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Research article

The prevalence and risk factors of *Spirocerca lupi* in domestic dogs in the Mekong Delta of Vietnam

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Abstract

Spirocercosis is caused by *Spirocerca* spp., which is a chronic disease and might cause life-threatening due to forming cancer in oesophagus in canid carnivores. There are limited studies involving spirocercosis in domestic dogs. Thus, this study aims to investigate the prevalence and analyse risk factors involved in the *S. lupi* infection in Mekong Delta in Vietnam. In total, 400 fecal samples from domestic dogs were collected from May 2020 to May 2021. The overall prevalence of spirocercosis in domestic dogs in the Mekong Delta was 10.50% by copromicroscope and PCR methods. PCR targeted to the housekeeping gene cytochrome c oxidase I (cox-1) was applied to identify species of *Spirocerca* spp. and analyse the phylogenetic tree. Outdoor dogs had 5.48 times (CI 95% = 2.45-11.690, $p < 0.001$) higher risks of *S. lupi* infection compared to indoor dogs. Besides, seasons and age showed a correlation to the increase the risk of *S. lupi* infection, while neither dog breeds nor gender influenced the prevalence of this species. The cytochrome c oxidase I (cox-1) gene sequence of *S. lupi* in the Mekong Delta showed the high homologues to the *S. lupi* isolates in India, Israel, and the North of Vietnam and belonged to the *S. lupi* genotype 2.

Keywords: Cox-1, Mekong Delta, Prevalence, Risk factors, *Spirocerca lupi*

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INTRODUCTION

Spirocerca lupi is the causative agent of spirocercosis that is chronic and might cause life-threatening parasitic diseases in canid carnivores such as dogs and foxes. To complete the life cycle, coprophagous beetles serve as the intermediate hosts, and canids act as the definite host. Besides, various paratenic hosts, including rodents, reptiles, hedgehogs, rabbits, poultry and birds can get *S. lupi* infection by the ingestion of coprophagous beetles containing a larvae stage of *S. lupi* (Rojas et al., 2020a). The adult stage of *S. lupi* is found in oesophageal fibrous nodules of canids, inducing the formation of fibrosarcoma and osteosarcoma in the oesophagus of dogs (Rojas et al., 2018; Rojas et al., 2020b).

The clinical symptoms of spirocercosis are characterized as weakness, hyper-salivation, regurgitation, vomiting, dyspnea, vertebral spondylitis, and hypertrophic osteopathy (Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004).

The spirocercosis has frequently occurred in many tropical and subtropical areas such as China (78.6%), Bangladesh 40%, India 23.5%, Kenya 85%, and Greece 10% (Mylonakis et al., 2001) in domestic dogs. Recently, studies have revealed the emergence of *S. lupi* in canid populations in many countries such as Mexico (Riodríguez-Vivas et al., 2019), and Hungary (Psáder et al., 2017). This might be explained due to the dramatic climate change, leading to the increase in the population of the intermediate host. In contrast to the epidemiological studies worldwide about *S. lupi* in dogs, the information about this species in Southeast Asia is sporadic (Hoa et al., 2021). This study therefore aims to investigate the infection rate analyzing risk factors and molecular characterization of *S. lupi* in dogs in the Mekong Delta by combining the copromicroscopic and molecular techniques.

MATERIALS AND METHODS

Sample size

To determine the sample size, the formula of Thursfield (2007) $N = \frac{1.96^2 \times pq}{d^2}$ was applied. In this formula, *N* stands for the required sample size, p indicates expected prevalence, *q* presents 1-*p*, and *d* refers desires absolute precision. As no studies have been conducted on the infection rate of spirocercosis in domestic dogs in Mekong Delta, the expected prevalence is assumed 50%. With d=5% at a 95% confidence interval and the expected prevalence at 50%, the minimum requirement is 384 samples. To improve the accuracy in the study, 400 fecal samples were randomly collected from dogs in the Mekong Delta (namely in An Giang, Ben Tre, Can Tho and Kien Giang).

