

Prevalence of *Hymenolepis nana* and *H. diminuta* from Brown Rats (*Rattus norvegicus*) in Heilongjiang Province, China

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Abstract: *Hymenolepis nana* and *Hymenolepis diminuta* are globally widespread zoonotic cestodes. Rodents are the main reservoir host of these cestodes. Brown rats (*Rattus norvegicus*) are the best known and most common rats, and usually live wherever humans live, especially in less than desirable hygiene conditions. Due to the little information of the 2 hymenolepidid species in brown rats in China, the aim of this study was to understand the prevalence and genetic characterization of *H. nana* and *H. diminuta* in brown rats in Heilongjiang Province, China. Total 114 fecal samples were collected from brown rats in Heilongjiang Province. All the samples were subjected to morphological examinations by microscopy and genetic analysis by PCR amplification of the mitochondrial cytochrome c oxidase subunit 1 (*COX1*) gene and the internal transcribed spacer 2 (*ITS2*) region of the nuclear ribosomal RNA gene. In total, 6.1% (7/114) and 14.9% (17/114) of samples were positive for *H. nana* and *H. diminuta*, respectively. Among them, 7 and 3 *H. nana* isolates were successfully amplified and sequenced at the *COX1* and *ITS2* loci, respectively. No nucleotide variations were found among *H. nana* isolates at either of the 2 loci. Seventeen *H. diminuta* isolates produced 2 different *COX1* sequences while 7 *ITS2* sequences obtained were identical to each other. The present results of *H. nana* and *H. diminuta* infections in brown rats implied the risk of zoonotic transmission of hymenolepiasis in China. These molecular data will be helpful to deeply study intra-specific variations within *Hymenolepis* cestodes in the future.

Key words: *Hymenolepis nana*, *Hymenolepis diminuta*, brown rat, mitochondrial gene, nuclear ribosomal RNA

Hymenolepiasis is a neglected zoonotic disease in humans, caused by cestodes *Hymenolepis nana* (dwarf tapeworm) and *H. diminuta* (rat tapeworm) [1]. *H. nana* and *H. diminuta* are globally widespread, but endemic to Asia, Southern and Eastern Europe, Central and South America, and Africa [2]. Epidemiological data have revealed that *H. nana* is more commonly reported as the cause of human hymenolepiasis than *H. diminuta*. More than 175 million cases of hymenolepiasis caused by *H. nana* have been reported in humans worldwide [3]. In contrast, only a few hundred people have been described to be infected with *H. diminuta* [4]. Generally, human cases of hymenolepiasis mostly appear asymptomatic; however, humans infected with these parasites are sometimes responsible for mild clinical symptoms, mainly including diarrhea, abdominal pain, anorexia, and vague gastrointestinal

manifestations [5,6]. Most seriously, infection of *H. nana* and *H. diminuta* ultimately can cause severe diseases, even life threatening conditions in immunosuppressed individuals with HIV [7,8].

Rodents are highly successful in adapting to a variety of environments throughout the world, which makes them extremely abundant. They are known as reservoirs or carriers of zoonotic bacteria, virus, and parasites, endangering public health by spreading various diseases via food or water destruction and contamination. Among them, brown rats (*Rattus norvegicus*) are the best known and most common, and usually live wherever humans live, especially in less than desirable hygiene conditions.

H. nana and *H. diminuta* have been detected in brown rats in many countries and areas. *H. nana* has been found in the Netherlands; 3.3% (1/30) in farms and 4.1% (2/49) in rural environments in 2016 [9]; 8.8% (10/112) in Brazil in 2016 [10]; 21.8% (7/32) in Taiwan in 2013 [11], and 100% (10/10) in Italy in 2015 [12]. *H. diminuta* has been found in the Netherlands; 50% (15/30) in farms, 10.2% (5/49) in rural environments, and 10.5% (4/38) in suburban environments in 2016

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[9]; 6.3% (2/32) in Taiwan in 2013 [11]; 30.5% (92/302) in Serbia in 2011 [13], and 62.5% (5/8) in India in 2009 [14]. In China, to date, only 2 studies reported hymenolepiasis in brown rats: 3.3% (5/151) for *H. nana* and 27.8% (42/151) for *H. diminuta* in Guangdong in 2004 [15] and 10.3% (6/58) for *H. nana* in Xinjiang in 2003 [16].

