

Original article

Prevalence and potential zoonotic risk of hookworms from stray dogs and cats in Guangdong, China

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ARTICLE INFO

Keywords:
Hookworm
Dog
Cat
PCR
Prevalence
China

ABSTRACT

Hookworm infection is globally prevalent among dogs and cats representing a major public health risk. Although previous studies have surveyed canine and feline hookworms in Guangzhou city, the status of these infection needs to be further explored in other regions of South China. To investigate the prevalence and zoonotic risk of canine and feline hookworms in eight cities (Guangzhou, Foshan, Shenzhen, Huizhou, Zhongshan, Shaoguan, Shantou and Chaozhou) of Guangdong province, China, we developed specific PCR methods based on ITS sequence for identifying three common hookworm species. The results showed that the prevalence of hookworms from stray dogs and cats was 20.23% (142/702) and 15.26% (47/308), respectively. The established PCR methods could identify *Ancylostoma ceylanicum*, *A. caninum* and *A. tubaeforme*. The mixed infections of *A. caninum* and *A. ceylanicum* were detected in stray dogs of Guangzhou and Shaoguan, with the rate of 8.3% and 21.2%, respectively. Among the stray dogs in Foshan, the infection rate of *A. ceylanicum* was higher than that of *A. caninum*. The stray cats in four of five investigated cities were infected with *A. ceylanicum*. The different region, age and rearing environments had an impact on the hookworm infection rates of stray dogs and cats. In conclusion, the reported higher infection rate of *A. ceylanicum* than other hookworm species in stray dogs and cats poses a potential risk to public health.

1. Introduction

Hookworms of the Ancylostomatidae include species of zoonotic parasites that inhabit the digestive tract of humans, dogs, cats and other animals, causing a series of symptoms such as intestinal bleeding, anemia, malnutrition and dermatitis (Tu et al., 2008; Hsu and Lin, 2012; George et al., 2016). According to one survey, about 576–740 million people worldwide are infected with hookworms, and more than half of them occur in Asia and the Pacific (Bethony et al., 2006). In Asia, the most affected countries are India, Bangladesh and Indonesia, with about 60 to 70 million people infected (De Silva et al., 2003; Bethony et al., 2006). China reported 46.6 million hookworm infections in 2010 (Yang et al., 2014). Hookworms that parasitize humans include *Ancylostoma duodenale* and *Necator americanus*, while *A. caninum*, *A. ceylanicum*, *A. braziliense*, *A. tubaeforme* and *Uncinaria stenocephala* live mainly in dogs and cats (Loukas et al., 2016).

There are significant differences in the distribution of different species of animal-derived hookworms worldwide. *U. stenocephala* is found in relatively cold climates in North America, South America and

Europe, while *A. braziliense* is mainly found in North America, Africa and Australia (Del et al., 2002; Palmer et al., 2007). *A. tubaeforme* is distributed worldwide and mainly infects feline animals. Cats infected with *A. tubaeforme* have been reported in Brazil, Italy, Qatar, the United States and China (Anderson et al., 2003; Labarthe et al., 2004; Gates and Nolan, 2009; Abu-Madi et al., 2010; Riggio et al., 2013; Shi et al., 2018). *A. caninum* is the most common hookworm in canids and is widely distributed around the world. It is also one of the most common hookworms among stray dogs in China (Liu et al., 2015). *A. ceylanicum* have been reported in dogs and cats of Malaysia, Laos, Indonesia, and Sri Lanka with infection rates ranging from 24% to 88% (Dissanaike, 1961; Scholz et al., 2003; Ngui et al., 2012a, 2012b). The prevalence of *A. ceylanicum* was 62% in dogs and cats of northeast Indian (Traub et al., 2007), and 53%–92% in dogs in Thailand (Traub et al., 2008). Other countries and regions in Asia, such as Fujian, Guangzhou, the Philippines, Solomon Islands and Fiji, have also reported *A. ceylanicum* infections in dogs and cats (Chen et al., 2012; Liu et al., 2014). *A. ceylanicum* is the only animal-derived hookworm species that can produce a patent infection in humans (Ngui et al., 2012a, 2012b) with

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<https://doi.org/10.1016/j.vprsr.2019.100316>