Collecting sample and microscopic identification

Fresh fecal samples were collected from domestic dogs with the help of owners. The samples were stored at 4°C until examination. The fecal samples were instantaneously examined or latest within 24 - 48 hours. Each fecal sample (2 grams) was crushed and homogenized in saturated Zinc sulfate ($ZnSO_4$). The homogenate was filtered through a sieve to remove debris, followed by centrifugation step (1000 x g) for 10 minutes. Then, the supernatant

was subjected to screen for the presence of *Spirocerca* spp. eggs by visualizing under the microscope. The identification of *Spirocerca* spp. eggs was based on the morphological characteristics which was described by Markovics and Medinski (1996).

DNA extraction and PCR amplification

The positive samples with *Spirocerca* spp. by coropmicroscopic method were subjected to DNA extraction. Feces samples (200 mg) were extracted by using QIAGEN stool kit (Germany). The specific primers including forward primer SF: 5'-TCTTTGTTGGTGGAGGTGCT-3' and reverse primer SR: 5'-GACCCCACACAGAAGTACCC-3' were designed by using PRIMER-BLAST, targeting the mitochondrial *cox-1* (cytochrome oxidase subunit 1) gene of *S. lupi*.

The PCR amplifications were conducted by using Bio-Rad thermal cycler C1000. Each PCR reaction was performed, comprising of 12.5 µl Master Mix 2X (Meridian Bioscience®), 0.5 µl of primer (25 µM), DNA template (2 µl), and DNase/RNase-free distilled water up to 25 µl. The cycling conditions include of initial denaturation at 96°C for 5 minutes (1 cycle), followed by 35 cycles of 95°C denaturation for 30 seconds, 58°C annealing for 1 minute, 72°C extensions for 1 minute 30 seconds, and a final extension 72°C for 7 minutes. After amplification, PCR products were loaded on 1.5% (w/v) agarose gel in 1X TAE 100V in 45 min with a 100 bp maker DNA (PhuSa Genomics). The gel was visualized under UV light (Biorad UV 2000) after being stained with ethidium bromide (0.5 µg/ml). Purified PCR products were delivered to Phu Sa Company for sequencing.

Data Analysis

The difference in the infection rate among various age groups of dogs and odd ratios were calculated by Chi-Square ($\gamma 2$) test using GraphPad Prism version 8.0.2. The results indicated to be the statistically significance at the p-value < 0.05. Nucleotide sequences were analysed by BioEdit software and BLAST in NCBI. Further, *cox-1* sequences of *S. lupi* were aligned with other reference sequences by using ClustalW. The Maximum Likelihood method and Tamura-Nei model have been used to analyse the genetic relationship among the sequences, then the phylogenetic tree was constructed by MEGA X. The *cox-1* gene sequence of *Spirocerca* spp. used in the phylogenetic analysis were obtained through Genbank. The nucleotide sequence of *S. lupi* in this study was deposited in Genbank with accession number OP592222. Isolates including the corresponding accession number are described in more detail in Table 1.

Table 1 Sequences of *cox-1* used to perform phylogenetic analysis.

Genbank accession no.	Species strain	Original
LC597860.1	*Spirocerca lupi*	Vietnam
OP592222	*Spirocerca lupi*	This study
MG957144.1	*Spirocerca lupi*	Israel
MH634010.1	*Spirocerca lupi*	India
MH634005.1	*Spirocerca lupi*	Israel
MF403001.1	*Spirocerca lupi*	Peru
MH633997.1	*Spirocerca lupi*	Israel
EF195133.1	*Spirocerca lupi*	Asia
MK577664.1	*Spirocerca lupi*	India
MH634011.1	*Spirocerca lupi*	Hungary
MH634012.1	*Spirocerca lupi*	Hungary
MT522373.1	*Spirocerca lupi*	Iran
KJ605486.1	*Spirocerca sp.*	Denmark
MT309693.1	*Spirocerca vulpis*	Switzerland
MT309674.1	*Spirocerca vulpis*	Portugal
MT309695.1	*Spirocerca vulpis*	Switzerland
MH633993.1	*Spirocerca vulpis*	Spain
AB243755.1	*Taenia solium*	

RESULTS

The infection rate of *S. lupi* in domestic dogs in the Mekong Delta

In total, 400 samples from domestic dogs were randomly collected to detect the presence of *Spirocerca* spp. by copromicroscope. *Spriocerca* spp. eggs were identified based on the morphological characteristics which were elongated with parallel sides and containing larvae (Figure 1). The measurements were documented based on the length and width of 100 eggs, in which the length ranged between 31.00-35.00 µm ($\bar{x}$= 33.49, SD= 1.30) and width 12.04-15.01 µm ($\bar{x}$=13.76, SD=0.80).

