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# PARASITOLOGICAL AND PATHOLOGICAL STUDIES ON *NEOSPORA CANINUM* IN EXPERIMENTALLY INFECTED DOGS

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***Abstract***

*Neospora caninum* is a protozoan parasite. It was isolated from brain of naturally infected rats and fed on puppies. Five to ten days post-infection, puppies began to excrete unsporulated oocysts. Sporulated oocycts were fed to rats. After death, fresh smears from intestines of puppies and brain smears stained with Giemsa stain from rats were taken for parasitological examination. Meanwhile, tissue specimens from the internal organs wer taken from puppies and rats for histopathological examination. However, only brains from rats were taken for electron microscopy examination.

Histopathological examination revealed meningoencephalomyelitis, hepatitis, nephrosis and occasional interstitial pneumonia in puppies and rats, in addition to myocarditis and myocitis in puppies. Tissue cysts and tachyzoites were present in lesions in the brain of rats. In puppies, tachyzoites were seen in the brain and in parasitophorous vacuoles within the cytoplasm of epithelium lining the small intestine.

Electron microscopy examination of the brain of rats revealed tachyzoites and tissue cysts.

Parasitological and pathological importance of *N. caninum* in puppies and rats was fully discussed.

## INTRODUCTION

*Neospra caninum* is a protozoan parasite recently described and related to Apicomplexa: Sarcocystidae. It was first identified by Dubey *et al.* (1988) who described and isolated the parasite from puppies having congenital encephalomyelitis. The protozoon is a cyst-forming parasite known to cause paralysis in young dogs (Bjerkas and Presthus, 1988). The coccidial life cycle of *N. caninuml* was first discovered by Mc Allister *et al.* (1998), who determined that dogs were the definitive host of the parasite. This parasite has a wide range of intermediate hosts. Natural infections had been found in cattle, sheep, goats, horses and deer, and had been proved to be a major cause of abortion in these animals (Dubey and Lindsay, 1996), as it can be transplacentally

transmitted. Experimental infections had been induced in mice, rats, dogs, foxes, goats, cats, sheep, pigs, rabbits and cattle.

The present studies were conducted to throw light on the parasitological and pathological importance of *Neospora caninum* in dogs and rats.

## MATERIALS AND METHODS

### I. Parasitological examination

Three puppies, 3 weeks-old were used. They were subjected to coprological examination, and proved to be free of any infection. They were kept in separate cages and fed fresh bread only; clear water was offered *ad-libitum*. Each puppy was injected with 40 mg methylprednisolone acetate (MPA) (Egyptian International Pharmaceutical Industries Co., A.R.E.). After having proved to be free from any parasitic infection, the puppies were fed the brains of naturally infected rats that had been previously examined several times to be infected with *N. caninum* and proved to contain tissue cysts of this parasite after examination of stained smears from brains with Giemsa stain. Faecal samples from each puppy were examined daily by using Sheather's solution, and continued till death of the puppy. Unsporulated oocysts were collected and placed in Potassium dichromate solution 2.5% to be incubated at 25°C till sporulation. Daily aeration of oocysts was carried out. Obtained oocysts were counted by the use of hemocytometer, then, after sporulation they were used to infect rats. Two puppies died 30 days post-infection, while, the third one died 32 days post-infection. Post-mortem examination was done for each puppy immediately after death.

Experimental infection of rats was carried out. For this purpose, 15 male rats obtained from special breeder and weighing 75-80 g each were used. They were divided into 3 groups each of 5. On the day of infection, 5 rats were injected, each with 2 mg MPA subcutaneously to raise their susceptibility to the infection; each of these rats was inoculated orally with $5 \times 10^4$ sporulated oocysts. Five rats were left as control without infection but injected with MPA, and the last five rats were also left as control without being given neither MPA nor oocysts. Infected rats were daily observed for the appearance of any clinical signs. Three rats of them died 7 days post-infection and the fourth died on 29th while, the fifth died 32 days post-infection. Post-mortem examina-

tion was carried out immediately after death of rats.

Smears from the brain were taken and stained with Giemsa stain and examined. On each occasion, one rat from each of the control group was sacrificed and stained smears from brain were examined. In case of presence of any parasitic stage, it was measured by using the ocular micrometer, then, illustrated.

