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Molecular Identification of *Trichuris suis* Worms and Eggs in Pig Feces, Infected Intestines, and Farm Environments in Japan

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Abstract

Trichuris suis is a common parasite in pigs that is thought to infect humans based on studies using recently developed molecular methods. However, the utility of such molecular methods for detection has not been examined. In the present study, we assessed PCR- and sequencing-based molecular methods for the detection of several stages of *T. suis*. PCR targeting the mitochondrial large ribosomal subunit was successful with amplification of DNA from the samples. The sequences of the isolates formed a phylogenetic cluster with isolates from China and Ecuador. The assay was effective by using DNAs extracted from a low number of eggs (> 20 eggs) and the fragments of worms after histopathologic analyses. This is the first report describing the molecular identification of *T. suis* from pigs in Japan.

Discipline: Animal Science
Additional key words: molecular detection, nematode, parasite, PCR

Introduction

Trichuris suis is a gastrointestinal parasite commonly found in pigs and wild boars. Infected pigs exhibit a variety of clinical symptoms, including dehydration, anorexia, diarrhea, and anemia, which result in significant economic loss in the swine industry (Batte et al. 1977, Roepstorff et al. 2011). The life cycle of *T. suis* is direct. Orally ingested embryonated eggs hatch in the small intestine, and the released larvae burrow into the intestinal wall of the cecum and proximal colon (Beer 1973), where they develop into mature worms and produce single-cell eggs after a 6- to 7-week prepatent period. Infective larvae develop within the eggs under appropriate temperature conditions. These eggs are highly resistant to extreme environmental conditions and drugs, and can survive for a long period, thus serving as a potential source of further infection.

In pigs, trichuriasis is typically diagnosed based on the detection of *T. suis* worms in the intestines during necropsy. Alternatively, eggs can be detected in feces using floatation methods with sucrose or saturated saline solution. Recovery of *T. suis* from humans in addition to pigs suggested that the disease is zoonotic (Cutillas et al. 2007, Nissen et al. 2012). However, the parasite is thought to be nonpathogenic in humans, and thus, the administration of *T. suis* has been interestingly demonstrated to be effective in inducing clinical remission against Crohn's disease or ulcerative colitis as inflammatory bowel diseases (Summers et al. 2003, Schölmerich et al. 2017). *Trichuris trichiura* is predominantly a human parasite, although adult worms,

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larvae, and eggs of *T. trichiura* and *T. suis* are difficult to morphologically distinguish. Thus, molecular techniques have been developed to enable a clear differentiation of the two species (Meekums et al. 2015). And the results of phylogenetic analyses have suggested that *Trichuris* spp. form geographic clusters, although sequence data thus far deposited (only from four countries—Denmark, the USA, Ecuador, and China) are insufficient to confirm this possibility (Meekums et al. 2015).

Veterinarians diagnose trichuriasis in living livestock on farms, but often examine the disease in dead animals by detecting worms in histopathologic analyses using paraffin sections. In such cases, it is difficult to identify the *Trichuris* spp. based on the morphology of worm fragments, especially for non-parasitology specialists. Moreover, it is difficult to detect only a few parasite eggs in feces. Therefore, molecular methods are needed to enhance the success and ease of identifying *Trichuris* parasites. In the present study, we evaluated molecular methods for the detection of eggs, worms, and paraffin sections of intestines of pigs infected with *Trichuris* spp. using recently published primers (Cutillas et al. 2007, Meekums et al. 2015). This is the first report describing the molecular identification of *T. suis* in pigs in Japan.

