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IDENTIFICATION OF TOXOPLASMA GONDII
AND ENCEPHALITOOZON CUNICULI
BY IMMUNOPEROXIDASE TECHNIQUES AND
ELECTRON MICROSCOPY, IN STORED,
FORMALIN-FIXED, PARAFFIN-EMBEDDED
TISSUE

By

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BJERKÅS, I. and T. LANDSVERK: *Identification of Toxoplasma gondii and Encephalitozoon cuniculi by immunoperoxidase techniques and electron microscopy, in stored, formalin-fixed, paraffin-embedded tissue.* Acta vet. scand. 1986, 27, 11—22. — The peroxidase-antiperoxidase (PAP), avidin-biotin-complex (ABC) techniques, and electron microscopy, were used to identify protozoan parasites in formalin-fixed material from routine necropsy cases. The material comprised paraffin blocks, that had been stored for up to 18 years, from 18 cases of suspected toxoplasmosis and encephalitozoonosis. With the ABC method a higher dilution of primary antibody could be used than with the PAP method. However, with both methods, a distinct reaction occurred with appropriate dilutions. The age of the material did not seem to effect the result. Frequently, phagocytized and necrotic parasites were also stained. Cystozoites (bradyzoites) of *T. gondii* were stained faintly compared to the endozoites (tachyzoites). A *Toxoplasma*-like parasite from a dog did not react with anti-*Toxoplasma* serum, and ultrastructurally it proved to be consistent with an unidentified cyst-forming sporozoan parasite previously reported in dogs. Electron microscopy based on paraffin-embedded tissue, seems to be a valuable method for identification of protozoan parasites, and thus provide a supplement or alternative to the immunoperoxidase methods.

protozoan parasites; histologic identification; immunohistochemistry; ultrastructure.

The advent of immunoperoxidase methods, and in particular, the most sensitive peroxidase-antiperoxidase (PAP) (*Sternberger et al.* 1970) and avidin-biotin-complex (ABC) methods (*Guesdon et al.* 1979, *Hsu et al.* 1981, *Wood & Warnke* 1981) has drama-

tically increased the possibility of establishing an etiological diagnosis in infectious disorders when only formalin-fixed tissue is available. This is relevant in connection with, for example, disorders due to protozoan parasites which are often difficult to demonstrate in sections treated by conventional histologic methods, either because the parasites are present only in small numbers in the sections, or because the parasites are difficult to distinguish from other structures. Likewise, in many cases identification of protozoa cannot be made solely on the basis of the light microscopic appearance.

Though *Andres et al.* (1981) and *Dutton et al.* (1984) were successful in applying the PAP method in toxoplasmosis, so far only limited efforts have been made to employ the new immunoperoxidase methods for the detection of other protozoan parasites. Apparently, no retrospective studies have hitherto been reported, and consequently little is known about the stability of protozoan antigens after storage in paraffin-embedded tissue, the usual means of storage practised in histologic laboratories.

Electron microscopy represents an alternative method for diagnosis when formalin-fixed tissue is available (*Scholtzsek & Mehlhorn* 1970, *Sulzer et al.* 1979), but reports on the fitness of paraffin-embedded and even stored tissue, for fine-structural identification of protozoan parasites, are apparently lacking.

This paper reports a retrospective study in which the PAP and ABC techniques were applied in demonstrating and identifying *T. gondii* and *E. cuniculi* in histologic sections from routine necropsy material. In addition, the identity of the parasites was confirmed by electron microscopic examinations based on deparaffinized material.

MATERIALS AND METHODS

The examinations were based on paraffin-embedded, formalin-fixed tissue from routine necropsy cases, in which *Toxoplasma*-like or *Encephalitozoon*-like structures were demonstrated in histologic sections.