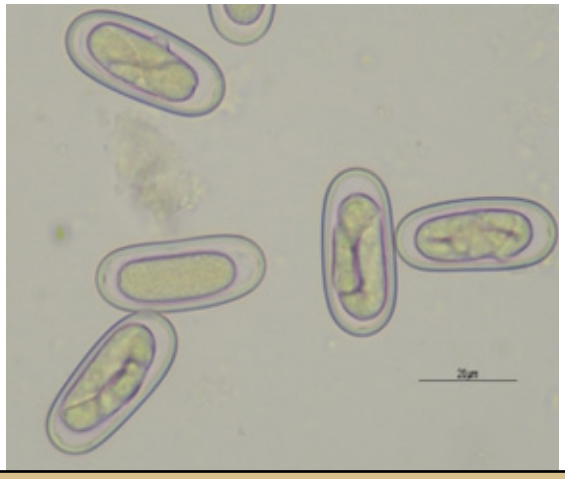

Figure 1 The morphological characteristics of *Spirocerca* spp. eggs in fecal samples, scale bar: 20 µm.

The overall prevalence of *Spirocerca* spp. in surveyed areas was 10.50% (42/400) (Table 2). The highest infection rate of *Spirocerca* spp. was found in surveyed dogs Kien Giang province with 12.37%, followed by An Giang and Ben Tre provinces with 10.89% and 10.11%, respectively. The lowest infection rate of *S. lupi* in Can Tho city was 8.85%; however, the difference in prevalence among surveyed areas was not significant ($p > 0.05$). All positive samples by morphological identification methods were further confirmed by PCR with specific primers for *S. lupi* targeting to *cox-1* gene. The results found that tested samples were positive with *S. lupi* with the successful amplification of PCR product around 520 bp. Therefore, the study revealed that dogs in Mekong Delta were infected with *S. lupi*.

Table 2 The prevalence of *Spirocerca* spp. infection in domestic dogs in Mekong Delta.

Regions	No. examined samples	No. infected samples	Infection rate (%)
An Giang	101	11	10.89
Kien Giang	97	12	12.36
Ben Tre	89	9	10.11
Can Tho	113	10	8.85
Total	400	42	10.50

Risk factors associated with S. lupi infection in domestic dogs

The results (Table 3) illustrated that the lifestyle of dogs were infected with *S. lupi*. The factor of lifestyle shows the most influence on the prevalence of spirocercosis in domestic dogs. Outdoor dogs had 5.48 times higher risk of *S. lupi* infection (OR=5.48, CI 95% from 2.45 to 11.69, $p < 0.001$) compared to indoor dogs. Seasons were the second-highest risk factor for *S. lupi* infection. The rainy season (from May to December) increased 2.46 times risk of *S. lupi* infection compared to the sunny season (CI 95%: 1.24 to 5.06, $p < 0.01$). The age of dogs increased the risk of *S. lupi* infection. The infection rate of *S. lupi* in adult dogs (>12 months) was 15.28%, which was significantly higher than that in young dogs (<12 months) at 11.54% ($p < 0.001$). With regard to regions, breeds and genders, there was no significant difference ($p > 0.05$).

Table 3 Analysis the risk factor of *Spirocerca lupi* in domestic dogs in Mekong Delta.

Factor	No. Positive	No. Examined	Prevalence (%)	OR (95% CI)	P value
Region					
An Giang	11	101	10.89	1.26 (0.52 to 3.15)	0.87
Kien Giang	12	97	12.37	1.45 (0.63 to 3.56)	
Ben Tre	9	89	10.11	1.16 (0.48 to 3.09)	
Can Tho*	10	113	8.85		
Breeds					
Foreign	9	118	7.63	0.62 (0.29 to 1.29)	0.22
Local breed	33	282	11.70		
Age					
>12 months	35	229	15.28		
	4.23 (1.88 to 10.14)	0.0003			
<12 months	7	171	11.54		
Lifestyle					
Outdoor	34	276	12.32	5.48 (2.45 to 11.69)	<0.0001
Indoor	8	124	6.45		
Season					
Rainy (May-November)	29	216	13.43	2.46 (1.24 to 5.06)	0.0087
Dry (December -April)	13	184	7.07		
Gender					
Male	22	204	10.78	1.06 (0.56 to 1.96)	0.8499
Female	20	196	10.20		