Materials and methods

1. Subject farm and piglets

The subject farm is located in the northern part of Akita Prefecture on the main island of Japan. The farm reared approximately 570 sows and 2,900 fattening pigs. Floors in the pens were soil covered with composted bark. In June 2017, three 4-month-old piglets (designated A-C) showed signs of wasting with watery diarrhea and then died. The affected piglets were not treated with any anti-parasitic drugs when disease was noted. The piglets were necropsied, with the small intestine, cecum, colon, and rectum being removed, fixed in 10% buffered formalin at room temperature for 4-7 days, and then sectioned and embedded in paraffin. Each tissue section was cut to a thickness of 3 μm, stained with hematoxylin and eosin (H & E) or periodic acid-Schiff (PAS), and then examined using light microscopy. Organs such as the heart, lungs, brain, spleen, liver, and kidneys were cultured for bacteria. Feces were collected from the rectum, and the eggs of *T. suis* were morphologically detected and purified by sugar floatation methods (Fujino et al. 2006). Adult *T. suis* worms were found in piglets A-C at necropsy. These parasites were used for molecular analyses of *Trichuris* spp. The feces were also used for the isolation of *Salmonella* and *Brachyspira* spp., and porcine epidemic diarrhea virus, transmissible gastroenteritis virus, porcine reproductive and respiratory syndrome virus, and porcine circovirus 2 were examined by PCRs.

2. DNA extraction

The eggs purified from piglets A-C were then pooled, and 10, 20, and 50 eggs were collected in PBS using a stereoscopic microscope. These eggs were subjected to three freeze-thaw cycles and then centrifuged at 5,440 × *g* for 3 min., and DNA was extracted from the supernatant. Adult *T. suis* worms (designated Nos. 1-3 from piglets A-C, respectively) collected at necropsy were used. The worms were cut into pieces, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Eggs were also collected from the bedding in four areas (samples designated No. 4-7) of the pig houses and then purified using sucrose floatation (Uga et al. 2000). DNA was extracted from 20 eggs from each sample using the same protocol described above. In addition, DNA was extracted from 10 serial sections of the colon from the infected piglets (designated No. 8-10 from piglets A-C, respectively) using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.

3. Molecular analyses

Using DNA samples from the 10, 20, or 50 eggs collected from feces (10-, 20-, and 50-egg samples), worms (Nos. 1-3), the 20 eggs collected from the beddings (Nos. 4-7), and colon sections (Nos. 8-10) as templates, PCR was performed using two primer pairs. We used forward primer TrirrnLF and reverse primer TrirrnLR targeting the mitochondrial large ribosomal subunit (Meekums et al. 2015), and forward primer NC5 and reverse primer NC2 targeting the 3′-5′ ends of the ITS1-5.8S-ITS2 flanking the 18S and 28S regions (ITS2) (Cutillas et al. 2007, Gasser et al. 1996). The sizes of products were 422 bp for the mitochondrial large ribosomal subunit region, and approximately 1,400 bp for ITS2 regions. PCR conditions were the same as those previously reported except that the number of cycles was changed to 40 (Cutillas et al. 2007, Meekums et al. 2015). PCR products were purified for sequencing using the Gel/PCR™ DNA Isolation System (Viogene, New Taipei, Taiwan) and then sequenced, aligned, and subjected to homology searching, as previously reported (Matsubayashi et al. 2014). Phylogenetic trees were constructed using the neighbor-joining algorithm, with evolutionary distances computed using the Tamura-Nei

model and 1,000 bootstrap replicates. The resulting trees were generated using MEGA 5 software (Tamura et al. 2011). No experiments involved the use of live animals. Thus, ethical approval of animal experimentation was not necessary. All examinations in this study were permitted by the farm owners and conducted as part of government affairs.

Results and discussion

No significant lesions were observed in most of the organ samples from the affected piglets at necropsy. However, white nodules were noted in the cecum, colon, and rectum, and the mucosa was slightly congested. Many white worms of *T. suis* were found on the mucosal surface. No significant diarrhea-causing bacterial or viral pathogens were detected (except for positive porcine circovirus 2 in one pig).

