FULL PAPER *Internal Medicine*

Nationwide Survey of *Leptospira* Antibodies in Dogs in Japan: Results from Microscopic Agglutination Test and Enzyme-Linked Immunosorbent Assay

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(Received 23 March 2009/Accepted 30 May 2009)

ABSTRACT. Leptospirosis is an infectious disease caused by *Leptospira interrogans sensu lato* and is common in both humans and animals. In the present study, serum samples were collected from 801 dogs across all 47 prefectures in Japan, and evaluated with a microscopic agglutination test (MAT), using 5 major *L. interrogans* serovars (Icterohaemorrhagiae, Canicola, Autumnalis, Hebdomadis, and Australis) as antigens, and an enzyme-linked immunosorbent assay (ELISA) using recombinant OmpL1 protein as the antigen. Across all dogs tested, 217 (27.0%) and 29 (3.6%) were MAT- and ELISA-positive, respectively. However, evidence strongly suggests that MAT also detected antibodies produced by vaccination. Of 243 dogs never inoculated with any canine vaccine, 41 (16.9%) from 23 prefectures were MAT and/or ELISA positive. The most commonly detected serovar was Icterohaemorrhagiae (22 dogs, 19 prefectures). Our results suggest that there are dogs with subclinical *Leptospira* infection throughout Japan. To the best of our knowledge, the present study is the first nationwide survey of *Leptospira* infection in dogs, and the findings are relevant not only for clinical veterinary medicine but also for public health.

KEY WORDS: canine, ELISA, epidemiology, leptospirosis, zoonosis.

J. Vet. Med. Sci. 71(9): 1191–1199, 2009

Leptospirosis is an infectious disease caused by the pathogen *Leptospira interrogans sensu lato* (referred to as *L. interrogans* hereafter) that affects many types of mammals, including humans, dogs, livestock, and those in the wild [13]. Dogs with leptospirosis show diverse clinical signs, with some developing acute renal failure, hepatic failure, and coagulation disorders, and others experiencing only pyrexia, anorexia, and vomiting [4, 5, 8, 16]. In addition, while leptospirosis can be fatal for some dogs, many cases are thought to be subclinical [10, 18], which implies that dogs can be carriers of *L. interrogans*. In humans, leptospirosis is usually an acute febrile disease with various symptoms. Some people develop icterus, hemorrhage, and renal failure, while others only show cold-like symptoms and then recover [13]. It is thought that humans contract leptospirosis through percutaneous exposure to water or soil contaminated with *L. interrogans* from the urine of carriers [13]. Dogs, including those kept as pets, can also be a source of infection in humans. Clarifying the geographical distribution of dogs infected with *L. interrogans* is thus likely to be useful in terms of both clinical veterinary medicine and public health. In Japan, epidemiological surveys of leptospirosis have been conducted in rodents in Hokkaido, Shizuoka, Aichi, Miyazaki, and Okinawa [14], and in dogs in Hokkaido [1], Yamaguchi [17], and Kagoshima [2]. However, to the best of our knowledge there has been no nationwide survey on *Leptospira* infection in dogs.

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Methods for confirming a diagnosis of canine leptospirosis include detecting specific antibodies in a patient's serum by a microscopic agglutination test (MAT) or an enzyme-linked immunosorbent assay (ELISA), or detecting pathogen in the patient's blood or urine by direct dark-field microscopy, culture, or polymerase chain reaction (PCR) [13]. MAT is the most common serological diagnostic method [13]; this involves introducing live *Leptospira* bacteria to a serum sample, and then looking for bacterial agglutination with dark-field microscopy. There are various serovars of *L. interrogans* [13], and an advantage of MAT is that it can identify each of these serovars. However, diagnosing leptospirosis with MAT requires using standard strains of all of the major serovars, which takes a significant amount of time. Another drawback of MAT is that it detects antibodies produced by vaccination.

OmpL1 is an outer membrane protein, identified by Haake *et al.* in 1993 [7], which has a molecular mass of approximately 31 kDa, is only present in pathogenic *Leptospira* spp., and is considered a primary target of the host immune response [7]. An ELISA utilizing recombinant OmpL1 protein as the antigen does not detect antibodies produced by vaccination [15], allows for the simultaneous testing of multiple samples, and does not require live bacteria, which implies the assays are safe.

In the present study, we determined the nationwide distribution of dogs with *Leptospira* antibodies by performing MAT and ELISA using dog sera samples collected from all 47 prefectures in Japan.

