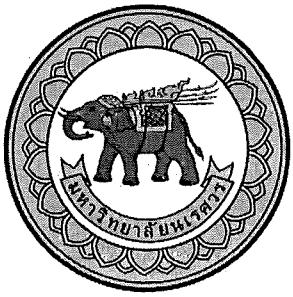

รายงานการวิจัยฉบับสมบูรณ์

โครงการวิจัย

การจำแนกเชื้อปรสิตที่ติดต่อจากสัตว์สู่คนในระดับโมเลกุลในสุนัข และแมวจากจังหวัดภาคเหนือตอนล่าง

(Molecular characterization of zoonotic parasites in cats and dogs from Lower Northern provinces of Thailand)

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งบประมาณประจำปีงบประมาณ พ.ศ. 2560

รายงานการวิจัยฉบับสมบูรณ์

การจำแนกเชื้อปรสิตที่ติดต่อจากสัตว์สู่คนในระดับโมเลกุลในสุนัข และแมวจากจังหวัดภาคเหนือตอนล่าง

(Molecular characterization of zoonotic parasites in cats and dogs from Lower Northern provinces of Thailand)

มหาวิทยาลัยนเรศวร

ABSTRACT

Zoonotic parasites of dogs and cats have a public health concern worldwide. We investigated the prevalence of zoonotic parasites in dogs and cats in lower Northern Thailand and identified the most prevalent species using molecular techniques. Fecal samples of 197 dogs and 180 cats were collected from 9 districts belonging to 9 provinces. The infection rate of intestinal parasites in dogs varied substantially within the region from 4% to 76.5%. The overall prevalence in cats varied similarly between 10-75%. Hookworms were the most prevalent parasite in dogs, while *Spirometra* was the most prevalent parasite in cats. Mixed-infection due to hookworms and *Spirometra* was common in both dogs and cats. Among hookworm infection in dogs and cats, *Ancylostoma ceylanicum* was the most prevalent, being 82.1% in hookworm infected dogs and 95.8% in hookworm infected cats. The liver fluke, *Opisthorchis viverrini,* was found in both cat and dog fecal samples. In lower Northern Thailand, dogs and cats were allowed to roam, hunt and eat small prey. Further, most dogs and cats had never be dewormed. All together our finding indicated that dogs and cats in lower Northern Thailand serve as reservoir hosts for many zoonotic parasites, predominantly hookworm, *Spirometra* and *O. viverrini* and potentially both animals can distribute the parasites to the environment and to inhabitants in communities.

Keywords: Parasitic zoonoses, Hookworm, *Ancylostoma ceylanicum, Ancylostoma caninum Spirometra, Opisthorchis viverrini*, Cats and Dogs

1. Introduction

Dogs and cats play a significant role as reservoirs for many zoonotic gastrointestinal parasites that have public health concern worldwide [1,2,3]. Common zoonotic parasitic diseases included sparganosis, opisthorchiasis, toxocariasis. Humans can become infected by infective stages of zoonotic parasites either via direct contact with a dog or cat or via contamination in food or water [4].

Dogs and cats are known as carriers of hookworm infections [5,6]. *Ancylostoma ceylanicum* (*A. ceylanicum*) is the most prominent hookworm in cats and dogs in Asia countries [2,7,8,9]. It was reported to be the second most common hookworm infection in humans that can lead to anaemia [10].

Human sparganosis is one of the parasitic zoonosis caused by the plerocercoid larvae of *Spirometra,* a common cestode found in cats and dogs. Human infections can be acquired through ingestion of raw or undercooked meat of intermediate hosts, for instance, snakes or frogs, or drinking contaminated water with larva of *Spirometra*. Cases of sparganosis have been reported in several countries [11,12]. In Thailand, during the period of 2001-2012, nine cases of human sparganosis have been diagnosed and the causative agent was identified as *Spirometra erinaceieuropaei* [13]. Thus, surveys about the spread of *Spirometra* infection in cats and dogs are crucial for public health concerns.

Dogs and cats are also recognized as reservoir hosts for zoonotic trematode parasite, *Opisthorchis viverrini* (*O. viverrini*), a main predisposing factor of cholangiocarcinoma. High infection rates of *O. viverrini* in cats and dogs were reported at opisthorchiasis endemic areas [1,14,15]. Recent information from the National Helminthic Survey revealed an overall reduction in presence of *O. viverrini* infection in Thailand. However, different situations had been found in the North region,where prevalence was 5.6% in 1980-1981 and increased to

10.0% in 2009 [16]. Dogs and cats might play a role in harboring and spreading the parasite in the environment, resulting in transmission to human.

