Factors Contributing to the Resistance of Chickens to Infection with Japanese *Fasciola* sp.

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ABSTRACT. Attempts were made to clarify the factors contributing to the resistance of chickens to infection with Japanese *Fasciola* sp. Infection was not successfully established in chickens by oral inoculation of metacercariae, nor by inoculation of excysted juvenile flukes into the body cavity or to the liver surface. Many metacercarial cysts were detected within two days in the feces of orally inoculated chickens. In the *in vitro* excystation test with chicken bile at 42°C, metacercariae emerged successfully. These results indicate that the major resistant factors may not act during the migration from the mouth to the liver. Histopathological examination of the liver of experimental chickens could not prove the effect of a resistant factor. Excysted flukes were cultivated at 37–42°C in RPMI1640 supplemented with calf serum, with the result that the survival rate of flukes fell with higher temperatures. When chicken serum was used instead of calf serum, flukes survived for a long period of time at 37°C, while all died within four days at 42°C. The higher body temperature of chickens than that of other mammalian hosts is considered to be the major factor contributing to the resistance of chickens to infection with *Fasciola* sp.—**KEY WORDS:** chicken, *Fasciola* sp., infection, resistance.

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Generally birds are resistant to infection with *Fasciola*. Experimental infection of chickens has been attempted by Ono and Kimura [9] and Dawes [1]. Ono and Kimura [9] inoculated 15 chickens with oral doses of 300–500 metacercariae of Japanese *Fasciola* sp. each, and found immature flukes in the liver parenchyma of three chickens 70–146 days after inoculation. Dawes [1] also inoculated six chickens orally with 50 metacercariae of *Fasciola hepatica* each, and recovered six and two juvenile flukes from the body cavities of two chickens, respectively, on day 3 after inoculation, and one juvenile fluke from the body cavity of another chicken on day 17. These studies indicate that the susceptibility of chickens is low and that flukes are unlikely to mature in chickens.

The factors influencing the considerable resistance of chickens to infection with *Fasciola* have not been studied. The present study was designed to examine some of these factors through *in vivo* and *in vitro* experiments. Chickens were inoculated with Japanese *Fasciola* sp. via three different routes, oral inoculation of encysted metacercariae, injection of newly excysted flukes into the body cavity, and surgical transfer of excysted flukes onto the liver surface, in order to examine if the infection is interrupted in the migratory course of *Fasciola* sp. between the mouth and liver. Excystment of metacercariae and cultivation of excysted juvenile flukes were attempted *in vitro* under the conditions imitated to those inside the chicken body.

MATERIALS AND METHODS

Chickens: Fifteen female White Leghorn chickens, 5 to 20 weeks of age, weighing 250–1,000 g, were used.

Metacercariae: Metacercariae of *Fasciola* sp. were obtained by experimental infection of *Lymnaea ollula* with miracidia hatched from eggs collected from the gallbladder of naturally infected cattle. Excystment of metacercariae was performed by a method described previously [5].

Inoculation of *Fasciola* sp. metacercariae: i) Six chickens were inoculated orally through a rubber catheter with 440–482 metacercariae without the cyst together with a small volume of saline containing penicillin G potassium salts at 500 units/ml and streptomycin sulfate at 1 mg/ml. ii) Six chickens were inoculated with 124–251 newly excysted flukes by injection into the body cavity with a small volume of saline. iii) Three chickens were inoculated with 129–139 newly excysted flukes by surgical transfer to the liver surface under anesthesia with lidocaine hydrochloride.

Fecal examination: Feces of two chickens orally inoculated with metacercariae through a catheter were collected for five days after inoculation. All feces collected in a day were suspended in a total volume of 1,000 ml distilled water and passed through a 30-mesh screen. The filtrate was examined microscopically for metacercariae.

Immunodiffusion test: Sera of the experimental chickens were collected weekly and examined for precipitating antibodies against *Fasciola* sp. by the double immunodiffusion test. The immunodiffusion test using antigens extracted with Triton X-100 was carried out by a method described previously [6].

Histological examination: Liver blocks of the experimental chickens were fixed in 10% formalin solution and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin.

Cultivation of newly excysted flukes: Excysted juvenile flukes were cultivated in RPMI1640 as a basal medium by a method described previously [4]. The number of excysted flukes used in each trial ranged from 42 to 76.

