

## Transmission of *Echinococcus ortleppi* at the Endangered Primate Rescue Center, Cuc Phuong National Park

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### Summary

According to the statistical report of the Endangered Primate Rescue Center, the prevalence of the cystic disease in four langur species (*Trachypithecus hatinhensis*, *T. delacouri*, *Pygathrix nemaeus*, *P. cinerea*) was on average 63.4% (N=41). Of which, five individuals were confirmed to be infected with *Echinococcus ortleppi*. To clarify the transmission route of infection in primates, we investigated the prevalence of intestinal helminths in leaves, the food for these primate species and in dog fecal samples collected in the surrounding areas of the center.

Only *Ancylostoma* spp. and *Isospora* spp. were detected in leaves samples, with the infection rate of 7.07% and 1.01% respectively. In addition, survey on 156 dog feces samples showed that the overall prevalence of gastrointestinal parasites was quite high (73.55%). However, *Echinococcus* eggs still remained undetected.

## Con đường truyền lây *Echinococcus ortleppi* ở Trung tâm Cứu hộ Linh Trưởng Nguy cấp (EPRC), Vườn Quốc gia Cúc Phương

### Tóm tắt

Tại Trung tâm Cứu hộ Linh trưởng Nguy cấp, đã ghi nhận 41 trường hợp nhiễm nang sán ở linh trưởng với tỷ lệ là 63.4%. Trong đó, năm cá được xác định là nhiễm *Echinococcus ortleppi*. Để xác định con đường truyền lây mầm bệnh cho linh trưởng, chúng tôi tiến hành xét nghiệm mẫu lá cây và mẫu phân chó thu thập xung quanh trung tâm. Đối với mẫu lá, phát hiện hai mầm bệnh là *Ancylostoma* spp. và *Isospora* spp. với tỷ lệ nhiễm lần lượt là 7.07% và 1.01%. Đồng thời khảo sát trên 156 mẫu phân chó thu thập xung quanh trung tâm thấy tỷ lệ nhiễm chung các loại ký sinh trùng khá cao, lên tới 73.55%. Tuy nhiên, nghiên cứu này vẫn chưa phát hiện được sự xuất hiện của trứng *Echinococcus*.

### Introduction

Cystic echinococcus (CE) is a zoonotic disease that is widely distributed in large parts of Europe and Asia (Ito & Budke 2017) that can develop asymptotically for years. The life cycle of *Echinococcus* involves dogs as the definitive host and some wild species such as dingo, wolf, jackal, red fox and hyenas (Dybicz et al. 2019). Cattle, sheep, goats and pigs are known to be susceptible as intermediate hosts to *Echinococcus ortleppi*.

The transmission of CE is typically accidental, as a result of ingestion of water, food and/or soil contaminated with pathogens from dog feces. When intermediate hosts (also primates or humans) eat these contaminated materials, metacestodes can live in several organs like the liver, kidneys or lungs where it develops into hydatid cysts. Cysts grow gradually in the body, which interfere with normal organ function and cause animals to suffer from intense pain. It can sometimes develop into a fatal cyst within the brain (Kvascevicus et al. 2016). To date, studies on cystic echinococcus are still very limited in Vietnam.

The Endangered Primate Rescue Center (EPRC) is located in northern Vietnam and houses more than 150 individuals of 15 primate taxa. The center provides sanctuary and rehabilitation to primates of the Genus *Nomascus*, *Nycticebus*, *Trachypithecus* and *Pygathrix* with their IUCN Red List status ranging from 'Vulnerable' to 'Critically Endangered'. Medical problems associated with these primates include enteritis, parasites and occasional trauma. However, the most significant disease today is hydatid cyst disease (Fig. 1). Hydatidosis due to *Echinococcus ortleppi* appears to be a common cause of death for the langur population in EPRC, which was first reported in 2009 (Plesker et al. 2009). The disease is not only posing a health risk to primates, but also affects humans as intermediate hosts, especially for those who live in endemic areas. In order to improve the effectiveness of disease prevention and treatment, the aim of this study was to investigate the prevalence of parasites in leaves and dog fecal samples in the surrounding areas of the center to find possible routes of transmission of the infection in primates with *E. ortleppi*.



