Ancylostomiasis in cats in Yogyakarta, Indonesia, and its causative genetic relations

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[2]Department of Internal Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Jl. Fauna No. 2, Sleman 55281, Yogyakarta, Indonesia. Tel.: +62-274-6492088, ♥♥email: indarjulianto@ugm.ac.id
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Abstract. *Dami JC, Damayanti LPE, Indarjulianto S, Priyowidodo D. 2023. Ancylostomiasis in cats in Yogyakarta, Indonesia, and its causative genetic relations. Biodiversitas 24: 2605-2611.* Ancylostomiasis is a zoonotic cat disease caused by one or more species of *Ancylostoma* spp. This study aims to determine ancylostomiasis in cats in Yogyakarta and its causative genetic relations. This research used ten cats with ancylostomiasis, based on the findings of *Ancylostoma* spp. eggs in their feces. Cats were examined clinically and laboratory, including blood profile and identification of *Ancylostoma* spp. eggs. The ITS1-5.8S-ITS2 region of *Ancylostoma* spp. was amplified by polymerase chain reaction (PCR) and sequenced; the results were compared with the Basic Local Alignment Search Tool (BLAST). As a result, physical examination showed that cats with ancylostomiasis suffered from diarrhea (100%), decreased appetite (70%), weakness (60%), anemia (60%), emaciation, and dull hair (10%). Blood examination showed that 5 (50%) cats had normochromic normocytic anemia, and 1 (10%) cat had hypochromic microcytic anemia. PCR tests, sequencing, and phylogenetic tests found that *Ancylostoma* spp. isolates had been successfully identified as *Ancylostoma braziliense* De Faria 1910, identical to *A. braziliense* from Australian isolate (DQ359149.1). Therefore, it was concluded that ancylostomiasis in cats in Yogyakarta was caused by *A. braziliense*.

Keywords: Ancylostomiasis, *Ancylostoma braziliense,* cats, PCR, Yogyakarta

INTRODUCTION

Cats and other felines act as definitive hosts for many intestinal parasites, some of which are responsible for several zoonotic diseases, such as ancylostomiasis. Ancylostomiasis is an infection caused by hookworm, *Ancylostoma* spp. (Raji et al. 2013). Hookworms like *Ancylostoma* spp. are blood-feeding parasites that inhabit the intestine of mammalian hosts, including cats, dogs, and humans. The close association between cats and humans is responsible for the high endemicity of some of these zoonotic diseases. The common species of *Ancylostoma* in cats are *Ancylostoma braziliense* De Faria 1910, *Ancylostoma tubaeforme* Zeder 1800, *Ancylostoma caninum* (Ercolani 1859) Hall 1913 (in some cases), and *Ancylostoma ceylanicum* (Bowman et al. 2010). Ancylostomiasis is also a neglected zoonotic parasitic infection commonly found in tropical and subtropical areas and occurs in warm and temperate climates, especially where there is adequate moisture (Brahmbhatt et al. 2015). Environmental contamination by *Ancylostoma* spp. eggs can transmit ancylostomiasis among animals and from animals to humans, their potential zoonotic risks, and clinical syndromes that can manifest in local populations as travelers (Traversa et al. 2014). Indonesia, as a tropical area, has many cases of hookworm infection along with the geographical location of Indonesia, which has warm and humid climates. Its condition has led soil-transmitted helminths such as *Ancylostoma* spp. to be well-grown (Ridwan et al. 2020).

Ancylostomiasis in cats can cause anemia, weight loss, poor hair coat, bloody diarrhea, vomiting, and death due to late treatment (Mello et al. 2020). Ancylostomiasis in cats can be transmitted in two ways, orally and percutaneously. Peroral infection occurs when the larvae enter with food or water, whereas in percutaneous infection, the larvae enter by penetrating the skin (Borthakur and Mukharjee 2011). In humans, *Ancylostoma* spp. can lead to cutaneous larva migrants (CLM) syndrome with specific clinical signs as a linear reddish lesion (serpiginosa), prominent and migrating in irregular patterns called creeping eruption (Tan and Firmansyah 2020). The infection is acquired via contact with soil or moist sand contaminated with the feces of hookworm-infected cats or dogs, with the feet, buttocks, thighs, and hands presenting as the most affected areas. Still, they can be found on any body part that has been exposed (Verzì et al. 2018).