MATERIALS AND METHODS

Strains of Leptospira serovars: Five *Leptospira* serovar strains, cultured and maintained as standard strains, served as the MAT antigens: Icterohaemorrhagiae (strain RGA), Canicola (strain Hond Utrecht IV), Autumnalis (strain Akiyami A), Hebdomadis (strain Akiyami B), and Australis (strain Akiyami C). All of the strains were provided by Prof. Atsuhiko Hasegawa, Nihon University. The strains were cultured at 28°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (*Leptospira* Medium Base EMJH; Difco, Detroit) containing 10% bovine serum albumin solution (*Leptospira* Enrichment EMJH; Difco).

Test samples: Serum samples were collected between November 2006 and October 2007 from 801 dogs taken to veterinary clinics. The inclusion criteria was an age >1 year, and never being inoculated with any canine vaccine (Group A, n=243), or inoculated at the last vaccination with a vaccine not containing *Leptospira* antigens (Group B, n=143), or inoculated with a *Leptospira*-containing or unidentified vaccine not within 11 months of the sampling day (Group C, n=415). While most dogs did not have any clinical abnormalities, some dogs in the study had been treated for injuries, otitis externa, or pyometra. We examined 4–40 dogs in each prefecture. Serum was collected and the following demographic information recorded: age, breed, sex, housing environment (indoor, outdoor, or mixed), if the dog is walked, if the dog drinks water from a natural source, if the dog plays in water, and the location of the house at which the dog is kept (urban, residential, or rural).

MAT: MAT was carried out in accordance with the guidelines provided by the World Health Organization (WHO) in *Human Leptospirosis*: *Guidance for Diagnosis, Surveillance and Control* [19]. In brief, the five *L. interrogans* strains were prepared to a concentration of 1×10^8/mL using phosphate-buffered saline (PBS; 1.37 M NaCl, 27-mM KCl, and 15-mM $KH_2PO_4 \cdot 12H_2O$; pH 7.2). The serum samples were diluted 10-fold with PBS, and serial dilutions up to 160-fold were prepared in plastic microtiter plates with 96 flat-bottomed wells (Thermo Fisher Scientific, Waltham). An equal amount of diluted serum and antigen (25 μL) was pipetted into each well, with final dilution rates of 20-, 40-, 80-, 160-, and 320-fold. Plates were incubated for 1 hr at 28°C and observed under a dark-field microscope. The final dilution rate of the sample for which the quantity of free bacteria was ≤50% of the control (i.e., without serum) was regarded as the agglutination titer. A sample with a titer of 1:80 or greater for at least one serovar was considered MAT positive.

ELISA with the recombinant OmpL1 protein: ELISA was carried out as per Okuda *et al.* [15]. In brief, purified glutathione S-transferase (GST)-OmpL1 protein was used as the antigen. The protein was extracted from *Escherichia coli* that had been transformed using the pGEX-6P-1 vector expressing the *OmpL1* gene in the *L. interrogans* serovar Icterohaemorrhagiae (strain RGA). GST-OmpL1 and GST (negative control) were diluted with 0.05-M carbonate buffer (pH 9.6) to a concentration of 25.0 nM; thereafter, 100 μL of each was transferred to a 96-well ELISA plate (F96 Polysorp Nunc-Immuno Plate; Nalge Nunc International, Roskilde). The plate was left overnight at 4°C for solid-phase binding, and twice washed with distilled water, and thrice washed with a washing solution [PBS + 0.05% (v/v) Tween-20]. The contents were blocked using a blocking solution [5% skim milk in PBS + 2% (v/v) Tween-20] for 1 hr at 37°C. The plate was washed with the washing solution three times, and 50 μL of the sample diluted 50-fold with the blocking solution was pipetted into each well. Thereafter, the plate was incubated on a plate shaker for 1 hr at 37°C, and washed with the washing solution three times. Subsequently, 50 μL of horseradish peroxidase-conjugated goat affinity-purified antibody to dog immunoglobulin G (IgG; Southern Biotech, Birmingham) diluted 50,000-fold with the blocking solution was pipetted into each well, and the plate incubated for 1 hr at 37°C. The plate was then washed with PBS three times, and 100 μL of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg) was pipetted into each well. The plate was shielded from light and incubated at 37°C for 40–50 min. The absorbance of each well [optical density (OD)] was measured at 405 nm using an ELISA plate reader. For each specimen, absorbance was measured in both the GST-OmpL1 and GST wells, and the difference of the average OD of these [(GST-OmpL1)–GST] was calculated. On the basis of a preliminary experiment and the findings of our previous study [15], samples with an average OD of ≥0.339 were determined to be ELISA positive.

Statistical analysis: Samples positive for at least one serovar were regarded as MAT-positive, while samples negative for all serovars were regarded as MAT-negative. MAT-positive and MAT-negative dogs were compared with respect to age using a Mann-Whitney test, and with respect to breed, sex, housing environment, going for a walk, drinking water from a natural source, playing in water, and housing location using chi-square tests. *Leptospira* vaccination history and the number of days between the last vaccination and sampling were compared between the MAT-positive and MAT-negative dogs using the chi-square and Mann-Whitney tests. A value of $P<0.05$ was considered significant.

