Prepatent and Patent Periods, and Production and Sporulation of Oocysts of *Eimeria subspherica* Isolated in Japan

Kenji ODA and Yumi NISHIDA

Division of Animal Husbandry, Research Institute for Animal Science in Biochemistry and Toxicology, 3-7-11 Hashimoto-dai, Sagami-hara-shi, Kanagawa 229, Japan

(Received 18 October 1990/Accepted 18 April 1991)

ABSTRACT. Eight calves were inoculated with 10^6 or 10^7 sporulated oocysts of *Eimeria subspherica*, Christensen, 1941. All the calves, except one, were successfully infected. Prepatent period was 10 to 11 days with a mean of 10.6 days and patent period was 1 to 5 days with a mean of 2.9 days. Peak oocyst discharge was found on days 11 and 12 after inoculation. The number of oocysts passed in the feces was very small and varied among calves, and no relationship was detected between the inoculum dose and the number of oocysts passed in the feces. No clinical symptoms were associated with the infection in any calves. Infectivity, reproductive potential and pathogenicity of *E. subspherica* were suggested to be low in the experimentally infected calves, even though relatively large numbers of oocysts were given. The sporulation process of *E. subspherica* oocysts was observed under a condition of 29°C with constant aeration. Completely sporulated oocysts increased in percentage following a sigmoid curve, and the expected 50% and 95% sporulation times were determined as 161 and 194 hours, respectively, by regression analysis.—**KEY WORDS:** bovine coccidia, *Eimeria subspherica*, sporulation.

— *J. Vet. Med. Sci.* 53(4): 615–619, 1991

More than 20 species of bovine coccidia have been named, but most of these species are described based on their characteristic nature only of oocysts in the feces of cattle. *Eimeria subspherica* was first described by Christensen in 1941 [1] and is known to be widely distributed in the world [7]. In Japan, also, we found *E. subspherica* oocysts in 4.5% of 1,015 fecal samples from cattle in a survey in 1985 [11]. However, there are a few reports on the biological characteristics of *E. subspherica*, so we carried out examinations in experimental infections of calves with *E. subspherica* isolated in Japan. The present paper describes the prepatent and patent periods, oocyst production, pathogenicity, and sporulation process of this species.

MATERIALS AND METHODS

Animals: Eleven Holstein calves of both sexes, less than 1 week old, were obtained from the farms near the institute. The calves were kept in individual pens, and they were given milk replacer for the first 3 or 4 weeks and completely weaned by about 5 weeks after birth. After weaned, they were given concentrated feed and timothy hay *ad libitum*. To prevent natural infections with coccidia, the pens and all the equipments and tools were sterilized as completely as possible with hot water prior to use. So, none of the calves passed oocysts of coccidia

before they were experimentally infected.

Oocysts: The strain of *E. subspherica* used was originally isolated from a naturally infected calf in Kanagawa Prefecture, Japan in 1985 and maintained by serial passage through calves in this laboratory. The oocysts used in the present study were not contaminated with those of other *Eimeria* species and were used within 3 months after sporulation. The sporulated oocysts suspended in a small amount of water were mixed with about 20 g of concentrated feed and were orally inoculated by a 30-ml plastic syringe.

Experimental groups: About 1 week after weaning, the calves were weighed and divided into 3 groups based on body weight; at this time the calves ranged from 49.0 to 72.0 kg with a mean of 57.1 kg in body weight. Four calves each in groups 1 and 2 were inoculated with 10^6 and 10^7 sporulated oocysts, respectively. Three calves in group 3 were not inoculated as controls.

Oocyst examination: Fecal samples were daily collected directly from the rectum of each calf, and 50 g of each fecal sample was examined for oocysts by the flotation technique with saturated sodium chloride solution. When oocysts were detected, the number of oocysts per gram of feces (OPG) was calculated by a plankton counting plate [13].

Body weight and feed intake: On days 5, 10, 12, 15, and 18 after inoculation, the calves were

weighed. Feed intake of each calf was recorded every day throughout the experiment.

Observation of oocyst sporulation: On day 11 after inoculation, the feces containing oocysts were transferred directly from the rectum of calves into iced water. Within 1 hour after the feces were transferred, oocysts were collected by the flotation technique with saturated sodium chloride solution under cold condition, and suspended in 2% potassium bichromate solution, and after then they were incubated with constant aeration at 29°C. Every 3 hours, 200 oocysts were randomly examined for development under a microscope at a magnification of $\times 400$ and classified into such morphological stages as the condensation of sporont, cleavage of sporont, sporocyst formation, and sporozoite differentiation (complete sporulation) according to Norton and Chard [10]. Percentages of completely sporulated oocysts were transformed into radians by the formula $\arcsin$ to obtain a straight line expressing the relation between sporulation rate and duration of time, and the expected 50% and 95% sporulation time in hours was calculated from the regression equation for this line.