Ancylostomiasis has gained major importance in veterinary as well as public health research. The prevalence of *Ancylostoma* spp. in cats in several countries has proven the importance of this disease. Research in Guangzhou, China, found *A. caninum* and *A. ceylanicum* in stray cats were 59,2% and 20,4%, respectively (Hu et al. 2015). Another prevalence of *A. ceylanicum* and *A. caninum* in

dogs and cats of the Lower Northern region of Thailand was 11.7% and 1.6%, respectively (Pumidonming et al. 2016). The incidence of ancylostomiasis in cats in Indonesia also varies in each region. The prevalence of ancylostomiasis in cats in Yogyakarta was reported by Anastasia (2014), with a prevalence rate of 6.8% (19/280).

Due to the high prevalence, pathogenesis, and zoonotic significance in cats, a proper identification process is needed to fulfill the accuracy of diagnosis. So far, veterinarians in Indonesia have diagnosed ancylostomiasis based on clinical symptoms and found *Ancylostoma* spp. eggs on microscopic examination. That is considered insufficient because it cannot provide a complete clinical examination of infected animals or assess the potential risk of zoonoses affecting human health (Rehman et al. 2017). Traditionally, identification of hookworm species in dogs and cats required coprological or postmortem examination of the adult worms based on morphological differences between the species of *Ancylostoma*. This is time-consuming, labor-intensive, and requires skilled personnel (Traub et al. 2004). However, recently molecular diagnostic tools such as the polymerase chain reaction (PCR) method have developed rapidly and could be an alternative diagnosis. This technique was applied to a large-scale epidemiological study and allowed feline hookworm identification to be conducted rapidly, with ease and accuracy. In addition, the PCR method has been used for *Ancylostoma* species differentiation in various countries (Palmer et al. 2007). The hookworm species previously thought to have been identified in Indonesian cats included *A. tubaeforme*, *A. braziliense*, and *A. ceylanicum*. However, this was reported in 1979, so updating the report on the *Ancylostoma* species in Indonesia is necessary. The specific purpose of this study is to diagnose ancylostomiasis in naturally infected cats and understand its causative genetic relations.

MATERIALS AND METHODS

Time and location

The study started from February to August 2021 and has been approved and declared to fulfill ethical eligibility by the Research Ethics Commission of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, with Certificate Number: 00012/EC-FKH/Int./2021. The study was performed at the Laboratory of the Department of Parasitology and the Department of Internal Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia.

Sample collection

A total of 137 cats with diarrhea were examined in this study. All cats underwent anamnesis, physical examination, and stool examination to identify *Ancylostoma* spp. eggs using native and flotation-centrifugation methods (Dami et al. 2021). Molecular procedures further characterized positive fecal samples. The hematological examination was also carried out to observe the number of erythrocytes, hemoglobin concentration, hematocrit value, mean

corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

DNA extraction, amplification, electrophoresis, and sequencing

DNA from the stool samples was extracted using the QIAamp DNA Fast Stool Mini Kit according to the manufacturer's protocol (QIAagen Strasse 1, 40724 Hilden, Germany). The internal transcribed spacer (ITS)-1, 5.8S, and ITS-2 regions of *A. caninum*, *A. braziliense*, *A. tubaeforme*, and *A. ceylanicum* were amplified by PCR using previously published three pairs of primers: Aca (5'-CTT TGT CGG GAA GGT TGG-3') and (5'-TTC ACC ACT CTA AGC GTC T- 3') (Traub et al. 2004), Ab (5'-CGT GCT AGT CTT CAG GAC TTT G-3') and (5'-CTG CTG AAA AGT CCT CAA GTC C-3') (Oliveira-Arbex et al. 2016), At-Ace (5'-CGT GCT AGT CTT CAG GAC TTT G-3') and (5'-CGT TGT CAT ACT AGC CAC TGC-3') (Liu et al. 2013). According to sequence information available on *GenBank,* the PCR was expected to amplify an approximate 404 bp region of *A. caninum*, 693 bp of *A. braziliense*, 679 bp, and 690 bp region of *A. tubaeforme* and *A. ceylanicum*, respectively.

