

## **The Characteristics of The Precocious Line Nn-P125 of *Eimeria necatrix***

H. ONAGA, T. KITAMURA, M. TAJIMA, AND T. NAKAMURA

*Nippon Institute for Biological Science, 9-2221-1 Shinmachi Ome, Tokyo 198-0024, Japan*

Received October 15, 1999 and Accepted January 10, 2000

Keywords: *E. necatrix*, Precocious, Attenuation

### **ABSTRACT**

The precocious line, Nn-P125, was assayed on its characteristics relevant to an attenuated live vaccine. The Nn-P125 line was attenuated to such an extent that all chickens did not exhibit any clinical signs by oral inoculation with  $9 \times 10^4$  oocysts/bird. In contrast, the same number of oocysts of the parent strain killed all the birds inoculated. The prepatent period of the line was shortened about one day as compared with that of the parent Nn strain. These characteristics of Nn-P125 were stable throughout repeated relaxed passages in chickens. The line has an immunological potential to the extent of that chickens received 25 or more oocysts/bird resisted the challenge conducted 4 weeks after vaccination. Chicks aged 3 days or more were found to be suitable for vaccination. Based on these results, the Nn-P125 line was recognized to be an appropriate seed of an attenuated live vaccine against *Eimeria necatrix* infection.

### **INTRODUCTION**

Outbreaks of coccidiosis caused by *E. necatrix* have usually been seen in breeder-pullet-flocks after 12 weeks of age. The disease frequently inflicts serious economic losses due to increased mortality and decreased yield of fertilized eggs. To prevent the disease, an artificial immunization relied on planned infections with field isolates or vaccination with attenuated live vaccines has been considered only an effective means, because coccidiostatics cannot be applied to breeder pullets aged

more than 10 weeks by the stipulation of the feed-safety-law. In Japan, no vaccine against *E. necatrix* is available, although the vaccine consisting of precocious lines of *E. tenella*, *E. acervulina* and *E. maxima* (Nisseiken chicken coccidial trivalent attenuated live vaccine named TAM) has been marketed. It has been known that the precocious line of *E. necatrix* selected by Shirley and Bellatti, 1984 retained immunogenicity appropriate to an attenuated live vaccine. Therefore, we have established a precocious line of *E. necatrix* named Nn-P125 aiming at developing an effective attenuated live vaccine. This paper describes the characteristics of Nn-P125 relating to an attenuated live vaccine.

## MATERIALS AND METHODS

### *Parasites:*

The Nn strain of *E. necatrix* was isolated from a field sample of chicken intestine in 1972, and has been maintained in our laboratory. The precocious line Nn-P125 was selected from the Nn strain by serial passage through chickens of the first oocysts produced during infections. As the result of 30 passages in chickens, the pre-patent period of the precocious line became about one-day shorter than that of the parent strain. The line proved to be of *E. necatrix* by the polymerase chain reaction on the ITS1 region of the parasite genome using the *E. necatrix*-specific primer in combination with those for other species that were prepared by the method of Schinezler et al. (1998).

### *Chickens:*

Specific-pathogen-free chickens (line M) obtained from the NIBS Laboratory Animal Research Station, Kobuchizawa, Japan, were used throughout the study.

### *Vaccination and Challenge:*

Vaccination of chickens with Nn-P125 line was conducted by in-feed administration with oocysts, while the challenge of immunized chickens was performed by oral inoculation with 50,000 oocysts of Nn strain/bird.

## RESULTS

### *Comparison of pathogenicities between the Nn-P125 line and Nn strain*

In groups of ten 52-day-old chickens, degrees of infection were compared



CHARACTERISTICS OF *EIMERIA NECATRIX* NN-P125

between the Nn-P125 line and Nn strain by oral inoculation with  $1 \times 10^4$ ,  $3 \times 10^4$  or  $9 \times 10^4$  oocysts/bird. During the 8-day observation period, all the chickens given the Nn strain showed severe clinical signs such as anorexia, depression and bloody droppings, whereas no clinical signs were observed in chickens inoculated with Nn-P125 line (Table 1). Mortalities were 60% and 100% in the groups received  $3 \times 10^4$  and  $9 \times 10^4$  oocysts of the Nn strain/bird, respectively. The relative body weight gains in chickens administered with Nn-P125 line or Nn strain were expressed as a percentage of the control value. The relative body weight gains of Nn strain-groups marked minus values. In the groups received the Nn-P125 line, mean body weight gains decreased in proportion to the increasing number of oocysts dosed. In the groups received  $3 \times 10^4$  or less oocysts/bird, the mean values did not differ significantly from the control values (Table 1). Oocysts were excreted in the feces of chickens from 6 days after inoculation with the Nn-P125 line, while from 7 days after inoculation with the Nn strain.

