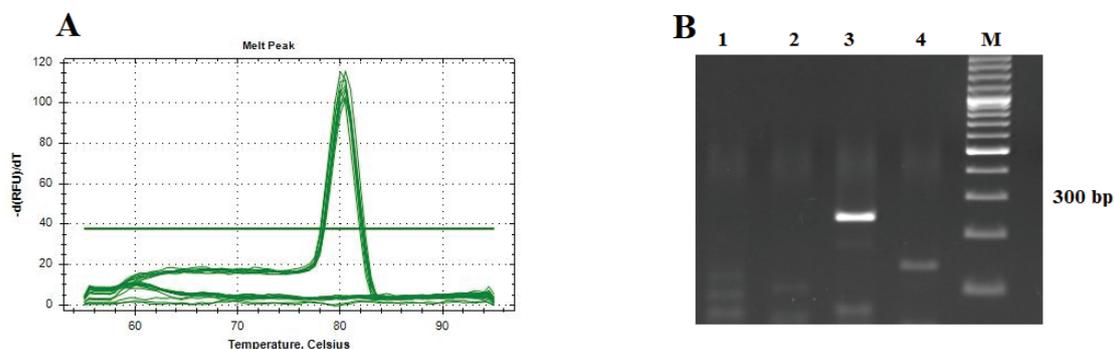
**Figure 4.** Demonstration of the coccidial stage (presumptive extraintestinal schizonts) within the cytoplasm (arrowhead)

We identified the types of *Eimeria* in the large intestine and brain tissue samples using specific primers with qRT-PCR. While *E. zuernii* was determined in both intestine and brain tissue samples, the other three types of *Eimeria* were not identified (Figure 5). Thereafter, we performed a specific qRT-PCR analysis for *E. zuernii* in the intestine and brain separately. We found that the specific gene region of *E. zuernii* was up-regulated in both intestine and brain tissue samples (Figure 6).

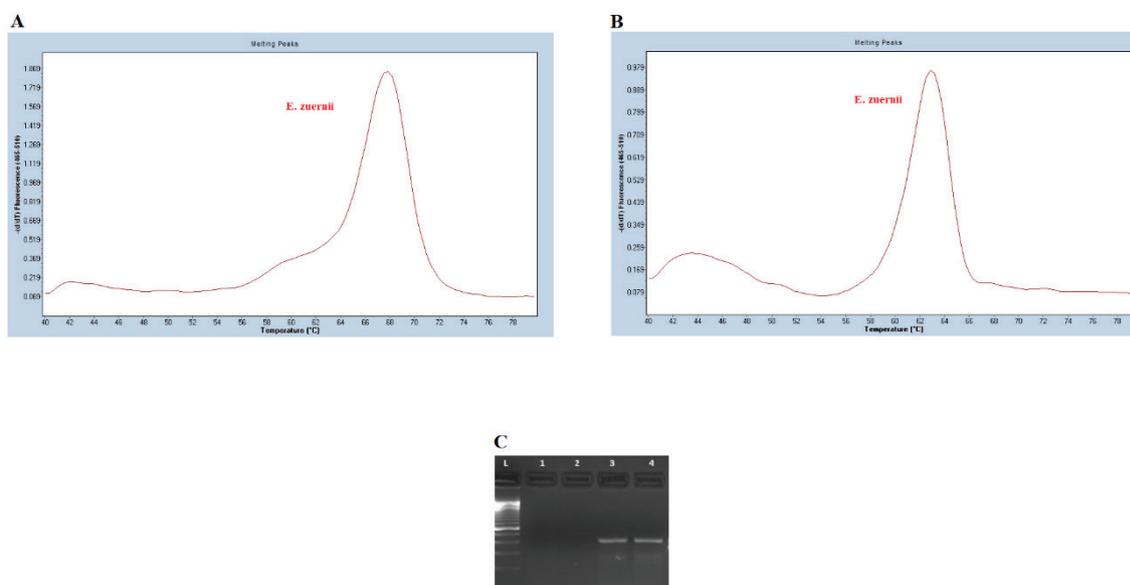
## DISCUSSION

This is the first study that the *E. zuernii* was determined in the brain parenchyma and brain capillaries of a calf with nervous coccidiosis by both histopathological and genetic methods. This form of the disease is less common and there has been no new published information on the pathophysiology of this form for over 30 years. Since the pathogenesis of nervous coccidiosis is still unknown today, the detection of *E. zuernii* in brain parenchyma and capillaries could give more insight into the pathogenesis of the disease. In this study, clinical findings, histopathological examination and especially qRT-PCR analysis results were employed for the diagnosis of coccidiosis infection. It should be noted that genetic analysis is a reliable method for diagnosing diseases as it gives more accurate and certain results.