DNA amplification by PCR was carried out under initial denaturation conditions at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by post-elongation at 72°C for 7 minutes, ending with a final extension at 4°C for two pairs of primer (At-Ace and Ab). For Aca primer, DNA amplification was carried out with predenaturation conditions at 96°C for 5 minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 90 seconds, followed by post-elongation at 72°C for 7 minutes, ending with a final extension at 4°C. The amplification results were followed by electrophoresis using 1.5% agarose gel for 45 minutes at 100 volts and visualized using a UV transilluminator. The PCR results were followed by sequencing and phylogenetic analysis.

PCR products were sent to PT. Genetica Science for purification and sequencing. The results were analyzed using the Basic Local Alignment Search Tool (BLAST). BLASTN analysis is carried out online through the website https://www.ncbi.nlm.nih.gov. Phylogenetic trees were constructed using molecular evolutionary genetics analysis (MEGA) version X. Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Neighbor-joining algorithms were conducted using the Tamura-Nei parameter distance analysis.

RESULTS AND DISCUSSION

Fecal examination, clinical symptoms, and hematology tests

The results of the microscopic examination showed that 10 out of 137 cats with diarrhea were positive for

Ancylostoma spp. eggs in the fecal samples. The ten infected cats were from different areas in Yogyakarta, such as Depok (40%), Mlati (20%), Ngaglik (30%), and Mantrijeron (10%). The transmission of ancylostomiasis in Yogyakarta areas is probably due to no-traffic restrictions between districts, sub-districts, or provinces. According to Anastasia (2014), the prevalence of ancylostomiasis in Yogyakarta has reached 6.8% (19/280), which is grouped into areas of infection as Yogyakarta city and Sleman districts within each case reached 47.4% (9/19) and Kulon Progo districts reached 5.3% (1/19) cases.

Based on the examination, the clinical symptoms that appeared in the study were generally the same as the clinical symptoms of ancylostomiasis described by Mello et al. (2020), such as weakness, poor hair coat, pale mucosa, vomiting, ascites, anemia, emaciation, and diarrhea. But, most cats did not exhibit specific clinical symptoms. In this study, diarrhea occurred in 10/10 cats, less appetite in 7/10 cats, weakness and lethargy in 6/10 cats, anemic mucosa in 2/10 cats, and 1/10 cats had emaciated hair dullness.

Hookworms are considered among the most pathogenic nematodes parasitizing dogs and cats. They may cause severe intestinal damage resulting in a fatal outcome, depending on different factors, including the species involved. Differentiation of clinical symptoms may be caused by the pathogenicity of the parasite as infection severeness, period of infection, and the nutrition or sensitivity of the infected cats (Morelli et al. 2021). Lack of appetite in cats may indicate the illness signs followed by fever, weakness, dehydration, stress, and vomiting. If these conditions continuously can lead to weight loss with a body condition score below normal (emaciate) and a lower immunity system. This condition can easily infect cats with various diseases. Pale mucous membrane indicated anemia or vasoconstriction shock (Subronto 2006). The specific clinical signs of diarrhea in this study were caused by both infective larvae (filariform) and adult worms that attach to the small intestine inducing blood loss. The blood loss is both direct consumption of blood by the parasite and bleeding around the attachment sites due to the secretion of anticoagulants by the worms to promote feeding. That is causing clinical signs and high death rates, especially in puppies and kittens with limited ability to compensate for blood loss (Idika et al. 2016).

