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## First report of the molecular detection of *Ancylostoma caninum* in Lahore, Pakistan: the threat from pets

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**ABSTRACT:** The molecular prevalence of *Ancylostoma caninum* was determined in stray and pet dogs in Lahore, Pakistan from July 2014 to August 2015. A total of 500 dog faecal samples were first evaluated using a sedimentation technique and further through a PCR assay targeting the ITS-2 region of the *A. caninum* genome. Overall, 130 (26%) samples were positive for hookworm infestation by microscopic examination. Only microscopically positive samples were processed for PCR and 89 (17.6%) were positive for *A. caninum*. Sequence analysis of amplicons showed 100% homology with *A. caninum* and the genotypes clustered in one clade with Brazilian *A. caninum* hookworms. There was a significantly ( $P < 0.05$ ) increased prevalence in male dogs younger than six months old. Labrador retrievers had higher *A. caninum* incidence compared to German shepherds and other breeds. To the best of our knowledge, this is the first report of the molecular prevalence of *A. caninum* in dogs in Pakistan. The novel results of the present study allow us to conclude that *A. caninum* is more prevalent in pet dogs, especially puppies, and this can be a potential threat for humans that come into contact with such animals. Therefore, the routine monitoring of pets, especially the more susceptible breeds, is essential for disease control.

**Keywords:** faecal; hookworms; PCR; sequencing; Labrador retriever; age; stray; dog

Ancylostomiasis is a neglected zoonosis that is spread throughout the tropics and subtropics. It is caused by the canine hookworm *Ancylostoma caninum*, which is a blood-feeding intestinal parasite found in almost all mammalian hosts including dogs and humans. The final hosts of *A. caninum* are dogs and the risk of zoonotic transmission is represented primarily by cutaneous larva migrans. Due to its high prevalence, pathogenesis and zoonotic signifi-

cance in dogs, *A. caninum* has assumed critical importance in the field of veterinary as well as public health research. In shedding millions of eggs, stray and pet dogs can contaminate soil in streets and public parks and in this way transfer disease to humans, especially children (Klimpel et al. 2010). As dogs are the primary host for *A. caninum* infection, there is an urgent need to investigate their role in harbouring infection and their potential in causing further

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transmission to humans. Human transmission from dogs has been reported from various parts of world such as Australia, the Philippines, South America, Israel, the US and Egypt (Bahgat et al. 1998).

To the best of our knowledge, there has been no study performed to determine the status of *A. caninum* in Pakistan. Therefore, we undertook this study to assess the prevalence of this pathogen in stray and pet dogs of various ages and both sexes using a polymerase chain reaction assay.

Our hypothesis was that a molecular-based diagnostic approach would yield a reliable confirmatory diagnosis since faecal microscopy, the routine method for the identification of hookworm infections, may result in inaccurate diagnosis due to the inability to morphologically distinguish *A. caninum* eggs from those of other nematodes (Monis et al. 2002).

The present study was designed to determine the molecular prevalence of *A. caninum* in Lahore, Pakistan for the first time.

## MATERIAL AND METHODS

This cross-sectional study was carried out on stray and pet dogs in Lahore, Pakistan from July 2014 to December 2015. A total of 1000 faecal samples were processed using the formol-ether sedimentation technique of Suwansaksri et al. (2003). Not all samples were used in all tested categories due to the unknown age and breed of some dogs. The samples from pet dogs were collected at various veterinary clinics and pet centres including the Pet Centre of the University of Veterinary and Animal Sciences (UVAS), Lahore and various pet clinics in Model Town, Cavalry grounds and the Defence Housing Authority Lahore, Pakistan. Street dogs were captured from streets, playgrounds and parks and were placed in experimental cages of the Pet Centre, UVAS for some days. After identification of gender and breed through physical examination and age determination based on teeth, faecal samples were collected directly from the rectums of these dogs. Special care was taken to prevent multiple sampling of individual dogs.

Egg sizes were determined using a calibrated ocular micrometer. Positive samples were preserved in 70% ethanol at  $-20^{\circ}\text{C}$  for further analysis.

DNA was extracted from all microscopically positive faecal samples with the AxyPrep™ multisource

genomic DNA mini-prep kit (Axygen-Biosciences, USA) with slight modifications including: (1) faecal samples were suspended in 350 instead of 250  $\mu\text{l}$  of PBS and vortexed for one minute; (2) the incubation period for egg lysis was increased from 15 to 25 min; (3) the centrifugation step after addition of wash buffer was reduced in duration from 10 to 1 min; (4) final elution of DNA was made in 100  $\mu\text{l}$  of lukewarm water instead of the elution buffer provided with the kit; (5) the standing time for the water on the column membrane was increased up to five minutes to increase elution efficiency. Working aliquots of 50  $\mu\text{l}$  of extracted DNA were kept at  $4^{\circ}\text{C}$  while stock DNA was stored at  $-20^{\circ}\text{C}$ .

