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Short Communication

Safe and Easy Monitoring of Anti-Rabies Antibody in Dogs Using His-Tagged Recombinant N-Protein

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SUMMARY: The virus neutralization (VN) test is a reliable indicator of adequate vaccination in animals. However, the VN test is tedious and complicated to perform. Enzyme-linked immunosorbent assay (ELISA), though rapid and simple compared to the VN test, is complicated and hazardous during preparation of the viral antigen. In an effort to overcome the disadvantage of ELISA, the recombinant His-tagged nucleoprotein (His-rNP) expressed in *Escherichia coli* was used as a safe antigen for ELISA (i.e., live virus was not used). Anti-rabies antibody levels were determined by fluorescent ELISA (FELISA) using His-rNP as an antigen. The presence of anti-rabies VN antibody was determined by the rapid fluorescent focus inhibition test (RFFIT). The VN titers by RFFIT were found to correlate well with the FITC-signal determined by the FELISA (r = 0.616). The sensitivity and specificity of the FELISA were 91.7 and 100%, respectively. This study showed that the His-rNP could be useful as an antigen of ELISA to test for anti-rabies antibody in vaccinated dogs. Several studies in Japan have investigated the antibody level in the sera of vaccinated dogs. A safe and convenient test using His-rNP would contribute to our understanding of the status of herd immunity among not only domestic dogs but also stray dogs in Japan.

In 1957, Japan successfully eradicated dog rabies, and has by now been a rabies-free country for over 45 years. Although rabies in domestic animals is controlled in many industrialized countries, in many parts of the world rabies still poses a significant public health problem for humans as well as domestic animals (1).

Rabies-free status in Japan has been sustained by strong legislative controls, including an animal quarantine inspection system and an annual vaccination program for domestic dogs. The virus neutralization (VN) test is a reliable indicator of adequate vaccination in animals. However, the VN test is tedious and complicated to perform, making it unsuitable for large-scale seroepidemiologic surveillance studies (2). Enzyme-linked immunosorbent assay (ELISA), though rapid and simple compared to the VN test, is complicated and hazardous during preparation of the viral antigen (3,4). In an effort to overcome the disadvantage of ELISA, the recombinant His-tagged nucleoprotein (His-rNP) expressed in *Escherichia coli* (5) was used as a safe antigen for ELISA (i.e., no live virus was used). The His-rNP was expressed by *E. coli* (DH5α) transfected with the pQE-9 vector plasmid harboring a full-length double-stranded cDNA coding for the NP gene of the CVS-11 strain according to the manufacturer's protocol (QIAGEN, Valencia, Calif., USA).

Serum samples were collected from 21 domestic dogs that visited the Veterinary Medical Center of Tokyo University (Table 1). Twelve dogs had been vaccinated 7 months previously, and nine dogs had no history of vaccination. The presence of anti-rabies neutralizing antibody was determined by the rapid fluorescent focus inhibition test (RFFIT) (6).

Anti-rabies antibody levels were determined by a fluorescent ELISA (FELISA) using His-rNP as an antigen. Due to the fact that the FELISA showed a very dynamic, wide range of FITC detection, it was not necessary to include several dilutions of serum samples. Preliminary experiments indicated that the serum sample diluted 1:5 was sufficient to obtain the required sensitivity and specificity. The 96-well microtiter plates were coated with His-rNP diluted in carbonate buffer (pH 9.5) (1 μg/100 μl/well) at room temperature (r.t.) for 2 h. After blocking with 0.25% bovine serum albumin (BSA) in phosphate-buffered saline without magnesium and calcium [PBS(–)] at r.t. for 1 h, 100 μl of each serum sample diluted with PBS(–) was placed in a His-rNP coated well then incubated at r.t. for 1 h. After washing the wells 4 times with 200 μl of 0.05% Tween 20 with 0.25% BSA in PBS(–), 100 μl of FITC conjugated sheep anti-dog IgG (BETHYL laboratories, Inc., Montgomery, Tex., USA) diluted with 0.25% BSA in PBS(–) was added, and the plates were further incubated at r.t. for 1 h. After washing the wells 4 times with 0.05% Tween 20 - 0.25% BSA in PBS (–), 100 μl of PBS(–) was added, and the intensity of the fluorescence was recorded using a fluorescence plate reader (FL500 Fluorescence Plate Reader, Bio-Tek Instruments, Inc., Winooski, Vt., USA).

