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NOTE

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Application of a real-time PCR assay for the detection of *Ascaris suum* DNA in the liver of experimentally infected chickens

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ABSTRACT. This study aimed to evaluate the sampling method for the detection of *Ascaris suum* larval DNA in chicken livers using real-time PCR. Chickens were inoculated with *A. suum* eggs of a single dose (Group A) or repeatedly low doses (Group B). White spots (WSs) were continuously observed on liver from day 3 after the last infection in Group B and day 14 in Group A. In Group A, larval DNA was detected in WS lesions (78.6%) at a significantly higher rate than in the remaining tissue samples (31.3%). In conclusion, applying WS lesions to the assay improved the detection rate of *A. suum* DNA in chicken livers, especially in the case of a single infection.

KEY WORDS: *Ascaris suum*, chicken liver, real-time PCR assay, white spot lesion

Ascaris suum, an intestinal nematode, is known to be distributed worldwide. Besides parasitizing in pigs as a definitive host, this roundworm can infect other animals, such as chickens and cattle, and is considered a paratenic host [7–9, 16]. In addition, this roundworm is a pathogenic agent causing ascarid larva migrans syndrome (ascarid LMS) in humans [5, 6, 12–14]. Humans can become infected by ingesting embryonated *A. suum* eggs from soil or contaminated vegetables/water [1, 2, 4], or eating raw or undercooked meat/offal containing infective larvae from paratenic hosts [3, 15].

In humans, diagnosis of *A. suum* infection is mainly conducted by serological assays such as the enzyme-linked immunosorbent assay (ELISA) [11, 13, 14]. Recently, an ELISA system to distinguish *A. suum* to *Toxocara* spp. infection was successfully developed for diagnosis in chickens [10]. However, this method cannot always identify the presence of larval contamination in meat/offal because serological tests reflect not only present infections, but also past ones. In a previous study, we evaluated a real-time PCR assay with high sensitivity and specificity in order to directly identify the existence of *A. suum* in meat and internal organs [9]. This study contained a limitation due to the processing sample amount: the maximum weight of applicable tissue for DNA sample preparation was 0.5 g. To apply this method for detecting *A. suum* DNA in domestic animals, such as chickens and cattle, a large size of liver should be subsampled for testing. Therefore, the sampling measurement is an important criterion.

The experimental studies of *A. suum* infection in chickens and calves showed the distribution of *A. suum* larvae and macropathogenicity in animal liver [8, 16]. The results showed that *A. suum* larvae were recovered from day 1 to day 3 in chicken liver [16] and day 5 in calf liver [8]. White spot lesions, produced by the interaction between the parasite and the host immune system [16], can be formed on the liver surface when *A. suum* larvae migrate to the liver [8, 16]. Thus, in this study we assessed the formation of white spot lesions on chicken liver and evaluated the sampling sites for the detection of larval contamination in two chicken groups: a single infection group representing an accidental infection model, and a trickle infection group representing a field infection model. The results of this study provide useful information in terms of meat inspection to prevent the risk of food-borne *A. suum* infection in humans.

A. suum-adults were obtained from swine intestines at a local abattoir in Japan. Worms were washed several times in saline (0.85%)

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and the lower part of the uterus was then removed to collect the fertilized eggs. The procedure of fertilized egg embryonation was described in detail in a previous study [9]. Embryonated eggs were then used for the experimental infection of chickens. As a positive control for real-time PCR assay, *A. suum* DNA was isolated from lung-migrating L3 larvae that were collected from experimentally infected male Japanese white rabbits (SLC, Shizuoka, Japan).