Toxoplasmosis

Cases of suspected toxoplasmosis included cats, hares, blue foxes, and a dog (Table 1). The sections from the hares were obtained from the Department of Wildlife Diseases, the National

Veterinary Institute, Oslo. The sections from the foxes were obtained from the Regional Veterinary Laboratory in Bergen. Liver sections from mice inoculated intraperitoneally or subcutaneously with the RH-strain of *T. gondii*, served as positive controls.

Encephalitozoonosis

The cases of suspected encephalitozoonosis included young blue foxes and a rabbit (Table 1) in which Encephalitozoon-like structures were stained by the modified Gram method (*Petri* 1969). The positive controls were spontaneous cases exhibiting a great number of parasites, which were identified on the basis of ultrastructural criteria in material fixed immediately post mortem in 3 % buffered glutaraldehyde.

Table 1. Case material.

Case no.	Host species	Year of necropsy	Immunoperoxidase studies	Electron microscopic studies
<i>Toxoplasma-like parasites</i>				
1	Cat	1968	Brain	Brain
2	Cat	—84	Myocardium, liver	Myocardium
3	Dog	—67	Brain, spinal cord	Spinal cord
4	Blue fox	—84	Kidney, lung	Kidney
5	„ „	—84	„ „	—
6	Hare	—80	Liver	Liver
7	„	—80	„	„
8	„	—80	„	—
9	„	—82	„	—
10	„	—82	„	—
11	„	—82	„	Liver
12	„	—82	„	—
<i>Encephalitozoon-like parasites</i>				
13	Blue fox	1982	Brain, kidney	Kidney
14	„ „	—82	Brain, kidney	Brain
15	„ „	—82	Kidney	—
16	„ „	—82	„	—
17	„ „	—78	„	—
18	Rabbit	—78	„	—

Immunoperoxidase methods

The specific rabbit anti-*Toxoplasma* serum, produced by inoculation of endozoites (tachyzoites) of the RH-strain, was

obtained from the Norwegian Defence Microbiological Laboratory, Oslo. The rabbit anti-Encephalitozoon serum was obtained from Testman A/S, Uppsala, Sweden). The PAP reagents (swine anti-rabbit antibody, horseradish peroxidase, and rabbit anti-horseradish peroxidase antibody) were obtained from Dakopatts A/S, Copenhagen, Denmark. Vectastain® ABC Kit (Vector Laboratories, Burlingame, California, USA) was used when applying the ABC technique.

P A P m e t h o d. The sections were cut at about 4 μm and placed for 30 min in an oven at 60°C. The sections were immediately transferred to a fresh xylene bath, and then hydrated in graded alcohols. The rehydrated sections were incubated for 30 min with 3 % hydrogen peroxide in methanol, in order to inactivate endogenous peroxidases. They were then immersed for 20 min in phosphate-buffered saline (PBS: 137 mmol/l sodium chloride, 2.7 mmol/l potassium chloride, 1.5 mmol/l potassium dihydrogen phosphate, 8 mmol/l disodium hydrogen phosphate-2-hydrate) pH 7.3, and incubated for 20 min with dilute normal swine serum to prevent non-specific background staining. Excess serum was blotted up around the sections. The sections were subsequently subjected to the following step-wise procedure; incubation for 30 min with the primary antiserum diluted in PBS, washing for 10 min in PBS, incubation for 30 min with dilute swine anti-rabbit serum (1:40), washing in PBS, incubation for 20 min with peroxidase-antiperoxidase complex, washing in PBS, and then in Tris-maleic buffer (200 mmol/l Tris-(hydroxymethyl)-aminomethane, 200 mmol/l maleic acid) pH 7.2, incubation for 10 min with enzyme substrate (0.5 mg 3,3'-diaminobenzidin-4HCl per ml, 0.01 fresh solution of 1 % hydrogen peroxide per ml, 50 mmol/l Tris-maleic buffer, pH 7.2), washing for 5 min with distilled water, and counterstaining for 2 min with hematoxylin. The sections were finally mounted in glycerin jelly.