### II. Histopathological examination

Tissue specimens from dead puppies and rats were taken from brain, heart, lungs, liver, spleen and kidneys, as well as, the skeletal muscles of thigh, the eyes and intestines of dead puppies. Specimens were fixed in 10% neutral buffered formalin solution, processed for paraffin embedding sections of 4-5 microns, then, prepared and stained with haematoxylin and eosin (Harris, 1898) and Giemsa stain (Schalm, 1965).

### III. Electron microscopy examination

Brains from experimentally infected rats were fixed in 2.5% cold glutaraldehyde, dehydrated in different grades of alcohol, then, embedded in epon. Smithin sections were prepared and stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate, then, examined by the transmission electron microscopy (Afifi *et al.*, 1996).

## RESULTS

### I. Parasitological examination

Faecal examination of experimentally infected puppies revealed first excretion of oocysts 5, 6 and 10 days post-infection. Unsporulated oocyst was ovoid in shape and measured 11.25 x 8.75 µm. The oocyst wall was smooth, colourless and appeared as if having a single layer, but sometimes more than one layer could be observed, no micropyle was noticed (Fig. 1).

Oocysts were sporulated 7-10 days post-incubation at 25°C. The sporulated oocysts measured 10 x 17.5 µm and contained two sporocysts each with four sporozoites; no residuum was apparent. Each sporocyst measured 10 x 8.75 µm, its wall was colourless and did not contain stiedae body, residuum was represented as many dis-

persed granules. Sporozoites were elongate, the nucleus was located slightly posteriorly (Fig. 2).

The excretion of oocysts was intermittent till the death of puppies. One day before death, the puppies were off food. Two puppies died 30 days post-infection, the third one died on the 32nd day after being depressed, showing muscle flaccidity and paralysis of hind limb (Fig. 3). Post-mortem examination revealed congestion of the brain, skeletal of thigh, small and large intestines. This picture was severe in case of the third puppy (Fig. 4). Single or multiple white areas 1 mm diameter were seen in brain and liver. Examination of fresh smears from small intestine revealed the presence of unsporulated oocysts in the ileum.

Infection of rats with sporulated oocysts excreted from puppies resulted in death of 3 rats 7 days post-infection and the fourth died after 29 days, the fifth died after 32 days. Before death, all rats showed rough hair coat, curled back, lethargy and loss of appetite (Fig. 5). Smears from brain of one rat died 7 days post-infection, revealed tachyzoites (Fig. 6). These tachyzoites were divided into 2 zoites that were found in parasitophorous vacuole. They were lunate-shaped and measured 2.5 x 1.25 μm each.

Gross examination of brain of a rat died 29 days post-infection, showed congestion. Also, examination of stained smears from brain of this rat and that died 32 days post-infection revealed tissue systs. The syst was spherical (11.25 um) to subspherical (12.5 x 11.25 μm) and contained bradyzoites which were surrounded with a distinct cyst wall (Fig. 7).

## II. Histopathological examination

In case of experimentally infected puppies, the main neural lesions consisted of non-suppurative meningoencephalomyelitis characterized by malacia, multifocal mononuclear cells infiltration around areas of necrosis. Several glial proliferation, severe oedema in the Virshow Runbin space and periastrocystes, vasculitis, perivascular inflammatory cells infiltration and demylination were seen (Fig. 8 a and b). Also, focal areas of non-suppurative leukocytic infiltration in the meninges were noticed. Meanwhile, tachyzoites were observed in lesions as groups or diffuse within these areas of tissue damages which were positive with Giemsa stain, also small cysts with thin wall (Fig. 9)

or with thick wall were detected. Myocarditis and necrosis of some of the cardiomyocytes with thrombosis of most of blood vessels were noticed in heart tissues. Cencerning the hepatic lesions, they consisted of infiltration in the portal area of mononuclear cells, variable foci of hepatocellular degeneration and necrosis. As well, occasional interstitial pneumonia was detected in lung tissues. Also, non-suppurative nephritis, myocitis of the thigh muscle were observed (Fig. 10). Examination of the intestines revealed severe degeneration, necrosis and sloughing of some lining intestinal epithelium. The lamina propria and submucosa of the small intestine were infiltrated by mononuclear cells with congestion, haemorrhages and occasional vasculitis. The ileum showed depletion in lymphocytes of the Pyer's patches. *Neospora caninum* tachyzoites were located within the cytoplasm of some lining intestinal epithelium. They resided in parasitophorous vacuole or located directly in the host cell cytoplasm. In Giemsa stained sections, the zoites were crescent-shaped or straight in appearance. The tachyzoites had visible nucleus (Fig. 11). Generally, tachyzoites were scarce in lesions detected in most of the examined internal organs.