RESULTS

Chickens were inoculated with *Fasciola* sp. metacercariae via three different routes: oral inoculation of encysted metacercariae, injection of newly excysted flukes into the body cavity, and surgical transfer of excysted flukes onto the liver surface (Table 1). None of the chickens showed clinical signs nor passed fluke eggs in the feces for 70 days after inoculation. In the oral inoculation group, 1 or 2 flukes were recovered from the liver parenchyma of three (Nos. 1, 3, 5) of the six chickens examined on day 70 after inoculation. Gross lesions consisting of localized tracks by migrating flukes

with haemorrhage were found in the liver of the three chickens. Precipitating antibodies against *Fasciola* sp. were also detected in sera of the three chickens. No lesions nor antibodies were found in the remaining three chickens (Nos. 2, 4, 6). Injection of juvenile flukes into the body cavity did not result in the recovery of any flukes, although liver lesions with haemorrhage were found in one (No. 11) of the six chickens examined. Antibodies were not detected in any of the six chickens. In the group subjected to surgical transfer of juvenile flukes to the liver surface, one fluke was recovered from the liver parenchyma of one (No. 13) of the three chickens examined. Liver lesions with haemorrhage and scars were found in all the three chickens. Antibodies were detected only in No. 13 chicken. Thus, infection of *Fasciola* sp. was not successfully established by the parenteral routes.

Flukes recovered from the chickens described above measured 5.0–6.5 and 2.2–3.5 mm in length and breadth, respectively. The genital organs, testis and uterus, were underdeveloped.

Fecal examination for metacercariae was carried out in two chickens (Nos. 3, 4) that were inoculated orally with 450 and 440 metacercariae, respectively (Table 2). Metacercariae were detected for two days after inoculation in each bird, but not thereafter. Total number of detected metacercariae was 107 and 134 in Nos. 3 and 4 birds, respectively. Most of the metacercariae detected left their cysts, which

Table 1. Infection of chickens with Japanese *Fasciola* sp.^{a)}

Inoculation method	Chicken No.	Age (week)	Inoculum size	Recovery of flukes ^{b)}	Gross lesion in liver	Appearance of precipitating antibodies (week)
Oral inoculation with metacercariae	1	5	482	1	+	7
	2	5	482	0	—	—
	3	10	450	1	+	8
	4	10	440	0	—	—
	5	20	480	2	+	5
	6	20	470	0	—	—
Injection of excysted juvenile flukes into body cavity	7	5	251	0	—	—
	8	5	159	0	—	—
	9	10	186	0	—	—
	10	10	161	0	—	—
	11	20	124	0	+	—
	12	20	195	0	—	—
Surgical transfer of excysted juvenile flukes to liver	13	18	131	1	+	6
	14	18	129	0	+	—
	15	18	139	0	+	—

a) Experimental chickens were autopsied on day 70 after inoculation. Fluke eggs were not detected in feces of any of the chickens during the experiment.

b) All flukes were recovered from the liver parenchyma.

Table 2. Detection of metacercariae in feces of two chickens orally inoculated with *Fasciola* sp.

Chicken No.	No. of metacercariae inoculated	No. of metacercariae detected in feces on days post-inoculation				
		1	2	3	4	5
3	450	88 (86) ^{a)}	19 (15)	0	0	0
4	440	106 (104)	28 (26)	0	0	0

a) Number of metacercarial cysts is shown in parentheses.

Table 3. Excystment of *Fasciola* sp. metacercariae

Bile ^{a)}	Temperature	No. of metacercariae tested	No. of excysted metacercariae (%)
Chicken	42°C	100	42 (42.0) ^{b)}
Cattle	37°C	94	50 (53.2) ^{b)}

a) Fresh bile was added at 10% to Hanks' balanced salt solution as medium [5].

b) Difference was not significant ($P>0.05$).

were passed in the feces.

The liver of the chickens used was examined histopathologically. Migrating juvenile flukes found in the parenchyma were surrounded by necrotic liver cells, but neither eosinophils nor lymphocytes were present. Track lesions were characterized by haemorrhage and infiltration of eosinophils. Around the tracks, a mild hyperplasia of connective tissues and remarkable infiltrations of lymphocytes were observed. In older tracks, a mixture of eosinophils, lymphocytes and mononuclear cells infiltrated. Marked hyperplasia of fibroblasts was noted around the older tracks.

The effect of chicken bile and a higher temperature on excystment of metacercariae was examined *in vitro* (Table 3). In a medium added with 10% chicken bile at 42°C, 42 (42.0%) of 100 metacercariae emerged. On the other hand, in a medium with cattle bile at 37°C, 50 (53.2%) of 94 metacercariae emerged. The results of these two experiments did not differ significantly from each other ($P>0.05$).

