-Original paper-

Title:

Evaluation of real-time PCR assay for the detection of *Ascaris suum* contamination in meat and organ meats.

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Abstract

Ascarid larva migrans syndrome (ascarid LMS) is caused by ascarid roundworms including *Ascaris suum*. In East Asia, ascarid LMS has been considered as a food-borne disease in adulthood who has the dietary habitat of consuming raw or lightly cooked meat and organ meats. To evaluate the potential risk of *A. suum* infection from these foods, *Ascaris* specific real-time PCR was developed. The assay could constantly detect *A. suum* DNA up to 10 fg. Its specificity was confirmed by non-amplification with *Toxocara canis* and *Toxocara cati* DNA. *A. suum* DNA could be amplified from not only a single larva but also mouse liver spiked with a larva. Moreover, the assay could detect *A. suum* DNA in experimentally infected mouse liver, and showed higher sensitivity than a conventional digestion method. This real-time PCR assay would be useful for detecting the *A. suum* larval contamination in meat and organ meats.

Key words: Food-borne disease, Parasite, Zoonosis, Real-time PCR, Meat, Organ meats

Practical application

It has been considered that one of the most important risk factors for ascarid LMS in humans is the consumption of raw or undercooked meat or organ meats of domestic animals infected with *A. suum*. Thus, we developed the novel real-time PCR with the high sensitivity and specificity in order to specifically detect *A. suum* DNA from animal tissues. This assay can be applied to the meat inspection procedure for the identification of parasite larval contamination that may adversely impact on public health as well as on animal health and welfare.

1. Introduction

Ascaris suum known as an intestinal roundworm in swine is distributed worldwide. Eggs are passed into the environment via the feces and become infective to animals after the period of embryonation. Once pigs ingest *A. suum* embryonated eggs, they hatched into larvae in the digestion system, then penetrate through intestinal mucosa, and migrate to the liver via hepatic portal system. From the liver, larvae migrate to the lungs via the venous system. Then larvae break out the alveolar capillaries and migrate up the bronchial tree to the pharynx where they are swallowed. They develop into adult worms in the small intestine after six to eight weeks post-infection.

The parasite can also infect to other animals such as chickens, cattle and humans (McCraw *et al.* 1971, Yoshihara *et al*. 2008). When *A. suum* infect to such inadequate hosts, larvae undergo the similar cycle as in pigs but reach to the intestine through the liver and lung (McCraw *et al.* 1974, Yoshihara *et al.* 2008). They remain in animal tissues with the infectivity (Permin *et al.* 2000) and do not grow to mature adults. In humans, although *A. suum* can sometimes grow up into adult worms (Nejsum *et al.* 2005, Arizono *et al*. 2010). However, the severe health problem is larva migrans syndrome (LMS) caused by the migration of larvae to various organs. This type of infection resulting a number of clinical symptoms such as eosinophilia, fever, coughing, enlarged liver or pneumonia is known as visceral larva migrans (VLM) (Izumikawa *et al*. 2011, Lamberton *et al.* 2015). Other type of infection resulting encephalopathy in the brain is known as neuro larva migrans (NLM) (Inatomi *et al*. 1999, Umehara *et al*. 2006).

The outbreak of VLM due to *A. suum* was firstly reported in Japan in 1996 (Maruyama *et al*. 1996). Since then, patients diagnosed in VLM caused by *A. suum* have been frequently

reported around the world (Hoenigl *et al.* 2010, Izumikawa *et al*. 2011, Pinelli *et al.* 2011, Miller *et al*. 2015). Humans get infection either by accidental ingestion of embryonated eggs in soil or contacted on raw vegetable that is grown in soil fertilized with *A. suum* contaminated-swine excrement (Matsuyama *et al*. 1998, Tokojima *et al.* 2004), or by eating raw or undercooked meat and organs that are parasitized by the parasite larvae (Izumikawa *et al*. 2011). In East Asia, the latter route is mainly considered as the cause of ascarid LMS due to the eating habit (Choi *et al.* 2012).

Diagnosis of *A. suum* infection in humans relies mainly on the immunological assays. The most recommended assay is the enzyme-linked immunosorbent assay (ELISA) using the Excretory/Secretory (ES) antigen derived from the third-stage larvae of *A. suum* (Pinelli *et al.* 2011*;* Schneider *et al.* 2015). However, it is an indirect method to detect *A. suum* infection, and cross-reaction with other parasitic infections is often observed. Although ELISA is useful for the diagnosis of *A. suum* infection in living animals, it cannot accurately evaluate the existence of larvae in meat and organ meats thought to be the cause of ascarid LMS. Therefore, the direct demonstration of *A. suum* larvae contamination in meat or organ meats is required to evaluate the actual risk of infection in those foods.