RESULTS

MAT: Across all of the 801 dogs tested (Groups A, B, and C), 217 (27.0%) were MAT-positive (i.e., positive for at least one serovar). For each demographic item, dogs for which there was no data were excluded from the analysis. The results of comparing MAT-positive dogs and MAT-negative dogs with respect to demographic items are shown in Table 1. While there was no significant difference between MAT-positive and MAT-negative dogs with respect to sex, there was a significant difference with respect to castration or spaying ($P=0.001$). Of the 211 MAT-posi-

Table 1. Variables associated with result of MAT

		MAT	
		Positive	Negative
Mean age (y) [SD]		7.7 [3.56]	7.4 [4.09]
Sex	Intact male	59	208
	Castrated male	38	66
	Intact female	50	168
	Spayed female	67	126
	Intact*	109	376
	Castrated or spayed	105	192
Housing*	Indoors	101	238
	Outdoors	68	237
	Both	42	88
Taken for a walk	Yes	196	509
	No	8	43
Location of the house*	Urban area	34	59
	Housing area	119	363
	Rural area	52	125
Drinks water from a wild source	Yes	112	320
	No	96	232
Playing in outside water	Yes	86	186
	No	122	362

* Significantly associated with result of MAT ($P<0.05$).

tive dogs, 101 were housed indoors, 68 were housed outdoors, and 42 were housed both indoors and outdoors. There was a significant difference between the MAT-positive and MAT-negative dogs with respect to the location of the house at which they were housed (P=0.048). There was no significant difference between the MAT-positive and MAT-negative dogs with respect to any other demographic item. Figure 1 shows the geographical distribution of MAT-positive dogs, which were found in 43 of the 47 prefectures. The number of prefectures with a prevalence rate within specific ranges was as follows: ≥60% to <80%, 1 (Hyogo); ≥40% to <60%, 7; ≥20% to <40%, 21; >0 to <20%, 14 (Fig. 1A). No prefecture had a prevalence rate of ≥80%. Figure 1B-F shows the geographical distribution of each serovar. The most commonly detected serovar was Icterohaemorrhagiae (172 dogs, 21.5%), followed by Autumnalis (42 dogs, 5.2%), Canicola (27 dogs, 3.3%), Hebdomadis (17 dogs, 2.1%), and Australis (9 dogs%). Forty dogs were positive for ≥2 serovars.

ELISA: Of the 801 dogs tested, 29 (3.6%) were ELISA-positive. Figure 2 shows the distribution of ELISA-positive dogs. These dogs were found in 18 prefectures, with Fukushima having the highest prevalence rate (3/12, 25.0%), followed by Shimane (4/20, 20.0%), and Yamaguchi (3/25, 12.0%).

Influence of vaccination: Table 2 shows the relationship between *Leptospira* vaccination and MAT results (grouped by serovar). The vaccination group included dogs inoculated with either a *Leptospira*-containing vaccine or an unidentified vaccine more than 11 months earlier than the sampling day (Group C, n=415). The unvaccinated group comprised dogs that had never been inoculated with any canine vaccine (Group A, n=243). With respect to vaccination rates, there was significant difference between MAT-negative and Icterohaemorrhagiae- or Canicola-positive dogs ($P<0.0001$ and P=0.0455, respectively). The vaccination rate was relatively high in Icterohaemorrhagiae- and Canicola-positive dogs. Group C dogs were divided into Icterohaemorrhagiae-positive (+) and Icterohaemorrhagiae-negative (–) groups; Figure 3 shows the number of days between the most recent *Leptospira* vaccination and the day of sampling (27 dogs for which the precise date for the most recent vaccination was not known were excluded). The mean number of days (± standard deviation) between the most recent *Leptospira* vaccination and day of sampling was 554.0 (± 400.8) days for the Icterohaemorrhagiae-positive dogs (n=133), and 1132.2 (± 1026.6) days for the Icterohaemorrhagiae-negative dogs (n=255); this duration was significantly shorter for the Icterohaemorrhagiae-positive dogs than for the Icterohaemorrhagiae-negative dogs ($P<0.0001$).

Leptospira antibodies in unvaccinated dogs: The MAT results of the present study may have been affected by antibodies produced by vaccination. To prevent any influence of antibodies produced by vaccination, we investigated *Leptospira* antibodies in dogs never inoculated with any canine vaccine (Group A, n=243). Forty-one (16.9%) of the dogs from this group were MAT- and/or ELISA-positive (Fig. 4). These dogs were found throughout Japan (23 prefectures). The most commonly detected serovar was Icterohaemorrhagiae (22 dogs, 19 prefectures), followed by Hebdomadis (7 dogs, 3 prefectures), Autumnalis (6 dogs, 4 prefectures), Australis (5 dogs, 5 prefectures), and Canicola (2 dogs, 2 prefectures). Eight dogs were MAT-positive for ≥2 serovars. The other seven dogs of the 41 dogs were only positive for ELISA but not MAT.