In the diagnosis of hymenolepiasis and differentiation of causative species, eggs recovered from host feces usually play an important role for identifying their morphological features [17]. However, PCR-based molecular techniques not only increase detection rates of parasites, but also provide the accurate species differentiation and their genetic characterizations [18]. Currently, the first and second internal transcribed spacer regions (ITS1 and ITS2) of nuclear ribosomal RNA gene can be helpful for resolving remarkable taxonomic issues and discriminating closely related genera and species [18]. Meanwhile, mitochondrial (mt) genome sequences have been proven to be useful and reliable genetic markers for population genetics and systematic studies [18].

Northeastern China's Heilongjiang Province is the biggest agricultural province and considered as the important commodity grain production base. To date, little information is available on *H. nana* and *H. diminuta* infections in these animals in this province [19]. During the period from April 2014 to March 2016, a total of 114 brown rats were captured using traps. They were collected from 4 different areas in Heilongjiang Province, including a granary in Xingren Town (n=23), a pig farm in Mingshui County (n=27), a pig farm in Qinggang County (n=27), and a sheep farm in Baoqing County (n=37). All the captured rats were euthanized by CO₂ inhalation. Fecal materials were collected directly from the intestine section of each rodent. Each sample was detected for the presence of *H. nana* and *H. diminuta* eggs using direct smear method by bright-field microscopy under ×100 and ×400. The present study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Har-

bin Medical University, P. R. China (no. HMUIRB20130009).

Fecal samples were sieved and washed with distilled water by centrifugation for 10 min at 1,500 g. Processed samples were stored in -20°C prior to being used in molecular analysis. Genomic DNA was extracted from approximately 180-200 mg washed fecal pellets using a commercially available kit (QIAamp DNA Mini Stool Kit, Qiagen, Hilden, Germany) according to the manufacturer-recommended procedures. Eluted DNA (200 µl) was kept frozen at -20°C until its analysis with PCR. All the DNA samples were detected for the presence of *H. nana* and *H. diminuta* by PCR amplification of a 391 bp nucleotide fragment of *COX1* gene and 671-741 bp *ITS2* region. The 2 sets of primers and PCR cycling parameters were used as previously described [20,21]. TaKaRa TaqDNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all the PCR amplifications. A negative control with absence of DNA was included in all PCR tests. All the PCR products mentioned above were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide before sequencing.

PCR products of *COX1* gene and *ITS2* region were sequenced in 2 directions with their respective PCR primers on an ABI PRISM3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA), using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). All the sequences obtained in the present study were compared with each other and reference sequences downloaded from GenBank database using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalX 1.83 (<http://www.clustal.org/>). The sequences with single nucleotide substitutions, deletions or insertions compared to published sequences were all further confirmed by DNA sequencing of at least 2 PCR products. Representative nucleotide sequences obtained in this study were deposited in the GenBank database under the accession nos. KY079336 for *H. nana* and KY079337 to KY079339 for *H. diminuta*.