All animal experiments were conducted in accordance with the guidelines for animal experimentation of the University of Miyazaki (2015-531 and 2014-017) and the relevant ethical guidelines of the Japanese Ministry of Education, Culture, Sports, Science and Technology. Thirty-seven locally-bred chickens (1 day old) were bought from the prefectural experimental stock farm (Miyazaki, Japan). The chickens were kept for 4 weeks to adapt to laboratory biosafety conditions and were then divided into two infected groups and an uninfected group (n=8) for inoculation. Group A consisted of chickens being infected with a single dose of 2,000 *A. suum* embryonated eggs (n=16), and group B were repeatedly inoculated twice a week for 6 weeks with a dose of 500 *A. suum* embryonated eggs (n=13). On 3, 7, 14 and 28 days after the last infection, three/four chickens from each infected group and two from the uninfected group were euthanized. Livers were inspected macroscopically, and the tissue samples were collected for DNA analysis. For livers with white spot lesions, 50 mg of the liver tissue containing just one white spot lesion was separated (the number of spots varied in individual chickens), weighed and put into 1.5 ml homogenizing tubes (BioMasher® II, Nippi, Tokyo, Japan). The remaining liver tissue without lesions was minced thoroughly, and 500 mg was subsampled, and put into 15 ml homogenizing tubes (BioMasher®sp, Nippi). Alkaline-lysis method was performed for extracting *A. suum* DNA from the liver tissue samples. For 500 mg of tissue samples, DNA extraction was performed following the protocol published by Nguyen *et al.*, 2016 [9]. The liver tissue was homogenized by BioMasher® sp in 15 ml tube with 1.8 ml of 50 mM NaOH, and then boiled for 30 min. After that, 200 µl of 1 M Tris-HCL (pH 8.0) was added and vortexed thoroughly. Finally, the mixture was centrifuged at 14,000 × *g* for 10 min, and supernatant was separated and stored at −20°C. For 50 mg of lesion samples, the procedure of DNA extraction was lightly modified. More specifically, 180 µl of 50 mM NaOH and 20 µl of 1 M Tris-HCl (pH 8.0) were applied to those samples. Homogenized tissue in NaOH was incubated at 95°C for 10 min by a heat block before adding Tris-HCl. Following this, the same steps were followed that were similarly performed for the treatment of the 500 mg tissue.

Fig. 1. White spot lesions on the liver in an experimentally infected chicken. Arrows show the white spot lesions.

A real-time PCR assay was applied to amplify the 82 bp DNA fragment of ITS1 region with the primer sets of forward primer 5′-TGCACATAAGTACTATTTGCGCGTAT-3′ and reverse primer 5′-CCGCCGACTGCTATTACATCA-3′. A TaqMan probe with the sequence of 5′-FAM-CGTGAGCCACATAGTAAATTGCACACAAAATG-TAMRA-3′ was designed in the sequence of the amplified product. The real-time PCR condition was described previously [9].

Statistical analysis of the differences in the positive rate of *A. suum* DNA detection between 50 mg lesion and 500 mg of the remaining samples in each group was done using Fisher's exact test with a significance level of 5%.

The results showed that the livers were normal in color and structure and several clear white spot lesions were recognized on the surface of livers in the two infected chicken groups (Fig. 1). As shown in Table 1, the white spot lesions were firstly found in livers of all four chickens on day 14, but just two out of four chickens on day 28 in the single infection group (group A). Meanwhile, these white spots were observed in all necropsy time points and in all infected chickens (except for two chickens on day 28) in the trickle infection group (group B). The number of white spots varied in each chicken, ranging from 1 to 16. In group B, the number of lesions reached a peak on day 3, declined sharply on day 7 and 14, and were mostly undetected on day 28 after the last infection. Apart from the observation of white spot lesions on the liver surface at necropsy, *A. suum* infected chickens did not show any other clinical symptoms.

In the single infection group (group A), 78.6% (11/14) of lesion liver samples and 31.3% (5/16) of the remaining tissue samples were found to be positive with *A. suum* DNA (Table 1). The results also revealed that the detection efficacy of *A. suum* in 50 mg of lesion liver samples was significantly higher than that in 500 mg of the remaining tissue ones ($P<0.05$). The detection rate of *A. suum* DNA in the remaining tissue samples declined significantly from day 3 (75.0%) to day 14 (25.0%), and no positive case was observed on day 28. Meanwhile, the positive rate in 50 mg lesion liver samples reached 77.8% on day 14 and maintained that percentage at 80.0% on day 28.

In the trickle infection group (group B), the results revealed that 78.9% (15/19) of white spot lesion samples and 100.0% (13/13) of the remaining tissue samples were positive for *A. suum* DNA in total (Table 1). In particular, the rate of *A. suum* DNA detection was highly constant until day 28 (100.0%). No significant difference was observed on the detection efficacy of *A. suum* DNA between 50 mg of the lesion tissue samples and 500 mg of the remaining ones, and the mean cycle threshold (Ct) values for the DNA amplification in almost all lesion tissue samples tended to be lower than that in the remaining ones.