A B C m e t h o d. The test was performed according to the procedure stated by the manufacturer (Vector Laboratories). In this procedure, normal goat serum was used to prevent non-specific background staining. After the incubation with primary antisera for 30 min, the sections were washed in PBS and incubated for 30 min with diluted biotinylated goat antibody to rabbit immunoglobulins. After washing in PBS, the sections were

incubated for 30 min with peroxidase conjugated biotin-avidin complex. The sections were then washed in PBS and Tris-maleic buffer and substrate solution was applied.

Control sections were included in which the primary rabbit antiserum was replaced by normal rabbit serum.

Ultrastructural studies

Tissue from most of the cases (Table 1) was prepared for electron microscopy. A small piece of tissue, corresponding to an area containing parasitic structures in the light microscopic sections, was cut out of the paraffin block. The tissue was deparaffinized, rehydrated in alcohols, post-fixed in osmium tetroxide, dehydrated in alcohols, and embedded in Epon. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Immunoperoxidase studies

Both in cases of suspected toxoplasmosis and encephalitozoonosis a distinct brown staining occurred when specific antisera were used. At optimal dilutions of primary antisera, background staining was minimal both with the PAP and ABC method. However, the ABC method was more sensitive than the PAP method. The age of the paraffin blocks did not seem to affect the results.

Toxoplasma-like parasites. In all cases except in the dog (Case 3), parasites were stained when anti-Toxoplasma serum was used as primary antiserum (Figs. 1, 2 and 3). The zoites were stained brown, with an even more distinct brown rim at the periphery, emphasizing the shape of the parasites. Endozoites were always strongly stained, and a staining along the membrane of the parasitophorous vacuole usually being observed. In addition, necrotic material and structures in the cytoplasm of macrophages were sometimes stained. However, there was no reaction when normal rabbit serum or anti-Encephalitozoon serum was used as primary antiserum. In the mice (control cases) and the hares (Cases 6—12), only endozoites were observed. Autolytic changes were relatively prominent in some of the hares, and endozoites could hardly be distinguished

in normally treated sections. Even in these cases they were easily demonstrated with the immunoperoxidase methods. In Case 1, only cyst stages were observed, while in Cases 2, 4 and 5, both endozoites and cyst stages occurred. The cystozoites (bradyzoites) were stained less strongly than the endozoites (Fig. 3). Extended incubation with primary antibody increased the intensity of staining only moderately. Usually, there was some staining of the cyst wall. In the dog (Case 3), neither endozoites nor cystozoites were stained.

Encephalitozoon-like parasites. In all cases of suspected encephalitozoonosis, parasitic organisms resembling spores were stained distinctly when anti-Encephalitozoon serum was used (Figs. 4, 5 and 6). Whether proliferative stages also were stained, could not be determined. In addition, there was a distinct reaction along the membrane of the parasitophorous vacuole (Figs. 4 and 6). No reaction was observed using normal rabbit serum, or anti-Toxoplasma serum as primary antiserum. Most spores that could be distinguished in the cytoplasm of macrophages, appeared to be stained (Fig. 5). This was in con-

Figure 1. Scattered endozoites (tachyzoites) of *T. gondii*, reacting with anti-Toxoplasma serum; mouse liver (control case). ABC technique; counterstained with hematoxylin. $\times 400$.

