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Original article

Finding of *Neospora caninum* in the wild brown rat (*Rattus norvegicus*)

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Abstract – Nine rats (16.4%) out of 55 (*Rattus norvegicus*) from cattle farms were seropositive to *Neospora caninum*. Two of the seropositive rats were also PCR positive but all were negative by immunohistochemistry and PAS staining. The brains of all the captured rats were homogenized and initially inoculated intraperitoneally into nude mice or into SPF ICR mice, which had been immunosuppressed with prednisolone. One mouse that was inoculated with brain material from a seropositive rat became infected with *N. caninum*, as demonstrated by the presence of a tissue cyst in the brain and confirmed by immunohistochemistry and PCR. This is the first finding of *N. caninum* in naturally infected farm rats. The findings show that natural *N. caninum* infection occurs in wild brown rats and thus rats may serve as a reservoir for the protozoan on the cattle farm.

***Neospora caninum* / rat / cattle farm / natural infection**

1. INTRODUCTION

Neospora caninum is known to cause neuromuscular paralysis in dogs and cattle, and also abortion or stillbirth in cattle, sheep, goats, horses and deer [3]. It has been reported in the United States [5], Australia [15], many European countries [2, 14, 18], and Asian countries such as Japan [12], Korea [7], Taiwan [13], Thailand [6] and Vietnam [4].

Since the great economic loss brought about by *N. caninum* is through the mid-term abortion of cattle, complete elucidation of the life cycle of this protozoan is deemed important. Besides the presence of infected cattle and dogs, we asked the question if there are any other animals in the cattle farm scene that might play a role in the maintenance of *N. caninum* in the nature. One obvious animal that is present on most cattle farms is the wild rodent. Although there are no reports of naturally occurring neosporosis in rodents, experimental infection of *N. caninum* in rats has been demonstrated [8]. Experimentally infected rats have also been shown to produce IgG against the protozoan [16]. This provides the basis for our seroprevalence study of naturally infected rats. We investigated the possibility that these rodents might be spontaneously infected with *N. caninum* and

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thus serve as a reservoir host as well as an indicator host for this protozoan on the cattle farm, by examination of seroprevalence, a histopathological observation, direct detection of the parasite DNA in brain tissues using PCR and indirect detection by inoculating the wild rat brain homogenates into SPF mice.

2. MATERIALS AND METHODS

2.1. Wild rodents

A total of 55 rats, all identified as *Rattus norvegicus*, were captured using single cage traps on six cattle farms, of which five were dairy and one was beef cattle farms. Four of the dairy farms and the beef cattle farm were located in Taichung County, Central Taiwan and one dairy cattle farm in Pintung County, Southern Taiwan, respectively. All the dairy cattle farms had a history of neosporosis-associated bovine abortion. The captured rats were killed with an overdose of ethyl ether anesthesia and necropsy was performed on them. For each captured rat, half of their brains, heart, lung, liver, spleen, pancreas, kidney, intestine and skin were fixed in formalin for histology. The other halves of the rat brains were homogenized for both intraperitoneal inoculation into nude mice or into SPF ICR mice and also for detection of the protozoan DNA by PCR. Their sera were collected and stored at –20 °C until use.

2.2. Laboratory mice

Six-week-old athymic nude mice (BALB/cByJ-nu) and four-week-old SPF ICR mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. The animals were housed in autoclaved cages and bedding, and were fed an autoclaved rodent diet (Lab Diet®, Castom Laboratory Animal Nutrition Products Contact) and autoclaved water, ad libitum. The ICR mice were injected subcutaneously with prednisolone succinate (Lyo-Donison®, China Chemical & Pharmaceutical, Hsinchu, Taiwan) at 3 mg/mouse/time, 6 and 3 days before the inoculation of the rat brain homogenate.

2.3. Detection of antibodies to *N. caninum*

Screening for antibodies against *N. caninum* in captured rat sera was carried out by the indirect fluorescence antibody test (IFAT) using tachyzoite antigen commercially obtained from Kyoto Biken, Uji-shi, Kyoto, Japan. The sera were screened at 1:50 dilution. For secondary antibody, FITC (fluorescein isothiocyanate)-conjugated affinity purified goat anti-rat IgG, obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) was used. The immunostained tachyzoites were observed under ultraviolet light with a fluorescence microscope. Diffused or peripheral staining of the tachyzoites was considered positive. Apical or polar staining of the tachyzoites was considered negative. Rat sera that were found to be positive at a 1:50 dilution were further checked for their antibody titer up to a 1:200 dilution.

