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Possibility of *Neospora caninum* infection by venereal transmission in CB-17 *scid* mice

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Abstract

CB-17 *scid* and BALB/c male mice were inoculated intraperitoneally with *Neospora caninum* to examine the possibility of its venereal transmission. Some of these mice were killed on days 7 and 20 post inoculation to examine the genital organs for presence of the parasite. The remaining *scid* male mice were housed with non-infected female mice from day 7 p.i. and kept with them for 14 days. These *scid* mice died between day 28 and 35 p.i. *N. caninum* DNA was detected in the testis of mice on day 7 p.i. and 20 p.i. by PCR and tachyzoite viability was determined by bioassay conducted by means of mouse inoculation. Microscopically, fewer tachyzoites were detected in the testis obtained on day 20 p.i, than in other organs. The inoculated BALB/c male mice survived until the end of the experiment with no clinical signs and *N. caninum* DNA was detected in the testis on day 7 p.i. but not on day 14 p.i. Five of 8 female *scid* mice housed with infected males became pregnant. Tachyzoites were detected in 3 of these mice and their neonates (n=3, 5 qnd 13, respectively). In 3 non-pregnant mice, no parasite was detected. Two of the 4 female BALB/c mice housed with infected male *scid* mice became pregnant but the parasite was not detected in them or in the neonates (n=3 and 13, respectively). These results indicate that the

33 tachyzoites were present in the genital organs of the immunodefecient mice from
34 day 7 p.i. and suggest that transmission may occur through mating with male
35 mice.

36 Key words: CB-17 *scid* mouse; *Neospora caninum*; Venereal transmission

A review concerning *Neospora caninum* (*N. caninum*) has stated that it causes severe neuromuscular disease and abortion, stillbirth and congenital infection in livestock and companion animals (Dubey and Linday, 1996). Interestingly, the presence of *N. caninum* DNA in semen from naturally infected bulls has been reported recently (Oretega-More et al. 2003; Caetano-da-Silva et al. 2004). These finding suggest the possibility of venereal transmission in neosporosis. Further, Serrano et al. (2006) demonstrated the potential of *N. caninum* in contaminated cattle semen to infect heifers via artificial insemination. Canada et al. (2006) demonstrated that cows artificially inseminated with frozen-thawed semen contaminated with *N. caninum* tachyzoites had no antibody production againsts *N. caninum*. Their results suggested that transmission by artificial insemination would be unlikely. We consider that these contradictory results could be due to host resistance. In mouse neosporosis, Long and Baszler (2000) hypothesized that induction of maternal type 1 responses against *N. caninum* could prevent vertical administration and demonstrated that modulation of type 2 cytokines through administration of anti-IL-4 monoclonal antibodies before pregnancy can reduce the frequency of vertical transmission. Several studies have demonstrated characterization of the immune response following *N.*

55 *caninum* infection in the pregnant mouse, and their findings suggested that
56 control of transplacental transmission and fetal loss are associated with bias of
57 the cell-mediated immune response during pregnancy (Kano et al. 2005; Omata
58 et al. 2004; Quinn et al. 2004; Rettigner et al. 2004).
59 In the present study, we used CB-17 *scid* mice, which are highly susceptible to *N.*
60 *caninum* infection, to determine the possibility of venereal transmission in
61 neosporosis, we examined the testis of mice experimentally infected with *N.*
62 *caninum* tachyzoites for the presence of the parasite and explored the possibility
63 of transmission from male to female through mating.
64 Eight-week-old female and male CB-17 *scid* and BALB/c mice were purchased
65 from Japan CLEA (Tokyo, Japan). Bovine angio-endothelial (BAE) cells were
66 cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine
67 serum (D-MEM10%FBS), and tachyzoites of *N. caninum* derived from sheep
68 (Kobayashi et al. 2001; Koyama et al. 2001) were maintained by continuous
69 passage in the BAE cells cultures and suspended in phosphate-buffered saline
70 (PBS).
71 The male mice were inoculated intraperitoneally with 2 x 10^5 *N. caninum*
72 tachyzoites. On day 7, and 20 post inoculation (p.i), respectively, 5 and 8 *scid*