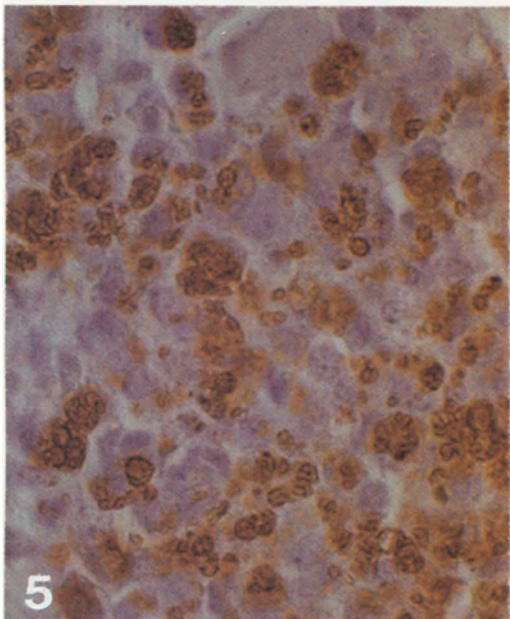
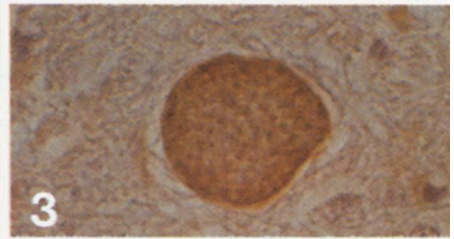
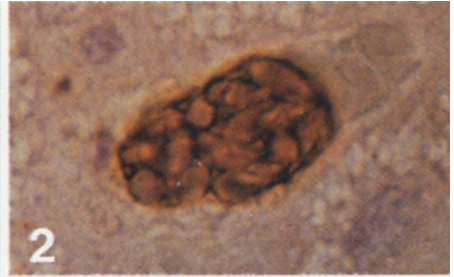
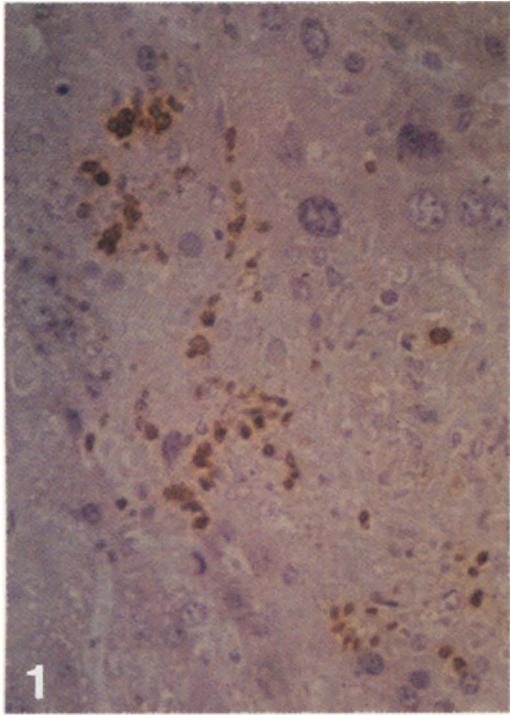
Figure 2. Parasitophorous vacuole containing endozoites of *T. gondii*, reacting with anti-Toxoplasma serum; liver of a mouse (control case). ABC technique; counterstained with hematoxylin. $\times 1,700$.