RESULTS

Morphology of sporulated oocysts: Sporulated oocysts were typically subspherical as shown in Fig. 1, but sometimes spherical or ellipsoidal. Smooth and colorless oocyst wall was composed of two layers. Micropyle, micropylar cap, and oocyst residuum were absent. Sporocysts were elongate ovoid with a small Stieda body and no sporocyst residuum. Measurements of sporulated oocysts are shown in Table 1, compared with those of previous authors.

Clinical symptoms: Some calves in the infected groups sometimes passed soften feces during the

Fig. 1. Sporulated oocyst of *E. subspherica*. bar=10 μ m.

experiment, but no relationship existed between inoculum dose and fecal conditions. No other clinical symptoms and changes in body weight gain and feed intake were observed in all the calves.

Oocyst discharge: Until day 10 after inoculation, no coccidian oocysts were detected in the feces of all calves. On days 10 and 11 after inoculation *E. subspherica* oocysts were first found in the feces of 3 and 4 calves, respectively (Table 2). One calf inoculated with 10^6 oocysts (No. 22) did not pass oocysts during the experiment. Mean prepatent period was 10.6 days, and patent period ranged from 1 to 5 days with a mean of 2.9 days. Peak oocyst discharge occurred on days 11 and 12 after inoculation, and the maximum OPG of 27,400 was found on day 13 in the calf inoculated with 10^6 (No. 21), followed by a OPG of 15,800 in the calf inoculated with 10^7 oocysts (No. 53) on day 12. The other calves in each infected group passed less than 2,000 OPG at the maximum discharge. No relationship existed between the inoculum dose and the number of oocysts passed in the feces. All the calves in the uninoculated control group did not pass oocysts of any coccidian species during the experiment.

Sporulation: At the start of incubation, oocysts were filled with a granular sporont. When oocysts

Table 1. Measurements of *E. subspherica* oocysts reported by previous authors and in the present study

Authors	Year	Reference No.	Oocyst size (μ m)			Length/width ratio		Number of layers in oocyst wall
			Length	Width	Mean	Range	Mean	
Christensen	1941	1	9 -13	8 -12	11.0 $\times$ 10.4	1.00-1.33	1.06 ^{a)}	1
Ernst and Courtney	1977	3	10 -13	9 -12	11.0 $\times$ 10.5	1.0 -1.2	1.06	2
Lee and Armour	1959	5	9.4-13.0	8.7-12.2	11.4 $\times$ 10.7	1.00-1.19	1.06 ^{a)}	Not given
Levine and Ivens	1967	6	11 -14	10 -13	12.7 $\times$ 11.8	1.0 -1.2	1.07	1
Majaro and Dipeolu	1981	9	13.8-27.0	11.4-24.6	17.4 $\times$ 15.6	Not given		Not given
Present authors	1990		13.0-15.7	10.5-14.0	14.5 $\times$ 12.3	1.05-1.34	1.19	2

a) The range and mean of length/width ratio are calculated with the width/length ratios given by the authors.

Table 2. Oocyst discharge in the calves inoculated with 10^6 and 10^7 *E. subspherica* oocysts

Group	Number of oocysts inoculated	Animal No.	OPG values (×100) on days after inoculation ^{a)}								
			10	11	12	13	14	15	16	17	18
1	10 ⁶	17	— ^{b)}	0 ^{c)}	10	20	6	—	—	—	—
		21	8	101	274	0	0	—	—	—	—
		22	—	—	—	—	—	—	—	—	—
		23	—	0	8	—	—	—	—	—	—
2	10 ⁷	49	—	4	0	—	—	—	—	—	—
		53	4	158	2	—	—	—	—	—	—
		55	—	2	4	—	—	—	—	—	—
		56	0	10	10	—	—	—	—	—	—

a) Until day 10 after inoculation, *E. subspherica* oocysts were not detected in all the calves.

b) Oocysts were not detected by the flotation technique.

c) OPG values were less than 400.

