Original Article

Relationship between Virus-Neutralizing Antibody Levels and the Number of Rabies Vaccinations: a Prospective Study of Dogs in Japan

Ippei Watanabe¹, Kentaro Yamada², Akira Aso³, Okio Suda⁴, Takashi Matsumoto¹, Takaaki Yahiro¹, Kamruddin Ahmed², and Akira Nishizono*¹

¹Department of Microbiology, Faculty of Medicine, and ²Research Promotion Institute, Oita University, Oita 879-5593; ³Oita Veterinary Medical Association, Oita 870-0901; and ⁴Suda Veterinary Hospital, Tokyo 191-0041, Japan

(Received July 4, 2012. Accepted October 16, 2012)

SUMMARY: A mass rabies vaccination of dogs has been conducted annually in Japan over the last 60 years. To assess both current levels of rabies virus-neutralizing antibody (VNA) in dogs and the rationale for current vaccination procedures, we used a rapid fluorescent focus inhibition test to determine VNA levels in 756 dogs that had visited animal hospitals in Japan. We found that 51.1% of the dogs that had received 1 rabies vaccination had protective VNA levels (≥0.5 IU/ml) with a geometric mean of 0.61 IU/ml. In contrast, 97.8% of the dogs that had been vaccinated at least twice had protective VNA levels with a geometric mean of 7.86 IU/ml. Furthermore, 97.9–100% of the dogs vaccinated at least twice retained protective VNA levels into the second year after the last vaccination. Although VNA levels in the dogs vaccinated at least twice tended to decline 2 years after the last vaccination, 78.9% retained protective VNA levels. Thus, the current rabies vaccination schedule provides adequate protection, but the registration system and vaccination schedule needs to be improved to ensure that increased numbers of dogs are vaccinated against rabies.

INTRODUCTION

Rabies, an acute and fatal encephalitis, is caused by the rabies virus. An estimated 55,000 people die from rabies each year worldwide; 99% of transmission is caused by dogs (the urban form) (1). Although rabies remains endemic in many countries, the virus has been eradicated in several others, including Japan. In 1950, the government of Japan enacted the Rabies Prevention Law to regulate and enforce a dog registration system, mandatory vaccination of dogs, management of unleashed dogs, stray dog regulations, a quarantine system for overseas dogs, and other practices (2–4). As a result, no cases of indigenous human rabies have been reported in Japan since 1954; further, it has not been reported in indigenous dog and cat rabies since 1956 and 1957, respectively (5).

Globalization has increased the travels of humans and animals from developing countries endemic with rabies, and thus, the risk of rabies entering Japan is growing. Natural disasters increase rabies risks as well. For example, the number of rabies cases in Japan grew during the years following the Great Kanto Earthquake of 1923 because of the increase in stray dogs (5). The number of stray dogs also increased following the huge earthquake and tsunami on March 11, 2011 (i.e., the Great East Japan Earthquake). The accidental introduction of a rabid animal to a large population of unvaccinated stray dogs promotes the rapid spread of rabies, thereby threatening Japan’s current rabies-free status.

Mass vaccination of dogs against rabies is a highly rational strategy for interrupting the natural transmission cycle of urban rabies. According to the World Health Organization (WHO), immunization of at least 70% of a dog population minimizes the risk of endemic rabies (1). However, the percentage of dogs that meet recommended virus-neutralizing antibody (VNA) levels in Japan is unknown. Furthermore, few studies have attempted to determine the adequacy of the current annual rabies vaccination program for dogs (6).

Therefore, we conducted a prospective, hospital-based study to evaluate the effects of the frequency of vaccination and intervals on serum VNA levels in pet dogs in Japan.

MATERIALS AND METHODS

Collection and storage of serum samples: A total of 756 serum samples were collected from dogs in 30 animal hospitals (28 from Oita Prefecture and 2 from Tokyo) between May 2010 and November 2011, along with information such as date of birth, number of rabies vaccinations, number of days since previous vaccination, and the sample collection date. Of the 756 serum samples, 649 were obtained from vaccinated dogs and 107 from unvaccinated dogs. Mean dog age was 6.2 years (range, 5 days to 19 years). Of the vaccinated
dogs, 249 were male, 280 were female, and the sex of 120 dogs was unknown. The sex of the unvaccinated dogs (age range, 0–12 months) was unknown. We were unable to confirm maternal protective VNA levels against rabies; however, since all subjects’ mothers had official records of multiple vaccinations against rabies, we assume that most also mounted protective VNA levels. The dog immunization formulations included 6 different commercially available rabies vaccines, all derived from the RC-HL strain.