Received 27 January 2019; Received in revised form 5 June 2019

Available online 18 June 2019

2405-9390/ © 2019 Published by Elsevier B.V.

varying symptoms ranging from gastrointestinal discomfort, abdominal pain, flatulence and diarrhea, however the rest are mainly restricted to dermatological lesions (cutaneous larva migrans) caused by migrating larvae (Bowman et al., 2010). In addition, *A. caninum* has been reported to migrate to the intestine of human causing severe eosinophilic enteritis (Prociv and Croese, 1996). Guangdong, one of the most economically developed areas in China, is densely populated, with a large number of pet including dogs and cats. Therefore, the zoonotic risk caused by canine and feline hookworms is worthy of attention. We have previously investigated the prevalence and zoonotic risk from hookworm of dogs and cats in Guangzhou city (Liu et al., 2013, 2014, 2015). However, little is known about situation of this infection in other cities of Guangdong province.

This study aimed to explore the prevalence and zoonotic risk from hookworms of stray dogs and cats in eight cities of Guangdong Province, China by standardization of a specific PCR method based on ITS sequences for identification of three common hookworms (*A. ceylanicum*, *A. caninum* and *A. tubaeforme*).

2. Materials and methods

2.1. Parasites and fecal samples

Adult hookworm samples (20 *A. ceylanicum*, 8 *A. caninum*, and 15 *A. tubaeforme*) isolated from dead dogs ($n = 2$) and cats ($n = 2$) were identified by Liu et al. (2014, 2015) and Shi et al. (2018), preserved in 75% ethanol and stored in our laboratory. The fecal samples from 702 stray dogs and 308 stray cats were collected in pet shelters of eight cities (Guangzhou, Foshan, Shenzhen, Huizhou, Zhongshan, Shaoguan, Shantou and Chaozhou) (Fig. 1). After the dog or cat defecated, the fresh feces was immediately collected and placed into the sampling bag, and the information of the sample (dog or cat, location, gender, age and environment) was recorded, and finally the sample was placed into the sampling box with built-in ice bag and brought to the laboratory. All fecal samples were stored in 2.5% (w/v) of potassium dichromate solution at 4 °C and prepared for the microscopic analysis (100 \times) after centrifugation and concentration of feces (10 g) by the flotation

Table 1

Specific primers of hookworms based on rDNA ITS.

Species	Primers	Fragment (bp)	Nucleotide sequence (5'-3')
<i>A. ceylanicum</i>	A.cey-F	268	AGCCTTATGGTTCCTTTGATCCTG CCTGACAGACAAGTGCCGAAC
	A.cey-R		
<i>A. caninum</i>	A.can-F	427	AGCATTAGGCTAACGCCCGA AACGAGTTTGCTGTTCATTGAGTCC
	A.can-R		
<i>A. tubaeforme</i>	A.Tub-F	170	AGCCTTATGGTTCCTTTGATCCTG CAGCGAAACGCGAACTTAGT
	A.Tub-R		

technique with saturated zinc sulphate.

2.2. Genomic DNA extraction

The adult hookworms preserved in 75% ethanol were repeatedly washed with double-distilled water (ddH_2O), and total genomic DNA were extracted using the Wizard® SV Genomic DNA Purification System (Promega, Guangzhou, China) according to the manufacturer's protocols. Moreover, genomic DNA of positive fecal samples identified by microscopic examination were extracted by a Stool DNA extraction kit (OMEGA, Guangzhou, China) according to the manufacturer's protocols. Extracted DNAs were then stored at $-20\text{ }^\circ\text{C}$ for use.

2.3. Primer design

The specific primers for amplifying rDNA ITS of three hookworms (*A. ceylanicum*, *A. caninum* and *A. tubaeforme*) were designed by software Primer Premier 5.0 based on their specific sequences (KF279132, KC755026 and JQ812691) deposited in GenBank. The designed primer sequences and their predictive amplification fragments are listed in Table 1.