Fig. 2. Sporulation process of *E. subspherica* oocyst at 29°C with constant aeration. ○—○: Condensation of sporont, ■—■: Cleavage of sporont, □—□: Sporocyst formation, ●—●: Sporozoite differentiation.

were allowed to develop under the condition as mentioned in the item of materials and methods, the sporont was completely condensed within the first 36 hours of incubation (condensation of sporont). At 72 hours after incubation, the sporont began to divide into 4 sporoblasts (cleavage of sporont); oocysts of this stage increased in percentage up to 28% at 90 hours and disappeared by 141 hours. At 78 hours, sporocyst formation began to occur and reached the maximum percentage of 98% at 126 hours; oocysts in this stage decreased gradually in percentage but were still present in small numbers at 210 hours. Sporozoite differentiation, the final stage of sporulation process with a large refractile globule in sporozoites, was first detected at 126 hours and

increased in percentage following a sigmoid curve, and finally reached the maximum percentage of 98% at 210 hours. The rates of each stage with time are shown in Fig. 2. The expected 50% and 95% sporulation time calculated from the regression equation were 161 and 194 hours, respectively (Fig. 3).

DISCUSSION

Morphological features of sporulated oocyst are necessary for valid identification of species of bovine coccidia [6]. In the original description of *E. subspherica* [1], however, morphological characteristics of sporulated oocysts have not been described

Fig. 3. Regression line for the complete sporulation rates in radian. Percentage data are transformed into radians by the formula $\arcsin$; the radians of 0.785 and 1.345 show 50% and 95%, respectively.

in details. Levine and Ivens [6], and Ernst and Courtney [3] described the morphological characteristics of the sporulated oocysts of this species in agreement with all the present findings with some exceptions. Levine and Ivens [6] described that oocyst wall was composed of a single layer, however, in the present study, oocyst wall was determined composed of two layers by observation of crushed oocyst. This agreed with the finding of Ernst and Courtney [3]. The difference in oocyst size among the authors [1, 3, 5, 6] including the present authors may be due to infra-specific variations and/or technical errors, although the size given by Majaro and Dipeolu [9] was apparently larger than the others.

There is only one report available on the experimental infections with *E. subspherica* in cattle, in which Ernst and Courtney [3] described that the mean prepatent and patent periods were 9.3 and 11.1 days, respectively, in infections of 2- to 3-weeks old calves with 10^6 to 58×10^6 oocysts. In the present study, the mean prepatent and the patent periods were 10.6 and only 2.9 days, respectively, in all the infected calves; the prepatent period was somewhat longer but the patent period was significantly shorter than those reported by Ernst and Courtney [3]. The calves used in the present study were 5 to 6 weeks old and completely weaned at the

time of inoculation, whereas in the study of Ernst and Courtney [3] the calves used seemed in unweaning judging from their age. So, this may be the reason for differences in the prepatent and patent periods of *E. subspherica* infections between the two studies.

Ernst and Courtney [3] did not describe oocyst production and clinical symptoms in the infected calves with *E. subspherica*, but they stated that as a result of their preliminary work, at least 10^6 oocysts were necessary to produce detectable patent infections. In the present study, one calf inoculated with 10^6 oocysts was not infected, and in the other successfully infected calves the number of oocysts passed in the feces was very small and varied. No clinical symptoms were associated with the infections in any calves.

Courtney *et al.* [2] reported that *E. wyomingensis* had a very short patent period of 2 to 5 days in the calves inoculated with 10^6 to 2×10^6 oocysts and some of them failed to pass oocysts. Ernst and Benz [4] also described that *E. wyomingensis* had a very short patent period and could not produce a large number of oocysts in the calves inoculated with 2×10^5 to 10×10^6 oocysts. For *E. alabamensis*, Soecardon *et al.* [12] described that at least 10^7 oocysts were necessary to produce consistent infections and that in the calves inoculated with 10^7 oocysts patent period was only 2 to 3 days and the number of oocysts passed in the feces was very small, although most of the calves inoculated with 8×10^7 to 10^8 oocysts passed more than 10^6 oocysts per gram of feces at peak oocyst discharge and had moderate diarrhea. These findings on *E. wyomingensis* and *E. alabamensis* infections almost agree with those on *E. subspherica* infections, showing that in these *Eimeria* species, inoculation of relatively large numbers of oocysts as 10^6 to 10^7 does not sufficiently produce heavy infections with long patent period, production of large numbers of oocysts, and positive clinical symptoms. Therefore, it is considered that in these species of bovine coccidia including *E. subspherica*, infectivity, productive potential of oocysts, and pathogenicity may be essentially low at least under experimental conditions.