Newly excysted juvenile flukes were cultivated *in vitro* in order to examine the effect of higher temperatures on the survival of flukes. Using RPMI1640 supplemented with 50% calf serum, excysted flukes were cultivated at 37°C, 40°C, 41°C and 42°C (Fig. 1). At 37°C, the survival rate was considerably high up to day 12 of cultivation (94.5% $\geq$), and the maximum survival time was 27 days. At 40°C, the survival rate was high up to day 6 (92.1%

Fig. 1. Survival *in vitro* of newly excysted flukes of *Fasciola* sp. at higher temperatures: ●, 37°C; ○, 40°C; ▲, 41°C; △, 42°C. Medium used was RPMI 1640 supplemented with 50% calf serum. Fig. 2. Cultivation of newly excysted flukes of *Fasciola* sp. in RPMI 1640 supplemented with 50% chicken serum: ●, 37°C; ○, 42°C.

≅), and thereafter decreased gradually. The maximum survival time was 12 days. At 41°C, the survival rate decreased rapidly from day 6 and became nil on day 10. At 42°C, all flukes died within 3 days.

Chicken serum was added to RPMI1640 at 50% and the medium was used to cultivate excysted flukes at 37°C and 42°C (Fig. 2). At 37°C, the survival rate was high up to day 6 (80.4% ≅), and thereafter decreased gradually. The maximum survival time was 24 days. At 42°C, all flukes died within 4 days.

DISCUSSION

The following conditions may contribute to the resistance of chickens to infection with *Fasciola* sp.: i) Metacercariae may be damaged when passing through the crop or the gizzard; ii) Metacercariae may be unable to excyst within the chicken body; iii) Excysted juvenile flukes may not penetrate the intestinal wall; iv) Flukes penetrating the intestinal wall could not easily reach the liver; v) Nutrition may be insufficient for flukes to develop in the chicken liver; vi) Cellular reactions may interfere with the migration and growth of flukes in the liver, to cause their death; and vii) The higher body temperature of chickens may block the growth of flukes.

Many metacercarial cysts were detected in the feces of chickens within two days after oral inoculation of metacercariae. This indicates that metacercariae were destructed during the passage through the digestive tract and/or that they emerged from their cysts in the digestive tract. In the present *in vitro* excystment test with chicken bile at 42°C, metacercariae emerged successfully, so, the latter situation may be the case.

We reported that the ligated intestine of chickens is highly resistant to penetration by *Fasciola* sp. flukes *in vitro* [7]. Many excysted flukes may die in the intestinal tract or in the course of migration through the intestinal wall. However, this is not a major factor of the resistance, because in the present study, infection of chickens was attempted via parenteral routes, namely, excysted juvenile flukes were inoculated into the body cavity or directly on to the liver surface, with the result that the infection was not successfully established. The major resistant factor may not be present in the migrating course from the mouth to the liver.

Fasciola sp. flukes could not grow sufficiently in the liver of chickens.

The body temperature of chickens is relatively higher than those of mammals. Excysted juvenile flukes of *Fasciola* sp. were cultivated *in vitro* at temperatures of 37°C, 40°C, 41°C and 42°C. The survival of cultivated flukes decreased remarkably as the temperature increased. The higher body temperature is considered to be the major factor that affects the survival of juvenile flukes in chickens. From the above reasons, birds generally may be resistant to infection with *Fasciola*.

Excysted juvenile flukes could be maintained for a considerable period of time in RPMI1640 supplemented with 50% chicken serum at 37°C, while all died within four days at 42°C. The result may also prove that the higher body temperature of chickens such as 42°C cause death of excysted juvenile flukes. Nutrition itself might be a minor problem or nil for the growth of flukes in the liver of chickens.

Qualitatively, the chicken liver was almost the same as of susceptible animals in the histological findings of *Fasciola* infection [2, 3, 12, 13]. Pigs are resistant to infection with *Fasciola* spp. Severe cellular reactions mainly with lymphocytes and eosinophils and encapsulation of migrating flukes occur in the liver of pigs in the early stages of infection [8, 10, 11], but such protective tissue reactions were not found in the chicken livers. A resistant factor could not be found by histopathological examination.

In the chickens inoculated with excysted flukes into the body cavity, liver lesions were found in only one of the six birds examined. In contrast, liver lesions were found in all the three chickens inoculated with excysted flukes on to the liver surface. If a fluke could penetrate the intestinal wall, it could not easily reach the liver, because some barriers, such as the air-sac, are present.

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