Effect of *Eimeria maxima* Infection upon the Invasion of *Salmonella* serovar typhimurium through the Intestine of Chicken

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ABSTRACT. Two experiments were conducted to examine invasion of salmonella through the intestine of chickens infected with *Eimeria maxima*. In experiment 1, $1.6\text{--}4.0 \times 10^5$ *Salmonella* serovar typhimurium (*S. typhimurium*) were injected into the small-intestinal loop established in *E. maxima*-infected and -uninfected chickens under anesthesia, and cardiac blood, spleen and liver were examined for the presence of *S. typhimurium*. The organism was more frequently recovered from these samples, particularly from spleen 10 days after coccidial inoculation, than from the samples of uninfected birds given only *S. typhimurium* into small-intestinal loop. When *S. typhimurium* was injected into small-intestinal loop or ligated cecum of intact chickens, more samples were positive for *S. typhimurium* from small-intestinal loop than those from ligated cecal group. Significant difference was seen in the spleen samples. In experiment 2, number of *S. typhimurium* in cardiac blood, spleen and liver was counted after injection of $1.8\text{--}8.4 \times 10^7$ *S. typhimurium* into small-intestinal loop of chickens 10 days after *E. maxima* inoculation. The counts in spleen were approximately 10^2 CFU/g 3 hr after the injection and were higher than those in liver and blood of both *E. maxima* infected and uninfected birds. In liver, the counts in *E. maxima* infected birds 30 min after the injection were significantly higher than those in uninfected birds. In cardiac blood, a few *S. typhimurium* were recovered from *E. maxima* infected birds, whereas no organism was found from uninfected birds until 2 hr after the injection. It is concluded that in chickens salmonella penetrated more readily through small intestine than ceca, and *E. maxima* infection enhanced the penetration.—**KEY WORDS:** chicken, *Eimeria maxima*, intestinal loop, penetration, *Salmonella* serovar typhimurium.

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When salmonellae are inoculated orally to susceptible chicken, majority of the organisms are often found in caudal ileum and ceca, and also detected in liver and spleen shortly after ingestion [5, 7]. A significant increase in number of *Salmonella* serovar typhimurium (*S. typhimurium*) in *Eimeria tenella*-infected ceca [1, 3, 4] and the increased penetration of the organism into the cecal wall by *E. tenella* infection [9] were reported. In *E. maxima* infected chickens, although numbers of *S. typhimurium* in the intestine were not significantly different from uninfected controls, the significant increase in the passage of *S. typhimurium* to liver was observed [13].

Penetration of salmonella through intes-

tinal wall of murine animals were reported [6, 11, 12], but limited papers are available in chickens [9, 10, 14]. In mice, the most primary site of salmonella penetration is the distal ileum, and Peyer's patches and lymph nodes are important parts for the route of penetration [6]. Since Payer's patches and mesenteric lymph nodes are not present in chickens, the liver and spleen have been examined for the penetration of salmonella through intestinal wall [1, 3, 9, 15]. Artificial loop of the intestine has been often used for studying pathogenesis and invasion of salmonella [8, 14].

The purpose of the present study is to examine the passage of salmonella to liver and spleen from the loop of small intestine

and ligated cecum, and the effect of *E. maxima* infection upon the invasion of *S. typhimurium*.

MATERIALS AND METHODS

Birds and diets: White Leghorn, Hy-Line®, day-old cockerels were purchased from a local commercial hatchery and kept in electrically heated battery brooders. The chickens were confirmed to be free of coccidial infection. For experiments, chickens were transferred to wired-floor batteries in an air-conditioned room with continuous artificial illumination where cleaning and steam disinfection were thoroughly performed. The chickens were given unmedicated basal feed [2] throughout the study.

Salmonella: The strain of *S. typhimurium*, L-55, supplied from the National Institute of Animal Health in Tsukuba, was originally isolated from a chicken in field outbreak of paratyphoid infection. The organism was grown in trypticase soy broth (Nissui Pharmaceutical Co., Tokyo) at 37°C for 18 hr, washed, and resuspended into sterile saline solution.

Coccidium: *E. maxima* strain used in this study was originally supplied from the National Institute of Animal Health in Tsukuba and maintained in the laboratory through chickens. Oocysts were separated from the feces of donor chickens 5 to 7 days after oral inoculation, sporulated and stored in 2.5% potassium dichromate solution at 4°C.

Surgical operation: Each bird was anesthetized with intramuscular injection of ketamine chlorate, 35mg per Kg of body weight. The right side of abdominal wall was incised. A small intestinal loop, 5cm in length, with Meckel's diverticulum in the middle was made, or one cecum was ligated at cervix.