In the present study, 2 cestode species were detected in

Table 1. Infection rates of *Hymenolepis nana* and *H. diminuta* in brown rats by microscopy and PCR

Collection site (county)	No. examined	No. positive for <i>H. nana</i> (%)			No. positive for <i>H. diminuta</i> (%)		
		By microscopy	<i>COX1</i>	<i>ITS2</i>	By microscopy	<i>COX1</i>	<i>ITS2</i>
Granary (Xingren)	23	3 (13.0)	3 (13.0)	1 (4.3)	-	6 (26.0)	2 (8.7)
Pig farm (Mingshui)	27	1 (3.7)	2 (7.4)	-	4 (14.8)	5 (18.5)	3 (11.1)
Pig farm (Qinggang)	27	-	-	-	2 (7.4)	4 (14.8)	2 (7.4)
Sheep farm (Baoqing)	37	-	2 (5.4)	2 (5.4)	-	2 (5.4)	-
Total	114	4 (3.5)	7 (6.1)	3 (2.6)	6 (5.3)	17 (14.9)	7 (6.1)

brown rats in the investigated areas, and *H. diminuta* (14.9%) showed a higher infection rate than *H. nana* (6.1%) (Table 1). Similar results occurred in brown rats in the Netherlands, with 10.2-50.0% for *H. diminuta* versus 0-4.1% for *H. nana* [9]. However, in a study of the endoparasites of brown rats conducted in Taiwan, *H. nana* (21.8%) was observed to be more common than *H. diminuta* (6.3%) [11].

H. diminuta eggs were observed in 5.3% (6/114) by microscopy while 14.9% (17/114) and 6.1% (7/114) at the *COX1* and *ITS2* loci by PCR, respectively. *H. nana* eggs were identified in 3.5% (4/114) by microscopy, while 6.1% (7/114) at the *COX1* locus and 2.6% (3/114) at the *ITS2* locus by PCR (Table 1). Here, PCR was observed to be 1.7 and 2.8 times as sensitive as microscopy in the diagnosis of *H. nana* and *H. diminuta* at the *COX1* locus, respectively. Not surprisingly, PCR-based detection was more sensitive than microscopy. It is known that microscopic techniques are closely related to the infection intensity of parasite eggs in feces. In fact, usually, egg numbers can be very low with sporadic egg shedding, leading to under-diagnosis of hymenolepiasis [22]. Thus, PCR is recommended to be used in the future epidemiological studies of human and animal hymenolepiasis, which not only increases detection rate but also helps us to understand their molecular characterizations.

In the present study, PCR had a higher detection rate at the *COX1* locus than at the *ITS2* locus in detecting eggs of *H. nana* and *H. diminuta*, with 6.1% vs 2.6% for *H. nana* and 14.9% vs 6.1% for *H. diminuta*. Many factors can influence the efficiency of PCR amplification, mainly including the primers and genetic structure of target fragments, the quality and quantity of DNA templates, and the quality and characterization of DNA polymerase used. Here, since the same DNA preparations and DNA polymerase were used to amplify the *COX1* gene and *ITS2* region of the 2 parasites, the degree of the primers binding DNA templates was likely to be the main reason for the ampli-

fication differences. The primers were originally designed from 'conserved' regions in the amplified genes. However, excessive mismatches in the binding regions of primer sequences might result in the failure of PCR amplification. In fact, numerous molecular data have confirmed both intra-specific genetic variations of *H. nana* and *H. diminuta* [23-25].

The highest infection rates of *H. nana* (13.0%) and *H. diminuta* (26.0%) occurred in the rats from a granary, while the lowest (5.4%) from a sheep farm for either of the 2 parasites (Table 1). The current study is the first report of *H. nana* and *H. diminuta* in brown rats from a granary in China. It is well-known that granaries often have a high intensity of rats, and provide suitable environments for arthropods as intermediate hosts of the 2 parasites. This might increase the opportunity of *H. nana* and *H. diminuta* infection in these animals. In particular, *H. diminuta* does necessarily depend on arthropods including flour or grain beetles to complete its life cycle. Thus, measures should be taken to protect grains from rodents and insects.