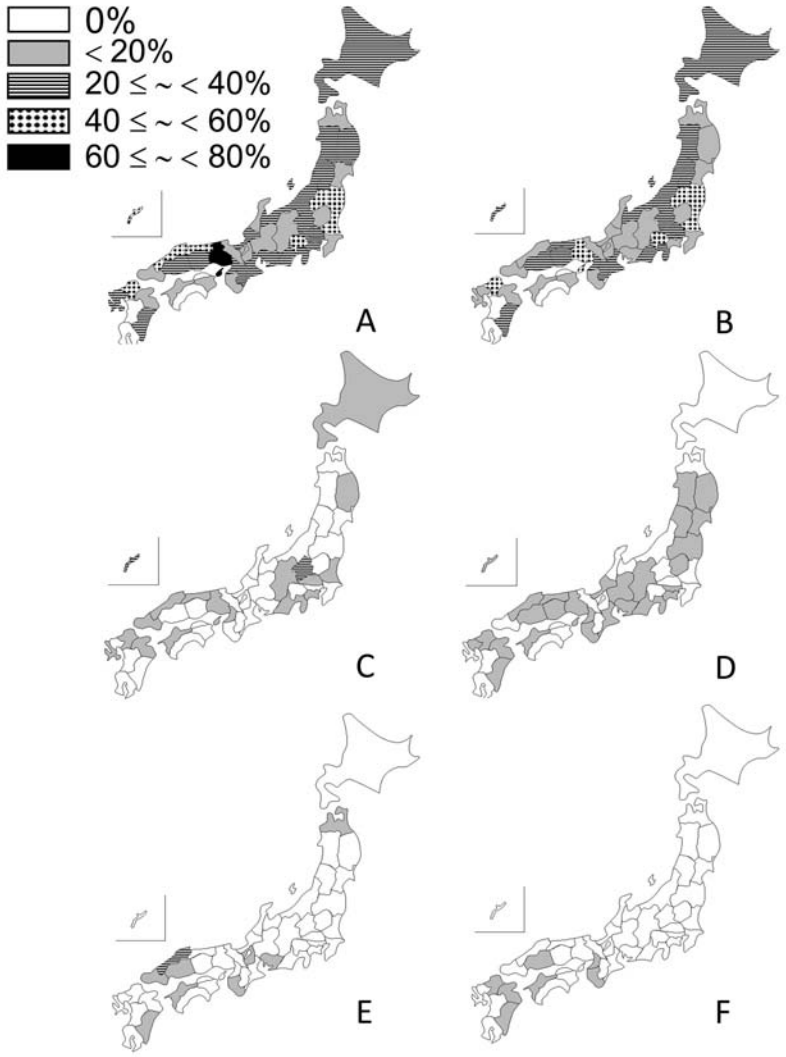

Fig. 1. Maps of Japan illustrating the geographical regions of *Leptospira* prevalence (%), as determined by MAT [All 5 serovars (A), Icterohaemorrhagiae (B), Canicola (C), Autumnalis (D), Hebdomadis (E), and Australis (F)]. Areas with prevalence rates of ≥60% to <80%, ≥40% to <60%, ≥20% to <40%, >0% to <20%, and 0% are shown as black, dotted, with horizontal lines, grey, and white, respectively.

Comparison of MAT and ELISA: Table 3 shows the relationship between the results of MAT and ELISA in dogs never inoculated with any canine vaccine (Group A, n=243). Of the 35 MAT-positive dogs, 3 were also ELISA-positive. In contrast, 8 of the 208 MAT-negative dogs were ELISA-positive. The sensitivity and specificity of ELISA relative to MAT were 8.6% and 96.2%, respectively. From the 3 dogs determined to be positive by both MAT and ELISA, 2 were Icterohaemorrhagiae-positive, and 1 was positive for both Icterohaemorrhagiae and Autumnalis at the same titer.