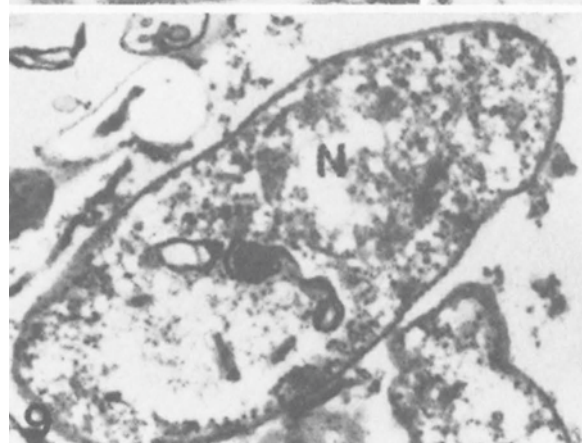
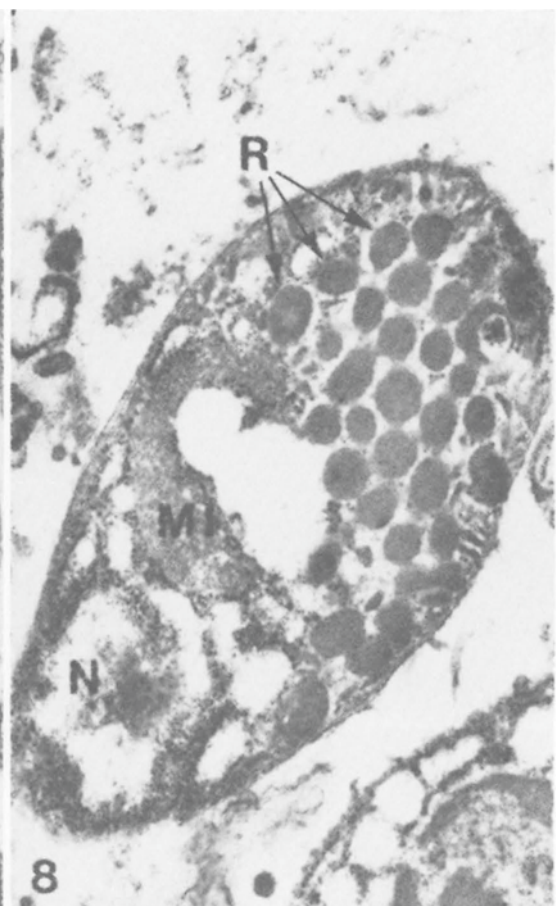
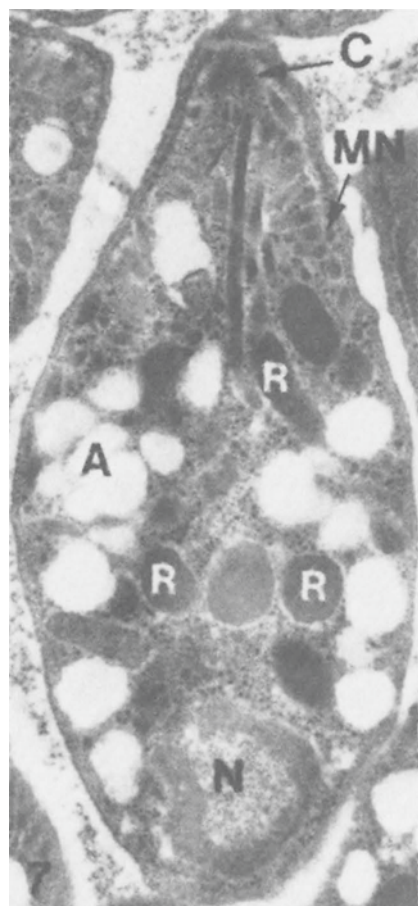
Figure 3. Cyst stage reacting with anti-Toxoplasma serum; brain of a cat (Case 1). Note the less intense staining of the cystozoites compared with the endozoites in Figs. 1 and 2. ABC technique; counterstained with hematoxylin. $\times 700$.

Figure 4. Parasitophorous vacuole with parasites reacting with anti-Encephalitozoon serum; kidney of a blue fox (Case 14). Note the strong reaction outlining the membrane of the parasitophorous vacuole. ABC technique; counterstained with hematoxylin. $\times 700$.

Figure 5. Numerous phagocytized parasites reacting with anti-Encephalitozoon serum; brain granuloma of a blue fox (Case 13). ABC technique; counterstained with hematoxylin. $\times 700$.

Figure 6. Parasitophorous vacuole with parasites reacting with anti-Encephalitozoon serum; kidney of a blue fox (Case 14). A few spores are also seen in the tubular lumen (arrow). ABC technique; counterstained with hematoxylin. $\times 1,700$.





trast to the many unstained spores occurring in sections stained by the modified Gram method. In the sections from the rabbit (Case 18), there was no definite increase in the intensity of background staining, although antibodies against rabbit immunoglobulins were used as secondary antibody.

