Diversity of human intestinal helminthiasis in Lao PDR

Somphou Sayasone a, b, Youthanavane Vonghajack c, Monely Vanmany b, Oroth Rasphone d, Smarn Tesana e, Jürg Utzinger a, Kongsap Akkhavong b, Peter Odermatt a,∗

a Department of Public Health and Epidemiology, Swiss Tropical Institute, P.O. Box, 4002 Basel, Switzerland
b National Institute of Public Health, Ministry of Health, Vientiane, Lao PDR
c Department of Parasitology, Faculty of Medicine, University of Health Sciences, Vientiane, Lao PDR
d Department of Radiology, Mahosot Hospital, Ministry of Health, Vientiane, Lao PDR
e Food-Borne Parasite Research Group, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

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Summary
Food-borne trematodiasis is an emerging public health problem, including in Lao PDR. We investigated the diversity of intestinal helminthes and polyparasitism in patients with hepatobiliary or intestinal symptoms in hospital and community-based surveys. Stool samples from 232 individuals aged ≥15 years were examined by the Kato-Katz method (three samples) and a formalin ethyl-acetate concentration technique (one sample). Opisthorchis viverrini and minute intestinal flukes (MIF) were common, with prevalences of 86.2% and 62.9%, respectively. Hookworm was the predominant soil-transmitted helminth (65.9%). The prevalences of Taenia spp., Strongyloides stercoralis and Trichuris trichiura were 22.8%, 10.3% and 8.6%, respectively. Additionally, 97 individuals were purged; O. viverrini and Haplorchis taichui were found in 95 and 76 participants, respectively. Other trematodes included Phaneropsolus bonnei (22.7%), Prosthodendrium molenkampi (14.4%), Haplorchis pumilio (5.2%), Haplorchis yokogawai (3.1%) and Echinococcosmus japonicus (3.1%). Co-infection with O. viverrini and MIFs was rampant (81.4%). Polytrematode infection is highly prevalent in Lao PDR and hence requires urgent attention.

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1. Introduction

Food-borne trematodes parasitising the liver, lungs and intestinal tract of humans are an emerging public health problem.1–3 It is assumed that over one-half of infections
Cholangiocarcinoma is a type of liver cancer that originates from the bile ducts. This cancer is common in parts of Southeast Asia due to the high prevalence of liver flukes that infect humans. The genera *Clonorchis* and *Opisthorchis* are responsible for more than 50% of cases of cholangiocarcinoma in the world. In northeast Thailand, approximately 60% of cases are attributed to the liver fluke *Opisthorchis viverrini*.

*O. viverrini* is endemic in all provinces of Lao PDR, with the highest prevalence rates found in central and southern parts of the country. In these communities, food-borne trematodiasis is a major health concern. Opisthorchiasis and haplorchiasis are the most common liver fluke infections, affecting up to 60% of the population in certain areas. In Lao PDR, stool analysis has revealed that a significant number of patients infected with *O. viverrini* have concurrent helminth infections, including soil-transmitted helminths.

Savannakhet Provincial Hospital, with 80 beds, is the largest hospital in Lao PDR and serves as a referral hospital for the southern and central parts of the country. All patients aged ≥ 15 years who were hospitalised with hepatic or intestinal symptoms such as icterus, stomach ache, abdominal pain (right hypochondrial quadrant), nausea, vomiting, or abdominal irritation were invited to participate in the study. Study participants underwent a physical examination by a general physician, along with a questionnaire to gather demographic data. All study participants were co-infected with soil-transmitted helminths.

In the present study, we assessed the diversity of trematode infections and intestinal polyparasitism during hospital-based surveys. Three stool specimens, collected over consecutive days, were obtained from each individual. One Kato-Katz thick smear (41.7 mg) was prepared from each specimen. Slides were allowed to clear for 30 min prior to examination under a light microscope. The number of eggs was counted and recorded for each parasite species separately.

In addition to *O. viverrini*, other food-borne trematodes with intestinal tropism occur in Lao PDR, including *Haematobium*, *Schistosoma*, and *Dicrocoelium*. Eggs of these parasites are often difficult to differentiate from those of minute intestinal flukes (MIFs) using light microscopy. Therefore, a quantitative FECT (formalin-ethyl-acetate concentration technique) was employed to distinguish between the different parasite species.