mice were killed to obtain the testis, epididymis, seminal duct and prostata. Half of the genital organs were used for histological examination and the rest minced in 1 ml of PBS. Half of the homogenate obtained was used for a polymerase chain reaction (PCR) using a *N. caninum* specific oligonucleotide primer as described by Yamage et al. (1996). The rest was inoculated intraperitoneally into 2 BALB/c mice to monitor for the presence of the live parasite. Briefly, the mice were killed on day 28 p.i and titers of serum IgG antibodies to *N. caninum* tachyzoites was examined by indirect immunofluorescent antibody test as described by Koyama et al. (2001). The parasite DNA in the brain and spleen of the mice was examined by PCR as described above.

For the histological examination in which the pathological features of the inoculated mice were examined, the genital organs were immediately placed in PBS containing 4 % paraformaldehyde. Tissue specimens were routinely processed and stained with hematoxylin-and-eosin for microscopic evaluation.

Another 8 infected male mice were housed with non-infected female mice from day 7 p.i and kept with them for 14 days. On day 22 after removing the males, the females were killed to obtain their brains, uterus, livers and spleens, which were examined for the presence of the parasite. For pregnant mice, the

91 neonates were also killed and their spleens and brains taken for examination.
92 The male *scid* mice which were not killed died between day 28 and 35 p.i. *N.*
93 *caninum* DNA was detected in the genital organs in all of 5 mice on day 7 p.i and
94 in those of 8 mice on day 20 p.i. by PCR and the viability of the tachyzoites was
95 confirmed by bioassay (Table 1). In the lung tissue obtained on day 20 p.i.,
96 severe pneumonia with necrosis, numerous tachyzoites and cysts were
97 observed, and in the urinary bladder of these mice, multifocal infected cells,
98 numerous tachyzoites and pseudocysts were present. In the testis,
99 multinucleated giant cell formations were observed in seminiferous tubules, but
100 few tachyzoites were detected (data not shown). In the seminal duct and
101 prostata, numbers of tachyzoites were observed. (Fig. 1). The BALB/c male mice
102 which were inoculated with the tachyzoites and survived until the end of the
103 experiment showed no clinical signs. *N. caninum* DNA was detected in the testis
104 in all of 10 mice on day 7 p.i, but not in that of 4 mice on day 14 p.i. The following
105 experiments were therefore conducted with *scid* mice.
106 Male mice were housed with female mice and as shown in Table 2, 5 of the 8
107 female mice became pregnant. In 3 of these dams (No. 1, 2, 5), *N. caninum* DNA
108 was detected in the brain and/or uterus (Fig. 2) and the presence of live

parasites was confirmed by bioassay by means of a mouse inoculation test. In the neonates (n=3, 13 and 5, respectively), *N. caninum* DNA was detected in the liver and brain and live parasites were also detected. The parasite was not detected in any organ in the other 2 dams, their neonates (n=3 and 12), or the 3 non-pregnant mice. Out of 4 BALB/c female mice housed with male mice, 2 mice became pregnant, and no parasite was detected in any of them.

The results in Table 1 indicate that tachyzoites migrate in the genital organs of mice during the first 7 days p.i and persist in the organs for at least until 3 weeks p.i. in immunodeficient mice, but not in immunocompetent mice. The number of the tachyzoites in the testis seemed to be fewer than that in the other somatic organs. As shown in Fig. 1, the tachyzoites were present in the seminal duct or prostata tissues. One possibility was also considered that some of them may have migrated into the semen. The results in Table 2 suggest that a small number of tachyzoites could still be transmitted by mating to immunodeficient female mice and then multiply in them, resulting in transplacental infection of the fetus. Whereas, when present in such small numbers, tachyzoites may fail to be transmitted by mating to immunocompetent mice. Since large numbers of tachyzoites were found in the lungs, urinary bladder and other organs in *scid*

male mice on day 20 p.i, it is possible that females housed in the same cages were infected through contagion or droplet infection. However, this possibility may be negligible because 2 non-pregnat female mice housed with males were not infected and infection was exclusively detected in the pregnant mice.