In case of experimentally infected rats, lesions varied in severity and intensity in various organs The most severe lesions were noticed in the brain, most of the examined cases showed moderate to severe multifocal meningoencephalitis which was characterized by multifocal areas of vascular congestion and occasional haemorrhage with perivascular infiltration of mononuclear cells. Oedema in Virshow Rubin spaces and periastrocytes, focal areas of necrosis and gliosis (Fig. 12) with demylination of the nerve fibres were distinct. Occasionally, groups of tachyzoites (Fig. 13) and small tissue cysts were identified in the brain tissue. Tissue cysts were few when present and were mainly found in the cerebrum of rats. They appeared as round to oval in shape, small cysts with a thin wall and few bradyzoites or with thick wall. Tissue cysts were not surrounded by a zone of host reaction, some cases showed degenerating bradyzoites accompanied with inflammatory cells aggregation. The main lesions in heart were manifested as few areas of myocardial degeneration and necrosis. As well, the lung showed occasional interstitial pneumonia. Examination of the liver revealed multiple granuloma formation consisting of aggregates of neutorphils, lymphocytes and macrophages in the portal and centrolobular interstitium and within the hepatic sinusoids. Hepatocellular degeneration and necrosis were also observed (Fig. 14). At the same time, the

spleen had mild lymphoid follicular hyperplasia and focal lymphocytic necrosis. As well, the kidneys revealed renal tubular degeneration with intraluminal casts formation.

Concerning rats of both control groups, they did not exhibit any abnormal clinical signs or histopathological changes in tissues, as well as, they were refractory for the presence of tachyzoites or cysts in brain smears or in histopathological preparations.

### III. Electron microscopy examination

Examination of brain of experimentally infected rats by transmission electron microscopy revealed the tachyzoites being surrounded by parasitophorous vacuoles (Fig. 15). In another section, Neospora cyst was detected. It consisted of a thick cyst wall surrounding the bradyzoites. Each bradyzoite showed subterminal nucleus, micropore and rhoptries (Fig. 16).

## DISCUSSION

*Neospora caninum* is an Apicomplexa that causes ascending paralysis and even death in naturally infected dogs (Dubey *et al.*, 1988a). In Egypt, Hassan *et al.* (2000), found *N. caninum* antibodies in 6.4% of sheep sera and in 3.24% of goat sera. In the present studies, the parasite had been isolated from brain of naturally infected rats that had been spontaneously discovered infected with *N. caninum* cysts. After feeding the brain of these rats to puppies, they excreted oocysts following a prepatent period of 5-10 days. The excretion of oocysts was intermittent and lasted till the death of puppies on the 30th or 32nd day post-infection.

McAllister *et al.* (1998), recorded that the prepatent period after infection of dog was 8 days and excretion of oocysts lasted for 7-19 days, as well as, experimentally infected mice died after infection.

The long patent period recorded in the present studies may be ascribed to the low severity of infection, or to the decreased number of cysts in brain of rats given to the puppies.

Lindsay *et al.* (1999a), found that the excretion of Neospora oocysts from infected dogs began after 5-10 days inclusive and on day 17 after ingestion of tissue cysts, as well as, oocysts sporulated within 24 hours at 37°C. Each oocyst contained 2