An ordinary PCR was developed to detect *A. suum* DNA in the tissue-embedded ascarid larvae (Ishiwata *et al*. 2003). However, the method was time-consuming, and unfortunately the sensitivity was not fully evaluated. The aim of this study is to develop a sensitive, specific and time-saving real-time PCR system in order to detect *A. suum* larval contamination in meat and organ meats and then to evaluate the risk of these foods for the parasite infection in humans.

2. Materials and methods

2.1. Parasites

Adult worms of *A. suum* were collected from swine intestines at a slaughterhouse in Japan. Adult worms of *T. canis* and *T. cati* were obtained from dogs and cats after administration of anthelmintics. *Ascaris lumbricoides* adult worms that were expelled in the feces of the human patient were identified ribosomal DNA typing by PCR-RFLP (Zhu *et al*. 1999). All worms were washed several times in saline (0.85%). For *A. suum*, *T. canis* and *T. cati*, the uterus was removed to collect the eggs. The eggs were then embryonated in 0.5N H_2SO_4 in culture flask at 25°C for 6-7 weeks. After embryonation, the eggs were stored at 4°C until being used. Larvae were then hatched mechanically, isolated free from egg shell contaminants (Takamiya *et al*. 1993). For DNA isolation by alkaline-lysis method, mechanically hatched *A. suum* larvae were fixed by 70% of ethanol (Wako, Osaka, Japan). Lung L3 larvae of *A. suum* were collected from male Japanese white rabbits (Kyodo, Kumamoto, Japan) using the Baermann method as described previously (Yoshida *et al*. 2012).

2.2. Experimental infection in mice

Eighteen C57BL/6 mice (SLC, Shizuoka, Japan) were divided into 4 groups. Three mice were used as control, and 5 mice in each of other 3 groups were inoculated with 100, 300, and 900 *A. suum* embryonated eggs orally. After 2 days post-infection, all mice were euthanized and the livers were collected. Then the liver of each mouse was minced thoroughly and 500 mg was subsampled for real-time PCR assay. The remaining tissue was weighted and digested by the pepsin/HCl digestion method to recover the larvae. The protocol of the experimental infection was approved by the Ethics Committee for Animal Experimentation of University of Miyazaki (2011-523-5).

2.3 DNA Extraction

DNA was extracted from lung L3 larvae of *A. suum*, mechanically hatched L3 larvae of *T. canis* and *T. cati* and adult worms of *A. lumbricoides* by PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) by following the instruction from manufacturer.

Alkaline-lysis method was also performed for extracting DNA from mechanically hatched L3 larvae of *A. suum*. Briefly, larvae were put into 2 mL tubes, followed by addition of 1.8 mL of 50 mM NaOH. After incubation at 95°C for 10 minutes, 200 µL of 1M Tris-HCl (pH 8.0) was added into each tube. The mouse liver samples that are spiked with *A. suum* larvae, and those of infected and uninfected mice were homogenated in 15 mL tubes provided by BioMasher®sp (Nippi, Tokyo, Japan) before adding 1.8 mL of 50 mM NaOH, and then boiled for 30 minutes. Then, 200 µL of 1M Tris-HCl (pH 8.0) was added into each tube. The mixture was vortexed thoroughly and centrifuged at 14,000 *g* for 10 minutes. Finally, the supernatant was separated and stored at -20°C.

2.4. Real-time PCR assay

The method in this study adopted the method reported by Pecson *et al.* (2006) which was developed for quantifying viable *Ascaris* eggs. A DNA fragment (82 bp) of the ITS1 region was amplified using real-time PCR 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. Amplification was performed in a total volume of 25 µL containing 1 µL of DNA template and the following PCR mixture: 12.5 µL of TaqMan® Fast Universal PCR Master Mix (Amplified Biosystems, Carlsbad, CA), 300 nM of both forward and reverse primers and 200 nM of TaqMan probe. The condition of real-time PCR was modified to 95°C for 20

seconds initially, then 40 cycles of 95°C for 1 second, 60°C for 20 seconds. The reactions with triplicate were performed on a StepOnePlus real-time PCR System (Amplified Biosystems). The results of amplification were analyzed by StepOne Software v2.2 (Amplified Biosystems).

