Experimental Parasitology 129 (2011) 355–361

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Human infections of fish-borne trematodes in Vietnam: Prevalence and molecular specific identification at an endemic commune in Nam Dinh province

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ARTICLE INFO

Article history:
Received 6 June 2011
Received in revised form 24 August 2011
Accepted 13 September 2011
Available online 19 September 2011

Keywords:
Food borne rematodes
Clonorchis sinensis
Haplorchis taichui
Haplorchis pumilio
Centrocestus formosanus
Molecular identification
Phylogeny
Vietnam

ABSTRACT

The prevalence of fish-borne trematodes in humans and their molecular identification was investigated in the Rang Dong commune of Nam Dinh province, Vietnam, between January 2009 and December 2010. A total of 405 people in this commune were interviewed on the habit of eating raw fish and all of their stool samples were collected using the Kato-Katz technique for examination of the presence of fish-borne trematodes. The worms (and eggs) were first morphologically examined, counted, described and identified, then the representative isolates were subjected for molecular species confirmation. A total of 385 adult flukes collected from 10 patients were morphologically identified to species and defined as *Clonorchis sinensis* (14.58%) in Opisthorchiidae family, *Haplorchis taichui* (32.29%), *Haplorchis pumilio* (52.08%) and *Centrocestus formosanus* (1.04%) in Heterophyidae family. A high rate (77.8%) of the interviewees was found to have the habit of eating raw fish. This habit was attributed to the high infection rate of fish-borne trematode in humans (22.72%; OR = 2.486). The infection rate of fish-borne trematodes in males was higher (29.3%) than that in females (16.0%) and increased by age, reaching the highest in the patients aged 40–59 years (28.2–28.7%). The infection intensity of fish-borne trematode was found light (336 EPG). Adult flukes were collected from a group of the patients with the highest intensity of infection and subjected to molecular and phylogenetic analysis using a portion (326 bp) of mitochondrial *cox1*. Phylogenetic tree inferred from *cox1* sequences using sequence data for 34 isolates of opisthorchid, heterophyid, fasciolid, paragonimid, schistosomid trematodes and taeniid cestodes revealed that they are distinct groups. The newly collected with the known clonorchid and heterophyid isolates form the well defined taxonomic groups, respectively, confirming that *C. sinensis* and *Haplorchis* spp. (*H. pumilio* and *H. taichui*) were among the collected samples.

1. Introduction

Fish-borne trematode infections comprising small liver flukes and minute intestinal flukes are known to be widespread in Asia including Vietnam (Chai et al., 2005, 2009; Olsen et al., 2006). Fish-borne trematodes still remain the zoonotic agents of heavily infected neglected tropical diseases causing a major public health problem in Vietnam (De et al., 2003; Dung et al., 2007).

Small liver flukes are regionally distributed (De et al., 2003; Le et al., 2006), with a remarkable feature of clear geographical separation in Vietnam that *Clonorchis sinensis* is found only in the North of the country, which is similar to that reported in the People's Republic of China (Yu et al., 2003; Yu and Xu, 2005) and *Opisthorchis viverrini* in the Southern provinces, which is similar to that in Thailand and Lao People's Democratic Republic (Sithithaworn and Haswell-Elkins, 2003; Waikagul and Radomyos, 2005). The potential overlapping zone is predicted in the middle part of Vietnam comprising Nghe An, Ha Tinh, Quang Binh and Quang Tri provinces (Le et al., 2006). However, up to now, there has not been any isolation of both species samples in any host and any particular province of these geographical regions (De et al., 2003; Le et al., 2006). These two small liver flukes (i.e., *C. sinensis* and *O. viverrini*) were found in so far 24 provinces in Vietnam (by stool examination; partly molecular confirmation) featuring the infection rate varying from 0.2% in Bac Kan province to 37% in Nam Dinh and Phu Yen provinces, with exception of Ha Tay province, up to 40% (De, 2004; Verle et al., 2003).

Other heterophyids have also been identified, giving rise to the preliminary country report that minute intestinal flukes were distributed in more than 18 provinces, including "*hot spot*" Nam Dinh province (De, 2004; Skov et al., 2009; Dung et al., 2007; Van et al., 2009). Adult worms collected from patients have been identified as *C. sinensis*, *Haplorchis taichui*, *Haplorchis pumilio*, *Stellantchasmus falcatus* and *Echinostoma* spp. From one heavily infected patient, for example, 1270 adult worms of *C. sinensis* and 29 worms of *H. taichui* and *H. pumilio* were collected (De, 2004).

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doi:10.1016/j.exppara.2011.09.005

Mitochondrial (mt) genomes of flatworms have been used as reliable sources for providing genetic markers to investigate inter and intra-specific variation for diagnosis, taxonomic identification and phylogenetic studies (Le et al., 2002; Hu and Gasser, 2006). The complete or near-complete mtDNA genomes for various zoonotic parasitic pathogens of public-health concern are now becoming available in the GOBASE database (O'Brien et al., 2009) (at: http://megasun.bch.umontreal.ca/ogmp/projects/other/mt_list.html), including many of helminths obtained by us and our collaborators (Le et al., 2002). Among mtDNA markers, cytochrome c oxidase subunit 1 (*cox1*) has been widely applied for taxonomic identification, diagnostic discrimination and phylogenetic analysis of a variety of trematode species (Le et al., 2000, 2002, 2006, 2008; Nguyen et al., 2009a).