Ultrastructural studies

Toxoplasma-like parasites. In contrast to the surrounding host tissue, the fine structures of the parasites were comparatively well preserved in most cases. In all cases but the dog, the fine structures were consistent with those of merozoites of *T. gondii* (Fig. 7). The nucleus, conoid, rhoptries, and the vacuoles of the amylopectin granules could be distinguished even in necrotic merozoites. The mitochondrion was usually less distinct. The subpellicular microtubules and the micronemes were fairly well preserved in most cases, but could hardly be distinguished in some of the hares. Division by endodyogeny was observed in several cases.

The parasites seen in the cats and hares had no more than 9 rhoptries, while the parasite in the dog (Case 3) frequently had 11, and sometimes more than 20, rhoptries (Fig. 8). In addition, this parasite contained only few amylopectin granules.

Encephalitozoon-like parasites. Both spores and proliferative stages of the parasites were studied. The spores

Figure 7. Electron micrograph of a cystozoite with fine structures consistent with *T. gondii*; myocardium of a cat (Case 2). A, amylopectin granules; C, conoid; MN, micronemes; N, nucleus; R, rhoptries. $\times 30,000$.

Figure 8. Electron micrograph of an endozoite that is different from merozoites of *T. gondii*; spinal cord of a dog (Case 3). Note the numerous rhoptries (R). MI, mitochondrion; N, nucleus. The section does not include the anterior end of the parasite. $\times 30,000$.

Figure 9. Electron micrograph of a microsporidian sporont in a parasitophorous vacuole; brain tissue of a blue fox (Case 13). Note the single nucleus (N). The absence of diplocarya in the developmental cycle suggests *Encephalitozoon*. $\times 25,000$.

Figure 10. Electron micrograph of a typical microsporidian spore in a renal tubular cell of a blue fox (Case 14). Note the coiled filament (arrows). $\times 39,000$. Inset: Higher magnification of the coiled filament. $\times 70,500$.

and sporoblasts were well preserved. A coiled filament could be distinguished in many of these (Fig. 10). The schizonts and sporonts were apparently very brittle structures, and many were so poorly preserved that no organelles could be distinguished. However, in some, a single nucleus could be observed (Fig. 9).

DISCUSSION

The immunoperoxidase methods appear to be very useful in demonstrating protozoa in histologic sections. In the present cases, the morphology of the parasite was so excellently demonstrated that any confusion with non-specifically stained structures appears inconceivable.

Though the ABC method had a higher sensitivity than the PAP method, the specificity was not essentially better at optimal dilutions of the primary antisera. The specificity will of course depend on the specificity of the antiserum which might contain antibodies against other agents, or the specific antibodies might cross-react with other parasites. Concerning *T. gondii* antibodies, cross-reaction with *Hammondia* spp. must be expected. Animals infected with *H. hammondi* develop antibodies against *T. gondii* (Christie & Dubey 1977). Araujo *et al.* (1984) found antigenic similarity between these two parasites. Likewise, *Hammondia* can probably not be differed from *T. gondii* on the basis of ultra-structural criteria. However, the pathogenicity of *Hammondia* is probably very modest (Dubey 1977). Whether there is cross-immunity between *E. cuniculi* and other parasites, is not known. However, nearly all mammalian microsporidian pathogens so far investigated, appear to belong to the genus *Encephalitozoon*. In addition, it seems likely that only one species is involved (Montrey *et al.* 1973, Barker 1975, Pakes *et al.* 1975, Shadduck *et al.* 1978, Botha *et al.* 1979, Mohn *et al.* 1981).

The less intense staining of the cystozoites compared with the endozoites of *T. gondii*, may be due to antigenic differences. Lunde & Jacobs (1983) found that antisera against endozoites of *T. gondii* reacted only partially with cystozoites, while antisera against cystozoites did not cross-react at all with endozoites. Hyperimmune sera made by inoculation of endozoites alone may contain antibodies almost exclusively against endozoites, since the antigens of the later developing cystozoites may not be accessible to the immune system because of sequestration inside

the cyst wall. Another reason for the different reactions may be that because the cystozoites are tightly packed and surrounded by a dense material, the antigens are rendered less available for antibodies in the immunological tests.