Sixty-eight ITS-2 region sequences of *A. caninum* were retrieved from the Genbank of the National Center for Biotechnology Information (NCBI) and were aligned using CLUSTAL W. Geneious R8.1.6 software (Kearse et al. 2012) was used for sequence alignment and to create the consensus sequence. Species-specific forward (5'-TCGGGGAAGGTTGGGAGTAT-3') and reverse (5'-AGCAGTAAGGCGGCATTCAT-3') primers were designed using Primer3 and Primer-Blast software (Ye et al. 2012) to amplify a 160-bp region. *In silico* validation of these designed primers was done using the NCBI BLAST tool (McGinnis and Madden 2004). A conventional PCR was designed for *A. caninum* DNA amplification. Gradient PCR on extracted DNA samples was used to ascertain the best annealing temperature for the primer pair. The gradient PCR was conducted in a 20  $\mu\text{l}$  volume with the final mixture consisting of 1X PCR buffer, 25  $\mu\text{M}$  dNTPs, 1 pmol of each primer, 1U Taq polymerase and 200 ng of DNA template. PCR cycling conditions were initial denaturation at  $95^{\circ}\text{C}$  for 5 min followed by 45 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $55-70^{\circ}\text{C}$  for 30 s, extension at  $72^{\circ}\text{C}$  for 30 s and a final extension at  $72^{\circ}\text{C}$  for 5 min. The optimised PCR conditions at an annealing temperature of  $68^{\circ}\text{C}$  was applied to all extracted DNA samples. PCR amplicons were visualised using 2% agarose gels under a UV transilluminator. PCR amplicons of the desired length were purified using ethanol precipitation and sequenced with an ABIPRISM DNA Sequencer. All chromatograms were viewed using Chromas LITE version 2.1.1 (Technelysium Pty Ltd) (Stephens et al. 2009). The similarity between generated and published sequences was determined using NCBI reference sequences with BLAST. A phylogenetic

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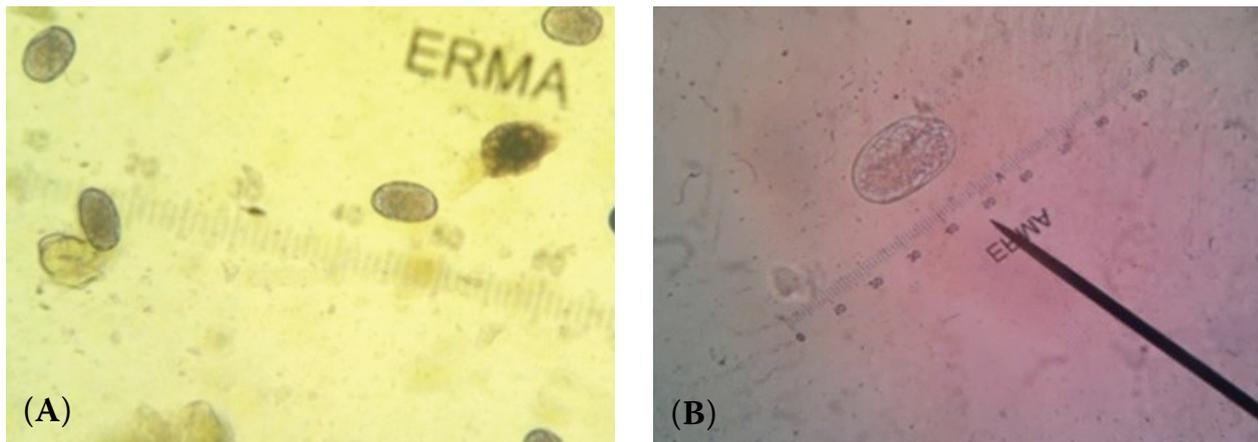


Figure 1. Micrometry of hookworm egg; (A) × 100; (B) × 400

tree was constructed using the neighbour joining method with 1000 bootstrap replicates and applying Kimura’s two-parameter correction using Geneious R8.1.6 software (Mulu et al. 2014). The chi-squared test was performed with SPSS software version 20 (SPSS Inc., Chicago, USA) at a threshold value of 5%. The concordance between the two techniques was measured by calculating the Kappa coefficient with SPSS version 17.0.