Sporulation process is one of the most important characteristics of coccidia and sporulation time can be a useful parameter for identification of species. The sporulation time of *E. subspherica* has been reported 96 to 120 hours at room temperature [1] and 120 to 144 hours at 27°C [5]. In the present

study, completely sporulated oocysts were first detected at 126 hours and reached the maximum percentage of sporulation at 210 hours at 29°C with constant aeration. However, it is difficult to compare these results to each other because of very wide range of sporulation time under different conditions. So, sporulation time should be determined under the standardized culture condition.

The sporulation process with time can be morphologically divided into four stages and a very accurate assessment of oocyst sporulation can be made by careful microscopical observation of the proportion of the final of the four stages [8]. The time expected for 50% and 95% oocysts to complete sporulation can be calculated by regression analysis following arcsine transformation of the percentages and 50% level is the most reliable because of its very small standard errors [10]. Actually, Norton and Chard [10] reported that 7 species of chicken coccidia, except one, could be significantly differentiated from each other by their 50% sporulation time, therefore, which was the most useful criterion for comparison of different species.

In the present study sporulation process of *E. subspherica* oocyst was observed every 3 hours under the condition of 29°C with constant aeration, which was suggested to be the best for assessment of oocyst sporulation in culture of chicken coccidia [8]. In this condition, no adverse effects, such as degeneration of oocysts, were detected in sporulation process, and the development of *E. subspherica* oocysts (Fig. 2) was similar in each stage to that of chicken coccidium *E. acervulina* [10], although it took longer time for each stage of *E. subspherica* oocyst to develop than *E. acervulina* oocyst. Completely sporulated oocysts increased in percentage following a sigmoid curve and finally reached the maximum of 98%. The expected 50% and 95% sporulation time of *E. subspherica* was determined to be 161 and 194 hours, respectively, by the

regression analysis. Therefore, 50% sporulation time estimated with the proportion of completely sporulated oocysts could be a useful parameter for identification of bovine coccidia.

REFERENCES

1. Christensen, J. F. 1941. The oocysts of coccidia from domestic cattle in Alabama (USA), with descriptions of two new species. *J. Parasitol.* 27: 203–220.
2. Courtney, C. H., Ernst, J. V., and Benz, G. W. 1976. Redescription of *Eimeria bukidononensis* Tubangui, 1931 and *E. wyomingensis* Huizinga and Winger, 1942. *J. Parasitol.* 62: 372–376.
3. Ernst, J. V. and Courtney, C. H. 1977. Prepatent and patent periods of the bovine coccidium *Eimeria subspherica* Christensen, 1941, with redescription of sporulated oocysts. *Proc. Helminthol. Soc. Wash.* 44: 97–98.
4. Ernst, J. V. and Benz, G. W. 1980. Attempts to produce experimental *Eimeria wyomingensis* infections in calves. *J. Parasitol.* 66: 625–629.
5. Lee, R. P. and Armour, J. 1959. The coccidia oocysts of Nigerian cattle. *Br. Vet. J.* 115: 6–17.
6. Levine, N. D. and Ivens, V. 1967. The sporulated oocysts of *Eimeria illinoisensis* n. sp. and of other species of *Eimeria* of the ox. *J. Protozool.* 14: 351–360.
7. Levine, N. D. 1973. Protozoan Parasites of Domestic Animals and of Man, 2nd ed. Burgess, Minneapolis, Minnesota.
8. Long, P. L. and Joiner, L. P. 1984. Problem in the identification of species of *Eimeria*. *J. Protozool.* 31: 535–541.
9. Majaro, O. M. and Dipeolu, O. O. 1981. The seasonal incidence of coccidia infections in trade cattle, sheep, and goats in Nigeria. *Vet. Q.* 3: 85–91.
10. Norton, C. C. and Chard, M. J. 1983. The oocyst sporulation time of *Eimeria* species from the fowl. *Parasitology* 86: 193–198.
11. Oda, K. and Nishida, Y. 1990. Prevalence and distribution of bovine coccidia in Japan. *Jpn. J. Vet. Sci.* 52: 71–77.
12. Soecardon, S., Ernst, J. V., and Benz, G. W. 1975. The prepatent and patent periods of *Eimeria alabamensis* and further description of the exogenous stages. *Vet. Parasitol.* 1: 19–33.
13. Tunoda, K. and Ishii, T. 1971. Technical Manual of Avian Coccidia. Keibyo Kenkyu-kai, Tokyo (in Japanese).