**Fig.1.** Lungs of a red-shanked douc langur (*Pygathrix nemaeus*) with white cysts. Photo: Vo Duy Thanh.

## Material and Methods

### Animals

In the EPRC, primates live in enclosures mimicking their natural habitat. Data of deceased langurs were precisely recorded by experts/veterinarians.

### Post-mortem examinations

After death, carcasses of primate individuals were kept under -20°C. A comprehensive necropsy procedure was conducted shortly thereafter. Tissues and cysts of interest would be collected and fixed in 70% ethanol, and sent to the Primate Genetics Laboratory of the German Primate Centre for PCR testing.

### Sample collection

The study was conducted at the Endangered Primate Rescue Center (EPRC), Ninh Binh Province from July to November 2019. The leaves samples used in the experiment were collected from the surrounding area of the center where leaves were frequently collected as food for primates, including those located at the foothills and along the forest edge as well as on hills higher than 10 m. During the same period, following verbal consent from owners, dog fecal samples were collected freshly off the ground, put into a sterile zipper bag, and sent to the laboratory. Households were randomly chosen among seven villages surrounding the center (Nga, Sam, Bai Ca, Ao Luong, Met, Dong Tam, Ky Phu). The majority of investigated dog breeds were Indochinese Dingo. The total number of leaves

and fecal samples for this study are 99 and 156 samples, respectively.

### Parasite examination

Fecal samples were examined for intestinal helminths using flotation technique with saturated sugar solution (SG: 1.28)(Dryden et al. 2005) using microscope, under 400x magnification for helminth eggs and 100x magnification for tapeworm eggs. The same procedure was used to examine leaf samples after leaves were cut into small pieces by a pair of scissors.

### DNA extraction method

#### For egg sample

*Echinococcus* suspected egg samples during fecal examination were collected and fixed in 70% ethanol. DNA was extracted from egg samples using QIAamp DNA Stool Mini kit (Quiagen, Germany), according to manufacturer's instructions.

#### For cyst sample

DNA was isolated from ethanol fixed cyst material as described by Dinkel et al. (1998). About 0.5 g of the cyst wall was cut into small pieces and digested in the presence of 2 mg/ml proteinase K in 500 µl of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulfate and 20 mM dithiothreitol. DNA was extracted with phenol chloroform isoamyl alcohol (25:24:1) and ethanol precipitation. After drying, the DNA was suspended in 200 µl TE-buffer (pH 7.6).

### PCR

#### For egg sample

Amplification of a fragment of the COX-1 mitochondrial gene was performed according to Correa et al. 2018. Briefly, an initial reaction at 95°C (5 min) followed by 35 cycles of 94°C (55 s), 54°C (55 s), 72°C (60 s), and final extension at 72°C (10 min). The primer set for the reaction was Eco1 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and Eco2 (5'-TAA AGA AAG AAC ATA ATG AA ATG- 3'). All positive PCR products were purified and subjected to sequencing using Gene JET PCR purification kit, Thermo and 1<sup>st</sup> Base – Malaysia and analysis using bioinformatics software (Sequencer v4.1.4, BLAST tool, Clustal X v2.1, MrBayes v3.2).