Table 1. Comparison of pathogenicities between *Eimeria necatrix* Nn-P125 line and Nn strain.

Coccidium	Inoculated Oocysts /bird <sup>a</sup>	died/ tested	Relative body wt. gain (%)	Bloody droppings at day			
				5	6	7	8
Nn-P125 line	$1 \times 10^4$	0/10	94.4	-	-	-	-
Nn-P125 line	$3 \times 10^4$	0/10	87.8	-	-	-	-
Nn-P125 line	$9 \times 10^4$	0/10	62.9 <sup>b</sup>	-	-	-	-
Nn strain	$1 \times 10^4$	0/10	-10.5 <sup>c</sup>	-	+2	+3	+3
Nn strain	$3 \times 10^4$	6/10	-15.0 <sup>c</sup>	+1	+3	+3	+3
Nn strain	$9 \times 10^4$	10/10	ND	+2	+3	ND	ND
Control	0	0/10	100.0	-	-	-	-

a: Ten 52-day-old SPF chickens/group were inoculated with one of these doses, and observed for 8 days. The mean body weight gain was significantly different from that of the control at  $p < 0.01$  (b) or at  $p < 0.001$  (c). Bloody droppings were scored, -: no droppings on the floor of a cage, +1: small amount, +2: moderate amount, +3: large amount.

*Histopathological findings in chickens inoculated with Nn-P125 line*

The 52-day-old chickens in a group were given  $3 \times 10^4$  oocysts/bird, and the duodenum, jejunum; ileum and cecum of 2 birds were sampled daily for 8 days and

## CHARACTERISTICS OF *EIMERIA NECATRIX* NN-P125

examined histopathologically. The results were similar in all four parts of the intestine, therefore, only findings in the jejunum are shown in Table 2. Schizont colonies of the Nm-P125 line were definitely fewer in number than those of the Nn strain. Tissue damages represented by hemorrhage and inflammation were also slight in the Nn-P125 line than in Nn strain. These findings coincided with the results of the aforementioned gross pathology. The major and minor diameters of the 2nd generation schizonts measured on 50 schizonts and the number of merozoites/schizont were significantly smaller in the Nn-P125 line than in Nn strain (Table 3).

Table 2. Comparison of histopathological findings between *Eimeria necatrix* Nn-P125 line and Nn strain.

Observation Items	Coccidium	Results obtained at post inoculation day							
		1	2	3	4	5	6	7	8
Average No. 2nd gen. Schizonts <sup>a</sup>	Nn-P125 line	0	0	1.5	2.5	9.0	3.0	4.5	7.5
	Nn strain	0	0	2.5	4.5	21.5	31.0	24.0	18.5
Degree of Hemorrhage <sup>b</sup>	Nn-P125 line	0	0	0	0	0.5	0	0.5	0.5
	Nn strain	0	0	0	1.0	2.0	3.0	3.0	2.0
Degree of <sup>b</sup> inflammation	Nn-P125 line	0	0	0.5	1.0	1.0	1.0	1.0	1.0
	Nn strain	0	0	1.0	2.0	2.0	3.0	3.0	2.5

The 52-day-old SPF chickens were given  $3 \times 10^4$  oocysts/bird, and the jejunums of 2 birds were examined histopathologically daily for 8 days. a: Numbers of the 2nd generation schizonts observed in a cross section of the jejunum were counted in 2 birds daily, and the average number is presented. b: Hemorrhage and inflammation was scored using following scoring system, 0: no gross lesions, 1: slight, 2: moderate and 3: severe. Table shows a mean value calculated with 2 birds/day.

Table 3. Comparison of major and minor diameters of the 2nd generation schizonts and numbers of merozoites/schizont between *E.necatrix* Nn-P125 line and Nn strain

Coccidium	Major diameter (µm)		Minor diameter (µm)		No. merozoites/schizont	
	Mean	SD	Mean	SD	Mean	SD
Nn-P125 line	20.44	2.10	15.40	1.93	20.48	1.93
Nn strain	39.68	5.45	29.60	4.79	67.66	11.63

### *Determination of minimal effective number of oocysts*

With fifteen 7-day-old chickens in each group, immunological potential of the Nn-P125 line was assayed by in-feed administration with 25, 50, 100, 200, 400, 1,000



## CHARACTERISTICS OF *EIMERIA NECATRIX* NN-P125

and 10,000 oocysts/bird. After the vaccination, chickens were reared in cages with litter on the floor for 4 weeks, and then 10 of 15 birds in each group were transferred to wire-floored cages and challenged with  $5 \times 10^4$  oocysts of virulent Nn strain. The vaccination procedure, even with the highest dose, exerted no adverse effect on chickens as shown by the mean body weight gains during the immunization period (Table 4). The patterns of oocyst excretion in the feces of chickens indicated that the chickens of all the groups underwent 3 recycled infections by ingestion of oocysts excreted (data not shown). There was no noticeable relationship between the the numbers of oocysts dosed and oocysts excreted.