The blood samples were obtained from dogs that had visited animal hospitals for vaccinations or other reasons. These dogs were not afflicted with any diseases related to the immune system. Verbal consent was obtained from each dog owner after a detailed explanation of the testing procedure was provided. We collected 10 ml of whole blood in plain glass tubes. After the sample was stored at room temperature for 15 min, the serum was separated from the blood by centrifugation at 2,000 × g for 10 min at 4°C. Serum samples were stored at −20°C and sent to our laboratory at Oita University. Frozen samples were thawed and serum complement was inactivated by incubation for 30 min at 56°C prior to VNA titration. This study was approved by the ethics committee of Oita University (M010003).

Determination of VNA levels: We used a rapid fluorescent focus inhibition test (RFFIT) (7,8) to determine the rabies VNA levels. First, serial serum sample dilutions were incubated in a 96-well plate with the challenge virus standard (CVS-11) rabies strain for 90 min at 37°C. BHK-21 cells were added to each well before incubating for 18 h at 37°C. Finally, the culture media was discarded and the cells were fixed in 90% acetone, stained with fluorescein isothiocyanate-conjugated anti-rabies N monoclonal antibody (Fujirebio Diagnostics, Inc., Malvern, Pa., USA) for 45 min at 37°C, and observed with a fluorescence microscope. The VNA levels were calculated based on comparison to the WHO standard serum. According to the WHO, VNA levels of ≥0.5 IU/ml provide adequate protection against rabies. This value is also recommended for dogs by the World Organisation for Animal Health (9,10).

Statistical analysis: Statistical analysis was performed using the Mann-Whitney U test to compare VNA levels. Correlations between variables were tested using Spearman’s rank correlation coefficient test. A graph was prepared with the DeltaGraph version 5 program.

RESULTS

Relationship between VNA levels and number of rabies vaccinations: We determined the rabies VNA levels in serum samples collected from dogs in animal hospitals in Japan. A VNA level of ≥0.5 IU/ml, the accepted protective level against rabies (1,9,10), was considered protective in this study. Table 1 shows the relationship between VNA levels and the number of rabies vaccinations. Of the 107 unvaccinated dogs (age range, 0–12 months), only 1 dog exhibited protective VNA levels. The geometric mean of VNA in 92 dogs that had received a single vaccination was 0.6 IU/ml; 51.1% of these dogs exhibited protective VNA levels. In contrast, the geometric mean of VNA in 557 dogs that had received multiple vaccinations was 7.86 IU/ml; 97.8% of these dogs exhibited protective VNA levels.

Duration of protective VNA after rabies vaccination: Next, we analyzed the duration of VNA in dogs that had received either a single or multiple rabies vaccinations and prepared a box and whisker plot to enhance our understanding of VNA distribution under each condition. As shown in Figs. 1A and 1C, of the dogs that had received a single vaccination, 33.3–57.7% exhibited protective VNA levels within 12 months of vaccination. The geometric mean of VNA in these dogs was 0.43–0.83 IU/ml; however, 13 months after the vaccination, 76.9% of the single vaccination dogs exhibited protective VNA levels.

In contrast, 97.3–100% of dogs that had received multiple vaccinations exhibited protective VNA levels for up to 24 months after the last vaccination (Figs. 1B and 1C). The geometric mean of VNA was elevated substantially within 18 months after the last vaccination.

Table 1. Relationship between the VNA levels and number of rabies vaccination

<table>
<thead>
<tr>
<th>No. of vaccinations</th>
<th>No. of samples</th>
<th>Average age (yr)</th>
<th>Average days after vaccination</th>
<th>Geometric mean of the VNA level (IU/ml)</th>
<th>No. of samples with VNA ≥ 0.5 IU/ml (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>107</td>
<td>0.22</td>
<td>—</td>
<td>0.12</td>
<td>1/107 (0.9)</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>2.25</td>
<td>156</td>
<td>0.61</td>
<td>47/92 (51.1)</td>
</tr>
<tr>
<td>2–</td>
<td>557</td>
<td>8.08</td>
<td>233</td>
<td>7.86</td>
<td>545/557 (97.8)</td>
</tr>
<tr>
<td>2</td>
<td>78</td>
<td>4.09</td>
<td>239</td>
<td>7.49</td>
<td>75/78 (96.2)</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>5.76</td>
<td>187</td>
<td>10.59</td>
<td>69/71 (97.2)</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>6.36</td>
<td>268</td>
<td>9.53</td>
<td>58/59 (98.3)</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>7.06</td>
<td>246</td>
<td>10.17</td>
<td>65/67 (97.0)</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>8.30</td>
<td>238</td>
<td>8.82</td>
<td>56/56 (100)</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>8.14</td>
<td>274</td>
<td>8.35</td>
<td>40/42 (95.2)</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>9.57</td>
<td>267</td>
<td>9.71</td>
<td>30/30 (100)</td>
</tr>
<tr>
<td>9</td>
<td>42</td>
<td>10.38</td>
<td>238</td>
<td>5.44</td>
<td>42/42 (100)</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td>11.22</td>
<td>209</td>
<td>8.55</td>
<td>42/42 (100)</td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>12.11</td>
<td>241</td>
<td>4.34</td>
<td>24/25 (96.0)</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
<td>12.96</td>
<td>236</td>
<td>4.71</td>
<td>19/19 (100)</td>
</tr>
<tr>
<td>13–</td>
<td>26</td>
<td>14.43</td>
<td>177</td>
<td>3.04</td>
<td>25/26 (96.2)</td>
</tr>
</tbody>
</table>

