NOTE *Avian Pathology*

Field Basis Evaluation of *Eimeria necatrix*-Specific Enzyme-Linked Immunosorbent Assay (ELISA) for Its Utility in Detecting Antibodies Elicited by Vaccination in Chickens

Hiroshi ONAGA[1)], Fumiya KAWAHARA[1)], Kazuhiro UMEDA[1)] and Shinya NAGAI[1)]

[1)]*Nippon Institute for Biological Science, 9–2221–1 Shinmachi, Ome Tokyo 198–0024, Japan*

(Received 2 February 2005/Accepted 20 May 2005)

ABSTRACT. *Eimeria necatrix*-specific ELISA, using a recombinant antigen (the cDNA-clone NP19 expressing protein), was utilized to detect antibodies against *E. necatrix* in breeder pullet flocks that had previously received an attenuated live vaccine to *E. necatrix*. Vaccinated flocks were discriminated significantly from non-vaccinated flocks by their antibody titers and antibody positive rates at 30–55 days post-vaccination. In addition, *E. necatrix*-oocysts were confirmed in fecal samples of vaccinated flocks using PCR in the case where the antibody positive rates rose. These findings implied that the vaccination prompted repeated infections, and consequently the chickens generated antibodies and secured their protection against virulent field-*E. necatrix*. Therefore, the ELISA was suggested to be a useful tool to estimate the immune state of chickens as a result of vaccination with a live *E. necatrix*-vaccine.

KEY WORDS: *Eimeria necatrix*, ELISA, vaccination.

J. Vet. Med. Sci. 67(9): 947–949, 2005

Coccidiosis induced by *E. necatrix*-infections has been known to cause severe economical loss in breeder chicken industries. Control of the disease relies exclusively on the protective immunity conferred on chickens. To immunize chickens against *E. necatrix*, a planned immunization program [1] with field isolates had been extensively performed in breeder pullet flocks; nonetheless, such measures assumed the risk of leading to outbreaks. The immunizing stages that depend on such non-attenuated coccidia were usually accompanied with an incidence of the infection, including the expression of clinical signs as a tendency recognizable at a flock level. The evidence of infection, however, indicated that the infections induced by vaccination actually continued to repeat. Repeated infections are the essential factor to elicit protective immunity in chickens, especially when receiving live coccidial vaccines. Recently, an attenuated live vaccine to *E. necatrix* (NECA, Nisseiken Co., Ltd., Japan) became available in Japan. The vaccine seed is a precocious line of *E. necatrix*, named Nn P-125, which bears a markedly milder pathogenicity than those shown by the wild type strains. The infections induced by vaccination are commonly expressed as being sub-clinical. Conversely, the lack of distinct clinical signs tends to elicit concern among vaccine-customers as to whether the vaccinated chickens are taking the relevant steps toward immunization. Therefore, an appropriate method to reveal the vaccinating outcomes is required. The polymerase chain reaction (PCR) has been developed to distinguish *E. necatrix* from other species accurately [9], and the method is assumed to serve for the present role. On the contrary, we have developed an ELISA that uses a recombinant antigen (NP19-clone protein) to detect antibodies against *E. necatrix* species specifically [10]. The purpose of the present study was to evaluate the ELISA for its ability to assess the immune condition on a flock basis after vaccination with a live attenuated vaccine against *E. necatrix*.

Fifteen breeder-pullet-flocks that received a commercial live vaccine NECA were taken for the survey. The flocks were comprised of 800–10,000 birds with their breeds of Chankee, Cobb and Boris-Brown, distributed throughout the nation in Japan. The vaccination was conducted on chickens at the age of 5–18 days, according to the manufacturer's instructions. For the non-vaccinated controls, 15 breeder pullet flocks consisting of Chankee and Cobb and 7 commercial layer chicken flocks using Juria were used. Sera were obtained 21 to 55 days post-vaccination (DPV) in vaccinated flocks, at the age of 30–61 days in unvaccinated breeder pullet flocks and 80–137 days in commercial layer chicken flocks. Ten sera samples were collected in each sampling time in all the flocks, therefore, the sum of samples was 120 in the vaccinated flocks for the quantitative analysis, 120 in those for the antibody-kinetic- analysis, 150 in the unvaccinated breeder pullet flocks or 70 in the layer chicken flocks. To confirm *E. necatrix*-oocysts in droppings of the vaccinated chickens, and thereby to secure the view that the infections certainly repeated or lasted, the PCR was conducted. For the PCR, fecal materials were collected 35, 55 and 42 DPV in the A (Cobb), B (Boris-Brown) and C (Chankee) flocks, respectively, as randomly from the floor of a chicken house at a volume of more than 2 kg. The collected materials were packed in a plastic bag and sent to our laboratory under kept in cooling condition.