The hematological tests resulted in ten blood samples indicating 6/10 (60%) cats had anemia, characterized by the lower rate of erythrocytes, hemoglobin, and hematocrit values. Meanwhile, on the physical examination of the mucous membranes were found 2/10 (20%) cats with anemic mucosa. According to Chaparro and Suchdev (2019), many anemias are initially asymptomatic. The diagnosis may be unexpected because the anemia developed over so long that the patient adjusted to the symptoms without realizing it. Therefore, the determination of anemia in animals is better supported based on the results of the hematological examination. The erythrocyte indices calculation can identify the classification of anemia; with mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) measurements (Brooks et al. 2022). Based on calculations, this study indicated that 6/10 cats had anemia, with 5/6 discovered as normocytic normochromic, and 1/6 cats had microcytic hypochromic. Campos et al. (2018) reported that 23.8% of infected dogs with ancylostomiasis had anemia normocytic normochromic, which is aligned with this study. This condition could be detected in the beginnings of a bleeding reaction as it has not stimulated regeneration in the bone marrow. Anemia is normocytic normochromic and may cause by acute bleeding, hemolysis, and metastatic infiltrative disease in the spinal cord (Campos et al. 2018). In this study, the anemic condition found in five cats indicated acute bleeding in response to *Ancylostoma* spp. infections in small intestines. Meanwhile, one cat had anemia microcytic hypochromic was caused by chronic bleeding. The decrease in MCV also indicated chronic blood loss, which commonly results in iron deficiency. Iron deficiency due to dietary constraints, inflammation, and infection may also cause microcytic anemia, as experienced by the cat in this study (Sazalli et al. 2016).

Molecular identification (PCR)

In veterinary clinics, the species identity of parasitic worms is often assumed based on the host of origin and may be complemented by specific morphological characteristics. However, for closely related species at the egg level or even larval level, certain hookworm species are morphologically indistinguishable (Fava et al. 2020). Therefore, selecting molecular methods such as PCR in identifying *Ancylostoma* species is one of the best ways to be considered. Furthermore, using PCR in this study aimed to differentiate *Ancylostoma* spp. based on the species level; this is useful for diagnosing ancylostomiasis and provides rapid and accurate therapy (Papaiakovou et al. 2017). Moreover, compared to microscopy, PCR presents several additional advantages that favor its usage for *Ancylostoma* identification, such as the large number of samples that can be processed and the high specificity and sensitivity of species-specific primers. Good detectability from minute quantities of DNA and successful amplification from fresh and preserved samples further enhance its suitability for field application. This PCR assay can detect all developmental stages of *Ancylostoma* (Rehman et al. 2017).

Therefore, for molecular identification, the DNA extract was used as a template for PCR amplifications at the nuclear internal transcribed spacer ITS1-5.8S-ITS2 region. Amplification results of the ten samples using Ab primer showed positive results. The DNA fragment of *Ancylostoma* spp. is specifically attached to the DNA template, indicated by the band formation with an amplicon length of about 693 bp. Meanwhile, negative results were shown in the Aca and At-Ace primers (Figure 1).
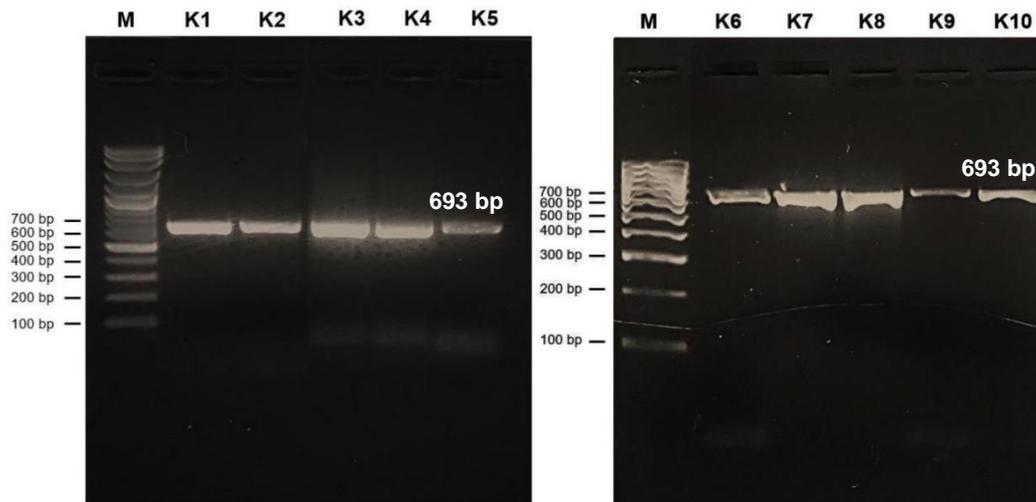
Figure 1. PCR amplification products of *Ancylostoma* spp. The PCR products were analyzed on a 1.5% agarose gel and visualized. Lane M: Marker (DNA ladder 100 bp), K1-K10: Samples