In PCR analyses using the TrirrnLF and TrirrnLR primers, a product of 420 bp was specifically amplified from the DNA obtained from the 20- and 50-egg samples, but not from the 10-egg sample. Using DNA isolated from the worms (Nos. 1-3) and 20 eggs from the bedding samples (Nos. 4-7), 420-bp amplicons were clearly detected from PCR analyses using the primers TrirrnLF and TrirrnLR (Fig. 1). The 420-bp amplicon was also detected in analyses of the 10 serial sections of infected colon from each piglet (Nos. 8-10). Thus, PCR analysis using the primers TrirrnLF and TrirrnLR appears to be useful for the detection of *T. suis* parasites in the egg and adult worm stages. In PCR analyses using primers NC5 and NC2, no clear amplifications were detected, and some nonspecific bands were observed on agarose gel electrophoresis, although the reason for this result is unknown.

We determined the sequences (approximately 400 bp) of all samples (Nos. 1-10) and found that the sequences of samples 1, 2, and 4-10 (Accession No. LC382030) were completely identical to those of China (e.g., Accession No. KU524494), and that of sample No. 3 (Accession No. LC382031) had 2-bp substitutions compared with samples from China (Accession Nos. KU524520 and GU070737). It remains unknown whether these two types sequences could be due to multiple copies in this gene region or infection with two genetical types of *T. suis*. We then constructed a phylogenetic tree using sequences of *T. suis* and other related parasites (Fig. 2). Three *T. suis* clusters were apparent. One clade was from Denmark, Ecuador, and the USA, whereas the isolates from Japan in the present study were included in a clade with the China and Ecuador isolates. Most of the isolates from China formed a single clade that was a sister to the China, Ecuador, and Japan clade. Although further studies using more samples from other countries are needed, it is not surprising that the Japanese isolates from the present study would be included with those from China, as pigs were transported to Japan from China after 1664 (Japan Pork Producer Association 2008). This is the first report describing the molecular identification of *T. suis* in pigs in Japan.

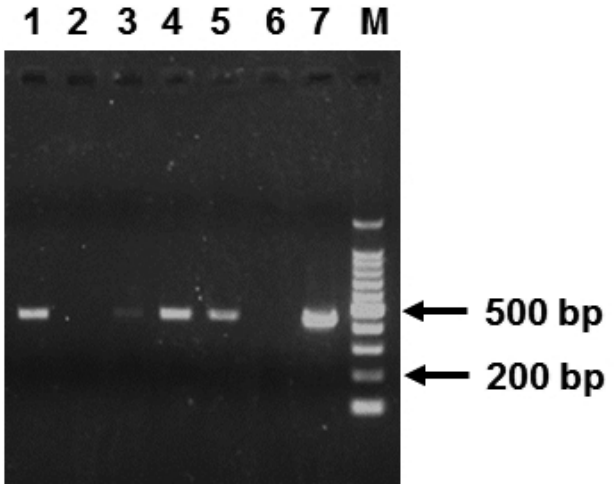

Fig. 1. Agarose gel electrophoresis of PCR samples amplified using primer sets TrirrnLF and TrirrnLR
Lane 1: DNA from 20 eggs
Lane 2: DNA from 10 eggs
Lanes 3-5: DNAs from paraffin sections of intestine from piglets A-C
Lane 6: TE buffer as a negative control
Lane 7: DNA from an adult worm from piglet A

In conclusion, we demonstrated the utility of PCR analysis for identifying *T. suis* worms and eggs, and environmental samples as well as the paraffin sections of infected intestine. These molecular methods are useful for detecting the parasite and identifying the species.

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Conflict of interest

None of the authors report any conflict of interest.