For ELISA, the antigen corresponding to the merozoite-protein of *E. necatrix* was prepared as the supernatant of sonicated cultured cells (sf21-cell line) that had been inoculated with the baculo-virus reconstructed with the cDNA clone, NP19. The clone NP19 was the DNA-fragment of 879 bp and the protein expressed to the clone was 35 kD in its molecular weight. The exact process to construct the clone and the nature of the protein were described previ-

ously [10]. This antigen was found not to possess any effective antigenicity to confer protection on chickens against *E. necatrix*-coccidiosis. Therefore, the ELISA-results do not mean the immune state of chickens, but mean that the birds previously received infections with *E. necatrix*. The ELISA-technique has also been described previously [10]. Briefly, an antigen-coated microtiter plate (Immuno-plate; Nalge Nunc International, Denmark) was reacted with 1:100-diluted test sera for 1 hr at 37°C, then washed and added to a 200 times dilution of goat anti-chicken IgG conjugate (Bethyl Laboratories Inc., TX U.S.A.). The diluted sera and all the reagents added to the well were usually 50 μl in volume. The plate was reincubated as above, washed, and received with 0.08% 22'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.006% H_2O_2 in 0.1 M citrate buffer, pH4.0. The plate was allowed to sit for 10 min at room temperature, and then treated with 0.1 N NaOH. The optical density (OD) was measured at 405 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Antibody positive and negative reference sera obtained respectively from *E. necatrix*-infected and non-infected specific-pathogen-free (SPF) chickens were used consistently as a pair of the same preparation in all plates tested. The results for the test sera were converted into ELISA (E) values with the following formula. E value = [OD of test serum - OD of negative-reference serum] ÷ [OD of positive reference serum - OD of negative-reference serum]. An E-value of 0.4 was selected tentatively as the cut-off-level to define chickens as antibody positive. This level was set at a point 4 times higher than that of the negative-reference serum.

The mean E-value was calculated in every flock, taking 10 samples for each, and then the mean E-values obtained were averaged in both categories of vaccinated and unvaccinated groups as shown in Table 1, and thereby termed the averaged mean E-values. Statistical analysis was accomplished with Student's *t*-test for averaged mean E-values, but with χ^2 for the mean positive rates.

For the PCR, oocysts were isolated from fecal materials with saturated salt flotation, and purified using sodium chloride solution containing 0.5% active chloride (Kanto Chemical Industry Co., Japan) for 20 min. The suspension of purified oocysts was put in a tube containing glass beads (0.5 mm in diameter), and then the tube was put on vortex-operation to obtain the sporocysts. The centrifuged sediment of sporocysts was suspended in 200 μl of InstaGene^{TM} Mattrix (Bio-Rad Laboratories), heat-treated at 56°C for 20 min, followed by 100°C for 8 min with a vortex-step between both heating operations, and then centrifuged. The supernatant was used as a PCR-sample at a dilution of 1:10 and 1:100 in distilled water. The techniques of PCR, including the sequencing patterns of primer pairs for the *E. necatrix* used were the same as those described by Schnitzler *et al.* [9] with slight modifications. The cycling programs were performed in a Gene Amp PCR System 9700 (PE Applied Biosystems) at 93°C for 1 min, followed by 35 cycles consisting of 93°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, and a final extension of 72°C for 5 min.

The results obtained are summarized in Tables 1 and 2. None of the clinical signs characteristic of *E. necatrix* were noted in any of the vaccinated flocks. The averaged mean E-value of the vaccinated groups was 1.62 ± 0.6 and the mean positive rate of chickens was 89%. These values were significantly different from those of the unvaccinated control groups (Table 1). The averaged mean E-values of younger and older unvaccinated groups were close together but did not show a statistical difference, indicating that the nonspecific reactions occasionally seen in sera of older chickens were not involved. In the assay to watch the antibody-kinetics (Table 2), both the mean E-values and the antibody-positive rates of chickens were gradually elevated in a synchronous manner after vaccination in all of the 3 flocks tested, reflecting the fact that an immunity-developing process occurred in the chicken flocks. The PCR conducted on the fecal materials from these vaccinated flocks showed the accumulation of *E. necatrix*-oocysts in a detectable quantity. These results suggested that recurrent infections with the *E. necatrix*-vaccine strain had occurred successfully in the vaccinated flocks, despite the lack of any noticeable clinical signs.

Protective immunity against coccidiosis has been known to develop in chickens relying on consecutive infections, even though the infections were extremely low in magnitude [2–6]. The intensity of infections was not always included in the factors stimulating immunity. In our previous study

Table 1. Mean ELISA-values in flocks that received or did not receive NECA

Group	Number of flocks	Range of ages on the day of the ELISA test (Days post-vaccination)	Averaged mean ELISA-value	Mean positive rate (%)
Vaccinated	12	38–49 (30–42)	1.62 ± 0.60[a)]	89[a)]
Unvaccinated	15	30–61	0.05 ± 0.04[b)]	4[b)]
Unvaccinated	7	80–137	0.04 ± 0.03[b)]	3[b)]

Ten sera samples were collected from each flock, and a mean ELISA-value was obtained on a flock basis before calculating in the two different categories of vaccinated and unvaccinated. The positive level was determined as ≥0.4 for the E-value. Small script a) and b) indicate a significant difference in the values between the groups with at a level of $p<0.001$.