Bacteriological examination: Mannitol lysine crystal violet brilliant green (MLCB)

agar (Nissui, Tokyo) was used to count number of *S. typhimurium* in the inoculum, wall of small intestine, cardiac blood, spleen and liver, or to confirm the presence of *S. typhimurium* in the cardiac blood, spleen and liver. Hajna tetrathionate (HT) broth (Nissui, Tokyo) was used for enriching *S. typhimurium* in the cardiac blood, spleen and liver samples.

At necropsy in Experiment 1, 1ml of cardiac blood from each bird was drawn aseptically and placed in a tube containing 9ml of HT broth. Approximately 1g of the right lobe of liver and whole spleen were taken aseptically. They were separately minced by pairs of scissors, and placed in a tube containing 9ml of HT broth. The broth was incubated at 37°C for 48 hr, and then one loopful of each broth was spread onto MLCB agar. MLCB agar plates were incubated at 37°C for 24 hr. Dark colonies 3 to 5mm in diameter characterized by convex surface with dark black center were counted. For identification, representatives from each of the tentatively identified colonies were tested with salmonella agglutinating serum to confirm its identity as *S. typhimurium*.

In Experiment 2, 1ml of cardiac blood were taken aseptically from each bird, and placed into glass tubes. The small intestine was obtained, washed with saline solution, and excessive solution was blotted with sterilized filter paper. The intestinal wall sample, the right lobe of liver, and whole spleen were weighed and minced in mortars aseptically. The initial dilutions of blood and organ samples were made by adding 9 volumes (V/W) of sterile saline solution and shaken thoroughly until homogeneous mixture. One ml of the mixture was then withdrawn and further diluted in saline solution by serial 10-fold steps. From each of the serial dilutions, including the initial dilution, 0.1ml of suspension was taken and spread on MLCB agar. *S. typhimurium*

colonies on the cultured plates were counted.

Statistical analysis: In Experiment 1, data on the number of birds positive for *S. typhimurium* were analyzed using the X^2 test. In Experiment 2, numbers of *S. typhimurium* were transformed into logarithm. When *S. typhimurium* was not recovered, addition of 1 to each number prior to taking logarithms, $\log_{10}(X+1)$, was made. Samples negative for *S. typhimurium* were given with $\log_{10} 1 (=0)$. Since the transformed data were not in a normal distribution, they were analyzed using the Wilcoxon's *U* test (Mann-Whitney's *U* test).

RESULTS

In Experiment 1, a total of 171 birds, 6 to 9 days old, were divided into 3 groups of 57 chickens each. Each bird in group 1 was inoculated with 1×10^5 sporulated oocysts of *E. maxima*, and 10 to 12 birds each were subjected to surgical operation to form a small intestinal loop on 3, 5, 7, 10 and 14 days after the coccidial inoculation. Birds in group 2 were subjected to the small intestinal loop only at the respective days. Birds in group 3 were subjected to cervical ligation of one cecum at the respective days. A

0.2ml of *S. typhimurium* suspension, 1.6 to 4.0×10^5 colony forming units (CFU), was injected into the small intestinal loop or ligated cecum of all chickens by a needle (0.4mm in diameter) immediately after the ligation. All chickens were necropsied 3 hr after the bacterial injection. The cardiac blood, spleen and liver from each bird were provided for bacteriological examination.

Comparing the penetration from small intestinal loop between *E. maxima*-infected (group 1) and -uninfected chickens (group 2), the percent positive for *S. typhimurium* in the cardiac blood, spleen and liver 3, 5, 7, 10 and 14 days after *E. maxima* inoculation was presented in Fig. 1. On 10 days after *E. maxima* inoculation, greater number of chickens were positive for *S. typhimurium*, especially in spleen, than those of uninfected chickens. On other necropsy days, however, there were no clear differences in percent positive for *S. typhimurium*. Fig. 2 shows the presence of *S. typhimurium* in the cardiac blood, spleen and liver in all of uninfected birds with the small intestinal loop (group 2) and those with ligated cecum (group 3) 3 hr after injection of *S. typhimurium*. Rate of chickens positive for *S. typhimurium* in spleen was significantly ($p < 0.05$) greater in birds with small intestinal loop

Fig. 1. Percent positive for *Salmonella* serovar typhimurium in cardiac blood, spleen and liver of chickens with small-intestinal loop constructed on 3, 5, 7, 10 and 14 days after *Eimeria maxima* inoculation. Chickens were necropsied 3 hr after *S. typhimurium* injection into the loop. ■ Infected with *E. maxima*. □ Uninfected control.

Fig. 2. Percent positive for *Salmonella* serovar typhimurium in cardiac blood, spleen and liver of uninfected chickens with small-intestinal loop (■) or ligated cecum (□) 3 hr after the injection of *S. typhimurium*. * Significantly different ($p < 0.05$).

than those in birds with ligated cecum. *S. typhimurium* passed from the lumen of intestinal loop more readily to spleen than to liver.