The positive staining along the membrane of the parasitophorous vacuoles both of *T. gondii* and *E. cuniculi*, and the cyst wall of *T. gondii*, could suggest the presence of antigenic deposits derived from the parasites. The cyst wall of *T. gondii* is thought to be formed by deposition of material from the parasites on the membrane of the parasitophorous vacuole (*Jacobs & Frenkel* 1982), and might therefore contain parasitic antigens.

The immunoperoxidase methods appeared particularly useful in demonstrating parasites in autolytic tissue containing only endozoites of *T. gondii*. Such parasites are extremely difficult to detect in routine sections. In the cases of encephalitozoonosis, the immunoperoxidase methods must be regarded as more specific than the modified Gram method. In addition, phagocytized and probably partly degraded spores were stained to a greater extent with the immunoperoxidase methods.

The negative result of the immunoperoxidase test on the parasite in the dog (Case 3), is in agreement with the ultrastructural findings, suggesting that this parasite is identical with the cyst-forming sporozoan found in Boxer dogs by *Bjerkås et al.* (1984). Nor did this parasite react positively with anti-*Toxoplasma* serum (unpublished).

All stages of the life cycle of *Encephalitozoon* contain only one single nucleus, and multiply within a parasitophorous vacuole of the host cell, in contrast to the parasites within the genus *Nosema* (*Cali* 1970). However, in the present cases, the proliferative stages were often insufficiently preserved for fine-structural analysis. On the other hand, the occurrence of typical microsporidian spores must be regarded as a fairly reliable criterion for diagnosis, since nearly all mammalian microsporidian pathogens so far investigated, are probably identical to *E. cuniculi* (*Montrey et al.* 1973, *Barker* 1975, *Pakes et al.* 1975, *Shaddock et al.* 1978, *Botha et al.* 1979, *Mohn et al.* 1981).

Paraffin blocks that had been stored for several years were still suitable for both electron microscopic and immunoperoxidase studies. These observations mean that it is possible to do ultrastructural in addition to immunohistochemical studies in most cases, even if there are few parasites present in the

tissue. Areas with parasites and/or lesions can be cut out from corresponding areas in the paraffin block, thus increasing the chance of detecting parasites.

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SAMMENDRAG

Identifisering av Toxoplasma gondii og Encephalitozoon cuniculi ved hjelp av immunoperoksydase-teknikk og elektronmikroskopi i lagret, formalin-fiksert, parafin-innstøpt vevsmateriale.

Peroksydase-antiperoksydase- (PAP) og avidin-biotin-kompleks-teknikk (ABC) ble benyttet til å identifisere protozoer i formalin-fiksert materiale. Materialet besto av parafinblokker fra 18 kasus hvor det var mistanke om toxoplasmosis eller encephalitozoonose. Enkelte blokker hadde vært lagret så lenge som 18 år. ABC-metoden var mer sensitiv enn PAP-metoden, men ved passende fortykning av primært antistoff ble det tydelig reaksjon ved begge metodene. Materialets alder syntes ikke å påvirke resultatet. Fagocytterte og nekrotiske parasitter ble også ofte farget. Cystozoitte (bradyzoitte) av *T. gondii* ble farget svakere enn endozoitte (tachyzoitte). En *Toxoplasma*-lignende parasitt fra hund reagerte ikke med anti-*Toxoplasma* serum. Elektron-

mikroskopisk undersøkelse tydet imidlertid på at den var identisk med en cyste-dannende sporozo som er beskrevet tidligere hos hund. Elektronmikroskopi basert på parafin-innstøpt materiale ser ut til å være en egnet metode for identifisering av protozoer og representerer således et supplement eller alternativ til immunoperoksydase-teknikkene.

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