18
Fig. 1. Duration of protective VNA levels in dogs after rabies vaccinations. Box and whisker plots of VNA during each period after the last vaccination of dogs that had been singly vaccinated (A) or multiply vaccinated (B). Asterisks indicate significant differences in the VNA levels of dogs that had received a single vaccination in the corresponding period since the last vaccination ($P < 0.01$, Mann-Whitney $U$ test). (C) This table lists the characteristics of the protective VNA duration in dogs. The number of dogs with VNA measuring $\geq 0.5$ IU/ml and the corresponding geometric mean (GM) of VNA during each period are indicated.

<table>
<thead>
<tr>
<th>Month since the last vaccination</th>
<th>0-3</th>
<th>4-6</th>
<th>7-9</th>
<th>10-12</th>
<th>13-15</th>
<th>16-18</th>
<th>19-24</th>
<th>25-27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no.</td>
<td>26</td>
<td>24</td>
<td>15</td>
<td>14</td>
<td>13</td>
<td>74</td>
<td>83</td>
<td>51</td>
</tr>
<tr>
<td>No. VNA $\geq 0.5$ IU/ml (%)</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>72</td>
<td>81</td>
<td>50</td>
</tr>
<tr>
<td>GM of VNA level (IU/ml)</td>
<td>0.83</td>
<td>0.50</td>
<td>0.43</td>
<td>0.61</td>
<td>0.58</td>
<td>8.32</td>
<td>7.56</td>
<td>8.17</td>
</tr>
</tbody>
</table>

(i.e., 7.56–8.78 IU/ml). Within each time period after the last vaccination (0–3, 4–6, 7–9, or 10–12 months), VNA levels differed significantly in dogs that received single versus multiple vaccinations. In contrast, VNA levels in multiply vaccinated dogs declined by 25 months following the last vaccination. The geometric mean of VNA in this group was 2.12 IU/ml, and 78.9% of those dogs maintained protective VNA levels. These data indicate that the VNA levels were maintained adequately for 2 years following the last vaccination in dogs that had been vaccinated at least twice.

Of the 557 multiply vaccinated dogs, 12 lacked protective VNA levels. The characteristics of the dogs with inadequate VNA levels are listed in Table 2 ordered according to most recent vaccination. Of this group, 5 serum samples (Nos. 1–5) were collected less than 1 year following the last vaccination, whereas 7 samples (Nos. 6–12) were collected more than 1 year after the last vaccination. Of the 12 dogs included in this study, 4 had an immune disorder. No direct correlations were observed between lower VNA levels and sex (Spearman’s rank correlation coefficient test).

Potential maternal antibody transferred in unvaccinated dogs: Of the 107 serum samples collected from unvaccinated dogs, 72 were obtained from dogs aged 90 days or less and 35 were obtained from dogs aged
91–365 days. As described above, we found that only 1 unvaccinated dog exhibited protective VNA levels whereas many unvaccinated dogs exhibited inadequate VNA levels. We considered the possibility that the VNA detected in unvaccinated puppies derived from maternally antibody transfer; however, the levels of antibody were insufficient for protection against rabies. To examine potential maternal transfer of antibody, we set the VNA level of 0.25 IU/ml as the cutoff value. The VNA levels in these puppies are listed in Table 3. Of the 72 unvaccinated puppies aged 90 days or less, 11 samples (15.3%) exhibited VNA levels of ≥0.25 IU/ml. In contrast, only 1 of the 35 unvaccinated puppies aged over 90 days (2.9%) exhibited VNA levels of ≥0.25 IU/ml.

### Table 3. VNA levels in unvaccinated dogs

<table>
<thead>
<tr>
<th>Age range (days)</th>
<th>No. of samples</th>
<th>VNA (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–90</td>
<td>72</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>91–365</td>
<td>35</td>
<td>61</td>
</tr>
</tbody>
</table>

#### DISCUSSION

The WHO recommends that at least 70% of the dog population should be immunized against rabies to minimize the risk of its reemergence (1), and this recommendation is particularly important in Japan, where rabies has been eradicated. A mass rabies vaccination of dogs is conducted in Japan each year, but neither the current status of rabies VNA in dogs nor the rationale for conducting an annual rabies vaccination program has been assessed. Therefore, we aimed to determine the actual VNA levels in dogs that had visited animal hospitals in Japan.