Serrano et al. (2006) demonstrated that *N. caninum* infection could be transferred to females by intrauterine inoculation with contaminated semen. Our results seem to support their finding and suggest that the possibility of transplacental transmission may be higher in immunodeficient mice than in immunocompetent mice. Caetano-da-Silva et al. (2004) reported that in PCR analysis of different semen straws from the same batch for *N. caninum* DNA, straws were not always positive, suggesting that parasite numbers were very low in the semen. We considered that immunocompetent mice may have protective capability against invasion by such a low number of parasites in the uterus mucosa. Thus, the occurrence of venereal transmission of *N. caninum* in mouse may be related to the potential absence of protective capability against in both male and female hosts.

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192 Figure Captions

193 Table 1. Detection of *N. caninum* in testis of BALB/c and CB-17 *scid* mice in

194 experimental infection.

195 Table 2. Detection of *N. caninum* in the tissues of dams and neonates of BALB/c

196 and CB-17 *scid* female mice mated with CB-17 *scid* male mice infected with the

197 tachyzoites.

198 Figure 1. Photomicrographs of male *scid* mice anterior lobe of prostate (A) and

199 seminal vesicle (B) on day 20 p.i. Bar= 50 µm, respectively. Note the cluster of

200 parasites (arrow).

201 Figure 2. Detection of N. caninum DNA in the tissues of dam (No. 1) and the

202 neonates of CB-17 *scid* female mice mated with CB-17 *scid* male mice infected

203 with the tachyzoites. Tissues of dam : lane 1, liver; 2, spleen; 3, uterus; 4, brain;

204 Tissues of male: lane 5, testis; 6, brain; Tissues of neonate 1: lane 7, brain; 8,

205 spleen; tissues of neonate 2: lane 9, brain; 10, spleen; tissues of neonate 3: lane

206 11, brain; 12, spleen; N, Negative control; P, parasite DNA. Numbers on the light

207 refer to the size in base pairs of the molecular size standard.