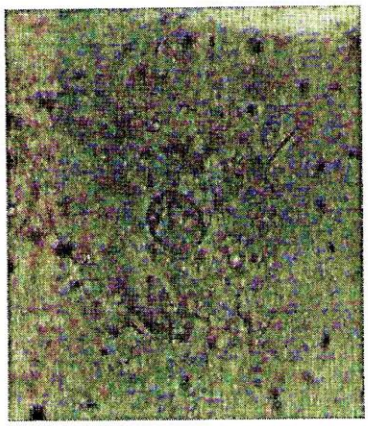


Fig. 1. Unsporulated oocyst of *N. caninum.* X 1250

Fig. 2. Sporulated oocyst of *N. caninum.* X 1250

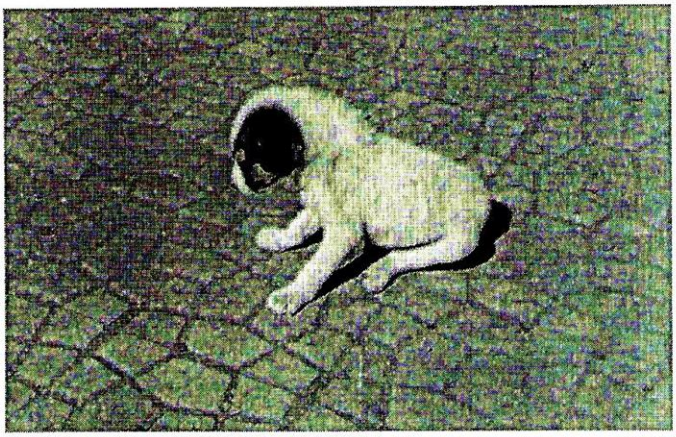


Fig. 3. Experimentally infected puppy with *N. caninum* showing paralysis of hind limb.

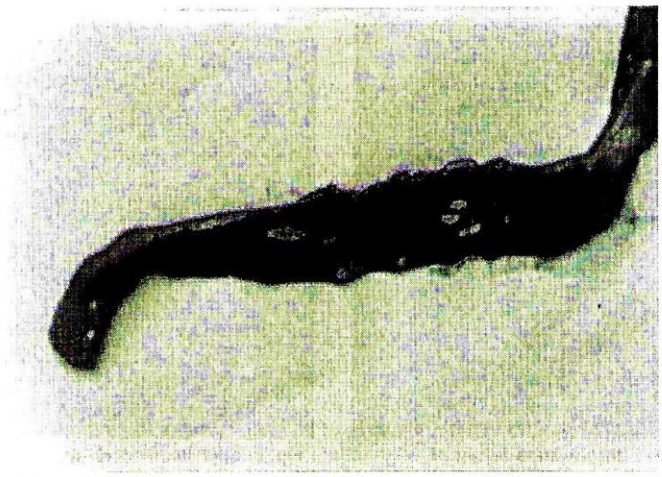


Fig. 4. Intestine of experimentally infected puppy showing congestion.

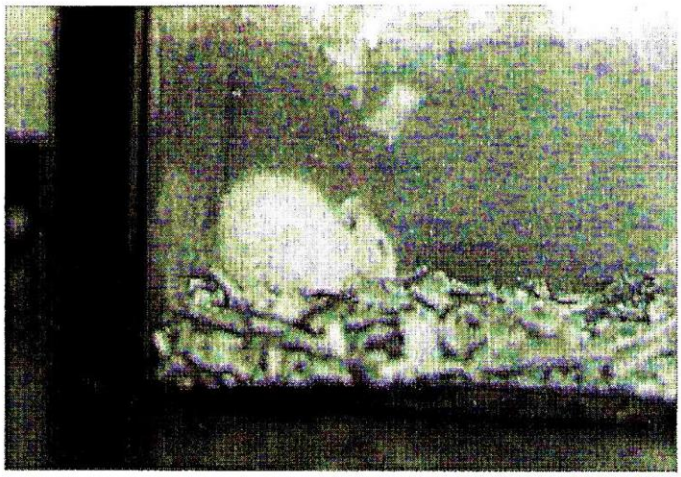


Fig. 5. Experimentally infected rat with *N. caninum* showing rough hair coat and curled back.

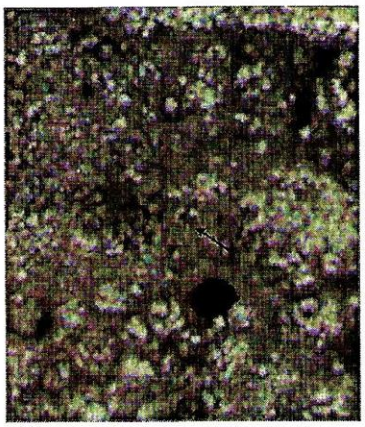


Fig. 6. Brain smear stained with Giemsa from experimentally infected rat showing tachyzoites x 1250