Although surveys of zoonotic gastrointestinal parasites in dogs and cats have been reported in Thailand, most of the studies focused in the Central or Northeastern area [1,5,17,18,19]. This is the first study to investigate zoonotic parasitic infection in dogs and cats in the lower Northern region of Thailand and also to identify species of the most prevalent parasite using molecular techniques.

2. Materials and methods

2.1. Ethical consideration

Animal procedures were reviewed and approved by the Animal Research Ethics Committee of Naresuan University, Thailand.

2.2. Study area

The study area covered the lower Northern part of Thailand consisting 9 provinces (Fig. 1). Borders of the lower northern Thailand connect to the upper Northern, the Central and the Northeast regions and in addition connect to Lao People's Democratic Republic and Myanmar. The climate is tropical and humid throughout the year. The communities where samples were collected were characterized by poor hygiene and containing inhabitants that migrated from Northeast area where consumption of raw or undercooked food is favoured.

2.3. Fecal sample collection

Dog and cat fecal samples were collected from nine communities located in nine provinces (Fig.1) between February and April 2014. Five grams of feces from each animal were collected and put into a sterile plastic container, packed into a box containing ice and transported to the Parasitology laboratory, Faculty of Medical Science, Naresuan University for microscopic examination and DNA extraction. A questionnaire of risk factors about

distribution of zoonotic parasites was handed out to cat and dog owners in the sampling communities.

2.4. Fecal sample processing and microscopic examination

Samples were screened for the presence of parasite worms under microscope and then all samples were microscopically examined again after processing with sucrose flotation method [20] and Formalin - ethyl acetate concentration technique (FECT) [21,22]. Briefly, for sucrose flotation method, fecal samples were thoroughly homogenized in the sucrose solution (50% w/v containing 650 ml hot water, 500 g sucrose and 6.5 ml water saturated phenol, specific gravity 1.2) to make a suspension and then centrifuged at 2,000 rpm for 10 min. After centrifugation, sucrose solution was added to the tube. When a slight positive meniscus was formed, a glass cover slip was placed over the tube and allowed to stand for 1 minute. Then the coverslip was removed to examine under a compound microscope using 10x and 40x objective lens.

Formalin - ethyl acetate concentration technique (FECT) was used for recovering heavy and operculated eggs. Briefly, fecal sample was mixed thoroughly with 0.85% normal saline and filtered through two layers of wet gauze over a disposable paper funnel into a 15 ml centrifuge tube. The tube was then centrifuged at 1500 rpm for 10 min. The supernatant was discarded and 7 ml of 10% formalin was added to the sediment. Two milliliters of ethyl acetate was added and subsequently the tube was centrifuged at 1500 rpm for 10 min. The plug of debris was removed and then the supernatant was discarded. The pellet was resuspended with 10% formalin and examined under a microscope at magnifications 100x and 400x to identify genus or species of eggs and larvae of parasites, based on their morphology.

2.5. Molecular procedures

PCR and sequencing were applied to characterize the most prevalent parasites in fecal samples. Microscopically, hookworm was the most prevalent parasite. To confirm and to identify the species of hookworm parasites, a forward primer RTHW1F (5'-GATGAGCATTGCWTGAATGCCG-3') and reverse primer RTHW1R (5'-GCAAGTRCCGTTCGACAAACAG-3') were used to amplify an approximately 380 bp section of ITS1, 5.8S and ITS2 regions of hookworm [5 Traub et al., 2008].

DNA was extracted from fecal samples using the following steps. Flotation part of two grams of dog or cat fecal samples after homogenization in the sucrose solution and centrifugation at 2,000 rpm for 10 min was harvested and centrifuged again at 15,000 rpm for 5 min at 4°C. The supernatant was discarded and 100 µl OOC-lysis buffer (600 mM EDTA, 1.3% (v/v) N-lauroylsarcosine, 2mg/ml Proteinase K) was added to the pellet and subjected to 3-5 cycles of freezing at -80°C for 20 min and thawing at 96-98°C for 1 hr in order to break parasite eggs. Thereafter, 400 µl OOC-CTAB buffer (2% (w/v) cetyl-trimethyl ammonium bromide, 1.4 M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris (hydroxymethyl) aminomethane) was added to the samples and incubated at 70°C for 1 hr. Then phenol/chloroform extraction method was used to extract and purify DNA. The purified DNA was used for Polymerase Chain Reaction (PCR). All PCR products were subjected to DNA sequencing. Cycle sequencing reactions were performed using a BigDye Terminator Cycle Sequencing kit version 3.1 according to the manufacturer's protocol (Applied Biosystems, USA), and each sample was analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were aligned and compared to published sequences of hookworm in order to identify the species. PCR and DNA sequencing at National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan.

