

Serological Survey of *Neospora caninum* Infection among Dogs in Japan through Species-Specific ELISA

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ABSTRACT. A seroepidemiological survey of *Neospora caninum* infection among dogs in Japan was conducted using species-specific enzyme-linked immunosorbent assay with recombinant surface antigen (Nc-SAG1t). Among 1,206 dogs examined, 126 dogs (10.4%) from 30 prefectures from Hokkaido to Okinawa were positive to *N. caninum* infections, which were more frequently detected in females than males. Siberian Huskies showed the highest positive rate compared with the other breeds. Dogs with pyometra and diabetes mellitus showed the higher positive rates than dogs with other diseases or without diseases.

KEY WORDS: canine, *Neospora caninum*, species-specific ELISA.

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Neospora caninum is an apicomplexan protozoan parasite which was originally identified in tissues of paralyzed dogs [7, 8]. To date, domestic dogs are the only definitive host known to excrete the oocysts of *N. caninum* [15]. *N. caninum* is considered to be the major cause of fetal abortion and neonatal mortality in cattle as well as a critical risk factor for canine neuromuscular paralysis in dogs [9, 10]. Several articles have reported clinical neosporosis in dogs [3, 4, 11]. Although ascending paralysis with hyperextension of the hind limbs in congenitally infected pups is the most common clinical manifestation of the disease in dogs, myocarditis, dysphasia, ulcerative dermatitis, pneumonia, and hepatitis may also occur [14]. Since canine neosporosis is a polysystemic protozoan disease, *N. caninum* infection can cause a wide variety of clinical syndromes.

Epidemiological studies for this parasite have been performed using serological method, including immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), comparative-inhibition ELISA, Western blotting, and direct agglutination. In most of these techniques, intact tachyzoite or tachyzoite-derived antigens are used for detection of antibodies [13]. One study from Japan revealed the presence of positive antibodies in 15 among 48 dogs (31.3%) reared in the dairy farms that had reported a case of abortion in the dog due to *N. caninum* infection and the presence of seropositive cattle, while the prevalence was 7.1% (14 of 198 dogs) among the dogs kept in urban areas [18]. This earlier study was performed by using IFAT with intact tachyzoites antigens. There are some concerns, however, that the use of whole tachyzoites antigens may result in several false-positive reactions due to cross-reactivity with closely related parasites, such as *Toxoplasma gondii*.

Recently, an ELISA approach using recombinant surface antigen 1 of *N. caninum* (NcSAG1t) has been shown to have high specificity and sensitivity for serodiagnosis [5]. The method has been used for serodiagnosis of *N. caninum* infection in cattle. In the present study, we conducted a seroepidemiological survey of *N. caninum* infection of dogs using species-specific ELISA. The primary aim of this survey was to determine the distribution of *N. caninum* infection in various breeds of dogs in Japan. The relationship between the *N. caninum* infection and clinical signs of the dogs was also examined.

Sera were collected from 1,206 dogs examined at animal hospitals located in 35 prefectures (Hokkaido, Aomori, Akita, Miyagi, Fukushima, Tochigi, Ibaraki, Saitama, Tokyo, Chiba, Kanagawa, Niigata, Fukui, Nagano, Yamaguchi, Shizuoka, Aichi, Gifu, Mie, Osaka, Kyoto, Nara, Wakayama, Hyogo, Tottori, Okayama, Yamaguchi, Kagawa, Tokushima, Kochi, Fukuoka, Nagasaki, Kumamoto, Miyazaki, and Okinawa) from July 2005 to July 2006. All the animals were household dogs that often spent time at outside. Information of sex, age, breed, clinical history and present illness was obtained from the veterinarians treating the dogs.

ELISA with NcSAG1t was carried out according to the protocol of Chahan *et al.* [5]. 96-well microplates were coated with glutathione S-transferase (GST) - NcSAG1t or GST only (negative control), at a concentration of 100 ng per well. The reaction was decided as positive when the difference between the absorbance of the antigen (GST-NcSAG1t)-containing well and that of the control antigen (GST)-containing well was greater than 0.1. The OD value 0.1 was calculated from the mean OD value plus 3-fold standard deviations of 30 specific pathogen-free dog sera.

Chi-square tests were performed to determine differences in the population based on breed, sex, age, and clinical problem (most recent clinical histories or present illness). *P*-values less than 0.05 were considered to be significant, but the

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level of statistical significant was corrected by Bonferroni-correction for the multiple hypotheses. Then, odds ratios (OR) were calculated for independent variables. Stat View Ver 5.0 (Hulinks) was used to analyze the results.