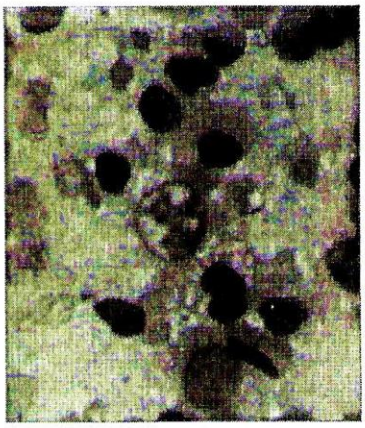
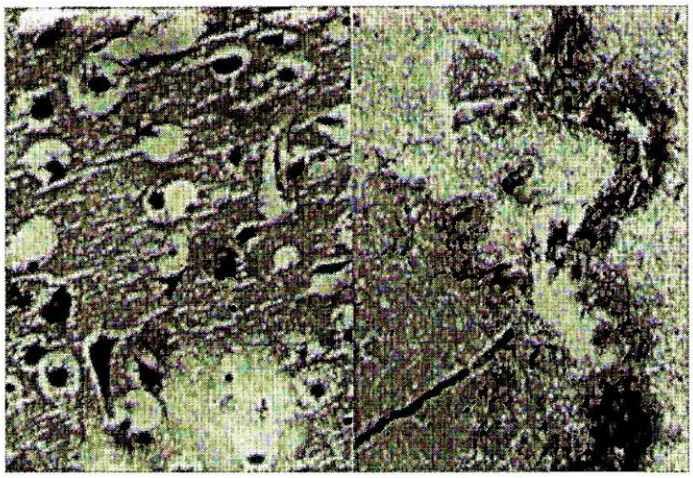


Fig. 7. Brain smear stained with Giemsa from experimentally infected rat showing tissue cyst containing bradyzoites x 1250



a b

Fig. 8. (a and b) Brain of puppy showing (a) oedema in the Virshow Rubin space and periastrocytes, malacia with degeneration and necrosis of the nerve cells, (b) vasulitis with perivascular inflammatory cells infiltration and demylination. (a) H and E x 400, (b) H and E x 250

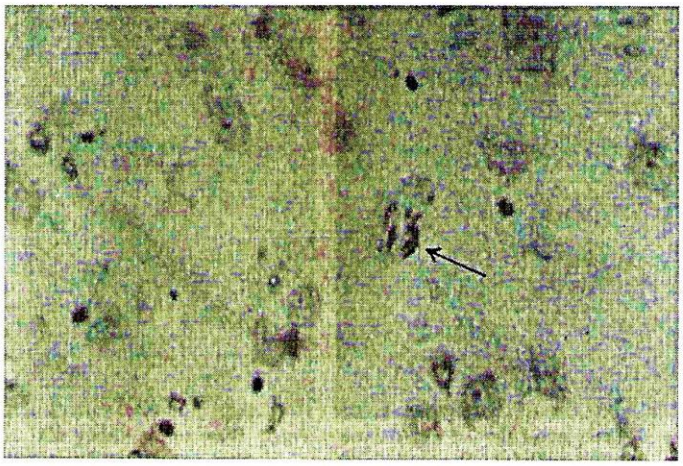


Fig. 9. Brain of puppy showing *N. caninum* cyst with thin wall (arrow) in between the degenerated nerve cells, Giemsa stain x 1000

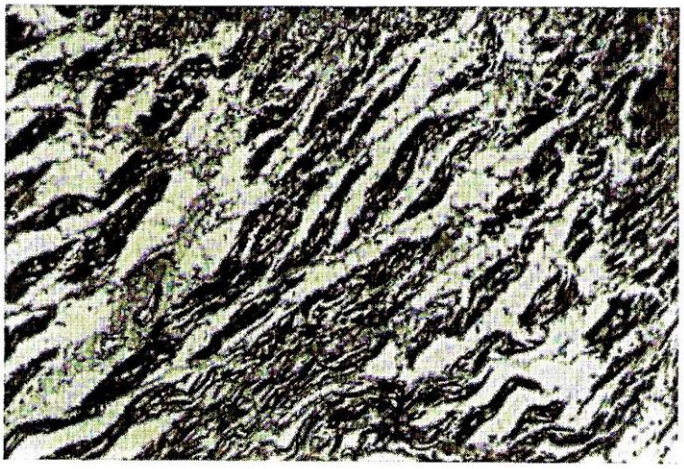


Fig. 10. Skeletal muscle of the thigh of puppy revealing degeneration and necrosis of the muscle fibres with infiltration of inflammatory cells. H and E x 250