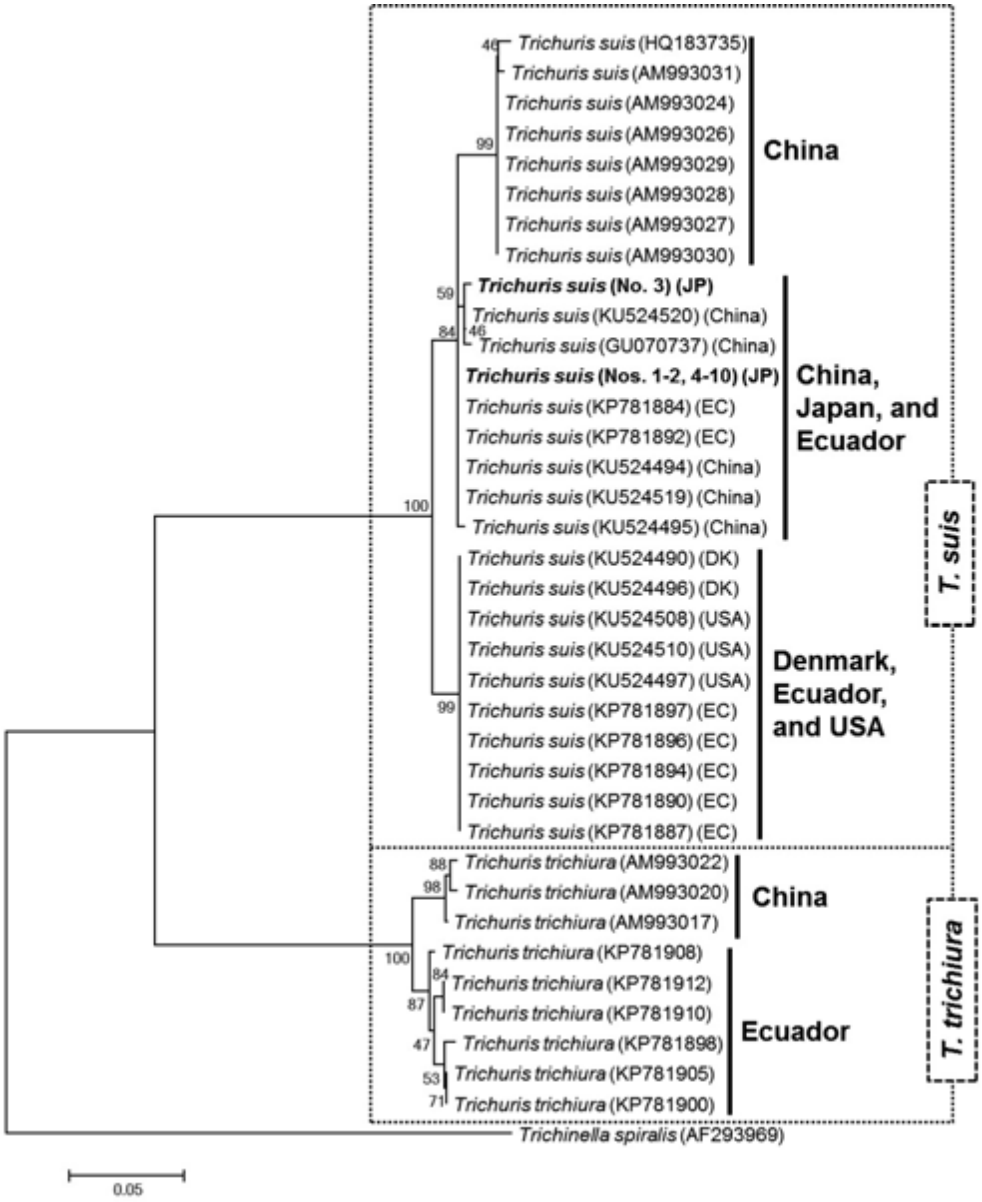

Fig. 2. Phylogenetic tree of *Trichuris* spp. inferred by the neighbor-joining method using partial ITS gene sequences

Sequences of *T. suis* in the present study were identical or highly similar to those of parasites from China and Ecuador. Accession numbers and derived countries are shown in parentheses, and accession numbers from Japan isolates are LC382030 from samples 1, 2, and 4-10, and LC382031 from sample No. 3. Scale bar represents substitutions per nucleotide, and bootstrap values are indicated (N = 1,000). Abbreviations; JP: Japan, EC: Ecuador, DK: Denmark.

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