DISCUSSION

In the present study, we explored the nationwide prevalence of *Leptospira* antibodies in dogs by using MAT with five major serovars found in Japan as antigens. MAT is the most commonly used serological diagnostic method, although it also detects antibodies produced by vaccination [13]; moreover, the duration of elevated antibody titer due to vaccination ranges from several weeks to four months [3, 8, 11]. Therefore, in aiming to prevent any influence of antibodies produced by vaccination, the inclusion criteria for dogs initially used in the present study included those dogs that had never received a *Leptospira* vaccine, or those that had not been inoculated with this vaccine in the 11 months preceding sampling. However, the results obtained from dogs included on these bases (Group A, B and C, n=801, Fig. 1) appeared to be affected by antibodies produced by

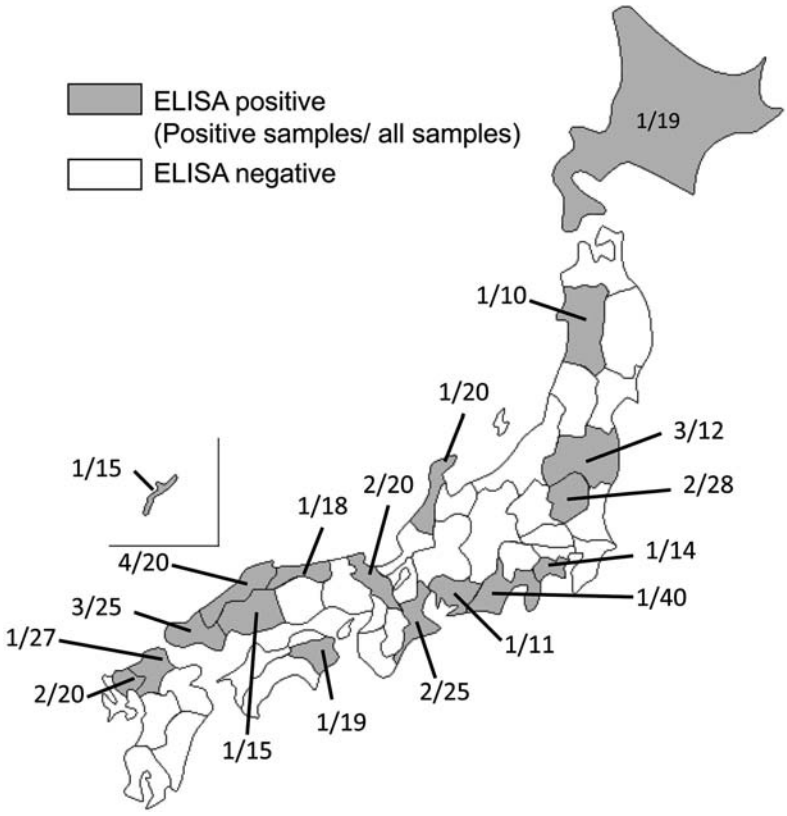

Fig. 2. Map of Japan illustrating the geographical location of 18 prefectures in which ELISA-positive dogs were found (grey, positive samples/all samples). ELISA-positive dogs were not found in 29 prefectures (white).

Table 2. Relationship between MAT results and vaccination

	Icterohaemorrhagiae[c)]		Canicola[c)]		Autumnalis		Hebdomadis		Australis	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Vaccinated[a)]	133	282	21	394	27	388	4	411	3	412
Unvaccinated[b)]	23	220	4	239	10	233	8	235	5	238
Total	156	502	25	633	37	621	12	646	8	650

a) Dogs inoculated with *Leptospira*-containing vaccine or unidentified vaccine more than 11 months from the last vaccination at the sampling time (Group C, n=415).
b) Dogs never inoculated with any canine vaccines (Group A, n=243).
c) $P<0.05$.

vaccination. There are three reasons for this, the first of which involves the main inactivated canine *Leptospira* vaccine currently used in Japan as containing 2 antigens (Icterohaemorrhagiae and Canicola). Recently, a company introduced a vaccine containing three antigens, the serovars Copenhageni, Canicola, and Hebdomadis. Copenhageni belongs to the same serogroup as Icterohaemorrhagiae, and they are very closely related serovars. There was a significant difference with respect to vaccination history (Group A versus Group C) between dogs positive for either Icterohaemorrhagiae or Canicola and MAT-negative dogs (Table 2). The vaccination rate for the Icterohaemorrhagiae- or Canicola-positive dogs was higher than for MAT-negative dogs, which suggests that antibodies produced by vaccination may have influenced the MAT results. In contrast, there was no significant difference with respect to vaccination history between the Hebdomadis-positive and MAT-negative dogs ($P=0.064$). We consider this to be because few dogs had been inoculated with the Hebdomadis-containing vaccine (as only one company has marketed this vaccine since 2000). For second reason, it also appears that the period between the most recent *Leptospira* vaccination and day of sampling may have influenced the MAT results, as this was significantly shorter for the Icterohaemor-