The results of the challenge are summarized in Table 5. In the unimmunized challenged control group, 7 of 10 birds died, and the relative mean body weight gain was -16.3. In contrast, only one bird died (the group received 100 oocysts/bird) in vaccinated groups and relative mean weight gains appeared to be improved compared with that of challenged controls.

Table 4. Relative body weight gains in the groups receiving different doses of Nn-P125 line oocysts during the immunization period.

No. of oocysts (dosed/bird)	Relative body weight gain (%)			
	0-1 wk	0-2 wks	0-3 wks	0-4 wks
25	87.6	102.9	105.4	94.5
50	90.0	103.4	97.8	118.0
100	75.4	93.7	102.3	113.4
200	85.0	97.7	86.0	109.1
400	79.4	92.0	96.8	115.9
1,000	92.1	106.7	94.0	109.8
10,000	75.2	97.3	90.7	129.0
Unimmunized	100.0	100.0	100.0	100.0

SPF chickens (7-day-old, 15/group) were given indicated numbers of oocysts, and kept on litter floor for 4 weeks.

### *Determination of chicken ages suitable for vaccination*

The effects of vaccination were compared between 3-day-old, 7-day-old and 14-day-old chickens by immunizing them as described above. The number of oocysts dosed was 100/bird in all the age groups. The chickens in all three groups were also given a trivalent attenuated live vaccine, TAM, when they were 3 days of age.

## CHARACTERISTICS OF *EIMERIA NECATRIX* NN-P125

Chickens vaccinated with Nn-P125 line at 3 days of age were challenged with Nn strain 32 days after vaccination, while those of other vaccinated groups were challenged 28 days after vaccination. As shown in Table 6, all the chickens tested developed a good protective immunity regardless of ages at which they were vaccinated. In addition, the TAM vaccine did not interfere with the immunizing effects of Nn-P125 line.

Table 5. The results of the challenge to chickens immunized with Nn-P125 line oocysts

Numbers of oocysts for immunization (dosed/bird)	died/ tested	Relative wt. gain	Mean lesion score <sup>a</sup>			
			Duodenum	Jejunum	Ileum	Cecum
25	0/10	90.4	0.2	0.2	0	0
50	0/10	65.0 <sup>c</sup>	0.4	0.8	0.8	0.5
100	1/10	51.8 <sup>c</sup>	1.6	1.2	1.6	1.1
200	0/10	84.2	0.1	0.2	0.2	0
400	0/10	78.8 <sup>b</sup>	0	0.1	0.2	0
1,000	0/10	79.5	0	0	0	0
10,000	0/10	68.0 <sup>d</sup>	0	0	0	0
UCC	7/10	-16.3 <sup>d</sup>	3.0	3.0	3.0	3.0
UUC	0/10	100.0	0	0	0	0

The immunized chickens were challenged with  $5 \times 10^4$  oocysts of *E. necatrix* Nn strain/bird and observed for 8 days. UCC: unimmunized challenged controls. UUC: unimmunized unchallenged controls. Significantly different from that of the control was observed at  $p < 0.05$  (b), at  $p < 0.01$  (c) or at  $p < 0.001$  (d). a: The lesion was scored using following degrees, 0: no lesions, 1: slight, 2: moderate and 3: sever.

### *Stability of attenuation of the line*

The Nn-P125 line has highly reduced pathogenicity and one-day-shortened prepatent period as shown by the aforementioned examinations. These characteristics were stable during five serial relaxed passages in 4-week-old chickens. The relaxed passages are still in progress to further confirm the stability of attenuation of the line.