3. Results

All together 377 dog and cat fecal samples were collected. Among the collected fecal samples, 197 were from dogs and 180 were from cats.

3.1. Parasitic infection in dogs

Out of 197 dog fecal samples examined by microscopy, 79 samples (40.1%) were positive for intestinal parasites. Among these positive samples, 57 (72.2%) samples were infected by one kind of parasite while, 22 (27.8%) samples were infected by a mixture of parasites. The parasites found in samples were hookworms in 42 samples (21.3%), *Spirometra* in 30 samples (15.2%), *Taenia* in 14 samples (7.1%), *Toxocara* in 7 samples (3.6%), *O. viverrini* in 6 samples (3.0%) and *Strongloides* in 3 samples (1.5%) (Table 1 and Fig. 2A). Hookworms were the most prevalent, followed by *Spirometra*. Co-infection of hookworms and *Spirometra* was the most common.

Among the 9 surveyed districts, the overall prevalence of parasitic infection in dogs varied regionally from 4% to 74.5% (Table 2). High prevalence (over 50%) of parasitic infection in dogs was found in the communities of Sukkhothai and Tak provinces, with the prevalence of hookworms being 39.2% and 40%, respectively. *Opisthorchis viverrini* was found in only 6 samples from 4 districts located in Sukhothai, Tak, Uthai Thani and Petchabun provinces (Table 3).

3.2. Parasitic infection in cats

Out of 180 cat fecal samples examined microscopically, 61 (33.9%) cats tested were positive for parasitic infection. Among these, 49 (80.3%) samples had infection from one kind of parasite and 12 (19.7%) samples had mixed infections. The parasites found in fecal samples were *Spirometra* in 36 samples (20.0%), hookworms in 25 samples (13.9%), *Toxocara* in 4 samples (2.2%), *O. viverrini* in 6 samples (3.3%) and *Trichuris* in 1 sample (0.6%) (Table 1

and Fig. 2B). *Spirometra* was the highest prevalent parasite. Mixed infection of *Spirometra* and hookworms was the most common in cats.

The overall prevalence in cats also varied regionally between 10 and 75%. Over 50% infection rates were found in Phitsanulok and Uttaradit provinces (Table 2). Hookworm and *Spirometra* were found in all 9 communities, while other parasites were found sparingly. For instance, *O. viverrini* were found only in samples from Sukkhothai, Phetchabun and Phitsanulok provinces while, *Toxocara* was found only in Uttaradit province (Table 4).

3.3. Molecular identification of hookworm infection in dog fecal samples

Microscopic examination revealed that hookworm was the most prevalent parasite in the study area. PCR amplification with hookworm specific primers showed a specific PCR product size at 380 bp (Fig.3) in 28 samples. All 28 PCR positive samples yielded clear and readable sequences. The sequences of 23 (82.1%) samples were identical to *A. ceylanicum* strain GD-M55 (KF279136) and 5 (17.9%) samples belonged to *A. caninum* GD-M45 (KC755026).

3.4. Molecular identification of hookworm infection in cat fecal samples

The PCR amplification with hookworm specific primers revealed that 24 samples tested positive with specific PCR product band at 380 bp. DNA sequences were obtained from all 24 samples. BLAST results revealed that 20 (83.3%) samples were infected with *A. ceylanicum* strain GD-M55, 1 (4.2%) sample was infected with *A. caninum* strain GD-M45 and 3 (12.5%) samples were infected with both *A. ceylanicum* strain GD-M55 and *A. ceylanicum* GD-M76.

3.5. Factors influencing the distribution of parasitic zoonoses in the study area

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In the lower Northern Thailand area, among 533 pet owners who answered the questionnaire, 434 (81.4%) fed their pets with cooked foods, 468 (87.8%) allowed their pets to roam freely, 371 (69.6 %) let their pets hunt and eat preys (birds, frogs, snake, etc.). Of the 533 pet owners, 510 (95.7 %) have never deworm their pets with anthelmintic drugs (Table 6).

4. Discussion

Parasitic infections in cats and dogs are considered an issue of global concern from the zoonosis point of view [2,3,8]. Important zoonotic parasitic diseases reported worldwide included spaganosis, toxocariasis, opisthorchiasis.