2.4. Specific PCR

The PCR amplification was carried out using the DNAs of the three hookworms as template. The DNAs from other common parasites in dogs and cats, including *Toxocara canis*, *Isospora*, and *Giardia lamblia*,

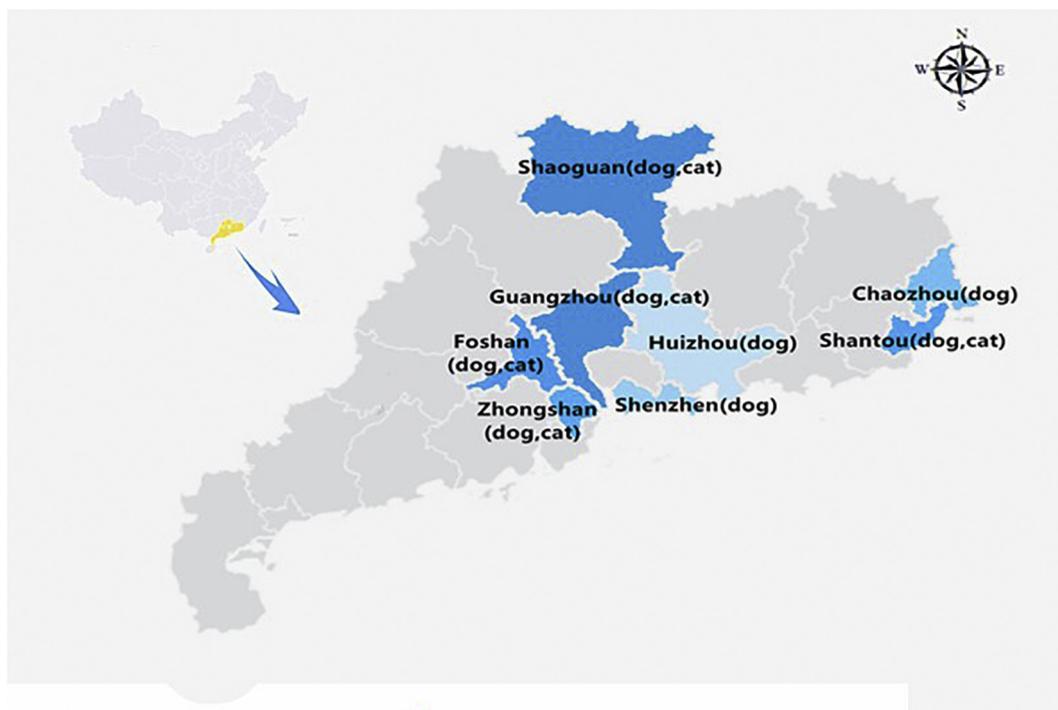


Fig. 1. Some cities of Guangdong province for canine and feline fecal sample collection in this study.

were used as control. The nucleic acid concentration was determined by micro-spectrophotometer with genomic DNAs of three hookworms identified, and then diluted tenfold at the concentrations of 10^1 to 10^8 . PCR were performed in 25 μ L, including 12.5 μ L of Premix Ex-Taq polymerase (TaKaRa, Dalian, China), 9.5 μ L of ddH₂O, 0.5 μ L of each primer AF/AR (50 μ mol/L), and 2 μ L of DNA sample. PCR cycling parameters were as follows: 1 cycle at 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, at 56–58 °C for 30 s, and at 72 °C for 45–60 s, followed by 1 cycle at 72 °C for 7 min. The PCR products were analysed by gel electrophoresis in 1.5% agarose gels stained with 0.2 mg/mL ethidium bromide, and visualized on a UV transilluminator.

2.5. Clinical detection and statistical analyses

The established PCR method was used to detect the DNA of all microscopically positive fecal samples for identifying species of hookworm and determine the infection status of stray dogs and cats in Guangdong. The chi-square test was conducted using SPSS software (Release 22.0 standard version, SPSS Inc., Chicago, USA) to compare the difference in the positive rate of hookworm under different conditions (including region, age, gender and feeding environment). Differences with $P < .05$ were considered significant.