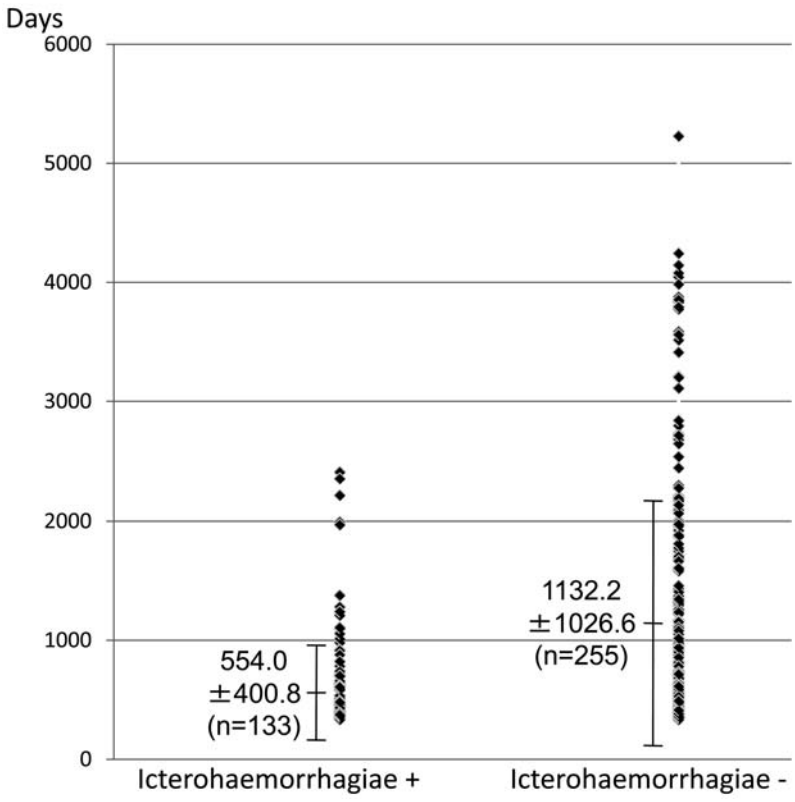

Fig. 3. Number of days since *Leptospira* vaccination plotted against MAT results for Icterohaemorrhagiae. Dogs inoculated with a *Leptospira*-containing or unidentified vaccine more than 11 months earlier than the sampling day (Group C) were divided into Icterohaemorrhagiae-positive (+) and Icterohaemorrhagiae-negative (–) groups. Twenty-seven dogs for which the precise date of the most recent vaccination was not known were excluded from the analysis. The mean (± SD) number of days since vaccination was significantly shorter for Icterohaemorrhagiae-positive dogs, 554.0 (± 400.8), than for Icterohaemorrhagiae-negative dogs, 1132.2 (± 1026.6) ($P<0.0001$).

rhagiae-positive dogs than for the Icterohaemorrhagiae-negative dogs (Fig. 3) of Group C. For third reason, relevant are the significant differences between MAT-positive and MAT-negative dogs with respect to castration, spaying, and housing environment (Table 1). Although there was no significant difference between these groups with respect to sex, there were relatively more MAT-positive dogs in the castrated or spayed group than in the intact group. Further, the number of MAT-positive dogs was greater in the indoor housing group than in either the outdoor or both indoor and outdoor groups. Compared to the other relevant groups, vaccination rates were also higher in both the castrated or spayed and indoor housing groups ($P=0.0098$ and $P=0.0006$, respectively). We consider that there were significant differences between MAT-positive and MAT-negative dogs with respect to these demographic items, because dogs either castrated or spayed or housed indoors tend to be vaccinated more strictly than intact or outdoor-housed dogs. Thus, there are three sets of findings strongly suggestive of the MAT results of the present study having been affected by antibodies produced by vaccination, even in dogs last inoculated more than 11 months earlier than the day of sampling. However, the prevalence rate of Canicola (3.3%) was much lower than that of Icterohaemorrhagiae (21.3%), despite there being a significant difference with respect to vaccination history between Canicola-positive and MAT-negative dogs (Table 2, $P=0.0455$). As discussed in more detail later, there is a possibility that the dominant serovar throughout Japan is Icterohaemorrhagiae, and that only this serovar continues to stimulate antibody production in vaccinated dogs. This idea may further help to explain why the MAT findings of the present study may have been influenced by vaccination history, despite the most recent vaccination being more than 11 months earlier than the day of sampling.