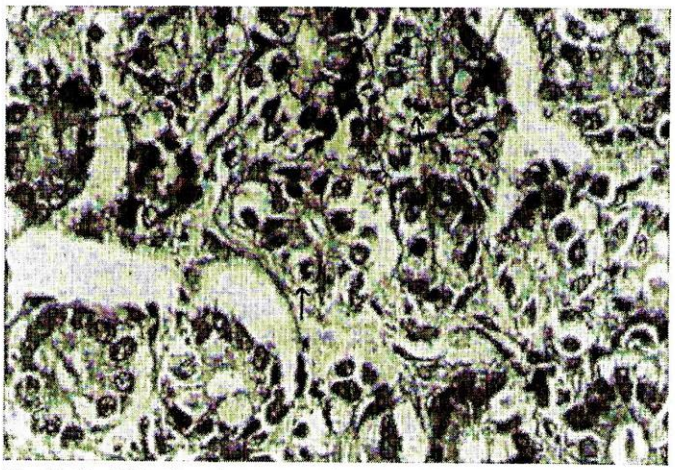


Fig. 11. Small intestine of puppy revealing tachyzoites in parasitophorous vacuole or present directly in the host cell cytoplasm within the lining epithelium (arrows) Giemsa stain x 400

Fig. 12. Brain of rat showing massive gliosis, necrosis of nerve cells and oedema in Virshow Rubin spaces and periastrocystes. H and E x 160

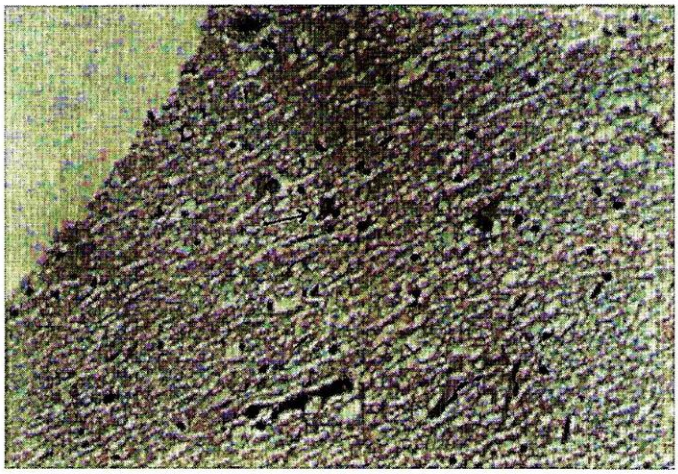


Fig. 13. Brain of rat showing aggregation of small groups of tachyzoites around degenerated nerve cells. H and E x 1000

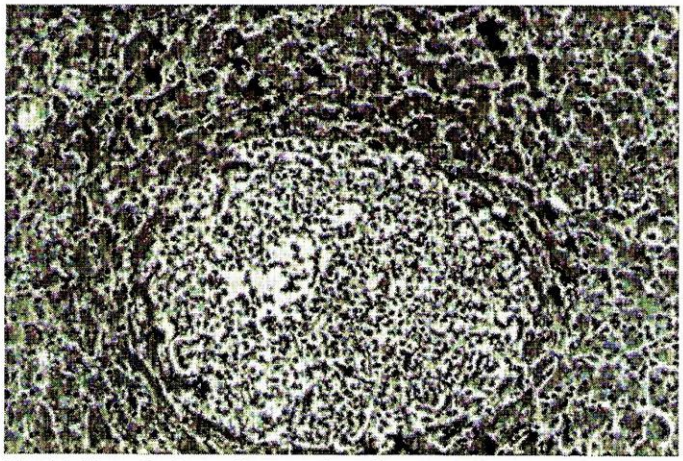


Fig. 14. Liver of rat revealing granuloma formation with aggregation of neutrophils, lymphocytes and macrophages. H and E x 160