There was no abnormal change observed on the liver surface and *A. suum* DNA was also not detected by real-time PCR method

Table 1. Detection of *Ascaris suum* DNA in the liver tissue of experimentally infected chickens

Group	Day after inoculation	No. of chickens	No. of WS positives	50 mg of white spot (WS) lesion tissue				500 mg of remaining tissue		
				Mean No. of WS (min–max)	Examined sample	Positives (%)	Ct value§ (mean ± SD)	Examined sample	Positives (%)	Ct value§ (mean ± SD)
A (Single infection)	3	4	0	0	0	-	-	4	3 (75.0%)	34.1 ± 2.3
	7	4	0	0	0	-	-	4	1 (25.0%)	34.1
	14	4	4	3.3 (1–6)	9	7 (77.8%)	32.5 ± 2.7	4	1 (25.0%)	35.9
	28	4	2	2 (0–4)	5	4 (80.0%)	34.7 ± 1.6	4	0 (0.0%)	-
	Total	16	6	21 (0–6)	14	11 (78.6%)*		16	5 (31.3%)*	
B (Trickle infection)	3	4	4	10 (6–16)	8	4 (50.0%)	31.0 ± 1.3	4	4 (100.0%)	34.5 ± 1.9
	7	3	3	2.7 (2–4)	5	5 (100.0%)	35.0 ± 1.6	3	3 (100.0%)	32.7 ± 1.4
	14	3	3	2.7 (2–3)	5	5 (100.0%)	34.1 ± 2.1	3	3 (100.0%)	36.4 ± 1.6
	28	3	1	0.3 (0–1)	1	1 (100.0%)	33.0 ± 0.3	3	3 (100.0%)	38.3 ± 0.7
	Total	13	12	4.4 (0–16)	19	15 (78.9%)		13	13 (100.0%)	

§Cycle threshold (Ct) value represents the mean and SD of Ct values in positives at each sampling point. Single infection: chickens were inoculated with a single dose of 2,000 *A. suum* eggs. Trickle infection: chickens were repeatedly inoculated twice per week for 6 weeks with a low dose of 500 *A. suum* eggs. *$P<0.05$.

in the uninfected chicken group.

Recently, awareness has grown of ascarid LMS in East Asia, especially in Japan, as a food-borne disease in adults who habitually consume raw or undercooked meat/offal [3]. Our previous study evaluated a real-time PCR assay with high sensitivity and specificity [9]. More specifically, this assay could detect *A. suum* DNA in 500 mg of mouse liver tissue spiked with one larva without the amplification of *T. canis* and *T. cati* DNA. In addition, this assay successfully detected *A. suum* in experimentally infected mice liver [9]. However, when this assay is applied for the detection of *A. suum* DNA in livestock animals, especially in a natural infection setting in which animals could only ingest a small number of eggs, sampling measurement should be taken into account. Notably, the migration of *A. suum* larvae into the liver can form white spot lesions on the liver surface [8, 16]. Based on macropathological features, the detection efficacy of *A. suum* DNA in 50 mg of liver samples containing white spot lesion was compared with 500 mg of liver tissue without lesions by real-time PCR assay. For instance, *A. suum* DNA was not detected in 500 mg of the remaining liver samples in all four chickens belonging to the single infection group on day 28, meanwhile 4 out of 5 lesion samples were positive with *A. suum* DNA (Table 1). For the trickle infection group, even though there was no significant difference of detection efficacy between the two samples, the mean Ct values for the detection of *A. suum* DNA in almost all lesion samples tended to be lower than that in the remaining samples, especially on day 28 (33.0 and 38.3) (Table 1). This indicated that the selection of 50 mg lesion samples gave a higher sensitivity for the detection of *A. suum* DNA in infected chicken liver by this real-time PCR assay. However, in the case of early infection with no observed lesions, 500 mg minced liver sample was the only selection for sampling.

The results of this study also demonstrated the superior advantage of the PCR assay compared to the traditional digestion method. The *A. suum* larvae were only recovered up to day 7 in chickens by the conventional digestion method, and larva was not found in the white spot lesions by pathological observation (data not shown), but *A. suum* DNA was still detected until day 28 by the real-time PCR in this study. This could be explained by larvae which were partly destroyed by the host immune system. Even though the detection of *A. suum* DNA did not provide direct proof for the infectivity of larvae to the new host, it provided information regarding the possibility of infection due to ingesting raw/undercooked meat/offal containing infective larvae from paratenic hosts.

This real-time PCR assay demonstrated with a high efficacy, the detection of *A. suum* DNA in experimentally infected chicken livers. White spot lesions on the chicken liver surface was clinical evidence of larval migration in chickens, while the real-time PCR assay indicated with a high possibility that the lesions contained *A. suum* DNA. Thus, it was confirmed that white spot lesions were the target of the sampling site for the detection of *A. suum* infection in chickens. The results of this study could provide useful information in terms of poultry inspection in order to prevent the risk of food-borne *A. suum* infection in humans. Moreover, the established method may contribute to improvements in public health by providing an intervention method for the control of *A. suum* infection in chickens.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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