Both the previous reports and our report accorded in noting similar clinical findings including, but not limited to, nystagmus, contractions in head and neck muscles, lateral recumbency and rowing movement in the front of the feet (Bowman, 2014; Hill and Ebbett, 2000; Jubb, 1988). The differential diagnosis of nervous coccidiosis comprises polioencephalomalacia, rabies, pseudorabies, lead poisoning, ethylene glycol poisoning, petroleum distillate poisoning, and clostridial enterotoxemia (Mackay and Van Metre, 2015). However, for the sake of differential diagnostics, seizures have a characteristic value for this form of the disease, as such characteristic seizures are not expected in other neurological conditions (Apley and Fajt, 1998). Seizures occur spontaneously or when the animal is excited or overactive. Twitching occurs in the facial and neck muscles, animals lie on the ground, and then epileptic seizures begin. Tonic and clonic contractions in the whole body and often opisthotonus and nystagmus occur. The duration of the sei-



**Figure 5.** A) Melting curves peak of qRT-PCR analysis for all types in the intestine and brain tissues. B) Gel image for PCR products in the brain tissues. Line 1; *E. bovis*. Line 2; *E. cylindrica*. Line 3; *E. zuernii*. Line 4; *E. ellipsoidalis*. M; DNA ladder



**Figure 6.** A) Melting curve peaks of *E. zuernii* in the brain tissue B) Melting curve peaks of *E. zuernii* in intestine tissue. C) Gel image for PCR products in the brain and intestine tissues. L; DNA ladder. Line 1 and Line 2; Negative control. Line 3; *E. zuernii* in the brain. Line 4; *E. zuernii* in the intestine

zures varies, but is approximately 5 minutes. After a short rest period (10-15 minutes), the seizures start again (Reppert and Kemp, 1972). As the disease progresses, the frequency of seizures increases and the interval between seizures becomes shorter (Reppert and Kemp, 1972). This type of seizure is a feature defined in neurological coccidiosis cases and is not seen in other neurological diseases included in the differential diagnosis (Apley and Fajt, 1998). From this, we infer that the animal of our case was experiencing the last stage of the disease as the duration of the seizures and intervals between seizures were only approximately 10 minutes and 1 minute respectively. Although the frequency of neurological findings decreased with the treatment, death occurred on the 4th day. The reason for this mournful end could be due to the fact that the disease was progressed sub-clinically, in other words, the apparent symptoms of enteritis like bloody faeces were not present. This possibly caused the postponement of the right and effective treatment which eventually gave rise to irreversible brain damage.

For the diagnosis of nervous coccidiosis, the age factor should also be considered. It is known that coccidiosis is most common in calves between 3 weeks and 6 months (Reppert and Kemp, 1972). It is rarely seen in calves older than 6 months and is usually subclinical (Joyner et al., 1966). It is noteworthy that the animals in the above reports (Bowman, 2014; Hill and Ebbett, 2000; Jubb, 1988) and in our study are 9 months old and over. In these animals, the de-

velopment of nervous findings may be due to i) the subclinical course of the disease, ii) possible delays in treatment accordingly and iii) extra-intestinal migration of *E. zuernii* to the brain and the damage it causes which overall constitutes the main emphasis of our study.

The pathophysiological mechanisms of the nervous signs have not been fully resolved yet, although many hypotheses (Isler, 1986; Isler et al., 1987a; Isler et al., 1987b) have been proposed about that. One of these hypotheses is that *E. zuernii* has been detected in faeces examination in more than 90% of the nervous coccidiosis cases (Arslan et al., 2015; Hill and Ebbett, 2000; Jubb, 1988) and some researchers believe that it is important to identify the *E. zuernii* to confirm the diagnosis of nervous coccidiosis (Reppert and Kemp, 1972). In line with this, we genetically proved the existence of *E. zuernii* in both intestinal and brain tissue specimens. From this, we believe that the probability of developing nervous coccidiosis is comparably high in animals with coccidiosis caused by *E. zuernii*.

Another assertion, it has been reported that the coccidia agents do not directly invade the central nervous system in cattle (Mackay and Van Metre, 2015). Likewise, all endogenous stages of the coccidial life cycle were claimed to occur only in the digestive tract in cattle (Blood and Henderson, 1968). On the contrary, extra-intestinal migration of *Eimeria* species is known in fish, birds and mammals (Ball et al., 1989).