3. Results

3.1. Prevalence of hookworm in dogs and cats

The detection rate of hookworm from stray dogs and cats in Guangdong by microscopy was 20.23% (142/702) and 15.26% (47/308), respectively. The detection rate of hookworm from stray dogs in Shaoguan, Guangzhou and Foshan was higher than that in other five cities, with a significant difference ($p < .05$) (Fig. 2). The detection

rate of hookworm from stray dogs in Foshan was higher than that in Chaozhou and Huizhou ($p < .05$). The detection rate of hookworm in young dogs was higher than that in adult dogs, with a significant difference ($p < .05$). The detection rate of hookworm in outdoor dogs was higher than that in indoor dogs and the difference was significant ($p < .05$). The difference in detection rate of hookworm from stray cats between different regions, genders, ages and feeding environments was not significant ($p > .05$).

3.2. Specificity and sensitivity of PCR

The amplified fragments of *A. ceylanicum*, *A. caninum* and *A. tubaeforme* were 268 bp (Fig. 3A), 427 bp (Fig. 3B) and 170 bp (Fig. 3C), respectively. No amplification was observed when the PCR was performed on genomic DNA of *Toxocara canis*, *Isospora* and *Giardia lamblia* and sterile ddH₂O. After determined by micro-spectrophotometer, the DNA concentration of *A. ceylanicum*, *A. caninum* and *A. tubaeforme* was 112.6 ng/ μ L, 102.7 ng/ μ L and 83.0 ng/ μ L, respectively. When the dilution of each sample was as low as 1:10⁷, they could still be detected by the PCR (Fig. 3 D ~ F). Therefore, the minimum detectable concentration of genomic DNA for *A. ceylanicum*, *A. caninum* and *A. tubaeforme* was 11.26×10^{-6} ng/ μ L, 10.27×10^{-6} ng/ μ L and 8.30×10^{-6} ng/ μ L, respectively.

3.3. Detection result of positive fecal samples by PCR

The PCR detection result of microscopically positive fecal samples for hookworm in stray dogs and cats is shown in Table 2. Mixed infection of *A. caninum* and *A. ceylanicum* was detected in stray dogs of Guangzhou and Shaoguan, with the infection rate of 8.3% and 21.2%, respectively. Among the stray dogs in Shenzhen, Huizhou and Chaozhou, all were single infections with *A. caninum*. While the