In conclusion, the co-occurrence of *Opisthorchis-like* eggs with other intestinal helminths is not only a challenge for diagnosis but may also aggravate morbidity. The study allowed validation of the quantitative formalin ethyl-acetate concentration technique (FECT) for diagnosing *O. viverrini* and MIFs.
movements) were collected and examined. Bottled drinking water was provided and patients were encouraged to drink as much as possible.

2.4. Worm collection

Diarrhoeal stool was poured into a 2 l bottle, filled up with tap water and stirred until the stool was homogenously mixed. After sedimentation for 10 min, the supernatant was discharged, water was added and stirred again. This washing procedure was repeated until the supernatant became clear. The sediment was examined for the presence of adult worms as follows. First, adult *Taenia* spp., *Echinostoma* spp. and *O. viverrini* worms were visually searched for. Second, the remaining sediment was examined with a stereomicroscope for the presence of MIFs. The number of species-specific parasites was recorded for each individual. Species identification was confirmed under a light microscope after specimens were coloured with carmine and mounted in Permount.

All individuals infected with *O. viverrini* and soil-transmitted helminth infections were treated according to national guidelines.18 An antispasmodic treatment and oral rehydration was provided in case of side effects due to drug administration.

2.5. Data management and statistical analysis

Data were double-entered and validated in EpiData version 3.1 (EpiData Association; Odense, Denmark). Statistical analyses were performed with STATA version 9 (Stata Corp.; College Station, TX, USA). Those individuals with complete data records were included in the final analyses.

Age was subdivided into five groups: (i) 15—25 years; (ii) 26—35 years; (iii) 36—45 years; (iv) 46—55 years; and (v) >55 years. Infections with hookworm, *Ascaris lumbricoides*, *Trichuris trichiura* and *O. viverrini* were grouped into light, moderate and heavy infections, respectively, according to Maleewong et al.19 and WHO guidelines20: hookworm, 1—1999, 2000—3999 and ≥4000 eggs per gram of faeces (epg); *A. lumbricoides*, 1—4999, 5000—49999 and ≥50000 epg; *T. trichiura*, 1—999, 1000—9999 and ≥10000 epg; and *O. viverrini-like*, 1—999, 1000—9999 and ≥10000 epg.

Fisher’s exact test and *χ²* test were employed to investigate associations between categorical variables. ANOVA was used to associate the parasite egg counts with either age group or sex. The arithmetic mean of worm counts was calculated for infected individuals in purged patients. Linear regression and Spearman’s correlation were used to investigate the relationship between number of adult worms of *O. viverrini* and MIFs and their egg counts in microscopic examination of stool samples. For all analyses the significance level was *P* = 0.05.

3. Results

3.1. Stool analysis

Complete parasitological data were obtained for 232 individuals, giving an overall compliance of 97.1%. The majority of subjects participated in the community survey (*n* = 213; 91.8%).

Table 1 summarises the results from the microscopic stool examination using either the Kato-Katz technique or FECT. Also shown are the pooled results, with all subsequent analyses performed on these pooled data. ‘*Opisthorchis-like*’ eggs were diagnosed in 217 individuals (93.5%). Examination of multiple Kato-Katz thick smears resulted in a significantly higher helminth infection prevalence compared with a single stool specimen examined by FECT: hookworm (62.9% vs. 37.5%; *P* < 0.001); *T. trichiura* (7.8% vs. 1.7%; *P* = 0.002); and *Taenia* spp. (21.1% vs. 9.9%; *P* = 0.001). With regard to diagnosing ‘*Opisthorchis-like*’ eggs, the two methods