To clarify the prevalence of *Leptospira* infection, we analyzed the results for dogs never inoculated with any canine vaccine (Group A). Dogs inoculated with a vaccine not containing *Leptospira* antigens at their last vaccination (Group B) were not included in this analysis, as complete vaccina-

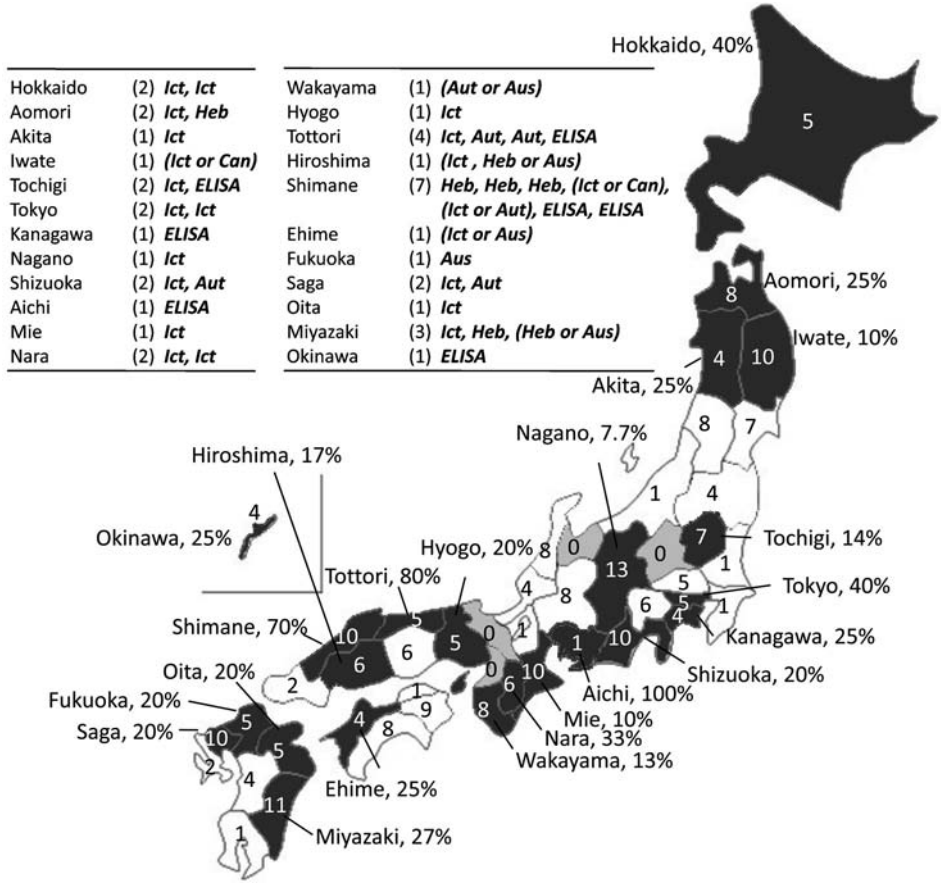

Fig. 4. Geographical distribution of *Leptospira* antibodies detected in unvaccinated dogs. The numbers of dogs never inoculated with any canine vaccine (Group C, n = 243) are shown for each prefecture. The prefectures where dogs with *Leptospira* antibodies were detected by MAT or ELISA are indicated by name, and the prevalence (%) shown. Listed for the relevant prefectures are the number of positive dogs found and the serovars detected (≥2 serovars were detected in some dogs): *Ict*, Icterohaemorrhagiae; *Can*, Canicola; *Aut*, Autumnalis; *Hed*, Hebdomadis; *Aus*, Australis.

Table 3. Evaluation of ELISA to detect anti-leptospiral antibodies in dogs sera as compared to MAT

		MAT		
		Positive	Negative	Total
ELISA	Positive	3	8	11
	Negative	32	200	232
	Total	35	208	243

Sensitivity = 8.6%, Specificity = 96.2%.

tion histories from birth were not known. Of the 243 dogs never inoculated, we found 41 from 23 prefectures to be MAT- and/or ELISA-positive (Fig. 4). Considering these results, and those obtained across all of the dogs tested in the present study (Fig. 1), it appears that the serovar Icterohaemorrhagiae exists throughout Japan. In contrast, it seems that the serovar Canicola is uncommon, and that the serovars Hebdomadis and Australis are mainly restricted to western Japan. Autumnalis positivity was also found mainly in the western part of Japan in unvaccinated dogs (Fig. 4), but throughout Japan across all of the dogs tested (Fig. 1D), and thus we cannot make any firm conclusions concerning the true distribution of the serovar Autumnalis.

There are a few reports of the prevalence of canine *Leptospira* antibodies in Japan [1, 2, 17]. A MAT survey in 1999 using eight standard serovars (the 5 used in the present study plus the Pyrogenes, Pomona, and Hardjo strains) found prevalence rates of 25.8% in Hokkaido (16/62), 40.0% in Shizuoka (30/75), 8.9% in Toyama (9/101), 10.0% in Hyogo (13/130), 15.0% in Okayama (9/60), and 29.0% in Okinawa (18/62) [1]. An earlier MAT survey (conducted in 1989) using the same antigens found a prevalence rate of 23.5% in Kagoshima (190/806) [2], while a much earlier rapid MAT (RMAT) survey (conducted in 1974) using only the Icterohaemorrhagiae and Canicola strains found a prevalence rate of 15.2% in Yamaguchi (99/650) [17]. In the present study, taking into account the results obtained across