## RESULTS

The overall positivity for faecal egg excretion was 26% (130/500). Eggs appeared oval, were covered with a thin envelope, and were 57.5–65 µm in length and 37–42.5 µm in width (Figure 1). In PCR analysis, only 17.6% (89/500) of samples were found to be positive for *Ancylostoma* with 160-bp-sized amplicons in all samples (Figures 2 and 3). The levels of PCR inhibitors were minimised by the

use of a commercial kit for DNA extraction, which also inhibited the attachment of DNA with faecal particles. The modified extraction protocol allowed successful completion of the entire process in approximately 35 min even though the consistency of faecal samples was variable. Non-diluted samples containing a minimum of 5.2 ng/µl of extracted DNA were successfully used for amplification (result not shown).

With regard to the higher specificity of PCR, we only used PCR-positive samples to compare results in pets and stray dogs of various ages and breeds.

Out of 89 PCR-positive dogs, 58 were pets and 31 were stray dogs (Table 1). Interestingly, therefore, pet dogs were more affected as compared to stray dogs. Similarly, PCR indicated a significantly increased prevalence of *A. caninum* ( $P < 0.05$ ) in male dogs compared to females. Further, young dogs aged one to six months showed a significantly higher incidence (30%) of disease than older dogs (12%), ( $P < 0.05$ ).

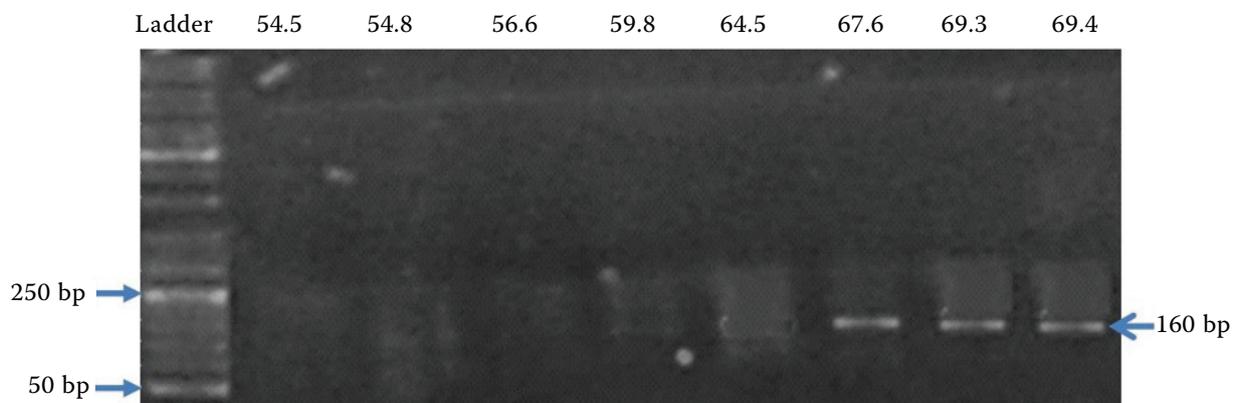


Figure 2. Gel image of *A. caninum* amplicons obtained after gradient PCR performed to select the best annealing temperature

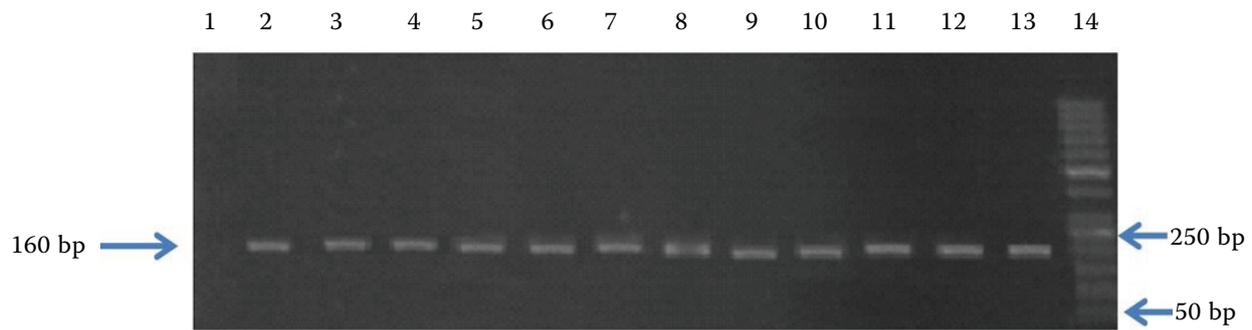


Figure 3. PCR amplicons obtained from positive DNA samples extracted from dog faecal samples on a 2% agarose gel. Lane 1: negative control, lanes 2–13: positive samples, lane 14: 50 bp ladder

The present study also compared the prevalence of *A. caninum* in five different breeds of pet dogs. The highest prevalence was found in Labrador Retrievers ( $P < 0.05$ ) (Table 1).