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Pooled results</th>
<th>Kato-Katz</th>
<th>FECT</th>
<th><em>χ²</em></th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trematodes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Opisthorchis-like eggs</em></td>
<td>217 (93.5)</td>
<td>216 (93.1)</td>
<td>204 (87.9)</td>
<td>3.61</td>
<td>0.057</td>
</tr>
<tr>
<td><em>Opisthorchis viverrini</em></td>
<td>200 (86.2)</td>
<td>ND</td>
<td>200 (86.2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Minute intestinal flukes</td>
<td>146 (62.9)</td>
<td>ND</td>
<td>146 (62.9)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Echinostomatidae</td>
<td>51 (22.0)</td>
<td>40 (17.2)</td>
<td>38 (16.4)</td>
<td>0.06</td>
<td>0.804</td>
</tr>
<tr>
<td><strong>Nematodes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hookworm</td>
<td>153 (65.9)</td>
<td>146 (62.9)</td>
<td>87 (37.5)</td>
<td>30.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>Strongyloides stercoralis</em></td>
<td>24 (10.3)</td>
<td>ND</td>
<td>24 (10.3)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Trichuris trichiura</em></td>
<td>20 (8.6)</td>
<td>18 (7.8)</td>
<td>4 (1.7)</td>
<td>9.35</td>
<td>0.002</td>
</tr>
<tr>
<td><em>Enterobius vermicularis</em></td>
<td>2 (0.9)</td>
<td>0</td>
<td>2 (0.9)</td>
<td>2.01</td>
<td>0.156</td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0.001</td>
<td>1.000</td>
</tr>
<tr>
<td><em>Capillaria philippinensis</em></td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0.001</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Cestodes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taenia</em> spp.</td>
<td>53 (22.8)</td>
<td>49 (21.1)</td>
<td>23 (9.9)</td>
<td>11.11</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Hymenolepis diminuta</em></td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>1.00</td>
<td>0.317</td>
</tr>
</tbody>
</table>

NA: not applicable; ND: not determined.
showed borderline statistical significance (93.5% vs. 87.9%; \( P = 0.057 \)).

There was a tendency for male study participants to have a higher infection prevalence for individual parasites. Statistically significant sex differences were found for \textit{O. viverrini} (males 91.6%, females 81.7%; \( P = 0.030 \)), \textit{Echinostomatidae} (males 29.9%, females 15.1%; \( P = 0.006 \)) and \textit{Strongyloides stercoralis} (males 18.7%, females 3.2%; \( P < 0.001 \)).

Figure 1 depicts age-specific prevalence curves for the different parasites investigated. None of the helminth prevalences were significantly associated with age. A distinct age peak of intensity of infection was found for \textit{O. viverrini} and \textit{Taenia} spp. in the 36–45 years and 26–35 years age groups, respectively (Figure 2). None of the other helminths showed a statistically significant association between intensity of infection and age or sex.

### 3.2. Polyparasitism and infection intensity

Among the 232 individuals with complete data sets, only 15 (6.5%) were free of intestinal parasites. Single-species parasitic infections were found in 23 individuals (9.9%). Over two-thirds of the participants (67.2%) harboured two to four different parasite species concurrently. Thirty individuals (12.9%) were infected with five different parasites. Six individuals (2.6%) were infected with six different parasites and two individuals (0.9%) (males aged 15 years and 28 years) harboured seven parasite species.

Table 2 summarises infection intensities, expressed in epg, of the different intestinal parasites either detected by the Kato-Katz or FECT, or after combining the results from both diagnostic approaches. The pooled results showed that 117 (50.4%) of the examined patients showed an infection with "\textit{Opisthorchis-like}" eggs of moderate intensity, whereas 20 individuals (8.6%) had a heavy infection.