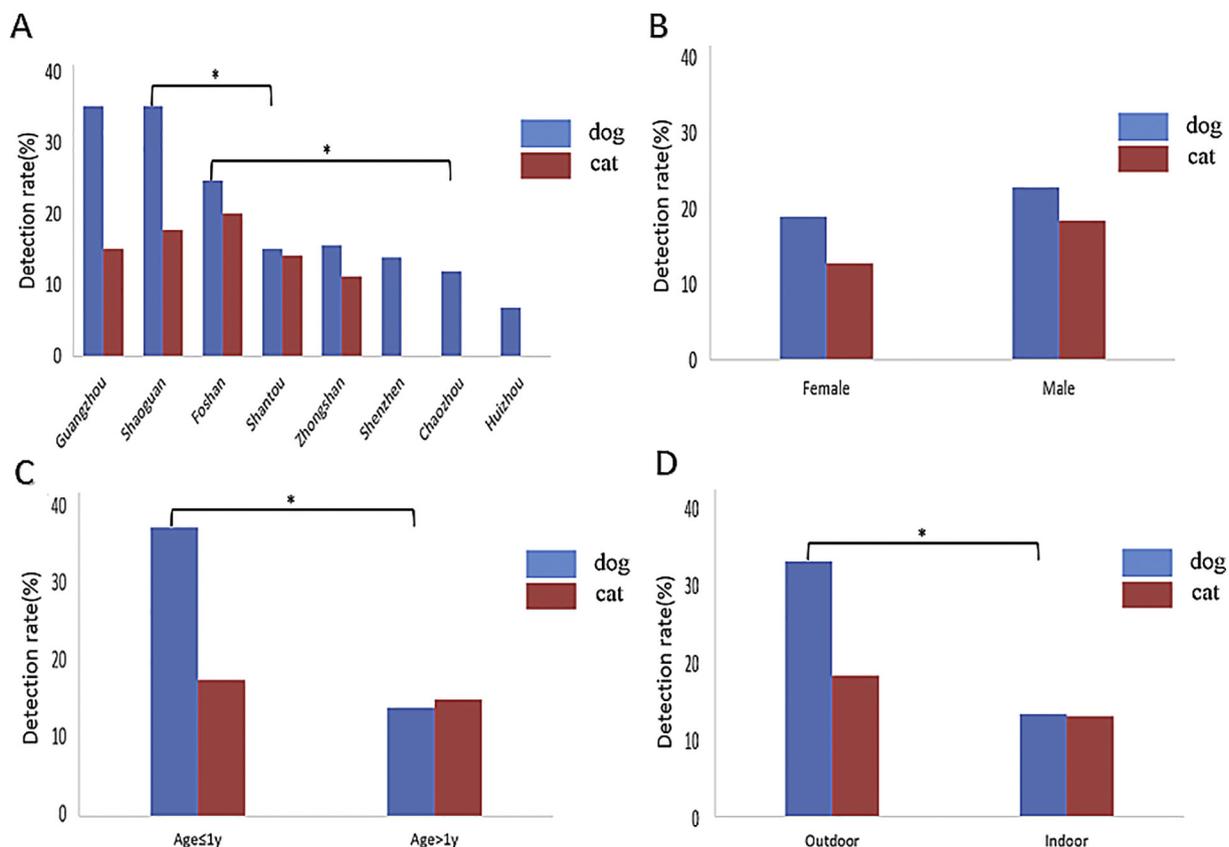


Fig. 2. Microscopic examination of fecal samples for hookworms in stray dogs ($n = 702$) and cats ($n = 308$) from different cities (A), gender (B), age (C) and feeding environment (D) in Guangdong province of China. * means $p < .05$.

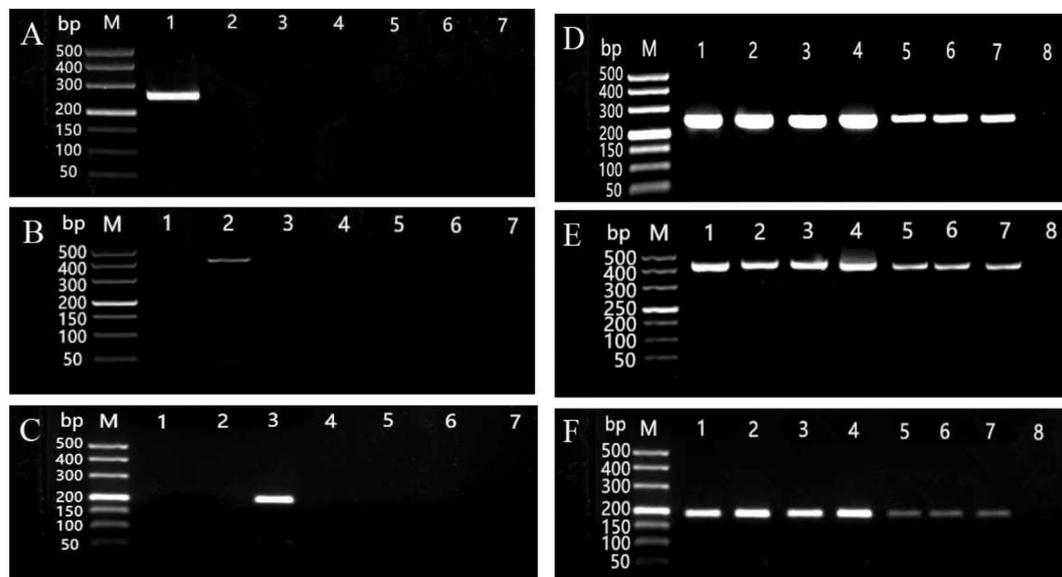


Fig. 3. The specificity and sensitivity of PCR for detecting canine and feline hookworms. A, B and C was the result of PCR specificity for *A. ceylanicum*, *A. caninum* and *A. tubaeforme*, respectively. M, MDL-500 DNA marker; 1, *A. ceylanicum*; 2, *A. caninum*; 3, *A. tubaeforme*; 4, *Toxocara*; 5, *Isospora*; 6, *Giardia lamblia*; 7, Sterilized ddH₂O. D, E and F was the result of PCR sensitivity for *A. ceylanicum*, *A. caninum* and *A. tubaeforme*, respectively. 1–8, A serial of dilutions of three hookworm genomic DNAs from 10¹ to 10⁸.

infection rate of *A. ceylanicum* (66.7%) was higher than that of *A. caninum* (33.3%) in the stray dogs of Foshan. The detection results of hookworms from cats showed that the stray cats in Guangzhou, Foshan, Zhongshan and Shaoguan were infected with *A. tubaeforme* while the cities affected by *A. ceylanicum* included Guangzhou, Foshan, Shaoguan and Shantou.