For establishing standard curve in real-time PCR, conventional PCR was used to amplify a fragment of the ITS1 region of *A. suum* DNA with primer sets of forward primer 5'-GGCAAAAGTCGTAACAAGGT-3' and reverse primer 5'-CTGCAATTCGCACTATTTATCG-3. PCR product was purified by SigmaSpin™ Sequencing Reaction Clean-up (Sigma-Aldrich, St. Louis, MO), measured DNA concentration by Qubit™ Fluorometer (Invitrogen), and used to construct a standard curve. The copy number of ITS1 region was calculated by the following formula:

Molecular weight (MW) of ITS1 fragment (g/mol) = (bp size of ITS1 fragment) x (330 Da x 2 nucleotide/bp)

Weight of one ITS1 molecule (g) = MW of ITS1 fragment (g/mol)/Avogadro's number ($6.02214199 \times 10^{23}$ molecules/mole)

Copy number of ITS1 region = quantity of DNA in reaction (g)/weight of one ITS1 molecule (g)

2.5 Digestion method

The remaining minced mouse liver left after subsampling for real-time PCR was digested in pepsin-HCl digestion fluid [1.2 % pepsin (1:10,000) (Nacalai Tesque, Inc. Kyoto, Japan) and 1% HCl (35%)] at 42°C for 1 hour under constant stirring in 100 mL glass beaker. The ratio between tissue (g) and fluid (mL) was approximately 1:10. Following digestion, fluid was filtered through a nylon mesh sieve with pore size of 100 µm. Then, the filtrate was filtered again

through a nylon mesh sieve with pore size of 10 μm. The larvae trapped on the nylon mesh sieve were collected, and the number of larvae in each sample was counted under a stereoscope.

The number of larvae in 500 mg of liver tissue was calculated based on the number of larvae recovered and weight of liver tissue examined.

3. Results

Sensitivity and specificity of real-time PCR assay

The sensitivity of the assay was determined by testing *A. suum* DNA with serial dilution from 100 pg to 100 ag (Table 1). Besides *A. suum* DNA, 100 pg of *T. canis*, *T. cati* and *A. lumbricoides* DNA were tested for evaluating the specificity (Table 1).

The assay could detect *A. suum* DNA up to 10 fg, equaling to 26 copies of ITS1 region. There was no amplification with 100 pg of *T. canis* and *T. cati* DNA. However, 100 pg of *A. lumbricoides* DNA was amplified in all samples of triplicate.

Detection of A. suum DNA extracted from larvae

To detect *A. suum* DNA extracted from actual larvae, the samples containing 1, 2, 5, 10 or 20 larvae were processed by the alkaline-lysis method, and 2.0 mL of lysate was obtained for each sample. Then, 1 μL (0.05 %) of the lysate was applied in triplicate to the real-time PCR as template DNA (Table 2). DNA extracted from one *A. suum* larva was amplified successfully with mean C_t value at 33.86, showing that 56.5 copies of ITS1 were existent in 0.05 % of the total lysate. The copy number of ITS1 in 1 μL of samples increased proportionally with the number of larvae used.

Detection of A. suum DNA extracted from larvae spiked into liver tissue

To evaluate the ability of the real-time PCR to detect *A. suum* larvae in meat and organs, template DNA was prepared from the mixture of larvae and animal tissue. In the experiment, 1, 2, 5, 10 or 20 larvae were spiked into 500 mg of uninfected mouse liver, and DNA was extracted

from the mixture by the alkaline-lysis method. One microliter of DNA lysate (0.05% of total lysate) was applied as template. As a result, single larva in 500 mg of liver tissue could be detected by the real-time PCR (Table 3). However, the samples containing 1, 2 and 5 larvae were amplified with the similar mean C_t value of around 35, whereas mean C_t value of samples containing 10 and 20 larvae were 33.90 and 31.17, respectively.

Larval recovery from experimentally infected mouse livers by the conventional pepsin/HCl digestion method

In order to detect migratory larvae in tissue, the digestion method is commonly used. After taking 500 mg of mouse liver for the real-time PCR assay, the rest of the liver was weighed and digested by pepsin/HCl digestion method. Then the number of *A. suum* larvae recovered was counted and adjusted the number as one in 500 mg of the liver (Table 4). The larvae were recovered in 8 mice inoculated with 300 or 900 eggs. No larva was recovered in any mice inoculated with 100 eggs and one mouse inoculated with 900 eggs.