Next in Experiment 2, a total of 72 chickens, 15 to 18 days old birds, were divided into 2 groups of 36 chickens each. In group 1, small intestinal loop was constructed 10 days after *E. maxima* inoculation (2×10^5 sporulated oocysts per bird). In group 2, only a small intestinal loop was constructed on the same day as uninfected control. Shortly after the ligation, *S. typhimurium* suspension (1.8 to 8.4×10^7 CFU per bird) was injected into the intestinal loop by a needle. Nine chickens each in each group were necropsied 0.5, 1, 2, and 3 hr after *S. typhimurium* injection. The mid-small intestinal wall, cardiac blood, spleen, and liver taken from each bird were provided for the bacteriological examination.

Mean counts of *S. typhimurium* per g or ml of the samples after the organism injection were shown in Fig. 3. In the small intestinal wall, mean *S. typhimurium* counts in both *E. maxima*-infected birds and uninfected controls were within 10^6 to 10^8 CFU and increased gradually. In spleen, 50 to

Fig. 3. Recovery of *Salmonella* serovar typhimurium from small intestinal wall (●, ○), cardiac blood (▲, △), spleen (●, ○), and liver (■, □) of *Eimeria maxima*-infected (closed symbols) and uninfected birds (open symbols). On 10 days after *Eimeria maxima* inoculation, chickens operated for small-intestinal loop, injected *S. typhimurium* immediately afterwards, and necropsied 0.5, 1, 2 and 3 hr later. * Significantly different ($p < 0.05$).

80% of samples were positive for *S. typhimurium* in both groups at any necropsy hour. Mean counts of *S. typhimurium* in *E. maxima*-infected birds were greater than those in uninfected controls, but not significant. In liver, more samples of *E. maxima*-infected group were positive for *S. typhimurium* than those of uninfected group. Mean counts also in *E. maxima*-infected group were higher than those in uninfected group. At 0.5 hr the difference was significant ($p < 0.05$); No liver sample was positive for the organisms in uninfected group. In cardiac blood, less than 30% of samples in *E. maxima*-infected group were positive for *S. typhimurium*. In uninfected group, no *S. typhimurium* was recovered until 2 hr. At 3 hr, the organism was recovered from only one sample. Those bacterial counts of spleen in both *E. maxima*-infected and uninfected chickens were higher than those of liver or cardiac blood.

DISCUSSION

In the present study, particular attention was paid to the site of *S. typhimurium* penetration in the intestine of chickens. When *S. typhimurium* was orally inoculated into chickens, the organisms were recovered most predominantly from ceca [5]. In Experiment 1, penetration of *S. typhimurium* from the mid-small intestinal loop was compared with that from the ligated cecum. Percent positive for *S. typhimurium* in spleen was greater when the organism was injected into the small intestinal loop than did in the ligated cecum. It indicated that the penetration of *S. typhimurium* from small intestine was greater than that from ceca of chickens. There may be a possibility that tall villi in small intestine contribute to greater surface area and the greater chances are given to *S. typhimurium* to penetrate.

Takimoto *et al.* [13] reported that the numbers of birds positive for *S. typhimurium* in the liver of chickens killed 7 days after *E. maxima* inoculation were greater than those of uninfected birds. Regardless of the infection of *E. maxima* in small intestine, the number of *S. typhimurium* in ceca was greater than that in small intestine when *S. typhimurium* was orally inoculated [13]. In the present study, the number of *S. typhimurium* in the small intestinal loop was similar between the *E. maxima* infected and the uninfected control chicken. Nevertheless, distribution of *S. typhimurium* in all of spleen, liver, and cardiac blood in *E. maxima* infected chickens was somewhat greater than that of uninfected chickens. This seems that coccidial lesions resulted in greater passage to the internal organs.

The lymphatic stream is reported to be the primary route for penetration of salmonella in mice; *S. typhimurium* was recovered most early from mesenteric lymph nodes after oral inoculation of the organisms [6].

However, lack of mesenteric lymph nodes in chickens leads to difficulty in investigation. Portal vein may be the major route. Many *S. typhimurium* were recovered from liver 0.5 hr after the injection into small-intestinal loop of *E. maxima*-infected chickens in this study. Higher bacterial counts in spleen than liver might be evident in large bacterium-trapping activity. Precise route of penetrations is still to be investigated.