In the present study, 7 *COX1* gene sequences of *H. nana* were obtained, which were identical to each other (KY079336) and had the largest similarity (99.2%) with that from *Mesocricetus auratus* (AB494472). 3 *ITS2* sequences of *H. nana* obtained here had 100% similarity with that from *Mus musculus* (HM536187). Likewise, at the *ITS2* locus, 7 *H. diminuta* isolates were successfully amplified and produced the same sequence (KY079339), which had the largest homology (99.9%) with that from *Rattus norvegicus* (AB494475). However, intra-specific variation was found in *H. diminuta* isolates at the *COX1* locus. 17 *H. diminuta* isolates produced 2 different *COX1* gene sequences (KY079337 and KY079338), both of which had the largest similarity with that sequence (AF096244). The results of homology analysis of *H. nana* and *H. diminuta* isolates at the *COX1* and *ITS2* loci were shown in Table 2. The result that no intra-specific variation was found in *H. nana* iso-

Table 2. Homology analysis in nucleotides at *COX1* and *ITS2* loci of *Hymenolepis nana* and *H. diminuta*

Species	Loci amplified	Accession no ^a	Accession no ^b	Homology (%)	Nucleotide (Position)
<i>H. nana</i>	<i>COX1</i>	KY079336	AB494472	99.23	T to G/20; T to C/317; T to C/335
	<i>ITS2</i>		HM536187	100.00	
<i>H. diminuta</i>	<i>COX1</i>	KY079337	AF096244	99.10	C to T/57; A to T/84; A to G/352; C to T/355
		KY079338	AF096244	99.75	C to T/118
	<i>ITS2</i>	KY079339	AB494475	99.86	A to T/691

^aAccession nos. indicating the novel sequences obtained in the present study.

^bAccession nos. indicating the sequences downloaded from GeneBank which have the largest homology with the sequences obtained in the present study.

lates at the 2 loci might be related to the small number of *H. nana* isolates analyzed here. A previous study indicated extensive intra-specific variations of *H. nana* at the *COX1* locus between 2 mouse-derived isolates as well as between human-derived and rodent-derived isolates [24]. A recent study revealed the presence of intra-specific variation of *H. nana* in the *ITS2* region [23]. In fact, hymenolepidid species were observed to have larger intra-specific variations at the *COX1* locus than at the *ITS2* locus based on phylogenetic analysis [25]. Here, all the 3 representative *COX1* gene sequences (1 from *H. nana* and 2 from *H. diminuta*) and 1 *ITS2* sequence from *H. diminuta* were not reported previously.

In the present study, because only a few *H. nana* and *H. diminuta* isolates were analyzed genetically, no genetic difference was found in geographical distribution. Mohammadzadeh et al. [26] also believed that genetic characteristic was not always related with geographical distribution. The same conclusion was drawn by sequence and phylogenetic analyses of other mt genes (*atp6*, *pnad5*, and *rrnS*) of 42 *H. nana* isolates from 7 provinces in China [27]. However, it was reported on Canary Islands in Spain that the *COX1* gene sequences of *H. diminuta* from 2 islands (Lazarote and Fuerteventura) were genetically distant from those from other islands [28].

The present study described the occurrence of *H. nana* and *H. diminuta* in brown rats in Heilongjiang Province, suggesting that rodents infected with both cestodes have the potential to transmit hymenolepiasis to humans. In fact, human cases of hymenolepiasis caused by *H. nana* ($n=14$) and *H. diminuta* ($n=1$) have been reported in the investigated areas [29,30]. Clinically, hymenolepiasis is often neglected or underreported due to more asymptomatic infections in humans. However, 2 death cases caused by *H. nana* in immunosuppressed individuals highlight the severity of hymenolepiasis [7,8]. The importance and necessity to carry out epidemiological investigations of human hymenolepiasis is undeniable in the future, including assessment of transmission dynamic and the burden of human hymenolepiasis attributable to zoonotic transmission.

In conclusion, our present study demonstrated the occurrence of *H. nana* and *H. diminuta* in brown rats in Heilongjiang Province, China, implying that rodents infected with the 2 *Hymenolepis* species have the risk of transmitting hymenolepiasis to humans. Molecular data will be helpful to deeply study intra-specific variations within *Hymenolepis* cestodes in the future.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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