## DISCUSSION

The present results indicated those characteristics of the Nn-P125 line that are relevant to an attenuated live vaccine. Regarding pathogenicity, this line has been attenuated to the extent of that, only minor reduction in body weight gains of chicken was induced without causing any clinical signs at the dosage level of  $9 \times 10^4$



CHARACTERISTICS OF *EIMERIA NECATRIX* NN-P125

oocysts/bird. The same dosage level of the parent Nn strain was completely fatal to chickens. The dosage level of  $1 \times 10^4$  oocysts of Nn-P125/bird exerted no adverse effect on chickens kept on litter floor during the 4-week immunizing period. Despite the Nn-P125 line had significantly low pathogenic and reproductive potentials, the line retained its immunological potential to chicks aged 3 days and older, as evidenced by challenge experiments performed 4 weeks after vaccination. This may have resulted from recycling of the attenuated oocysts following vaccination. Furthermore, a dosage level as low as 25 oocysts/bird conferred a good protective immunity on chickens. The 4-week immunizing period adopted in the present study seemed not to be too long, because it has been suggested that troubles caused by *E.necatrix* usually occurred in chickens 8 weeks of age or over (McDougald et al. 1990).

Table 6. Comparison of immunizing effects resulting from different ages at vaccination

Age at vaccination	died/ tested	Relative body wt. gain (%)	Mean lesion score <sup>a</sup>			
			Duodenum	Jejunum	Ileum	Cecum
3-day-old	0/10	70.2 <sup>b</sup>	0.8	0.8	0.6	0.2
7-day-old	0/10	86.8	0	0	0	0
UCC	7/10	-16.3 <sup>c</sup>	3.0	3.0	3.0	3.0
14-day-old	0/10	89.4	0.2	0.2	0.1	0.1
UCC	2/10	3.4 <sup>c</sup>	2.8	3.0	2.6	2.0
UUC	0/10	100.0	0	0	0	0

The chickens in all groups were vaccinated with 100 oocysts/bird, and they were also vaccinated with the TAM vaccine when they were three days of age. The chickens vaccinated with Nn-P125 line at 3 days of age were challenged with 50,000 oocysts of Nn strain/bird 35 days post vaccination, while other vaccinated groups received the challenge 28 days post vaccination. a: The lesion was scored using following degrees, 0: no lesions, 1: slight, 2: moderate and 3: sever. Significantly different from that of the control was observed at  $p < 0.01$  (b) or at  $p < 0.001$  (c).

The in-feed administration of oocysts was adopted in the present study, since the preliminary examination showed that in-feed vaccination surpassed the vaccination via drinking water in that fluctuation in immunity developed by 4 weeks post-vaccination was minimal in the in-feed vaccination. However, other delivery methods may also be applicable, because uniform protective immunity in chickens is

boosted by recycling of the attenuated oocysts following vaccination.

An attenuated multivalent vaccine containing oocysts of *E.necatrix* and other species of coccidia has been recognized to exert good coccidiosis-controlling effects on chickens including breeder pullets (Shirley and Millard 1986; Bushell et al. 1989; Evans et al. 1989; Norton, Catchpole and Evans 1989). The present study revealed that the Nn-P125 line is able to serve as a safe and effective vaccine for breeder chickens due to its appropriate attenuation and immunological potential.

## REFERENCES

- Bushell,J.E., Harding,R.B., Evans,N.A. & Shirley,M.W. 1989. Coccidiosis control in chickens using a live attenuated vaccine. II. Field trial results. *In: Coccidia and Intestinal Coccidiomorphs, Vth International Coccidiosis Conference, Tours (France)*, 689-692, INRA Publ..
- Evans,N.A., Harding,R.B., Roberts,S. & Shipley,M.W. 1989. Coccidiosis control in chickens using a live attenuated vaccine. i. Experimental studies. *In: Coccidia and Intestinal Coccidiomorphs, Vth International Coccidiosis Conference, Tours (France)*, 683-688, INRA Publ.
- McDougald,L.R., Fuller,A.L. & McMurray,B.L. 1990. An outbreak of *Eimeria necatrix* coccidiosis in breeder pullets: analysis of immediate and possible long-term effects on performance. *Avian Dis.* 34: 485-487.
- Norton,C.C., Catchpole,J. & Evans,N.A. 1989. Performance of an attenuated coccidiosis vaccine in floor pen challenge studies. *In: Coccidia and Intestinal Coccidiomorphs, Vth International Coccidiosis Conference, Tours (France)*, 677-682, INRA Publ.
- Shirley,M.W. & Bellatti,M.A. 1984. *Eimeria necatrix*: selection and characteristics of a precocious (and attenuated) line. *Avian path.* 13: 657-668.
- Shirley,M.W. & Millard,B.J. 1986. Studies on the immunogenicity of seven attenuated lines of *Eimeria* given as a mixture to chickens. *Avian Path.* 15: 629-638.
- Schinizler,B.E., Thebo,P.L., Mattsson,J.G., Tomley,F.M. & Shirley,M.W. 1998. Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic *Eimeria* species of the chicken. *Avian Path.* 27: 490-497.