Among 1,206 dogs examined, 126 dogs (10.4%) showed positive by ELISA. Positive antibodies were detected in the dogs from 30 out of the 35 prefectures of Japan from Hokkaido to Okinawa.

Potential risk factors for *N. caninum* infection, including sex, age and breed were analyzed (Table 1). Sex was a statistically significant risk factor for *N. caninum* infection ($p < 0.0001 < 0.05/3 = 0.0167$). *N. caninum* infections were more frequently detected in the females (14.8%, OR=2.34) than the males (6.8%, OR=0.43) and the sex unknown group (8.8%, OR=0.83). Positive rates of older dogs were comparatively higher than those of younger dogs; however, the differences of the positive rates among different ages were not statistically significant ($p = 0.0317 > 0.05/7 = 0.007$). When the breeds consisting of respective 20 or more dogs were analyzed for *N. caninum* infections, it was cleared that breed was also a statistically significant risk factor for *N. caninum* infection ($p = 0.0003 < 0.05/14 = 0.0036$). Siberian huskies showed the highest positive rate of 42.9%

(OR=6.85). Shetland Sheepdogs (18.4%, OR=1.99), Shibas (17.6%, OR=1.90) and Golden Retrievers (14.2%, OR=1.48) were also showed higher rate than the other breeds. Miniature breeds showed comparatively lower positive rates: Maltese (5.9%, OR=0.53), Pomeranian (9.5%, OR=0.90), Yorkshire Terrier (7.1%, OR=0.65) and Miniature Dachshunds (8.5%, OR=0.78).

Infection rates in the dogs having a certain kind of diseases in which respective 10 or more cases were recognized are shown in Table 2. Among 574 dogs with clinical problems, 72 (12.5%, OR=1.54) were positive for *N. caninum*, while 54 (8.5%, OR=0.65) dogs were positive among 632 dogs without present or past clinical problems. The prevalence rates of the dogs with respect diseases showed significant differences ($p < 0.0001 < 0.05/15 = 0.0033$). The dogs with pyometra showed the highest positive rate of 44.7% (OR=7.87), followed by the rate of 35.7% (OR=4.92) in the dogs with diabetes mellitus. The dogs with renal failure (21.4%, OR=2.37) and seizure (20.0%, OR=2.17) also showed higher positive rates.

In the present study, the total *N. caninum* positive proportion was 10.4% (126/1206) in the dogs obtained from 35 prefectures of Japan from Hokkaido to Okinawa. This indi-

Table 1. Risk factor for *N. caninum* infection among the dogs in Japan

Factors	Number of dogs examined	Number of dogs positive	Percent positive (%)	Odds Ratio	95% CI Lower-Higher
Sex					
Male	631	43	6.8	0.43	0.29-0.64
Female	541	80	14.8	2.34	1.59-3.42
Unrecorded	34	3	8.8	0.83	0.25-2.74
p -value (chi-square) $< 0.0001 < 0.05/3 = 0.0167$ **					
Age (years old)					
< 2	99	8	8.1	0.74	0.35-1.56
> 2, <5	238	13	5.5	0.44	0.24-0.79
> 5, <8	236	23	9.7	0.91	0.56-1.46
> 8, <11	310	35	11.3	1.13	0.74-1.70
> 11, <14	212	33	15.6	1.79	1.16-2.74
> 14	93	12	12.9	1.30	0.69-2.45
Unknown	18	2	11.1	1.07	0.24-4.72
p -value (chi-square) $0.0317 > 0.05/7 = 0.0071$ **					
Breed*					
Siberian Husky	21	9	42.9	6.85	2.83-16.60
Shetland Sheepdog	38	7	18.4	1.99	0.86-4.62
Shiba	51	9	17.6	1.90	0.90-4.00
Golden Retriever	120	17	14.2	1.48	0.86-2.56
Shih Tzu	69	8	11.6	1.13	0.53-2.42
Mix	331	34	10.3	0.97	0.64-1.48
Pomeranian	21	2	9.5	0.90	0.21-3.91
Miniature Dachshunds	94	8	8.5	0.78	0.37-1.66
Yorkshire Terrier	28	2	7.1	0.65	0.15-2.89
Labrador Retriever	71	5	7.0	0.63	0.25-1.61
Beagle	48	3	6.3	0.56	0.17-1.83
Maltese	34	2	5.9	0.53	0.13-2.23
Welsh Corgie	29	1	3.4	0.30	0.04-2.23
Others	251	19	7.6	0.65	0.39-1.08
p value (chi square) $0.0003 < 0.05/14 = 0.0036$ **					

*: Breed including 20 or more dogs.