all of the dogs examined (Fig. 1), the prevalence rates for particular prefectures were 21.0% in Hokkaido, 37.5% in Shizuoka, 37.5% in Toyama, 64.2% in Hyogo, 40.0% in Okayama, 20.0% in Yamaguchi, 0% in Kagoshima, and 46.6% in Okinawa. Thus, our results are similar to those previously reported for Hokkaido, Shizuoka, and Yamaguchi, which may imply that the findings of previous studies were also affected by antibodies produced by vaccination. Indeed, Akuzawa *et al.* mentioned the possibility of their results being affected by vaccination [1]. However, we must consider limitations of the present study beyond this affect, including the small numbers of dogs and veterinary clinics in each prefecture. For example, in August and September 2006, a cluster of human leptospirosis occurred in Miyazaki prefecture, and Koizumi *et al.* recently reported that 6 of 8 hound dogs suspected of leptospirosis showed high titers of *Leptospira* antibodies by MAT in northern part (Nobeoka) of Miyazaki prefecture [12]. However, there was no veterinary clinic in this area cooperating in the present study, although three veterinary clinics have cooporated in the prefecture. Thus, further studies are necessary to clarify the prevalence of canine *Leptospira* infection in Japan.

In the present study, we compared results obtained using MAT with those obtained using ELISA with recombinant OmpL1 as the antigen; this was done for dogs never inoculated with any canine vaccine (Group A). The sensitivity and specificity of ELISA relative to MAT were 8.6% and 96.2%, respectively (Table 3). A study of 137 serum samples using ELISA that detects *Leptospira* antibodies in dogs, with recombinant LipL32 protein as the antigen, found the sensitivity and specificity relative to MAT with seven standard serovars (Icterohaemorrhagiae, Canicola, Autumnalis, Hebdomadis, Pomona, Grippotyphosa, and Javanica) as the antigens to be 96.9% and 97.3%, respectively [6]. The specificity was similar in the present study, but the sensitivity was markedly lower. The vaccination history and clinical condition of the dogs tested with ELISA using the LipL32 protein were not reported, and it may be the case that the LipL32 ELISA detected antibodies produced by vaccination. Therefore, the low sensitivity of ELISA used in this study is not related to procedure of the assay but to the properties of the antigen, i.e., the recombinant OmpL1 protein. However, we have experienced canine leptospirosis cases, showing that the antibodies detected by OmpL1 ELISA decrease faster than those of MAT after their recovery (Wada Y. and Okuda M., unpublished observation). This phenomenon might explain the low sensitivity of OmpL1 ELISA in the present study. Therefore, this assay will not necessarily a suitable method for epidemiological surveys.

Interestingly, we found eight samples that were ELISA-positive, but MAT-negative. One possible explanation for this is that the antibody titers for serovars other than those tested in the present study were elevated. For example, a canine leptospirosis case showing the highest titer against the Castellonis serovar was reported in Miyazaki [12]. Furthermore, it is thought that ELISA detects both agglutinating and non-agglutinating antibodies, whereas only the former are detected by MAT. Indeed, the detection of non-agglutinating *Leptospira* antibodies in the sera of dogs in the early stage of infection by an indirect ELISA has been reported [9]. It is therefore possible in the present study that ELISA detected non-agglutinating antibodies.

One of the most important inclusion criteria used in the present study was vaccination history, which was typically well documented. Of the 41 MAT- and/or ELISA-positive dogs never inoculated with any canine vaccine (Group A, Fig. 4), none showed any of the clinical signs, such as renal or hepatic failure, thereby suggesting a possible case of leptospirosis. We consider that the antibodies present in these dogs were produced by subclinical infection.

In the present study, we have revealed a nationwide existence of *Leptospira* antibodies in dogs. This is particularly the case for the serovar Icterohaemorrhagiae, but not for the serovar Canicola, which appears to be relatively uncommon. Moreover, we have demonstrated that the results of epidemiological surveys using MAT can be affected by vaccination, even when this occurs more than 11 months earlier than the day of sampling. Nevertheless, the present study is limited in examining only a small number of samples from each prefecture, meaning that the results may not fully reflect the true prevalence in each of these. It is also possible that the antibody titers for serovars other than the five tested may have been elevated in the ELISA-positive and MAT-negative samples. Thus, further studies using greater sample numbers and more serovars are warranted. Moreover, the results obtained in the present study can be confirmed by detecting the pathogen itself by means of PCR or cultivation.

ACKNOWLEDGMENTS. This study was supported by Fort Dodge K.K. and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (19580369). The samples were provided by Kyoritsu Seiyaku Corporation, and we would like to thank Dr. Masami Mochizuki and Mr. Ken Kuriki.

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