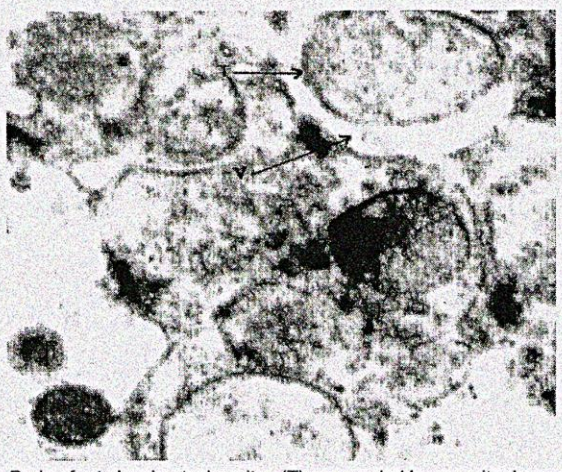



Fig. 15. Brain of rat showing tachyzoites (T) surrounded by parasitophorous vacuole (V). x 18000

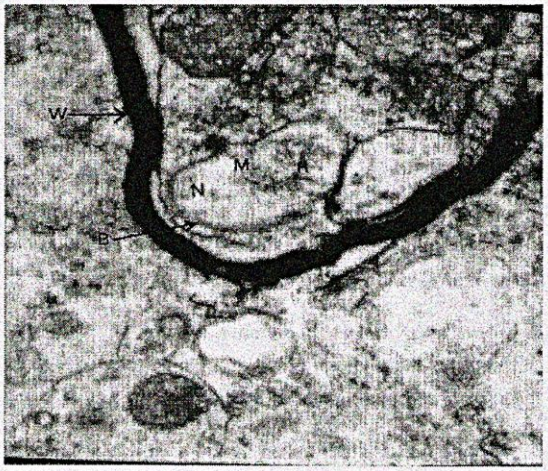



Fig. 16. Brain of rat showing tissue cyst surrounded by cyst wall (W) and containing bradyzoite (B) which consisted of subterminal nucleus (N) micropore (M) and rhoptries (R) X 11000

sporocysts each with 4 sporozoites. Accurate details about Neospora oocysts were explained by Lindsay *et al.* (1999b). They gave clear appearance on the definite characteristics of the oocysts.

In the present studies, unsporulated oocysts measured 11.25 x 8.75 µm and did not contain micropyle. They sporulated 10 days post-incubation at 25°C. The difference in the sporulation time in the present study may be ascribed to the low temperature degree applied. The sporulated oocyst measured 17.5 x 10 µm and contained 2 sporocysts each measured 10 x 8.75 µm and contained 4 sporozoites.

McAllister *et al.* (1998), stated that unsporulated oocysts were 11-12 µm. The measurements were in agreements with those found in the present study. Experimental infection of puppies revealed that the puppies were off food before death and one of them showed myonecrosis, encephalitis and paralysis of hind limb then died 32 days post-infection. This fact was in agreement with Dubey and Lindsay (1996). They stated that young dogs infected with *N. caninum* developed hind limb paresis that developed into progressive paralysis. Hay *et al.* (1990), recorded difficulty swallowing and paralysis of the jaw in dogs. After death of puppies, post-mortem examination revealed congestion of small and large intestines. As well, examination of fresh scrapings from intestines revealed unsporulated oocysts in the ileum.

Pathological lesions reported in puppies were necrosis in brain and liver (Dubey *et al.*, 1988a), granulomas reaching 1 cm in diameter in visceral tissues (Dubey *et al.*, 1988b). In the present study, most of the lesions observed in the examined tissues from infected puppies and rats were similar to those seen in exprimentally infected dogs and kittens detected by Dubey and Lindsay (1989) and in naturally infected dog detected by Uggla *et al.* (1989).

The diversity of neural lesions consisting of malacia, neuritis, gliosis and focal perivascular infiltration of mononuclear cells in the rats infected with *N. caninum* may be useful in the study of neosporosis in other animals.

As well, this study revealed tachyzoites in many cells including neural and intestinal cells in puppies. Dubey and Lindsay (1996) reported hind limb paralysis in dogs infected with *N. caninum.* They stated that tachyzoites should be looked for in central nervous system. However, tachyzoites detected in this study in parasitophorous vasu-

oles were seen in the cytoplasm of the lining epithelium of the small intestine of the experimentally infected puppies. From the extent of our knowledge, this was considered to be the first reported case of Neospora tachyzoites in intestine of dogs. Gray *et al.* (1996), detected a case of visceral neosporosis in mare. They stated that most lesions were confined to intestines and mesenteric lymph nodes. Tachyzoites penetrated host cells by active invasion and became intracellular within 5 minutes by contact with host cells (Hemphill *et al.*, 1996). This was in agreement with the present study in which tachyzoites were located within the host cell cytoplasm.