2.4. Detection of antibodies to *T. gondii*

Screening for antibodies to *T. gondii* in captured rat sera was checked by using the commercially available latex agglutination test (Toxocheck-MT, Eiken Chemical, Tokyo, Japan). Briefly, 5 μL of test sera were added to 35 μL of dilution buffer, which contained 1% choline chloride, as supplied by the manufacturer. The diluted sera were then diluted two fold in a 96-well microplate. A 1:16–1:2048 dilution was made. *T. gondii* antigen-coated latex particle suspension (25 μL) was added to each well and the agglutination in the microplate well was observed overnight. Agglutination of latex particles after reacting with a serum titer of 1:64 or above was considered as positive.

2.5. Demonstration of *N. caninum* from captured wild rats

For the inoculation of each sample into two mice, halves of the brain of the seropositive rats were homogenized in 10 mL PBS and then inoculated intraperitoneally at a dose of 1 mL/time/mouse/2 days for three times into SPF ICR mice that had been immunosuppressed with prednisolone or into male BALB/cByJ-nude mice. When the inoculated ICR mice died, their brains were homogenized and then were inoculated intraperitoneally into nude mice. The remaining 4 mL of the brain homogenate was used for PCR. When the inoculated ICR mice died, their brains were homogenized and then were inoculated intraperitioneally into nude mice.

2.6. Histopathological observation and immunostaining of *N. caninum* tissue cyst

At necropsy, the brain, heart, lung, liver, spleen, pancreas, kidney, intestine and skin of the rats and mice were fixed in 10% PBS buffered formalin solution. Tissue sections were made using routine histological techniques and 5 μm thick sections were prepared. They were then stained with hematoxylin and eosin (H&E) and were examined under a light microscope. The sections of the mouse brain containing the cysts were probed using *N. caninum* bovine positive and negative control serum obtained from Kyoto Biken at a 1:200 dilution, a dog polyclonal antibody against *N. caninum* at a 1:50 dilution and a bovine polyclonal antibody against *N. caninum* at a 1:200 dilution. These bovine and canine positive sera were from naturally infected animals and had been confirmed to be positive by IFAT as described above in Section 2.3. All the aforementioned *N. caninum*-antibody positive bovine and canine sera used were also found to be negative for *T. gondii* antibody as confirmed by the latex agglutination test (Toxocheck-MT, Eiken Chemical, Tokyo, Japan). Immunostaining of the histological cyst were also carried out using *T. gondii* antibody positive as well as negative bovine serum at a 1:64 dilution as confirmed by the latex agglutination test (Toxocheck-MT, Eiken Chemical, Tokyo, Japan). Both the *T. gondii* positive and negative sera were confirmed to be negative for *N. caninum* by IFAT as described in section 2.3. FITC-conjugated affinity purified goat anti-bovine IgG and FITC-conjugated rabbit anti-dog IgG obtained from Jackson ImmunoResearch Laboratories at 1:800 dilutions were used as a secondary antibody to confirm the presence of *N. caninum*.

2.7. Polymerase chain reaction (PCR) confirmation of *N. caninum*

Two different preparation methods for PCR were used. A portion of the brain of the wild rats as well as that of the rat brain-inoculated nude mice and of nude mice inoculated with brains from SPF ICR mice were homogenized in PBS followed by squeezing three times through a 23-gauge needle. The homogenate was then directly subject to PCR reaction. Another portion of the brain homogenate was layered onto a 35% isotonic Percoll gradient followed by centrifugation to isolate *N. caninum* tissue cysts [11]. Parasite DNA was extracted using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene®, Taipei, Taiwan). DNA was eluted with TE buffer (0.01 M Tris base and 0.001 M EDTA). The PCR assay was carried out as described by Yamage et al. [19]. The primers used were CAGTCAACCTACGTCTTCT (Np 6) and GTGCGTCCAATCCTGTAAC (Np 21). The condition of the PCR was 35 cycles with denaturation (94 °C, 1 min), annealing (50 °C, 1 min) and primer extension (72 °C, 1 min). DNA from *N. caninum* tachyzoites used as a positive control in the PCR was obtained from Kyoto Biken, Japan. Aliquots of amplification products obtained in the PCR test were analyzed by 2% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under

Table I. Seroprevalence of *N. caninum* antibody in cattle farm rats.

Cattle farm	*N. caninum* antibody positive wild rats / No. of rats examined on that farm[a] (%)
A (Taichung)	0/7 (0)
B (Taichung)	0/4 (0)
C (Taichung)	0/1 (0)
D (Taichung)	0/2 (0)
E (Taichung)	1/16 (6.3)
F (Pintung)	8/25 (42.9)
Total	9/55 (16.4)

[a] Indirect fluorescence antibody test. Rat sera were diluted to 1:50 for IgG detection.

ultraviolet light. The bands were sequenced in an ABI prism 377 DNA sequencer.