#### For cyst sample

Polymerase chain reaction A cestode specific PCR (cs PCR) was done as described by Dinkel et al. (1998; 2004) in 50 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM of MgCl<sub>2</sub>, 200 µM of each dNTP, 20 pmol of each primer and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems). Amplification was done for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 55°C and elongation for 30 s at 72°C). For identification of genotypes and species of *Echinococcus* a semi-nested PCR assay specific for *E. canadensis* G6/7 and *E. ortleppi* as described in Dinkel et al. (2004) was performed. For the first PCR (g5/6/7), the 50 µl reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of MgCl<sub>2</sub>, 200 µM of each dNTP, 25 pmol of each primer, and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems) for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 53°C and elongation for 40 s at 72°C). To discriminate between *E. ortleppi* and *E. canadensis* G6/7, the semi-nested PCRs for *E. ortleppi* (g5 PCR) and *E. canadensis* G6/7 (g6/7 PCR) were used in a second step, both in a 50µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of MgCl<sub>2</sub>, 200 µM of each dNTP, 25 pmol of each primer, and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems) for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 60°C and elongation for 30 s at 72°C). For all PCR's, target sequence for amplification is a part of the mitochondrial 12S rRNA gene. For subsequent gene sequencing, two additional PCR's were performed as described by Bowles et al. (1992) and Bowles & McManus (1993) with the target sequences of a part of the mitochondrial cox 1 and nad 1 genes. All amplification products were resolved on a 1.5% ethidium bromide stained agarose gel.

**Results**

During the period from 1994 to 2018, 41 deceased langurs of the species *Trachypithecus delacouri*, *T. hatinhensis*, *Pygathrix nemaeus*, *P. cinerea* were necropsied. The necropsy records have shown that the prevalence of the cystic disease in langurs of four species was on average 63.4% (Table 1). Of which, *P. cinerea* has the highest prevalence rate, at 87.5%. Five cysts from randomly selected individuals were subjected to PCR for molecular identification. All samples showed positive to *E. ortleppi*.

Ninety-nine leaves samples were collected within 20 km radius of the center, scattered from the ground to the hills. Eight out of 99 leaf samples were infested with *Isopora* spp. and *Ancylostoma* spp., at 1.01% and 7.07% respectively. However, *Echinococcus* eggs were not found. (Table 2).

**Table 1.** Post-mortem results of langur species at the EPRC, 1994-2018.

Species	Number of necropsied individuals	Number of langurs diagnosed with cysts	Prevalence of cysts (%)	Number of PCR positive for <i>E. ortleppi</i>
<i>Trachypithecus delacouri</i>	4	2	50	-
<i>Trachypithecus hatinhensis</i>	11	6	54.5	-
<i>Pygathrix nemaeus</i>	10	4	40	2
<i>Pygathrix cinerea</i>	16	14	87.5	3

**Table 2.** Parasitic prevalence in leaf samples collected as a food source for primates.

Location/ height	Number of samples	<i>Isospora</i> spp. positive	<i>Ancylostoma</i> spp. positive
2-20 km at 0-10 m	55	0	4
2-20 km at > 10 m	44	1	3
<b>Total</b>	<b>99</b>	<b>1</b>	<b>7</b>

The prevalence of intestinal helminths in 156 dog fecal samples is quite high, accounting for 73.55% (n=114). *Ancylostoma* spp. has the highest infection rate (68.39%), followed by *Toxocara* spp. (25.81%), *Trichuris* spp. (10.97%) and *Isospora* spp. (7.1%). The eggs of *Taenia* spp. are also found in six samples (3.87%) (Table 3). These species are all common intestinal parasites, which had been recorded previously in Vietnam.

**Table 3.** Gastrointestinal parasites identified in dog fecal samples at the area around the Endangered Primate Rescue Center.

Parasite species	No. of positive samples	Infection rate (%)
<i>Taenia</i> spp.	6	3,87
<i>Ancylostoma</i> spp.	106	68,39
<i>Toxocara</i> spp.	40	25,81
<i>Trichuris</i> spp.	17	10,97
<i>Isospora</i> spp.	11	7,1

## Discussion

*E. ortleppi*'s life cycle includes dogs as definitive hosts and cattle as intermediate hosts. These are all common and widely distributed animals, but the infection rate of *E. ortleppi* in the world is negligible. To the best of our knowledge, only ten human cases of *E. ortleppi* infection were recorded from some parts of the world (i.e. Argentina, Brazil, Mexico, South Africa, Netherlands, Poland, France, China, India, and Vietnam) (Grenouillet et al. 2014; Rojas et al. 2014; Dybicz 2019; Shi et al. 2019). It is suggested that the low incidence may be due to the fact that cattle are mainly slaughtered in locations away from residential areas, and dog access to these areas is limited, thereby inhibiting transmission to the local dog population (Nguyen Van De & Duyet Le Van 2017). However, cases of hydatid cysts have been recorded in the EPRC for many years, suggesting the existence of *Echinococcus* pathogens in the direct environment and finding out the cause of the disease is necessary.