Concerning tissue cysts of *N. caninum*, Dubey *et al.* (1988a), stated that they were round to oval in shape and had been observed only in neural tissues. Dubey and Lindsay (1996) found that his parasite was capable of producing grossly visible necrotic lesions in few days and caused cell death by the active multiplation of tachyzoites.

In the present study, tissue cysts were not surrounded by a zone of host reaction but, some cases showed inflammatory cells aggregation around degenerative cysts and bradyzoites. Dubey *et al.* (1996b), suggested that some tissue cysts ruptured and the subsequent host reaction caused foci of inflammation. Dubey and Lindsay (1996) stated that degenerative to inflammatory lesions may be found throughout the visceral organs of infected dogs, but were most common in the central nervous system, heart, skeletal muscles and liver. In this respect, Walsh *et al.* (2000), detected severe lesions in various organs in mice infected with *N. caninum*, but definitive tissue cysts of the parasite were not identified in the brain or other tissues.

In conclusion, the present studies emphasized the parasitological and pathological effects caused by *N. caninum* in experimentally infected puppies. Encephalitis in rats and severe neuromuscular lesions in puppies due to the destruction of large numbers of neural cells were the most common lesions encountered. As well, tissue cysts of *N. caninum* were detected only in the neural tissues and tachyzoites in host cell cytoplasm, or present diffuse or in groups in the tissue.

From the zoonotic point of view, there has been no clear evidence that this protozoan parasite could be transmissible to man and further studies are needed to emphasize its role between animals and man.

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# دراسات تجريبية طفيلية وباثولوجية لطفيل نيوسبورا كانينم فى الكلاب

**ماجدة عبد الحى الرفاعى، أحمد أنور وهبة، جيهان جميل شهاب**

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نيوسبورا كانينم من الطفيليات الأولية، تم عزل هذا الطفيل من مخ الجرذان المعدية طبيعياً ثم تم عدوى الكلاب تجريبياً. بعد مرور خمسة إلى عشرة أيام بعد العدوى بدأ ظهور الحويصلات الغير متجرثمة (١١,٢٥ × ٨,٧٥ ميكرون) فى براز هذه الكلاب. وقد تم عدوى جرذان تجريبياً بهذه الحويصلات المتجرثمة (١٧,٥ × ١٠ ميكرون).

عقب النفوق، أخذت مسحات من أمعاء الكلاب ومسحات من مخ الجرذان وتم صبغها بصبغة جيمسا للفحص الطفيلى، فى نفس الوقت، أخذت عينات من الأنسجة المختلفة من الأعضاء الداخلية من الكلاب والجرذان للفحص الهيستوباثولوجى. كما تم فحص مخ الجرذان بالميكروسكوب الإلكترونى.

وقد أسفر فحص المسحات من الأمعاء الدقيقة للكلاب عن وجود الحويصلات الغير متجرثمة بينما مسحات المخ المصبوغة أثبتت وجود التاكيزويت وحويصلات النيوسبورا كانينم.

أظهر الفحص الهيستوباثولوجى لكل من الكلاب والجرذان وجود إلتهاب المخ السحائى وكذا التهاب بالكبد والكلى. كما لوحظ فى بعض الحالات ظهور التهاب رئوى بينى بالإضافة إلى وجود التهاب بالقلب والعضلات فى الكلاب. وقد وجدت حويصلات طفيل النيوسبورا والتاكيزويت بأنسجة مخ الفئران، وأيضاً التاكيزويت فقط بأنسجة المخ وبداخل الخلايا الطلائية المبطنة لجدار الأمعاء الدقيقة فى الكلاب.

فى نفس الوقت، أثبت فحص مخ الجرذان بالميكروسكوب الإلكترونى عن وجود التاكيزيوت وحويصلات هذا الطفيل.

وقد تم مناقشة الأهمية الطفيلية والباثولوجية لطفيل نيوسبورا كانينم فى كل من الكلاب والجرذان.