208

Figure

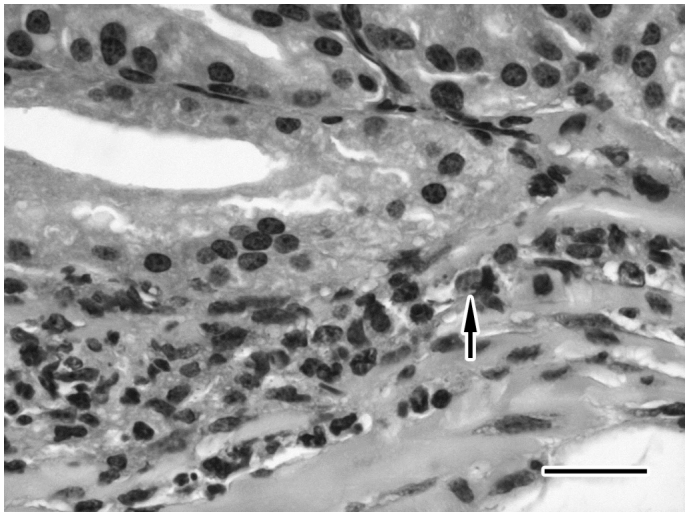

Fig.1A

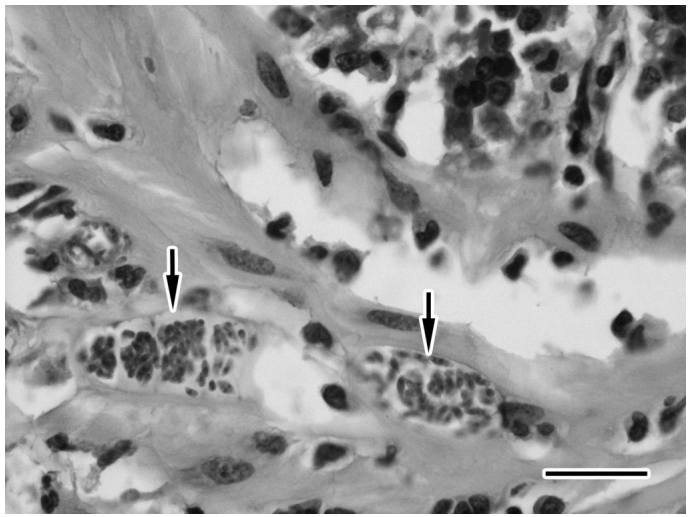

Fig.1B

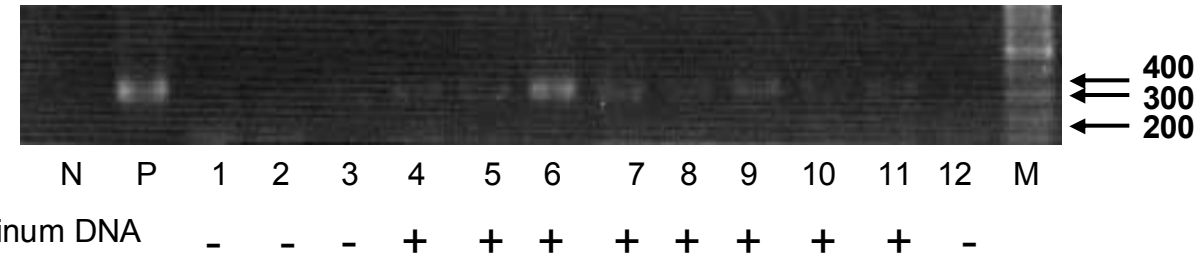

Fig.2

Table

Sampling	mice	PCR	Mouse inoculation test
On day 7 p.i.	scid	5 / 5*	5 / 5
	BALB/c	10 / 10	10 / 10
On day 14 p.i.	BALB/c	0 / 4	0 / 4
On day 21 p.i.	scid	8 / 8	8 / 8

* No. positive/ Total no.

Table 1

mice	No.	PCR				Mouse inoculation test	
		dam		neonates		dam	neonates
		uterus	brain	liver	brain		
scid	1	+	+	2 / 3	3 / 3	+	3 / 3
	2	+	+	10 / 13	10 / 13	+	10 / 13
	3	-	-	3 / 3	0 / 3	-	0 / 3
	4	-	-	N. P		-	
	5	-	+	1 / 5	0 / 5	+	5 / 5
	6	-	-	0 / 12	0 / 12	-	0 / 12
	7	-	-	N. P		-	
	8	-	-	N. P		-	
BALB/c	1	-	-	0 / 3	0 / 3	-	0 / 3
	2	-	-	0 / 13	0 / 13	-	0 / 13
	3	-	-	N. P		-	
	4	-	-	N. P		-	

No. positive/ Total no.

N. P: non-pregnant

Table 2

*** Revision Note**

Dear Dr. Mansfield,

Thank you for your letter, the referees comments.

We are very pleased to learn that our manuscript is reconsidered for publication after major revison.

We have made revisions in accordance with the comments of reviewers.

Regarding the comments of reviewer #1, we have added Fig. 2 which is a photo of PCR gel and changed Fig.1 in which tachyzoites were depicted in tissues. We have added the details of mouse inoculation bioassay as described in lines 78-82. We have changed the term "tachyzoite DNA" to "N. caninum DNA".

Regarding the comments of reviewer #2, we have added "in mouse" in line 140.

We have changed Table 2, because of mistyping.

With best regards,

Sincerely

Yoshitaka Omata