Table 2. Movement of ELISA-values in 3 breeder pullet flocks after vaccination with NECA

Frock (Chicken breed)	Age at day of vaccination	Days after vaccination until tested with ELISA	Mean E-value	Number of birds positive / number of birds tested
A (Cobb)	5	28	0.28 ± 0.37	2 / 10
		35	1.99 ± 1.36	8 / 10 *
		42	1.97 ± 0.07	10 / 10
		49	1.67 ± 0.31	10 / 10
B (Boris Brown)	9	34	0.13 ± 0.14	1 / 10
		44	0.04 ± 0.04	0 / 10
		49	0.28 ± 0.42	2 / 10
		55	1.22 ± 0.73	8 / 10 *
C (Chankee)	18	21	0.07 ± 0.11	1 / 10
		28	0.32 ± 0.41	2 / 10
		35	0.27 ± 0.23	3 / 10
		42	0.85 ± 0.87	5 / 10*

* The star marks indicate that the fecal oocysts sampled at those ages were proven to contain *E. necatrix* oocysts with the PCR.

[10], we inoculated oocysts of the *E. necatrix*-precocious line Nn P-125 to SPF chickens that were raised on a litter floor, and the subsequent antibody responses were examined with the ELISA. Antibodies to *E. necatrix* were detected 14 days post-inoculation (DPI), created a peak in titer at 21 DPI, and then declined to one-third the OD-level from that of the peak by 56 DPI. Moreover, the assay at 56 DPI revealed that several chickens turned out to be serologically negative. All of the birds, however, exhibited good protection against the challenge conducted with a virulent strain at 56 DPI. These findings implied that once *E. necatrix*-infections are confirmed in a flock, the chickens are regarded as having become immune. Hence, the *E. necatrix*-specific ELISA is an available means to judge chickens vaccinated as they develop immunity. As the condition to ensure the good immunity for a flock, the underneath levels of a mean E-value and a positive rate are supposed to be set as 0.8 and 50%, respectively, when the data of antibody kinetics were taken into consideration. It would be desirable that the assessment of chickens can be made as remaining a spare of time that is long enough to allow the revaccination procedure if needed. The chicken ages susceptible to *E. necatrix*-related outbreaks when being raised on the litter floor have been defined as those older than 8 weeks [7, 8]. According to the present surveys, the best time to conduct the ELISA for the flocks that received *E. necatrix*-vaccine is considered to be appropriate at around 5–7 weeks post- vaccination.

By employing PCR, it was confirmed that the vaccinated chickens underwent repeated infections with *E. necatrix*, therefore, the PCR is also expected to be a suitable tool to analyze the vaccination outcomes. Nonetheless, the preparation of oocyst-samples from the field-sampling materials was a relatively mundane task; consequently, the PCR is not feasible when one needs to cope with a large number of samples concurrently.

The *E. necatrix*-specific ELISA supplied the system to examine the chicken's immune condition as the reflection of the vaccinating effect. For other pathogenic *Eimeria*, oocyst-detection and oocyst-per-gram- counting have been performed widely at a practical field of vaccination, because species other than *E. necatrix* are multiplicative in oocyst production, which makes it easy to conduct the test. However, to modernize check system it would be desirable to supply a similar ELISA system for these species.

REFERENCES

1. Edgar, S. A. 1986. p. 617. *In*: Research in Avian Coccidiosis (McDougald, L. R. and Long, P. L. eds), Univ. Of Georgia.
2. Davis, P. J., Barratt, M. E. J., Morgan, M. and Parry, S. H. 1986. pp. 618–633. *In*: Research in Avian Coccidiosis (McDougald, L. R. and Long, P. L. eds), Univ. Of Georgia.
3. Hein, H. E. 1976. *Exp. Parasitol.* **40**: 250–260.
4. Johnson, J. K., McKenzie, M. E., Perry, E. and Long, P. L. 1986. pp. 634–641. *In*: Research in Avian Coccidiosis (McDougald, L. R. and Long, P. L. eds), Univ. Of Georgia.
5. Joyner, L. P. and Norton, C. C. 1973. *Parasitology* **67**: 333–340.
6. Joyner, L. P. and Norton, C. C. 1976. *Parasitology* **72**: 115–125.
7. Mattiello, R., Boviez, J. D. and McDougald, L. R. 2000. *Avian Dis.* **44**: 711–714.
8. McDougald, L. R., Fuller, A. L. and McMurray, B. L. 1990. *Avian Dis.* **34**: 485–487.
9. Schnitzler, B. E., Thebo, P. L., Mattsson, J. G., Tomley, F. M. and Shirley, M. W. 1998. *Avian Pathol.* **27**: 490–497.
10. Tajima, O., Onaga, H. and Nakamura, T. 2003. *Avian Dis.* **47**: 309–318.