Here, we reported the total prevalence of intestinal parasites in lower Northern Thailand as 40.1 % (79/197) in dogs and 33.9 % (61/180) in cats, respectively. Zoonotic parasites found included hookworms, *Spirometra*, *Toxocara*, *O. viverrini*, *Taenia*, *Strongyloides* and *Trichuris*. The prevalence of parasitic infection in dogs and cats varied within the lower Northern regions. In dogs, hookworm infestation was the most common in the lower northern area while, *Spirometra* was the second most prevalent. Conversely, in cats, *Spirometra* was the most prevalent parasite and hookworms were the second most prevalent parasites. Mixed-infection of hookworms and *Spirometra* was also recorded in both cats and dogs. Among hookworms, *A. ceylanicum* was the most prevalent in both dogs and cats. This finding was consistent with other investigators who demonstrated the high prevalence of *A. ceylanicum* in Thailand and in other Asian countries [2,5,9,10,18]. *Ancylostoma ceylanicum* is a potential zoonotic parasite known to produce potent infections in humans [10,23]. From our current investigation, the results revealed the presence of *A. caninum* about 5% in dogs and 1% in cats. Although its detection rate was lower comparing to that of *A. ceylanicum*, this parasite can result in eosinophilic enteritis and chronic abdominal pain in human [6,24].

In addition, our results clearly demonstrated that *Spirometra* was also highly prevalent in dogs and cats in the study area. High rates of *Spirometra* infection was a reflection of the fact

that most dogs and cats roam freely and had access to intermediate hosts as food sources. This high infection rate of *Spirometra* consequently leads to a high risk of sparganosis in local residents who have the habit of eating undercooked meat. Human non-proliferative sparganosis is endemic in Thailand with about 53 cases previously being reported [13].

Infection due to a mixture of hookworm and *Spirometra* that is common in both dogs and cats leads to a high of risk of sparganosis, larva migrans, eosinophilic enteritis, and anaemia in these communities. The larva migrans can also be caused by *Toxocara* [25] which were also found in feces of dogs and cats examined in this survey. Human can be infected by the ingestion of embryonated eggs that could be present in soil contaminated with dog or cat feces [4,17].

In the present study area, *O. viverrini* was also detected in dogs and cats. The infection rate of this parasite in cats and dogs in the lower Northern area was relatively less than the rate in the Northeastern region of Thailand where *O. viverrini* infection in humans is high [1,15]. However, it is important to emphasize that the inhabitants in these communities originally migrated from the Northeastern area. It could be the reason behind the existence of *O. viverrini* infection in cats and dogs in the lower Northern Thailand. Dogs and cats could potentially be infected through ingestion of raw or undercooked cyprinoid fish which is infected with *O. viverrini* metacercariae. The metacercariae develop into adult worms and then produce eggs in the gall bladder of dogs and cats. These eggs are then released to the environment along with feces and spread to aqua-ecology by rains. High infection rate of *O. viverrini* infected dogs and cats can also increase the occurrence of infected snails and cyprinoid fish which consequently increase the incidence of *O. viverrini* infection in humans.

Most cat and dog owners fed their pets with cooked meals. However, they allowed their pets to roam, to hunt and to eat small preys (birds, snakes, rodents and fish). Infective stage of zoonotic parasites, for instance, plerocercoid of *Spirometra* and metacercaria of *O. viverrini*

[15,26] can be present in those animals and causes infection in dogs and cats. From our investigation we found that dogs and cats host many zoonotic parasites however, over 95 % of dog and cat owners have never deworm their pets with anthelmintic drug.

In conclusion, we reported the prevalence of zoonotic intestinal parasites in dogs and cats in the area of lower Northern Thailand. We detected a variety of parasites, namely, hookworms, *A. ceylanicum*, *A. caninum*, *Spirometra*, *Taenia*, *Toxocara*, *O. viverrini*, *Strongyloides*, and *Trichuris*. Hookworms and *Spirometra* were the most prevalent parasites. Among hookworms, *Ancylostoma ceylanicum* was the most common. These infections are considered neglected because relatively little attention has been devoted for their surveillance, prevention and treatment. Educational and awareness program about these zoonotic parasites along with an appropriate anthelmintic program for both dogs and cats is urgent need to reduce the environmental contamination and consequently, the incidence of zoonotic parasitic infections. Additionally, large-scale and rigorous studies would be required to infer the results at the national level. All of these should be made a continuous task to reduce the prevalence of zoonotic parasites and to prevent their spread from highly endemic areas to those of lower prevalence.

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