3. RESULTS

Nine (16.4%) out of the 55 farm rats examined showed the presence of *N. caninum* antibodies as measured at a 1:50 dilution, while the rest of the 46 captured rats were negative (Tab. I). Antibody titers to *N. caninum* of the rat sera were found to be generally low and their correlation to the PCR results are shown in Table II. Only 3 out of 9 rats showed an antibody titer of 1:100. Five out of the 55 farm rats showed antibody to *T. gondii*, with one of them also being seropositive for *N. caninum* (Tab. II). Many thick wall cysts of *Sarcocystis* sp. were observed in the stomach sphincter muscle of one of the wild rats (Fig. 1). This rat was found to be seronegative for *N. caninum* as well as *T. gondii* antibody. Two of the 55 brains of the captured wild rat homogenates that were subjected to PCR showed the specific amplification products of 328 bp (Fig. 2). One of two *N. caninum* positive brain homogenates had been processed for isolating *N. caninum* tissue cyst using a 35% isotonic Percoll gradient but not the other. The DNA sequence of the amplicon showed 94% homology with the gene of the *N. caninum* Austria 1 strain when checked against the data of the gene bank. No cyst was seen in the prepared tissue sections of all the wild rats by histology and PAS stain.

Furthermore, despite that all the inoculated mice were examined for *N. caninum* by histology and PCR, only one cyst, with a 1 μm thick wall in the brain of a nude mouse was observed (Fig. 3a). That mouse had been inoculated six months before, with the brain of a prednisolone-treated ICR

Table II. Comparison of *N. caninum* and *T. gondii* serum titer of wild rats with the PCR result.

		IFAT for *N. caninum*[a]						Total
		Seronegative (< 1:50)		Seropositive				
				1:50		1:100		
LA test for *T. gondii*[b]		+	–	+	–	+	–	
PCR for *N. caninum*	+	0	0	0	2	0	1[c]	3
	–	4	42	1 (1:128)[d]	3	0	2	52
Total		4	42	1	5	0	3	55

[a] IFAT; Sera diluted to 1:50 and 1:100. Diffused fluorescence on tachyzoite considered positive.
[b] Latex agglutination test for *T. gondii*. Reciprocal serum titer of 1:64 or above considered positive.
[c] Protozoan DNA detected in the mouse that was inoculated with the rat brain.
[d] LA test for *T. gondii* show reciprocal serum titer of 1:128.

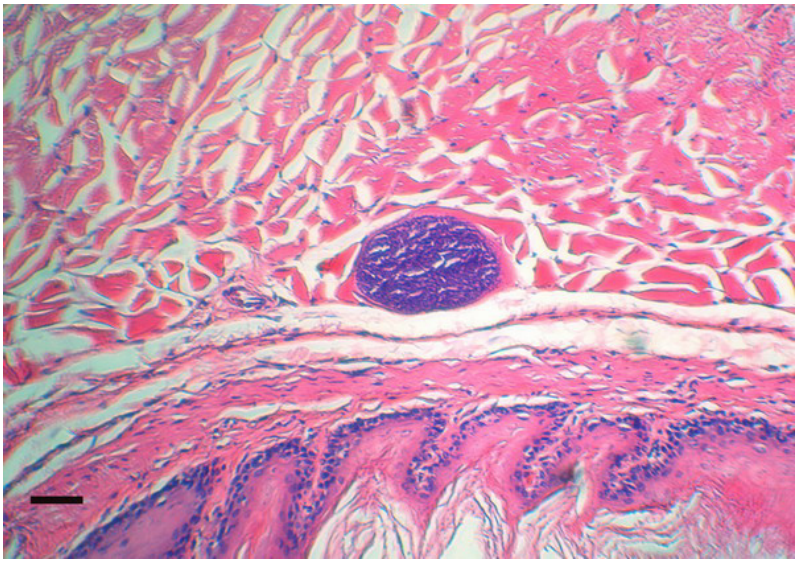

Figure 1. A thick wall cyst of *Sarcocystis* sp. was observed in the stomach sphincter muscle of one of the wild rats. H & E. stain. Bar = 50 µm.