All samples were sequenced for analysis using BLAST. Sequencing is the final step to determine the amplified nucleotide sequence. Sequence results as a chromatogram product was analyzed using MEGA X and alignment with the Clustal W program. The analysis continued to BLASTN to compare the nucleotide sequences of a recent study (query) with the GenBank NCBI (subject). One sample (K9) could not be sequenced, indicated by sloping peaks, and not separated from each other or double peaks on the chromatogram results. This may be affected by the process of DNA isolation and sample conditions that affect the extraction results. According to Yudianto et al. (2019), the visualization results of thick and thin target bands in positive samples show the quality of isolated DNA due to contamination, a small amount of target DNA, and DNA degradation.

The BLASTN analysis discovered max score and total score within different grades in each sequence, 1210-2018 of 97-100% query coverage from nucleotide sequences aligned with the database. The rate of E-value was 0.0, indicating the homology among sequences was high or nearly identical. All samples were identified as *A. braziliense* with 99% similarity or close identical with *A. braziliense* sequences from the Australian isolate in the GenBank (accession no. DQ359149.1). These results are the same as previous studies by Oliveira-Arbex et al. (2016), which have identity results of 99-100% for *A. braziliense*.

Sequence alignment aims to observe the similarity of nucleotide sequences from two different organisms and the possibility of being derived from the same ancestral sequence (common ancestor). Sequence alignment will show sequence positions that do not change (conserved) and which develop (divergent) or become different from the common ancestor (Phillips et al. 2000). The nucleotide alignment of *A. braziliense* from this study with an isolate from Australia (DQ359149.1) and Brazil (DQ438063.1) from GenBank using offline Clustal W in MEGA X software, the results showed that the species *Ancylostoma*

spp. of all the research samples was *A. braziliense*; this was known because the nucleotide sequences between the sample sequences and the sequences of Australian isolates and Brazil have similarities of 99-100%. Furthermore, no differences in nucleotide sequences and interspecies sequence variations were found between the study and GenBank isolates (Figure 2). That shows there is no gene mutation. Mutations in genes can lead to the appearance of new alleles and provide the basis for the evolution of new variations within species. It is known that a species has a close relationship with other species if it has a low level of genetic variation; on the contrary, if the genetic variation is high, it can lead to the formation of new species (speciation) (Hopf et al. 2017).

Sequencing results can be followed by phylogenetic analysis. Phylogenies are high-utility tools to test evolutionary and biogeographic hypotheses, inform conservation strategies, and reveal the age and evolutionary histories of traits and lineages (Bogusz and Whelan 2017). Phylogenetic analysis in this study aims to understand diversity by reconstructing a phylogenetic tree; it could describe the genetic relations between sample organisms based on their evolutionary relations with the sequences (rows of the alignment) of comparison organisms from GenBank or describe the close relations between species being compared (Hall 2013). The phylogenetic tree was constructed using the Neighbor-joining method and statistically tested using the bootstrap method for 1,000 repetitions. Phylogenetic tree construction was made by comparing the sample sequences (9/10) from the study with the sequence data from the six isolates in GenBank (accession no. DQ359149.1, DQ438063.1, JQ812691.1, KP844730.1, DQ381541.2, HQ262055.1) (Figure 3).