Detection of A. suum DNA in experimentally infected mouse livers

Template DNA was isolated from 500mg of mouse liver by alkaline-lysis method. DNA of *A. suum* was detected in all liver samples of mice inoculated with 300 and 900 eggs and 4 of 5 mice inoculated with 100 eggs (Table 5). Moreover, the mean Ct values and the copies of ITS1 region were relative to the number of eggs inoculated.

4. Discussion

A. suum has been regarded as an improtant causative species of ascarid LMS resulting from the migration of ascarid roundworms (Maruyama *et al*. 1996, Pinelli *et al.* 2011). Humans become infected with *A. suum* through the ingestion of infective eggs from the soil or

contaminated vegetables (Matsuyama *et al*. 1998, Tokojima *et al.* 2004), or the ingestion of larvae in meat and organ meats (Izumikawa *et al*. 2011, Choi *et al.* 2012). Herein, we reported that the development of a sensitive and specific real-time PCR assay allowing rapid and reliable identification of *A. suum* larvae in animal tissue.

ITS rDNA sequence is the target gene often chosen to detect pathogens because of its high copy number. In addition, genetic diversity of this sequence could be used to distinguish the nematode species which are closely related and/or with morphologically similar (Chilton *et al*. 1994, Ishiwata *et al.* 2003). In this study, we established a real-time PCR assay targeting ITS1 region of *A. suum* DNA to detect the contamination of *A. suum* larvae in animal tissue. Specific amplification was resulted with no detection of *T. canis* and *T. cati* DNA. However, *A. lumbricoides* that possess an extreme similarity of the ITS1 regions (98.67% of homology) with *A. suum* (Zhu *et al.* 1999; Splicker *et al.* 2013) was amplified by this system. As result of the effects of mass chemotherapy and improving water, sanitation and hygiene in developed countries, the prevalence of *A. lumblicoides* infection has been decreased dramatically (Jones 1983, Kobayashi *et al.* 2006). It is uncommon and cases are generally believed to have been imported (Mandell *et al.* 2005). Therefore, the risk of contamination by *A. lumbricoides* eggs could be neglected in these areas. However, *A. lumbricoides* contamination needs to be considered when applying our system in developing countries where *A. lumbricoides* infection is commonly found.

Comparing the results between applying extracted DNA from larvae themselves and the mouse liver spiked with the same number of larvae, copy numbers of ITS1 region of the latter samples were obviously lower than those of the former samples. For example, the mean copy number was 56.5 for one larva samples (Table 2), while that was 21.9 for liver tissue samples

spiked with one larva (Table 3), i.e. approximately 2.6 times lower copy number was observed in the latter samples. The PCR reaction in the latter samples could be explained by the interference of PCR by the inhibitors, such as heme etc.; and the excess amount of host DNA originated from liver tissue in the latter sample. Although the efficiency for detecting larvae was reduced when it is applied on liver tissue, the sensitivity of the developed real time PCR was still enough for detecting one larva.

Ishiwata *et al.* (2003) succeeded *A. suum* DNA amplification from 50 mg of pig liver tissue embedded with *A. suum* larvae. In this study, to extract *A. suum* DNA from the liver tissue, samples were firstly homogenated by BioMasher®sp (Nippi, Tokyo, Japan) that can be applied to the maximum weight of tissue, 500 mg. Our developed system can examine the DNA in ten-times more tissue than previous method. However, considering the weight of meat and organ meat, 500 mg of tissue is extremely small and is not sufficient to evaluate larval contamination in meat and organ meats fully. Migration of *A. suum* larvae through the liver causes hemorrhage, fibrosis, and accumulation of lymphocytes present to white spots (Nakagawa *et al.* 1983). It has been known that white spot formation on liver is characterized as a typically macropathological feature in *A. suum* infection and their presence indicates recent *A. suum* infection/reinfection (Yoshihara *et al*. 2008). Therefore, we could apply this real-time PCR system in terms of inspection procedure by subsampling 500mg of tissue sample with white spot lesions.