Table 1. Prevalence of hookworm egg excretion by microscopic examination of faeces and PCR. Values are presented as positive/total (%  $\pm$  SE)

Factor	Microscopic examination of faeces	<i>P</i> -value	PCR	<i>P</i> -value
<b>Life style</b>				
Pets	66/89 (74.15 $\pm$ 0.046)	–	58/89 (65.1 $\pm$ 0.051)	–
Stray	23/89 (25.84 $\pm$ 0.04)		31/89 (34.83 $\pm$ 0.051)	
<b>Sex</b>				
Male	90/330 (27% $\pm$ 0.02)	0.08	77/330 (23.23 $\pm$ 0.02)	0.04
Female	40/170 (23.5 $\pm$ 0.03)		12/170 (7.06 $\pm$ 0.02)	
<b>Age</b>				
1–6 months	90/250 (36 $\pm$ 0.03)	0.03	63/250 (25.2 $\pm$ 0.03)	0.01
6–12 months	20/120 (16.67 $\pm$ 0.03)		10/120 (8.3 $\pm$ 0.02)	
More than 1 year	20/130 (15.38 $\pm$ 0.03)		16/130 (12.31 $\pm$ 0.03)	
<b>Breed</b>				
Labrador	110/390 (28.21 $\pm$ 0.02)	0.91	80/390 (20.5 $\pm$ 0.02)	0.03
German shepherd	10/70 (14.29 $\pm$ 0.04)		6/70 (8.5 $\pm$ 0.03)	
Terriers	8/30 (26.67 $\pm$ 0.02)		3/30 (10 $\pm$ 0.09)	
Others*	2/10 (20 $\pm$ 0.13)		0/10	
Overall	130/500 (26 $\pm$ 0.02)	–	89/500 (17.8 $\pm$ 0.02)	–

\*Rottweiler, Bullmastiff, Bulldog, etc.

The *A. caninum* ITS-2 region was selected as a diagnostic marker because of its small size (221 bp) and its high copy number on various chromosomes (Chilton 2004). In addition, this region has higher inter-species differences when compared with ITS-1, which ranges from 0.9% to 13.2% among *Ancylostoma* species. Our ITS-2 sequences (UVAS\_1711379\_21 and UVAS\_1708317\_31) showed 100% homology with *A. caninum* Genbank sequences and clustered with a Brazilian isolate of *A. caninum* (e Silva et al. 2006) (Figure 4). DNA amplicons clustered with most of the retrieved sequences from Genbank and seemed to be clustered within a single clade. The intra-species variations were either nonexistent or minute for ITS-2 sequences. There were some deviations in sequences, which might be due to the dissimilar or unavailable aligned sequence of DNA.

## DISCUSSION

The faecal samples were collected from stray and pet dogs of various ages and breeds of both genders. More samples were positive by conventional microscopy than by PCR. This may be attributed to the greater chance of false positive results by microscopy due to the near identical morphology of *A. caninum* eggs to those of other hookworms such as *Ancylostoma duodenale* and *Ancylostoma braziliense* (Georgiev 2000).

Moreover, in comparison to microscopy, PCR presents with several additional advantages which favour its usage for *A. caninum* identification, such as the large number of samples that can be processed and the high specificity and sensitivity owing to species-specific primers. There is also no need for experienced personnel such as those required for the microscopic identification