Analysis of the phylogenetic tree of S. lupi in domestic dogs

The phylogenetic tree (Figure 2) illustrates that the sequences of *S. lupi* from the Mekong Delta Vietnam (OP592222) was grouped with *S. lupi* from Israel, India, and another Vietnamese isolate from the North of Vietnam. Those isolates belonged to genotype 1 (Rojas et al., 2018), while the isolates of *S. lupi* from Hungary were formed a separate group, and were classified as genotype 2 (Rojas et al., 2018), the sequences of *S. vulpis* established a distinct clade from other sequences of *S. lupi*.

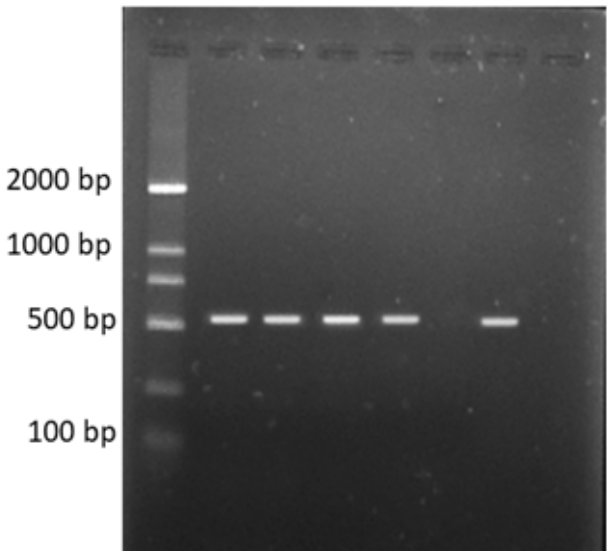

Figure 2 Agarose gel electrophoresis of PCR amplifications targeting to cox-1 sequence of Spirocerca lupi. Lane 1: DNA ladder 100bp, lane 2, 3, 4, 5: samples, lane 6: negative control and lane 7: positive control (520 bp).

Analyzing the pairwise distances revealed that the *cox-1* sequence of *S. lupi* in Mekong Delta was highly similar to *S. lupi* isolates in India, in Israel (isolate Q- MG957144.1), and in North of Vietnam with 97.92%. However, it was different from isolate L, C of *S. lupi* from Israel and Peru (4.12 - 6.25%) and Hungarian isolates from dogs with 8.33%. The interspecific variations between *S. lupi* and *S. vulpis* varied from 6.25 to 11.45%.

DISCUSSION

S. lupi is a typical spirurid parasite associated with oesophagus sarcoma of canids. The presence of *Spirocerca* spp. eggs in canids was normally confirmed as *S. lupi* because it is the ubiquitous species of canids. However, the identification of *Spirocerca* species should be more cautious due to the new described *Spirocerca* species in various canid hosts- *S. vulpis* originating from red foxes (Rojas et al., 2018). Recently studies in Africa, Europe, Asia, and America have shown a rise in the number of canids (Riodríguez-Vivas et al., 2019; Rojas et al., 2020a) suffering from the infection with the new species, *S. vulpis*. Therefore, this study has been conducted based on both egg morphological features and molecular data to identify *Spirocerca* spp in domestic dogs. The length and width of *S. lupi* eggs ranged between 31.00-35.00 µm ($\bar{x}$= 33.49, SD= 1.30) and 12.04-15.01 µm ($\bar{x}$=13.76, SD=0.78), respectively. Those measurements were compatible with morphological data of *S. lupi* eggs in the studies of Rojas et al. (2018), Hoa et al. (2021). Moreover, the PCR results target to *cox-1* gene which has been frequently used to differentiate S.lupi and S.vulpis (Hoa et al., 2021) were consolidated for morphological identification results. Thus, domestic dogs in Mekong Delta were infected with *S. lupi*, which was in line with achieved results of Hoa et al. (2021) who identified the presence of the *Spirocerca* spp. population in Vietnam.