Recently, a number of intensive epidemiological and morphological studies have been conducted, mainly those under the FIBOZOPA project sponsored by the DANIDA (Denmark), leading to various reports published in the international journals about nationwide distribution of the fishborne trematodes. These included investigations on presence of the small liver flukes and heterophyid trematodes in Vietnamese freshwater fishes (Skov et al., 2009; Chi et al., 2008); in fish farms in the Mekong Delta (Thien et al., 2007); in cultured and wild fish in An Giang Province (Thu et al., 2007); in cultured and wild-caught freshwater fish from the Red River Delta (Phan et al., 2010a); in domestic animals in a highly endemic area of North Vietnam (Nguyen et al., 2009b); even, in raw fish dishes served in restaurants in Nam Dinh province and Hanoi city (Tran et al., 2009), and most recently in humans living in the pond-rich communes of Nam Dinh provinces (Phan et al., 2010b). The neglected fish-born trematodes, thus, are becoming the most important foodborne causative agents of the zoonotic parasitic diseases attracting public concern in Vietnam and worldwide (Chai et al., 2009; De et al., 2003; Phan et al., 2010b).

Despite a number of reports as listed above published, almost all of these studies have focused on searching for presence, morphological examination, and infection rate of fish-borne trematodes found in fisheries, humans and environment. Regarding to systemic investigations, data of fish-borne trematode infections in humans with molecular species identification conducted for whole communes are lacked or partly available. Additionally, the habit of eating raw fish is still, indeed, traditionally common in many areas of Vietnam, giving the link to the transmission circle between the infected fishes (food supplier) and humans (consumers). In order to provide a directory implementation for the improved control of fish-borne trematodiasis in human, the present study is aimed to assess overall prevalence and molecular taxonomic speciation of fish-borne trematode infection in human in a locality of plentiful fish ponds and dense population having raw fish eating habit, the Rang Dong commune of Nam Dinh province of Vietnam.

2. Materials and methods

2.1. Study area

A cross-sectional study was conducted during January 2009 and December 2010, in the coast-proximal Rang Dong commune, Nghia Hung district of Nam Dinh province, Vietnam. There are 9000 inhabitants in 220 households and 200 fish ponds in this commune. This means that every household has its own fish pond giving rise to the inhabitants frequently exposing to raw fish provision and contamination with the fish-borne parasitic pathogens. Moreover, inhabitants in this commune have very high frequency of eating raw fish, often two times a week from fishes caught from their own ponds. One hundred of households in the commune were randomly selected for the study. All the household members including children over six years were enrolled for investigations. Their stool samples were collected and examined. All the chosen individuals were interviewed using an available questionnaire for their habit of eating raw fishes.

2.2. Stool examination and treatment

Stool samples were examined by Kato-Katz technique (Katz et al., 1972) to find fish-borne trematode eggs and to count number of eggs (all trematode eggs found in feces) in individual fecal sample. No otherwise criteria given elsewhere, the intensity in this study was based on number of eggs counted by EPG (eggs per gram feces) from which three levels of infection are inferred as: light (EPG < 1000), moderate (between 1001 and 9999) and heavy (over 10,000) described in Yu et al. (2003).

All the people whose stool examination was positive (excluding pregnant women) were treated according to the WHO's guidelines as nematode infected persons with a single dose of 400 mg albendazole and trematode infected people with praziquantel 25 mg/kg bodyweight, three times a day (WHO, 1995).

From 10 patients, who have the highest infection intensity, during the treatment course with praziquantel, all fish-borne adult trematodes were collected for species identification. Brief description: Patients were given praziquantel three times, at mid-night, 5 AM and 10 AM; and after the last dose, they were given 30 g magnesium sulfate ($MgSO_4$) with boiled water for purgation. Adult flukes were collected directly from feces and the collected adult flukes were morphologically identified to species after stained with acetic carmine (Miyazaki, 1991). Fresh worms from each taxonomic group were subjected to species confirmation by molecular identification (see Le et al., 2006; Van et al., 2009).

2.3. Data analysis

All morphological results on the data sheet were encoded in Excell. Statistical analysis was carried out by using EPI INFO 6.0 and SPSS software. The *R x C chi* square (χ^2) test was used at statistical significance level of 0.05 (Olsen et al., 2006). For molecular analysis, the multiple alignments of sequences were performed and specific identification was confirmed by comparison with the known sequences of the corresponding species in GenBank or by reference to our previous published data as described in details in Le et al. (2008) and Van et al. (2009).

2.4. Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from a fresh individual from each group of morphologically identical worms collected from the infected peoples using Qiagen genomic DNA extraction kit (Qiagen, USA). The extracted genomic DNA was diluted to a working concentration of 100 ng/μl and 1 μl of this was used as template in the PCR of 50 μl volume. PCR was applied to obtain a fragment of *cox1* using the JB3F–JB4.5R primer pairs, and additionally, ITS-2 using the 3SF–BD2R primer pairs for internal control, respectively, as previously described (Le et al., 2008; Van et al., 2009). PCR products were visualized on 1% agarose gel stained with ethidium bromide, and photographs were digitally recorded using Dolphin-Doc (Wealtec, USA). The ITS-2 amplification is aimed to check the DNA integrity of the particular species concerned (see Van et al., 2009), whilst the *cox1* amplification generated fragments for purification and subjected to sequencing for nucleotide comparative analysis for molecular identification.

2.5. Sequence analysis and reconstruction of phylogeny

The PCR products purified using a QIAquick Purification kit (Qiagen Inc.) were subjected to direct sequencing using Big-Dye