The intensity of fluorescence corresponding to the level of anti-viral nucleoprotein antibody was expressed as the FITC-signal ([FITC intensity of wells with sample serum] − [FITC intensity of the wells without sample serum (FITC-signal of background)]). The FITC-signals of serum samples obtained from vaccinated and non-vaccinated dogs varied from 685.8 to 2363.8 and from 53.3 to 543.8, respectively (Table 1). In

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Table 1. Profiles of serum samples used in this paper

Sample	Immunization[1)]	Species	RFFIT titer[2)] (IU/ml)	FS[3)]
1	+	Samoyed	16 (0.1)	685.8
2	+	Pug	113 (0.8)	1411.8
3	+	Hybrid	143 (1.0)	2363.8
4	+	Labrador retriever	16 (0.1)	1140.3
5	+	unknown	81 (0.6)	857.8
6	+	Dachshund	21 (0.2)	894.8
7	+	Pug	81 (0.6)	1610.3
8	+	Hybrid	48 (0.3)	2073.8
9	+	Hybrid	58 (0.4)	1312.8
10	+	Dalmatian	818 (5.8)	2252.3
11	+	Kai	57 (0.4)	1744.3
12	+	Akita	525 (3.8)	1462.3
13	–	Beagle	<5 (0.0)	53.3
14	–	Labrador retriever	<5 (0.0)	134.3
15	–	Hybrid	<5 (0.0)	530.8
16	–	Hybrid	<5 (0.0)	288.3
17	–	Hybrid	<5 (0.0)	404.8
18	–	Beagle	<5 (0.0)	225.8
19	–	Beagle	<5 (0.0)	320.8
20	–	Labrador retriever	<5 (0.0)	259.8
21	–	Hybrid	<5 (0.0)	543.8

[1)]: Vaccinated at April, 1999 and bled at November, 1999.
[2)]: Titer of RFFIT was 50% focus-forming dose (FFD_{50}). The RFFIT titer of serum samples was expressed as IU per ml (IU/ml) by comparison with the result obtained using the reference standard.
[3)]: FS = FITC-signal.

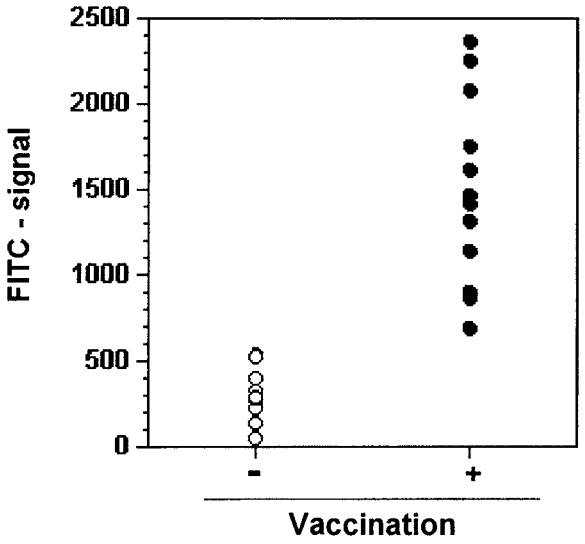

Fig. 1. Comparison of FITC-signal of antibody reacted with the recombinant His-tagged nucleoprotein between vaccinated and non-vaccinated dogs.

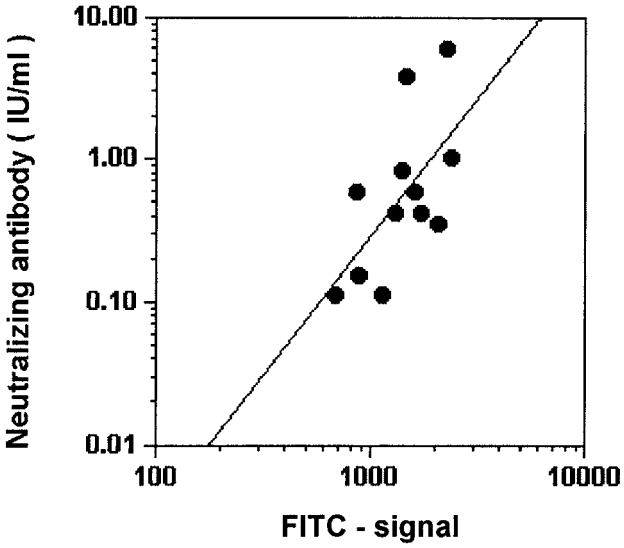

Fig. 2. Comparison of serum anti-rabies neutralizing antibody (IU/ml) and anti-rabies nucleoprotein antibody (FITC-signal). $Y = 1563.3X^{1.942}$, $r = 0.616$.

all cases, the FITC-signals of vaccinated dogs were greater than those of non-vaccinated dogs (Fig. 1). The negative cutoff point was set at 803.5 by calculating the arithmetic mean of FITC-signal plus a standard deviation (SD) of 3, using the FITC-signal of non-vaccinated dogs (M = 306.8 and SD = 165.6).

All the serum samples of vaccinated dogs were shown to be positive by the RFFIT test, in which sera with a titer greater than 0.1 IU/ml were considered positive (6,7). The VN titers by RFFIT test were found to correlate well with the FITC-signal determined by the FELISA ($r = 0.616$) (Fig. 2). However, one serum sample tested positive by the RFFIT (VN titer by RFFIT test = 0.1 IU/ml) but negative by the FELISA (FITC-signal was 685.8). Thus, the sensitivity and specificity of the FELISA were 91.7 and 100%, respectively. This study, therefore, shows that the His-rNP could be useful as an antigen in ELISA used to test for anti-rabies antibody in vaccinated dogs.

Several studies in Japan have investigated the antibody level in the sera of vaccinated dogs (2,4). A safe and convenient test using His-rNP would contribute to our understanding of the status of herd immunity among not only domestic dogs

but also stray dogs in Japan.

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