4. Discussion

As one of the tropical diseases, hookworm infection is often neglected (Traub, 2013). With the deepening of the research on hookworm disease in recent years, more and more people are calling on society to pay attention to it. The 2010 Global Disease Burden (GBD) research report pointed out that disability-adjusted life years (DALY) caused by intestinal nematode infections ranked first in the tropical diseases, and hookworms ranked first in intestinal nematodes (Pullan et al., 2014). There are two main reasons for the great harm caused by hookworm disease. First, it can cause moderate or severe anemia in the host. Recent studies have shown that anemia caused by hookworms is more pronounced in children and pregnant women (Brooker et al., 2008; Smith, 2010). The other is that the combined infection with hookworm and malaria can cause important co-morbidity effect, and can lead to the death of the host when heavily infected (Brooker et al., 2006, 2007; Righetti et al., 2012). Canine and feline hookworm may

cause zoonotic disease particularly cutaneous larva migrans. *Ancylostoma braziliense* is most often associated with dermatological lesions with long serpiginous and persistent tracks underneath the skin, while *A. tubaeforme* seems not to penetrate human skin or cause only a little skin penetration (Bowman et al., 2010). *A. caninum* can cause folliculitis (Opie et al., 2003; Rivera-Roig et al., 2008), myositis (Little et al., 1983), erythema multiforme (Vaughan and English, 1998), eosinophilic pneumonitis (Schaub et al., 2002), and occasionally eosinophilic enteritis (Prociv and Croese, 1996). Also, *A. caninum* has been suggested to cause diffuse unilateral subacute neuroretinitis in humans (Garcia et al., 2008). *A. ceylanicum* is the only species to easily mature into adults in human intestine (Bowman et al., 2010), causing eosinophilia, iron deficiency anemia, abdominal pain, occult blood in stools, nausea, and watery diarrhea (Chung et al., 2012; Hsu and Lin, 2012).

As China's urbanization continues to accelerate, the number of people raising dogs and cats has risen sharply, especially in Guangdong, one of the largest economic provinces. In Guangdong, which is located in South China, hookworm disease is endemic. From 2013 to 2015, the epidemiological survey of hookworm in dogs and cats in Guangzhou showed that the infection rate of *A. ceylanicum* was 22.67% - 40.2% (Liu et al., 2013, 2014, 2015). Hu et al. (2015) used a multiplex PCR method to detect hookworm infection in dogs and cats in Guangzhou and found that the infection rate of *A. caninum* and *A. ceylanicum* was 34.8% and 17.8%, respectively. To understand the epidemiological data of canine

Table 2

The PCR identification of microscopically positive fecal samples for hookworm in stray dogs and cats from eight cities of Guangdong province.

City	Positive samples		<i>A. ceylanicum</i> (%)		<i>A. caninum</i> (%)		<i>A. tubaeforme</i> (%)		Mixed infections (%)	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Guangzhou	36	9	36.1	44.4	55.6	0	0	55.6	8.3	0
Foshan	21	11	66.7	36.4	33.3	0	0	63.6	0	0
Shenzhen	11	-	0	-	100	-	-	-	0	-
Huizhou	5	-	0	-	100	-	0	-	0	-
Zhongshan	12	7	33.3	0	66.7	0	0	100	0	0
Shaoguan	33	12	30.3	66.7	48.5	0	0	33.3	21.2	0
Chaozhou	9	-	0	-	100	-	0	-	0	-
Shantou	15	8	40	100	60	0	0	0	0	0
Total	142	47	33.1	51.1	59.9	0	0	48.9	7.0	0