We determined that approximately 50% of singly vaccinated dogs did not exhibit protective levels of serum VNA within 1 year of vaccination, which indicates that single vaccination dosing is insufficient for establishing herd immunity. A previous study also demonstrated that the VNA titer dropped to <0.5 IU/ml in >50% of dogs vaccinated within 120 days after a single vaccination (11). In the current study, however, the VNA levels in dogs that had received a single vaccination were higher 13 months after a single vaccination than those with a history of at least 2 vaccinations (Fig. 1B), which indicates that the current annual vaccination schedule is sufficient for protection against rabies, although, a biennial vaccination schedule could be considered. In Texas, the vaccination schedule has shifted from yearly to triennial and effectively increased dog and cat vaccination rates significantly (13). Several factors require careful consideration if the mandatory vaccination schedule in Japan is changed in the future, including confirmation of sustainable VNA levels, the introduction of a simple serological survey method, and measures implemented to protect low responders. We recommend that VNA levels in dogs are checked every year to confirm the need for vaccination. However, the methods used to measure the rabies VNA level, such as virus neutralization assays and ELISA, are inconvenient and require specialized laboratories and equipment, and trained personnel. Recently, we developed a rapid, safe, easy, and inexpensive VNA test for rabies based on a combination of an immunochromatographic technique and a competitive immunoassay with neutralizing monoclonal antibodies (14,15). This semi quantitative test determines within 60 min whether a dog has protective VNA levels, without using a live virus and expensive equipment. This may represent a convenient method for determining whether dogs require booster immunization.

The Ministry of Health, Labour and Welfare of Japan reported the registration of 6.88 million dogs in 2009; 74.3% had been vaccinated against rabies (16). However, in 2009, the Japan Pet Food Association reported that the number of breeding dogs in Japan was 12.32 million (17). When unregistered dogs are considered in the total, only 41.5% of dogs are vaccinated against rabies, which pushes the immunization level far below the required 70% level recommended for rabies epidemics prevention in a population. The Ministry of Agriculture, Forestry, and Fisheries of Japan reported that about 5 million doses of rabies vaccine were produced in 2011 (18), which indicates that insufficient vaccine availability to cover all dogs in Japan. Therefore, we suggest in the event of an outbreak of rabies in Japan, unvaccinated dogs or singly vaccinated dogs should receive vaccine priority above dogs that have been vaccinated at least twice.

In Japan, about 50,000 stray dogs visit animal health centers each year (16) and approximately 5,000 dog bite cases are reported (19). Unvaccinated stray dogs may be a risk for the circulation of rabies and its transmission to humans. An understanding of rabies seroprevalence in stray dogs is important for an accurate assessment of the risk of rabies reemergence in Japan, but its seroprevalence was not surveyed in this study. Ogawa et
al. reported that only 27.7% of stray dogs that were captured by rabies prevention officers and brought in to the Hyogo Prefecture Animal Well-being Center in Japan in 2006 and 2007 exhibited protective VNA levels (20). Additional seroprevalence surveys should be conducted on stray dogs from other geographical areas to determine the actual state of rabies immunity in the dog population of Japan.

We detected maternal rabies antibodies in 15.3% of unvaccinated puppies less than 3 months in age, whereas we detected these antibodies in only 1 unvaccinated dog 3 or more months in age. The rabies vaccines that are produced for dogs in Japan are inactivated, and thus, maternal antibodies do not interfere with the vaccine response in puppies (21). However, maternal antibodies can interfere with the immune response to live attenuated rabies vaccines (22,23). Therefore, the future introduction of live vaccines for mass dog immunization should consider the timing of vaccinations in puppies.

The results of this study suggest the current annual rabies dog vaccination program is adequate given that all dogs are registered and vaccinated each year. However, as suggested above, the rates of rabies vaccination in dogs of Japan are supposed to be far less than 70%. More than 60 years have passed since the introduction of the Rabies Prevention Law in Japan, and Japanese lifestyles and the relationships between humans and pet dogs have changed dramatically during this period. Therefore, the present may be the appropriate time period to consider updating the rabies prevention system.

Acknowledgments We thank Suda Veterinary Hospital, Oita Veterinary Medical Association, and Kyoritsu Seiyaku Corporation for sample collection, and Ms. Kazuko Noguchi for RFFIT.

Conflict of interest None to declare.

REFERENCES