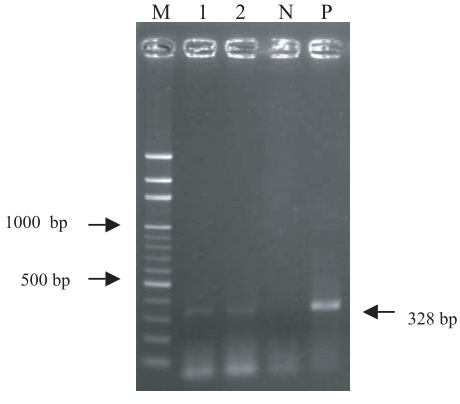

Figure 2. PCR products of *N. caninum* from the hemogenate of the brains of the two captured wild rats. Lane M shows the 100 bp marker ladder. Lane 1 shows the PCR product from the brain homogenate of the farm rat. Lane 2 shows the PCR product from the brain of another rat that had been processed for *N. caninum* tissue cysts using 35% isotonic Percoll gradient. Lane P shows the positive control derived from *N. caninum* tachyzoite. Lane N shows the negative control from the brain of uninfected SPF rats.

mouse, which was in turn previously inoculated with the brain of a captured wild rat. The rat, whose brain was used in the inoculation of the prednisolone-treated ICR mouse, showed an *N. caninum* antibody titer of 1:100 by IFA but no antibodies to *T. gondii* (< 1:16). The *N. caninum* positive nude mouse was emaciated and depressed. A histopathological lesion in its brain, such as gliosis, was minimal. When the brain histological section containing the cyst was immunologically stained with an anti-*Neospora* polyclonal antibody from either the cattle, dog or the bovine positive control serum obtained from Kyoto Biken (Fig. 3b), immunofluorescence of the cyst was observed. The cyst did not show any fluorescence when stained with the anti-*T. gondii* bovine serum that was positive for the *T. gondii* antibody but negative for the *N. caninum* antibody. Moreover, when the brain was subjected to the PCR test, a specific amplicon of 328 bp, which was indicative of *N. caninum,* was observed (data not shown). The remaining mice brains were negative by the PCR test.

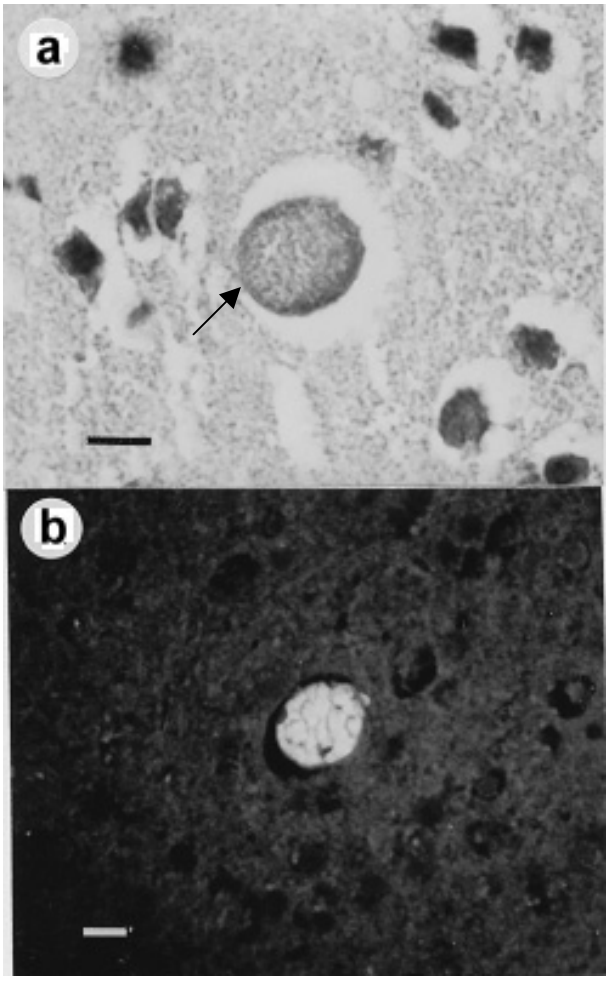

Figure 3. *N. caninum* cyst in the brain of a nude mouse that was inoculated with the brain of a prednisolone-treated ICR mouse six months before. The ICR mouse was inoculated with the brain of the captured rat three months before sacrifice. H & E. stain (Bar = 10 μm). An immunofluorescence stain using *Neospora*-specific bovine polyclonal antibody as the primary antibody and FITC-conjugated goat anti-bovine IgG as the secondary antibody. Bar = 10 μm.