and feline hookworm in Guangdong province of China, this study investigated fecal samples from 702 dogs in eight cities and 308 cats in five cities. The results showed that the total detection rate of hookworm was 20.23% (142/702) in dogs and 15.26% (47/308) in cats. The infection rate of canine hookworm in eight cities ranged from 6.67% to 34.38%, among which Shaoguan had the highest infection rate and Huizhou had the lowest infection rate. This may be related to the geographical location and unbalanced economic development of the two cities. Shaoguan is located in the mountainous area of northern Guangdong, and its economy is underdeveloped. Huizhou has a relatively developed economy and less population flow, and its population density is much lower than in Guangzhou and Shenzhen, which may explain why the infection rate of hookworm is lower. The infection rate of feline hookworms in the five investigated cities was not significantly different, ranging from 10.94% to 19.64%. Through the comparison of different sexes, ages and feeding environments, it was found that young dogs are more susceptible to hookworm than adult dogs, which is consistent with findings of previous studies (Little et al., 2009; Sowemimo, 2009), suggesting that young dogs might have acquired the infection prenatally. In addition, outdoor dogs had a higher hookworm infection rate than indoor dogs. This is in agreement with the results of previous studies in which a higher prevalence was documented among stray dogs than housed dogs (Sowemimo, 2009) and may be due to outdoor dog scavenging habits, which make them more vulnerable to natural infection than indoor dogs.

Among the eight cities where canine hookworm was investigated, mixed infections of *A. caninum* and *A. ceylanicum* were found in Guangzhou and Shaoguan, especially in the latter, with the proportion of mixed infections as high as 21.2%. In Shenzhen, Huizhou and Chaozhou, there were only single infections of *A. caninum*, indicating that this hookworm is the dominant species in these cities. Among the five cities where feline hookworm was investigated, the cities with *A. tubaeforme* infection were Guangzhou, Foshan, Zhongshan and Shaoguan, while the cities affected by *A. ceylanicum* were Guangzhou, Foshan, Shaoguan and Shantou. The results indicated that *A. tubaeforme* or *A. ceylanicum* was the dominant species of cats in these cities. For the whole of Guangdong province, the hookworm infection in dogs is mainly *A. caninum*, accounting for 59.9% of positive samples. However, the geographical distribution of feline hookworm is different, the *A. tubaeforme* is mainly in the area of the Pearl River Delta (Guangzhou, Foshan and Zhongshan), while *A. ceylanicum* is mainly in the northern (Shaoguan) and eastern (Shantou) regions of Guangdong province.

Some molecular techniques such as RFLP-PCR and qPCR have already been standardized for the determination of hookworm species (George et al., 2016; Papiakou et al., 2017). However, each of these methods has its own advantages and disadvantages. The RFLP-PCR has a great risk of contamination because of its cumbersome steps, while the qPCR requires expensive fluorescent dyes and real-time fluorescence quantitative PCR instrument. In this study, a specific PCR method was established and used to identify hookworm species. The results showed that the specific PCR method established can detect the ITS sequences of three hookworms, *A. ceylanicum*, *A. caninum* and *A. tubaeforme*, with a minimum detectable DNA concentration of 11.26×10^{-6} ng/ μ L, 10.27×10^{-6} ng/ μ L and 8.30×10^{-6} ng/ μ L, respectively.

In conclusion, the investigation of the infection status and hookworm species in stray dogs and cats indicates that potential zoonotic hookworm infection in Guangdong should be considered and the necessary prevention and control measures should be implemented in this area to ensure public health and safety. In addition, the established specific PCR method is a sensitive, accurate, and relatively cost-effective tool that can help in the future monitoring efforts of canine and feline hookworm infection.

Declaration of competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical statement

All animal experiments and husbandry involved in this study were treated in accordance with the guidelines of the South China Agricultural University Animal Care and Use Committee, which operates under the Animal Welfare Law and Regulations of the Department of Health and Human Services. The South China Agricultural University Animal Care and Use Committee has approved all protocols of this study.

Acknowledgements

This work was supported by a grant from National Natural Science Foundation of China (grant no. 31672541) and the Science and Technology Planning Project of Guangdong Province, China (grant no. 2014A020214005). We would like to thank the humane shelter personnel to help collecting all of the samples.

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