The first case of *E. ortleppi* infection in Vietnam was reported in the langurs housed at EPRC (Plesker et al. 2009). Later, two human cases were also detected in Thanh Hoa Province, Vietnam (Nguyen Van De & Duyet Le Van 2017). These data suggest the existence of zoonotic cycles of *Echinococcus* pathogens in the environment among neighboring provinces. In the EPRC, primates are kept by pair or in groups in separated enclosures. Each enclosure was built to mimic their natural habitat, with a safety distance of at least 1.5 m away from visitors. Therefore, it is impossible for the animals at the center to come into contact with definitive hosts.

Eighteen out of 55 leaves samples from the foothills and forest edge were collected around the landfill and places which dogs and cats frequently showed up (villages Nga, Bai Ca, etc.) making them easy targets to get contaminated. Even for those collected on hills higher than 10 m, with less presence of dogs, 3 samples were still found to be positive with hookworms. During collecting leaves as food for primates, staff could not bring the leaves with them all the time and had to put on the ground, and this may be the reason why they could easily get contaminated with pathogens. Additionally, leaves are collected in the forests around the center where wild animals have access, which is a potential source of diseases. *Echinococcus* eggs can survive for a prolonged period of time (Federer et al. 2015; Veit et al. 1995). High prevalence of intestinal parasites in primates of the center was also reported (62.63%), in which *Strongyloides* spp. constituted the highest proportion (48.48%), followed by *Trichuris* spp. (31.31%), *Ancylostoma* spp. (8.08%) and *Capillaria* spp (5.05%) (Bui Khanh Linh et al. 2018). This was mostly the case that primates frequently ingested contaminated food sources, thus they were vulnerable to these parasitic diseases. However, it is very difficult to determine the source of infection as there is little correlation between the number of investigated samples and the daily primate food intake. On the other hand, 155 fecal samples of dogs were examined, in which the overall infection rate was quite high (73.55%). The figure for *Ancylostoma* spp. was the highest (68.39%), followed by *Toxocara* spp. and *Trichuris* spp. Similarly, previous studies on the prevalence of intestinal parasites in dogs in Hanoi and Ho Chi Minh City showed five common species, namely *Spirocercalupi*, *Toxocaracanis*, *Ancylostoma* spp., *Trichuris vulpis*, *Taenia* sp. (Quyen et al. 2015; Nguyen Phi Bang et al. 2016). In our study *Isopora* and *Taenia* eggs were also identified, at the rate of 7.1% and 3.87% respectively. The prevalence of parasite infection in fecal samples was higher than that of leaf samples. However, *Echinococcus* spp. still remained undetected, probably due to the small number of samples or because of its small size (30-43µm in diameter), which might have been missed during examination.

During our investigation, we observed that each household normally owned one to three dogs. In addition, dogs were not routinely dewormed or kept in cages, posing the high risk of spreading pathogens in the environment. In addition, they could become infected if they accidentally ingest intestinal organs with hydatid cysts from cattle. It is also observed from our study location that after being slaughtered at local slaughterhouses, unused cow parts are thrown away or consumed by the butcher's dogs, which might contribute to the circulation of pathogens in the dog population.

Another hypothesis is that primates might be exposed to pathogens in the wild before being rescued to the Center. Thus, we are going to have a follow-up plan on primate individuals born and raised in the center to determine whether there is a case of infection in this group.

## Conclusion

*Echinococcus* eggs have not been found in our current study. However, considering high prevalence of intestinal helminths in leaf and dog fecal samples collected in the surrounding areas of the center, more research needs to be conducted on a larger scale (e.g. further testing of dog fecal samples, consideration of cases of hydatid cysts in local cattle slaughterhouses etc.) to confirm the circulation of pathogens in this region.

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