Testis, ovary, peritoneum, spleen, kidney, liver, gallbladder, bladder (Pellérdy, 1974) and gill filaments (Dykova et al., 1983) are some regions where the coccidia agents parasitize extra-intestinally in fish. Chapman et al. (2016) have found the *Caryospora cheloniae*, a coccidial pathogen of mariculture, in the brain, gastrointestinal system, lung, thyroid and kidney tissues of green sea turtles (*Chelonia mydas*). Kogut and Long (1984) reported that *Eimeria* sporozoites were detected in chickens and turkeys' blood. In this case, contrary to the above-mentioned reports (Blood and Henderson, 1968; Mackay and Van Metre, 2015) about cattle, in the histopathological examination and genetic testing of meront form of the *E. zuernii* were found in the brain parenchyma and the brain capillaries of the calf with nervous coccidiosis. To our knowledge, this is the first report submitted. Detection of the *E. zuernii* in the capillaries of the brain reinforces our hypothesis that the agent can travel to the brain through enteric blood vessels. Considering the above statements (Ball et al., 1989; Chapman et al., 2016; Dykova et al., 1983; Kogut and Long, 1984; Pellérdy, 1974), detection of the agent in the brain parenchyma shows that there can be an extra-intestinal migration in cattle as well. In addition, the existence of the agent in the brain may be responsible for the occurrence of nervous symptoms. Additionally, Isler et al. (1987b) histopathologically observed fewer coccidial forms in the descending colon and rectum of calves with nervous coccidiosis compared to enteric coccidiosis. We speculate that the migration of these agents to the brain may be the reason for the low count of coccidial forms in the intestines.

Brain oedema and congestion have been reported in nervous coccidiosis (Reppert and Kemp, 1972; Fanelli, 1983; Clayburg 1970), but none of them reported the presence of *Eimeria* agents in the brain of cattle (Fanelli, 1983; Hill and Ebbett, 2000; Jubb, 1988; Mackay and Van Metre, 2015; Radostits and Stockdale, 1980; Reppert and Kemp, 1972). In this case, we detected neuronal necrosis and oedema and *Eimeria*-like agents histopathologically in the brain parenchyma. Immunohistochemistry (IHC) could have been an alternative way for us to show the presence of *E. zuernii* in the brain tissue. However, to our knowledge there is not any produced antibody for this particular parasite currently, therefore we could not perform IHC. For this reason, and also in the light of

clinical findings, stool examination findings and genetic analysis results, we identified the factors that we observed with PAS staining in the brain parenchyma as possible *Eimeria* factors. The reasons for detecting no *Eimeria* agents in the brain of cattle in any of the reports published (Fanelli, 1983; Hill and Ebbett, 2000; Jubb, 1988; Mackay and Van Metre, 2015; Radostits and Stockdale, 1980; Reppert and Kemp, 1972). until this time can be listed as follows: (i-) The incidence of nervous coccidiosis is low which causes a limited opportunity for a thorough study. Accordingly, there is no published article about the pathophysiology of the disease for more than 30 years. (ii-) Enteritis form of the disease is easily diagnosable and an immediate treatment prevents nervous findings. (iii-) Compared to the past, today is much more advanced in terms of technical facilities used in diagnosis as until now, to our knowledge there have been no studies using qRT-PCR methods for the detection of *Eimeria* agents in the brain tissue.

In conclusion, for the first time in the world, *E. zuernii* in the brain capillary and brain parenchyma was detected in the calf with nervous coccidiosis using histopathological and qRT-PCR methods in this case report. Localization of the *E. zuernii* in the brain parenchyma may be responsible for neurological signs in the disease. Additionally, detecting the agents in the brain capillaries signifies that these agents have reached the brain through enteric blood vessels. This finding also ignores the statement that the whole life cycle of coccidiosis is restricted to the digestive tract in cattle. We believe that this finding is an important contribution to the pathogenesis of the disease and it will shed light on other studies which will be done in the future. We strongly recommend the qRT-PCR analysis for the detection of *Eimeria* spp. in the brain tissue in animals with nervous coccidiosis. We suggest the use of antibiotics that can cross the blood-brain barrier, such as fluoroquinolones and sulfonamides, for the treatment. Similarly, injection of these antibiotics directly into the cerebrospinal fluid should also be considered.

#### CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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