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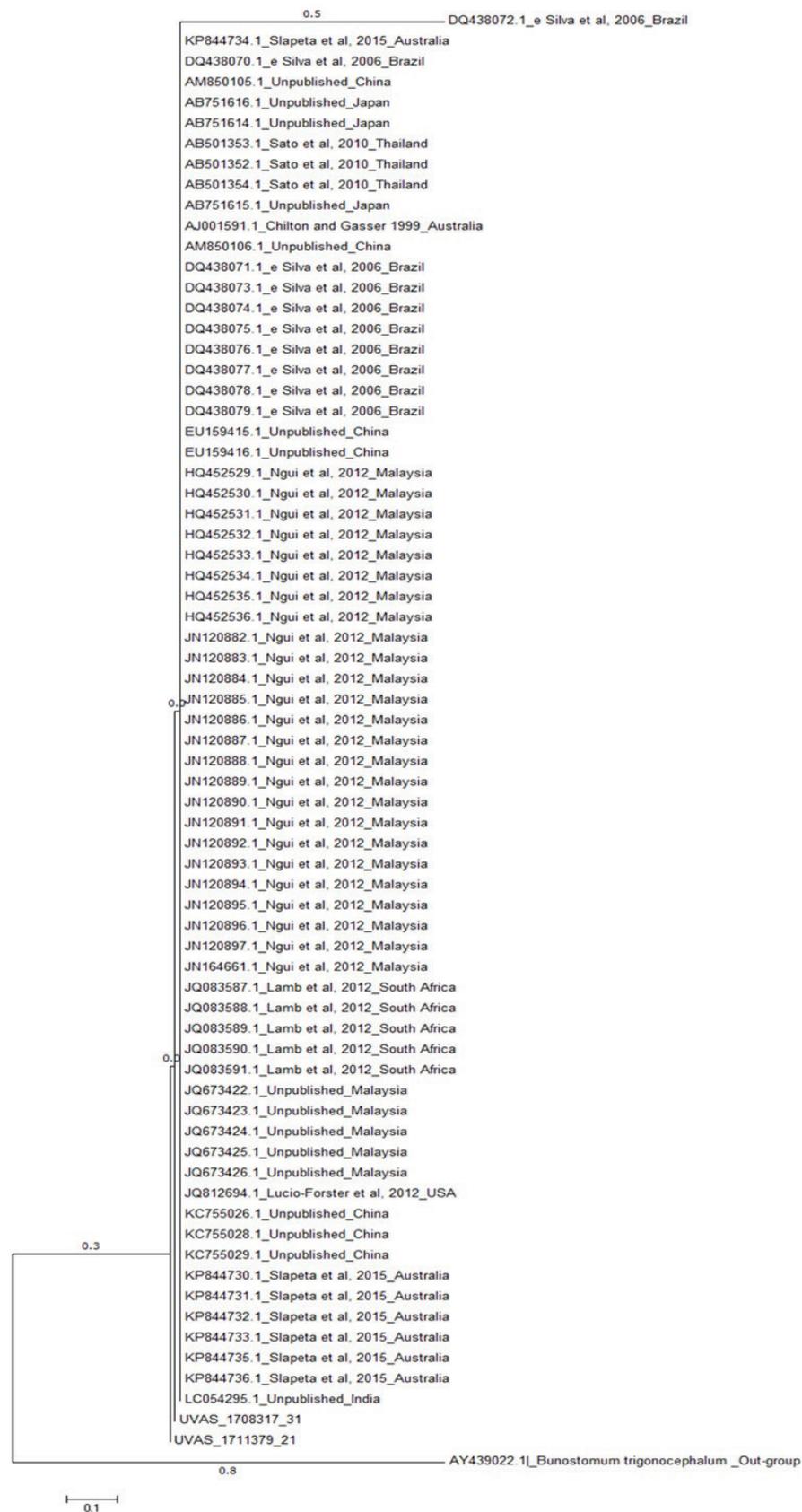


Figure 4. Phylogenetic tree of ITS-2 sequences from the present study (UVAS\_1711379\_21 and UVAS\_1708317\_31) with ITS-2 reference sequences from the Genbank database of *A. caninum*

of *Ancylostoma* eggs. Moreover, good detectability from minute quantities of DNA and successful amplification from both fresh and preserved samples further enhance its suitability for field application. This PCR assay can be employed to detect all developmental stages of *Ancylostoma*.

Pet dogs were found to harbour an increased incidence of disease compared to stray dogs. This is in agreement with previous studies (Oliveira-Sequeira et al. 2002) that also reported a higher incidence of *Ancylostoma* in pets as compared to stray dogs in Brazil. This may be due to the differing lifestyles of pet and stray dogs. *Ancylostoma* infection is disseminated through contaminated food, water and soil; pets are usually kept in confined spaces and so may have higher chances of harbouring infections than stray dogs who move around without restrictions.

The increased incidence in male as compared to female dogs could be due to the different pathophysiology of worms in the two genders (Schmidt et al. 2016). The increased incidence in young dogs as compared to adults, meanwhile, could be due to lower immunity in young pups and trans-placental infection as well as trans-mammary transmission of *Ancylostoma* eggs (Lefkaditis 2001; Ramirez-Barrios et al. 2004).

A further novel finding of this study was the increased incidence in Labrador retrievers as compared to other breeds such as German shepherds. This finding could be significant for future control of ancylostomiasis in more susceptible breeds and further transmission to humans.

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