** : The level of statistical significant was corrected by using Bonferroni-correction.

Table 2. Infection rates of *N. caninum* among different disease states

Diseases*	Number of dogs	Number of positive	Percent of positive (%)	Odds Ratio	95% CI Lower-Higher
I. No illness/Healthy	632	54	8.5	0.65	0.45–0.95
II. Illness	574	72	12.5	1.54	1.06–2.05
1) Pyometra	38	17	44.7	7.87	4.03–15.36
2) Diabetes mellitus	14	5	35.7	4.92	1.62–14.91
3) Renal Failure	14	3	21.4	2.37	0.65–8.61
4) Seizures	15	3	20.0	2.17	0.60–7.80
5) Chronic mitral valve disease	21	4	19.0	2.05	0.68–6.19
6) Filariasis	46	8	17.4	1.86	0.85–4.08
7) Urolithiasis	12	2	16.7	1.73	0.37–7.97
8) MGT**	26	4	15.4	1.58	0.53–4.65
9) Mast cell tumor	11	1	10.7	0.85	0.11–6.74
10) Babesiosis	22	2	9.1	0.85	0.20–3.70
11) Allergy/Atopy	29	1	3.4	0.30	0.04–2.23
12) Otitis Externa	22	0	0.0	–	–
13) Extoparasites	20	0	0.0	–	–
14) Others	284	22	7.7	0.66	0.41–1.07

p-value (chi-square) <0.0001 <0.05/15 = 0.0033#

*: Disease including 10 or more cases.

** :MGT: Mammary gland tumor.

#: The level of statistical significant was corrected by using Bonferroni-correction.

cates that canine *N. caninum* infection has a widespread geographic distribution in this country; however, it is difficult to compare this percentage to those reported in previous studies, because in our study canine antibodies against *N. caninum* were detected by a species-specific ELISA using recombinant NcSAG1t and most of the previous studies were performed by IFAT using intact tachyzoites antigens [1, 2, 12, 16, 18, 19]. Further studies are required to confirm the relationship between the positive antibodies detected by the ELISA and the practical exposure to *N. caninum*.

Sex was a statistically significant risk factor for *N. caninum* infection and female dogs were more frequently infected than male dogs. In previous studies conducted in Iran and Austria, seropositivity rates did not differ between males and females [16, 19]. On the other hand, the report from Hungary showed more male dogs had antibodies to *N. caninum* than females [12]. The reason for the higher infection rate in the female dogs found in our study is currently unknown.

Age has been shown to be a risk factor for *N. caninum* infection in seroepidemiological studies from Austria and Iran [16, 19]. We also found that the positive rates of older dogs were comparatively higher than those of younger ones; however, the differences were marginal. A higher percentage of infection in the older dogs may be due to the horizontal transmission that occurs between dogs and cattle.

Breed was also a risk factor for *N. caninum* infection, and Siberian huskies showed the highest positive rate of 42.9% with OR of 6.85 in the present study. Shetland Sheepdogs, Shibas and Golden Retrievers were also showed higher rate. A previous serological survey carried out in Japan reported that 17 Shetland sheepdogs, all older than 7 months, were all seropositive for *N. caninum* [18]. Since it is reported that

transplacental transmission of *N. caninum* is a confirmed mode of natural transmission in dogs [6], breed appears to be an important risk factor for *N. caninum* infection. Earlier seroepidemiological studies revealed that the farm dogs showed higher *N. caninum* infection rates than the household dogs [12, 16, 18]. Although all the dogs examined here spent some time at outdoors, information on the contact rate of each dog with ruminants such as cows and goats was not available in this study. Miniature breeds such as Maltese, Pomeranians, Yorkshire Terriers, and Miniature Dachshunds, showed comparatively lower infection rates. In Japan, these dogs are generally household animals and the contact chance with ruminants is less frequent than outdoor dogs. Beef infected with *N. caninum* can be an additional cause of infection for dogs with this parasite. Although information on the diet of each dog was not collected in the present study, nutritional information should be analyzed in the future.

In the present study, the relationship between *N. caninum* infection and diseases was analyzed. The prevalence rates of respective diseases showed significant differences. The dogs with pyometra and diabetes mellitus showed higher positive rates than the others. Although these dogs had never shown clinical features of *N. caninum* infection, it could cause unexpected clinical features since neosporosis is a systemic protozoan infection. In cattle, *N. caninum* is considered to be the major cause of fetal abortion and neonatal mortality. This may be related to the higher affinity of this parasite with reproductive organs in animals. On the other hand, pyometra itself might lead to *N. caninum* infection. In this study, pathological examination was not performed. Thus, it was not possible to evaluate the exact relationship between the presence of infection and each dis-

ease. Further studies are required to clarify the exact pathogenesis of this important infection.

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