4. DISCUSSION

In our study, nine (16.4%) out of the 55 farm rats examined showed the presence of *N. caninum* antibodies. In the titration of the rat IgG, we observed that the antibody titers were generally very low, with six of the seropositive rats showing only 1:50 antibody titers and three showing 1:100. These antibody titers were much lower than those reported (between 1:800 and 1:6400) in France [16]. This discrepancy might be because our rats were spontaneously infected while those reported by others were experimentally infected. In experimental infections, the number of the infesting protozoan might be higher than that of natural infections. This might also help to explain why

we could not find the protozoan in the histological section of the rat brains.

Experimental infection of *N. caninum* in nude mice has been reported by [17]. In that report, only aggregates of *N. caninum* tachyzoites but not cysts were observed in the nude mouse brain. In our study, we also observed aggregates of tachyzoites but enclosed in a cyst with a comparatively thin 1 μm thick cyst wall, in the brain of a nude mouse. The difference between our sample and that of the previously reported case is that our nude mouse had been infected for six months before necropsy while theirs only for 2 to 4 months. This suggests the possibility that it takes some time for *N. caninum* to develop the typical cyst structure in the brain of a nude mouse. In other animals, *N. caninum* tissue cyst walls have been reported to be up to 4 μm thick, presumably depending upon how long the infection has existed [3]. In most tissue cysts, the cyst wall is 1 to 2 μm thick. Since the tissue cyst wall in our study was not very conspicuous, it might be difficult for a complete cyst wall structure to develop in immunodeficient mice.

N. caninum is widely recognized as being antigenically distinct from *T. gondii* [3]. One out of 9 rats seropositive for *N. caninum* and 4 out of 46 rats seronegative for *N. caninum* were also found to contain antibody to *T. gondii*. This indicates that there was no cross-reaction between *N. caninum* and *T. gondii* among the rats. In our immunostaining experiment, we demonstrated that the observed cyst in the nude mouse was indeed *N. caninum* because it was stained positive by both bovine and canine sera that had been shown to positively stain the *N. caninum* tachyzoite but negative for the *T. gondii* antibody. Furthermore, that cyst was negative to the staining by canine and bovine sera, which had been proven to contain *T. gondii* antibody but not *N. caninum* antibody. Moreover, the serum of the rat that was found to be infected with *Sarcocystis* sp. did not react positively with the *N. caninum* antigen. This shows the remote possibility of antigenic cross reactivity between *N. caninum* and *Sarcocystis* sp. Experimental infection of *Sarcocystis neurona*, *S. muris* and *S. cruzi* in rabbits has also demonstrated that the animals did not develop antibody titers to *N. caninum* in the IFAT [3].

In the present study, we showed for the first time that cattle farm rats can be naturally infected with *N. caninum*, indicating that the rodent host might help in maintaining the life cycle of the protozoan on the farm. Since the life cycle of *N. caninum* in the nature has not been completely elucidated, our finding implies that the wild rat on the cattle farm can serve as a reservoir host. These seropositive rats were caught in farms with cattle that showed high seroprevalence against *N. caninum* (data not presented). Many wild rats live on these cattle farms and might feed on the contaminated food and water. When wild rats are infected with *N. caninum*, they naturally become a transmission source for other animals. The dog has been reported as a definitive host of *N. caninum* [9, 10]. Infection of *N. caninum* in farm dogs is thought to be through the ingestion of aborted fetal materials that contain tachyzoites or cysts of the protozoan. However, our finding of infected rats on the cattle farm suggests that the dog might also be infected through the ingestion of wild rats. Besides controlling the farm dog, it has been suggested that *N. caninum* infected cattle should be culled to interupt the life cycle of *N. caninum* on the cattle farm [1]. The wisdom and effectiveness of the culling measure will become questionable because we reveal that the rodent can become a source of *N. caninum* infection for farm dogs or stray dogs that scavenge on the cattle farm premises.

Many serologically positive cattle for *N. caninum* had a history of abortion, indicating that *N. caninum* is a major cause of bovine abortion. Thus, ingestion of infected rats by dogs can help maintain the life cycle and contribute to the contamination of the cattle farm. Besides the control of dogs, rodent control in cattle farms should also

be considered as a measure to prevent the spread of infection.

Although this study demonstrates that *N. caninum* could infect wild rats on the cattle farm, it is still unknown how the rat really becomes infected. One can conjecture that the rats might probably be infected through the ingestion of *N. caninum* oocysts shed in the infected farm dog feces or by feeding on aborted bovine fetuses or placenta that might contain the tachyzoite or the cyst. However, such speculation needs further study. It is also suggested that a serological survey of farm rats might reveal the extent of the contamination of *N. caninum* on the cattle farm.

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