REFERENCES

1. Arakawa, A., Baba, E., and Fukata, T. 1981. *Eimeria tenella* infection enhances *Salmonella typhimurium* infection in chickens. *Poult. Sci.* 60: 2203-2209.
2. Arakawa, A., and Ohe, O. 1975. Reduction of *Clostridium perfringens* by feed additive antibiotics in the ceca of chickens infected with *Eimeria tenella*. *Poult. Sci.* 54: 1000-1007.
3. Baba, E., Fukata, T., and Arakawa, A. 1982. Establishment and persistence of *Salmonella typhimurium* infection stimulated by *Eimeria tenella* in chickens. *Res. Vet. Sci.* 33: 95-98.
4. Baba, E., Yaono, M., Fukata, T., and Arakawa, A. 1985. Infection by *Salmonella typhimurium*, *S. agona*, *S. enteritidis* or *S. infantis* of chickens with caecal coccidiosis. *Br. Poult. Sci.* 26: 505-511.
5. Brownell, J. R., Sadler, W. W., and Fanelli, M. J. 1969. Factors influencing the intestinal infection of chickens with *Salmonella typhimurium*. *Avian Dis.* 13: 804-816.
6. Carter, P. B., and Collins, F. M. 1974. The route of enteric infection in normal mice. *J. Exp. Med.* 139: 1184-1203.
7. Fanelli, M. J., Sadler, W. W., Franti, C. E., and Brownell, J. R. 1971. Localization of salmonellae within the intestinal tract of chickens. *Avian Dis.* 15: 366-375.
8. Giannella, R. A., Formal, S. B., Dammin, G. J., and Collins, H. 1973. Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. *J. Clin. Invest.* 52: 441-453.
9. Hikasa, Y., Baba, E., Fukata, T., and Arakawa, A. 1982. The invasion of *Salmonella typhimurium* into the cecal wall of chickens infected with *Eimeria tenella*. *Zbl. Bakt. Mikrobiol. Hyg. A* 253: 344-354.
10. Popiel, I., and Turnbull, P. C. B. 1985. Passage of *Salmonella enteritidis* and *Salmonella thompson* through chick ileocecal mucosa. *Infect. Immun.* 47: 786-792.

11. Takeuchi, A. 1967. Electron microscope studies of experimental salmonella infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *Am. J. Pathol.* 50: 109-136.
12. Takeuchi, A., and Splinz, H. 1967. Electron microscope studies of experimental salmonella infection in preconditioned guinea pig. II. Response of the intestinal mucosa to invasion by *Salmonella typhimurium*. *Am. J. Pathol.* 51: 137-161.
13. Takimoto, H., Baba, E., Fukata, T., and Arakawa, A. 1984. Effects of infection of *Eimeria tenella*, *E. acervulina*, and *E. maxima* upon *Salmonella typhimurium* infection in chickens. *Poult. Sci.* 63: 478-484.
14. Turnbull, P. C. B., and Richmond, J. E. 1978. A model of salmonella enteritis: The behaviour of *Salmonella enteritidis* in chick intestine studied by light and electron microscopy. *Br. J. Exp. Pathol.* 59: 64-75.
15. Turnbull, P. C. B., and Snoeyenbos, G. H. 1974. Experimental salmonellosis in the chicken. I. Fate and host response in alimentary canal, liver and spleen. *Avian Dis.* 18: 153-177.

要 約

鶏の腸管における *Salmonella* serovar typhimurium の侵入に及ぼす *Eimeria maxima* 感染の影響：馬場栄一郎，山元美奈子，深田恒夫，荒川 皓（大阪府立大学農学部家畜内科学教室）—— *Eimeria maxima* が感染した鶏の腸管におけるサルモネラの侵入の様相を調べた。 *E. maxima* 感染鶏および非感染鶏を麻酔し，小腸中央部に設けたループに *Salmonella* serovar typhimurium (S. typhimurium) $1.6-4.0 \times 10^5$ CFU を注入し，血中，脾，肝への移行を検査した。各材料からの S. typhimurium の検出率は非感染対照鶏の小腸ループの場合よりも高く，とくに *E. maxima* 投与後10日の脾で著明であった。一方，コクシジウムに感染していない鶏の小腸ループあるいは結紮した盲腸に S. typhimurium を注入した場合の菌の移行は，小腸ループの方が高く，脾での検出頻度には有意差がみられた。つぎに， *E. maxima* 投与後10日の鶏の小腸ループに， S. typhimurium $1.8-8.4 \times 10^7$ CFU を注入して経時的に心血，脾，肝での菌数を測定した。脾の菌数は， *E. maxima* 感染鶏，非感染鶏ともに肝や血中よりも高く，注入後3時間まで平均約 10^2 /g の菌数を示した。肝では，注入後30分に *E. maxima* 感染鶏に比べて有意に高い菌数を示した。心血では， *E. maxima* 感染鶏から少ないながら菌が検出されたが，非感染鶏では注入後2時間まで検出されなかった。以上の成績から，鶏では結紮した盲腸よりも小腸ループからの方がサルモネラは侵入しやすく， *E. maxima* 感染はこれを助長することがわかった。