The construction of the phylogenetic tree showed that *A. braziliense* from the study (K1-K8 and K10) is in the same clade with *A. braziliense* Australian isolate (DQ359149.1) and clustered with a Brazilian isolate of *A. braziliense* (DQ438063.1). These results are in line with the alignment DNA results, that revealed no differences

were found among newly obtained sequences of *A. braziliense*, which means there were no variations of interspecies sequences in the isolates from the study and isolates from *GenBank*, which also means that there were no gene mutations in the study samples. Sequence alignment will show sequence positions that do not change (conserved) and which develop (divergent) or become different from the common ancestor (Castresana 2000). In conclusion, the local variant of *A. braziliense* was phylogenetically similar to the Australian isolate and may derive from a common ancestor.
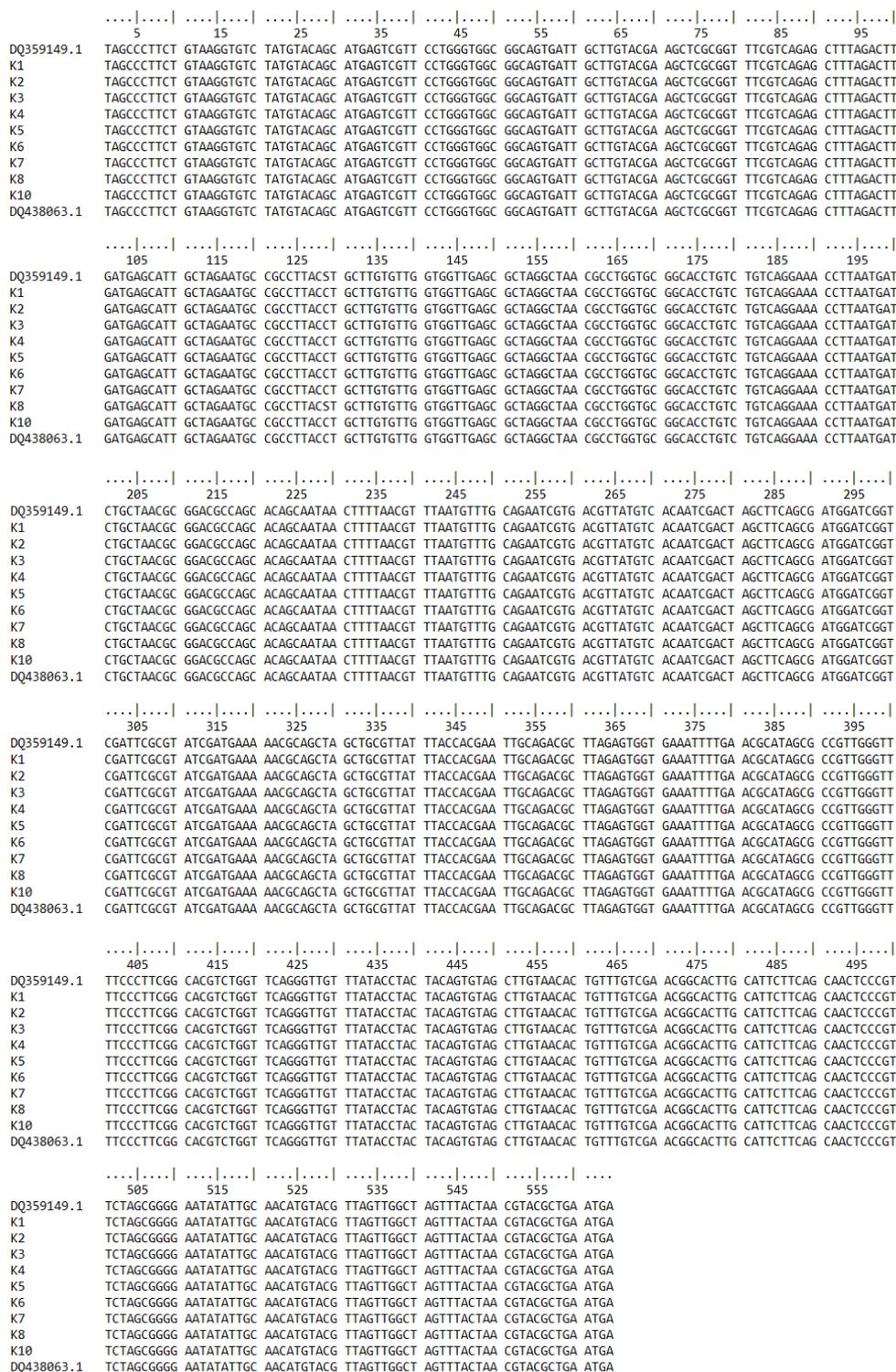
Figure 2. The alignment result of nucleotide sequences between the samples and the GenBank from *Ancylostoma braziliense*
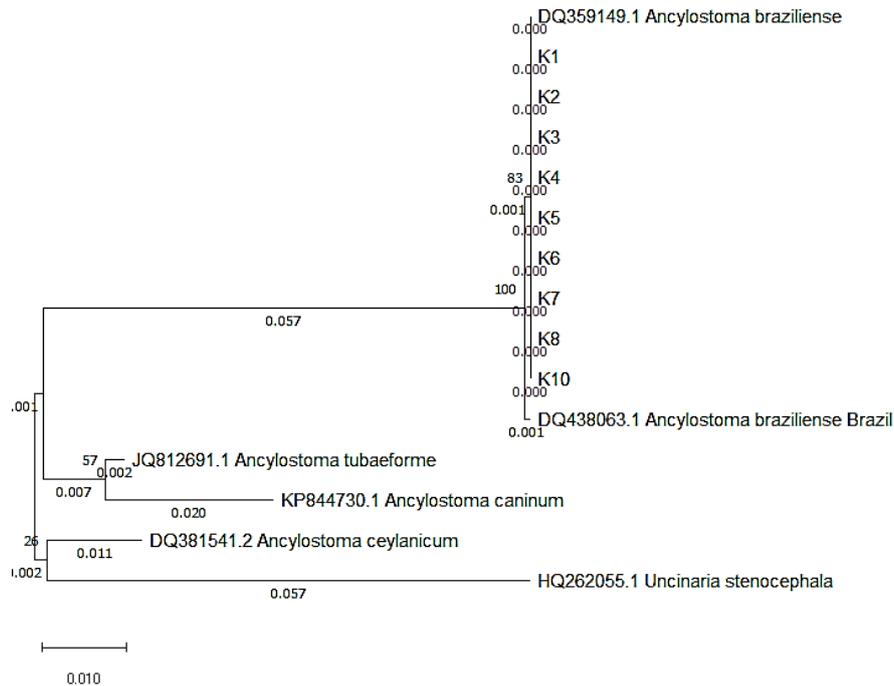
Figure 3. Construction of the phylogenetic tree of *Ancylostoma* spp. in the ITS1-5.8S-ITS2 region target using the Neighbor-joining method