The overall prevalence of *S. lupi* infection was 10.50%, which was lower than that by many studies in Kenya (38%) (Brodey et al., 1977), South Africa (13%) (Minnaar et al., 2002) , and North Vietnam (17.7%) (Hoa et al., 2021).

The difference in prevalence of *S. lupi* infection was attributed to many elements such as region, season, lifestyle, and age of dogs (Oryan et al., 2008; Riodríguez-Vivas et al., 2019).

Of many risk factors, the lifestyle of dogs was considered the most influential factors with regard to the increase in the *S. lupi* incidences. For instance, outdoor dogs had 5.48 times higher risk of *S. lupi* infection compared to indoor dogs. The outdoor lifestyle of dogs might facilitate them to ingest the intermediate hosts such as coprophagous beetles or paratenic hosts such as lizards, poultry, and rodents (Rojas et al., 2020b) that carried the larvae L3 stage of *S. lupi* than indoor dogs did. Outdoor dogs in rural areas were found to be more accessible to the livestock stools and agriculture-by-products which were feeding source of dung beetle population or the environment for development of paratenic hosts as rodents than indoor dogs.

The season was the next concerning risk factor of *S. lupi* infection. Although the season factor was not directly influence to the host-parasite interaction, it intimately involved in the population development of coprophagous beetles- intermediate host of *S. lupi*. In fact, the temperature and moisture has been proved as the two important elements in the size, reproduction, and activity of coprophagous beetles (Vessby, 2001). Moreover, the study by Araújo et al. (2022) found that the abundance of species and quantities of coprophagous beetles population correlated to the summer season. The rainy season in Mekong Delta in Vietnam commences in May and ends in September/October; therefore, it shares some similarities to the summer months (June-August) in terms of the climate conditions such as temperature and high humidity (Tran et al., 2015). Thereby, the development of coprophagous beetles in the rainy season might cause the rise of the incidences of spirocercosis in dogs compared to the dry season.

Another risk factor associated with the infection of *S. lupi* was the age of canines. Our study indicates that adult dogs (> 12 months old) had higher incidences of *S. lupi* infection (OR= 4.23, CI 95% 1.88 to 10.14) compared to young dogs. These data are coincident with previous studies of Oryan et al. (2008) and Riodríguez-Vivas et al. (2019). The embryonated eggs of *S. lupi* only hatch when they are ingested by coprophagous beetles and it takes two months to transform to larvae stage L3 (infective stage) and become encysted (Oryan et al., 2008). The larvae stage is liberated from intermediate hosts after being swallowed by canid hosts. The prepatent period of *S. lupi* is approximately 6 months (Marchiondo, 2019). Furthermore, young dogs were usually kept in houses that also prevented them from incidentally ingesting intermediate hosts. Although previous studies illustrated that the occurrence of spirocercosis endemic was affected by the geographical factor, there was no difference in the infection rate of *S. lupi* among surveyed areas in this study. It can be interpreted as investigated regions had high similarities in the altitude and climate features. Moreover, there was no gender predilection for *S. lupi* infection. Our findings were consistent with those by other reports in literature (Wandera, 1976; Fox et al., 1988; Oryan et al., 2008). Besides, the study found that the infection rate of *S. lupi* in domestic dogs was not affected by breed factors ($p > 0.05$).

Differentiation *S. lupi* and *S. vulpis* might confuse by applying only morphological identification (Hoa et al., 2021). The molecular analyses would be effective tools to consolidate the morphological results. Due to analyzing the

phylogenetic tree based on the *cox-1* sequence, *S. lupi* formed a separate cluster to *S. vulpis* (Figure 3). The genetic differences of *S. lupi* and *S. vulpis* have been described in previous studies (Rojas et al., 2018). The isolate of *S. lupi* in the Mekong Delta belonged to the main group (genotype 1) including the isolates from the North of Vietnam, Israel, India (isolate Q), Peru, Asia, and Iran while the isolates of *S. lupi* from Hungary (genotype 2) formed a distinct cluster. The classification genotype 1 and 2 of *S. lupi* were investigated by Rojas et al. (2018). Therefore, the genetic diversity of Spriocerca spp might be influenced by geographical locations or definitive hosts (Rojas et al., 2018).