Migration pattern of *A. suum* in mice has been evaluated by experimental infection studies (Slotved *et al*. 1998, Lewis *et al*. 2006, 2007, Dold *et al*. 2010). In C57BL/6 mice, larvae first appeared in the liver 6 hours post-infection and then accumulated gradually in the organ in 1-2 days post-infection, although larval recovery in migratory phase showed big variation for each individual (Lewis *et al*. 2007, Dold *et al*. 2010). In this study, we collected the liver from

experimentally infected mice on second day post-infection, and evaluated the copies of ITS1 gene and larval burdens in 500 mg of liver samples for both samples by the real-time PCR assay and the digestion method. In general, there was direct proportion between the copies of ITS1 region detected by real-time PCR assay and the number of larvae recovered by digestion method in mice inoculated with 300 or 900 eggs. Even though no larva was recovered by digestion method, our system could detect *A. suum* DNA in 4 out of 5 mice inoculated with 100 eggs. Moreover, this system could also detect at least 22.04 copies of ITS1 gene in one 100 eggs-infected mouse liver sample (data not shown), equivalent to the detection limit of our real-time PCR system determined based on the result in using liver tissue samples spiked with *A. suum* larvae. Hence, there would be at least one larva migrating to the liver in 4 out of those 5 mice. However, one of five those samples became negative by real-time PCR assay. Considering the detection limit of our assay, no larva could be included in liver tissue used to DNA isolation in real-time PCR negative mouse.

The real-time PCR system was developed in this study to detect *A. suum* larval contamination in the liver that has a risk of transmission of *A. suum*. This assay proved to have high sensitivity and specificity. Application of the assay on the meat inspection procedure may contribute to improvements on public health as well as on animal health and welfare. Besides, this assay would also be applied for evaluation soil or sandpit environment contaminated with parasite eggs.

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Table 1. Results of a real-time PCR assay performed on DNA extracted from *A. suum*, *A. lumbricoides*, *T. canis* and *T. cati*

Species	Amount of DNA	Ct value (mean±SD)	Copy number of ITS1 region*	Number of positive reactions in triplicate
A. suum	100 pg	20.22±0.02	296,876.1	3
	10 pg	23.80±0.14	29,893.3	3
	1 pg	27.21±0.39	3,408.0	3
	100 fg	30.55±0.26	393.4	3
	10 fg	34.81±0.57	26.2	3
	1 fg	35.11	-	1
	100 ag	-	-	0
A. lumbricoides	100 pg	21.23 ± 0.05	181,281.8	3
T. canis	100 pg	-	-	0
T. cati	100 pg	-	-	0

*Copy number of ITS1 region in 1 µL of DNA extraction was calculated based on the mean quantity of IST1 fragment in reaction.

Table 2. Detection of *A. suum* DNA extracted from larvae by real-time PCR

Number of larvae	Ct value (mean ±SD)	Copy number of ITS1 region*	Number of positive reactions in triplicate
0	-	-	-
1	33.86 ± 0.18	56.5	3
2	33.63 ± 0.73	71.0	3
5	31.61 ± 0.23	253.8	3
10	30.33 ± 0.64	626.3	3
20	29.25 ± 0.22	1220.8	3

*Copy number of ITS1 region in 1 µL of DNA extraction was calculated based on the mean quantity of IST1 fragment in reaction.

Table 3. Detection of *A. suum* DNA extracted from the liver tissue spiked with *A. suum* larvae

Number of larvae spiked	**Ct value (mean ±SD)**	**Copy number of ITS1 region***	**Number of positive reactions in triplicate**
0	-	-	-
1	35.32 ± 0.44	21.9	3
2	35.29 ± 0.56	22.6	3
5	35.76 ± 1.13	18.9	3
10	33.90 ± 0.53	57.0	3
20	31.17 ± 0.13	339.0	3

*Copy number of ITS1 region in 1 µL of DNA extraction was calculated based on the mean quantity of IST1 fragment in reaction.

Table 4. Larval recovery from the liver of mice experimentally infected with *Ascaris suum* eggs by the pepsin/HCl digestion method

Dose (Number of eggs)	**Number of mice**	**Mean larval burden* (range)**	**Positive mice (%)**
900	5	13.6 (0-29)	4 (80%)
300	4	5.3 (2.4-10)	4 (100%)
100	5	0 (0-0)	0 (0%)

* The number of larvae recovered from each samples were adjusted as one recovered from 500 mg of liver tissue.

Table 5. Detection of *A. suum* DNA in 500 mg of liver from mice inoculated with the parasite eggs

Dose (Number of eggs)	**Number of mice**	**Ct value (mean ±SD)**	**Copy number of ITS1 region***	**Positive mice (%)**
900	5	29.44±1.68	14,200.9	5 (100%)
300	5	31.99±3.20	1160.3	5 (100%)
100	5	35.79±3.26	142.3	4 (80%)
0	3	-	-	0 (0%)

*Copy number of ITS1 region in 1 µL of DNA extraction was calculated based on the mean quantity of IST1 fragment in reaction.