Ancylostoma braziliense is known as the most frequently implicated etiological agent and is responsible for CLM. The contact is through the soil, contaminated by the feces of cats and dogs (Scharf 2017). *A. braziliense* can also penetrate the skin considerably faster than either *A. ceylanicum* or *A. caninum*; penetrates the dermis and results in a local inflammatory reaction as it migrates through the skin and dermal tissues (Coello et al. 2019). In humans, CLM is characterized by the appearance of a single linear, pruritic, and slow-growing lesion that advances at a rate of 1-2 cm per day (Torres-Chablé et al. 2015). Therefore, precautions must be taken to protect cats and humans from infection with *Ancylostoma* spp., such as *A. braziliense*. So far, *Ancylostoma* spp. infection was associated with a lack of veterinary attention. The lack of veterinary care, such as vaccination and deworming affects the health and welfare of animals and the health of the owner and others.

This study showed that *Ancylostoma* spp. had spread between countries like Indonesia. However, it is impossible to know whether transmission has occurred. One of the factors may be related to the transportation of cats between countries through trade and migration routes. Another fact is spreading of these parasites may be favored by current climate changes because these parasites have periods of development and survival in the environment (Gordon et al. 2017). Risk reduction between countries about this disease can be done by quarantine and strict inspection of animals entering or leaving a country to prevent nematode transmission from infected areas to others (Mackenstedt et al. 2015).

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