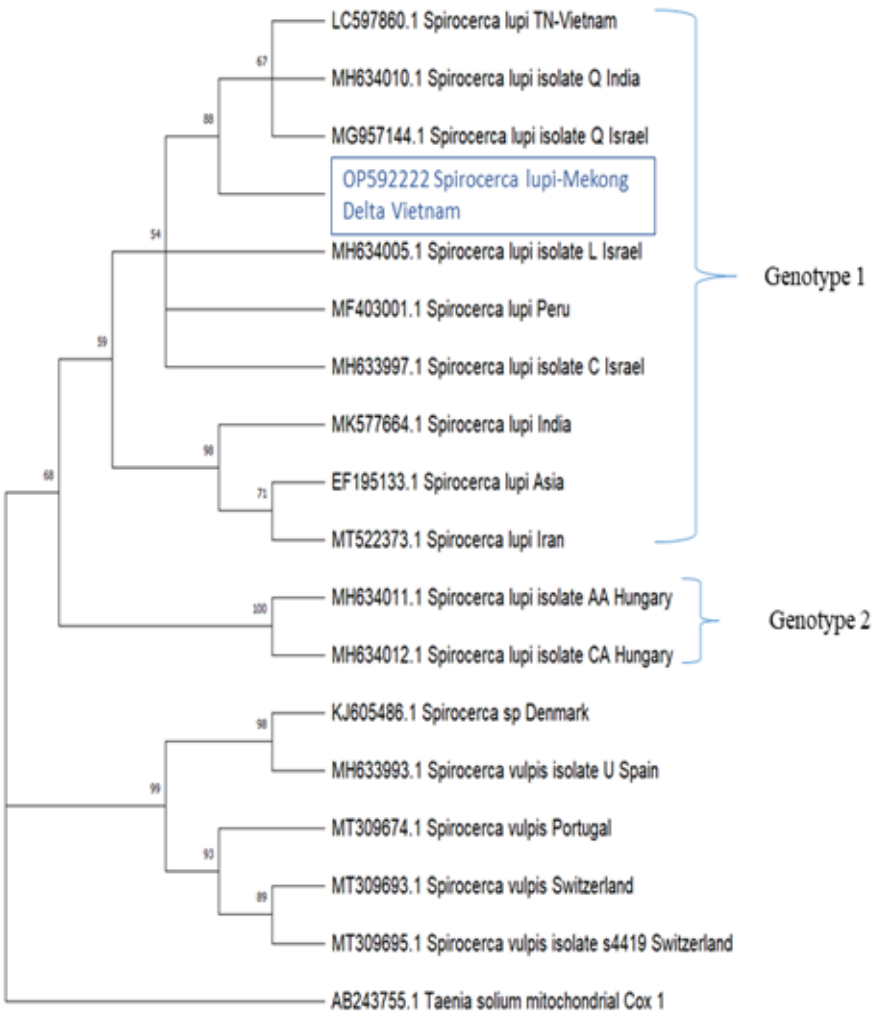

Figure 3 Phylogenetic tree was constructed from *cox-1* sequence of *Spirocerca* spp. by applied Maximum Likelihood method, Tamura-Nei model. Bootstrap analysis was performed using 1000 replicates. The numbers in the branches indicated the bootstrap values. Taenia solium AB243755.1 was used to outgroup.

CONCLUSIONS

To our knowledge, this is the first molecular biology-based investigation of the prevalence and risk factors of *S. lupi* in the Mekong Delta. Despite the fact that *S. lupi* infestation in domestic dogs was still moderate, infected dogs were at a significant risk of getting sarcoma. To conduct additional research, it is preferable to combine molecular biology and radiographic testing to discover *S. lupi* at early stage and offer appropriate therapies.

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AUTHOR CONTRIBUTIONS

Tran Nguyen-Ho-Bao: Conceptualization, performed experiments, supervision, original draft preparation, editing and finalization.

Chuc Thi Nguyen: Investigation, performed experiment, data analyzing, interpretation of study, writing and editing the manuscript.

Bang Phi Nguyen: Investigation, statistic analyzing, contribution in finalization.

Hung Huu Nguyen: Conceptualization, supervision, methodological development, interpretation of study, writing manuscript.

CONFLICT OF INTEREST

We have no conflict of interest.

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