### 3.3. Characteristics of purged individuals

From the 107 individuals invited for the purging study, 10 were excluded upon clinical examination as they were...
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Table 3  Number (%) of individuals with adult helminths recovered after purgation among 97 study participants in a community and hospital-based survey in Lao PDR.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Overall (n = 97)</th>
<th>Community (n = 82)</th>
<th>Hospital (n = 15)</th>
<th>χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver flukes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Opisthorchis viverrini</em></td>
<td>95 (97.9)</td>
<td>82 (100)</td>
<td>13 (86.7)</td>
<td>11.03</td>
<td>0.001</td>
</tr>
<tr>
<td>Minute intestinal flukes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Haplorchis taichui</em></td>
<td>76 (78.4)</td>
<td>69 (84.1)</td>
<td>7 (46.7)</td>
<td>10.29</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Phaneroplosus bonnei</em></td>
<td>22 (22.7)</td>
<td>20 (24.4)</td>
<td>2 (13.3)</td>
<td>0.92</td>
<td>0.336</td>
</tr>
<tr>
<td><em>Prosthodendrium molenkampi</em></td>
<td>14 (14.4)</td>
<td>13 (15.9)</td>
<td>1 (6.7)</td>
<td>0.89</td>
<td>0.344</td>
</tr>
<tr>
<td><em>Haplorchis pumilio</em></td>
<td>5 (5.2)</td>
<td>5 (6.1)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td><em>Echinococcus japonicus</em></td>
<td>3 (3.1)</td>
<td>3 (3.7)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td><em>Haplorchis yokogawai</em></td>
<td>3 (3.1)</td>
<td>3 (3.7)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Cestodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taenia saginata</em></td>
<td>18 (18.6)</td>
<td>18 (22.0)</td>
<td>0</td>
<td>4.10</td>
<td>0.043</td>
</tr>
<tr>
<td>Other <em>Taenia</em> spp.</td>
<td>5 (5.2)</td>
<td>4 (4.9)</td>
<td>1 (6.7)</td>
<td>0.08</td>
<td>0.782</td>
</tr>
</tbody>
</table>

NA: not applicable.

Severely sick (6 with severe kidney stone disease and 1 each with liver tumour, ascites or liver cirrhosis) or pregnant (n = 1). Hence, 97 individuals were purged, 82 (84.5%) in the community-based survey and 15 (15.5%) in the hospitals. Lao Loum was the main ethnic group and patients were aged 15—75 years. The illiteracy rate among females in the community survey was significantly higher than among males (45.0% vs. 34.2%; χ² = 4.09, P = 0.043), whilst only one of the participants was illiterate in the hospital study. Subsistence farming was the main occupation of study participants both in the community and the hospital-based surveys (89.0% and 67.7%, respectively).

3.4. Number and species of worms collected after purgation

Table 3 shows the species-specific prevalence of intestinal parasites recovered from study participants after purgation. Adult *O. viverrini* flukes were diagnosed in 95 individuals (97.9%). Additionally, adult worms of six different species of MIF were identified, with *Haplorchis taichui* being the most common (78.4%). The prevalences of *Phaneroplosus bonnei* and *Prosthodendrium molenkampi* were 22.7% and 14.4%, respectively. Significantly higher prevalences were observed in the community-based study compared with hospitalised patients for *O. viverrini* (100% vs. 86.7%; P = 0.001), *H. taichui* (84.1% vs. 46.7%; P = 0.001) and *Taenia saginata* (22.0% vs. 0%; P = 0.043).

Seventy-nine individuals (81.4%) harboured *O. viverrini* and at least one species of MIF concurrently. Conversely, single infections both with *O. viverrini* and MIF were rare, with prevalences of 16.5% and 2.1%, respectively. Two of the parasites were significantly associated with age group, namely *P. molenkampi* (χ² = 10.63, P = 0.031) and *P. bonnei* (χ² = 9.18, P = 0.050), with a peak prevalence in the 36—45 years age group. Figure 3 shows species-specific age—prevalence curves of adult flukes following purgation.

Table 4 summarises the total number of flukes collected from the 97 purged individuals, including arithmetic mean counts and 95% confidence intervals (CIs). Very high total counts were recorded for *O. viverrini* (17 755; community 14 802, hospital 2953) and *H. taichui* (15 555; community 14 530, hospital 1025). The mean fluke count for *O. viverrini* was 186 (community 182, hospital 206) and the mean count for *H. taichui* was 207 (community 214, hospital 146).

Figure 4 depicts the association between the number of adult *O. viverrini* flukes recovered after purgation and the infection intensity as expressed by epg upon microscopic stool examination using FECT. Linear regression analysis showed a significant positive association, with a regression
The validity of FECT for diagnosis of *O. viverrini* and MIFs was assessed considering purgation as the diagnostic ‘gold standard’. The sensitivity of FECT for the discovery of *O. viverrini* and MIF eggs was 96.8% (92/95) and 85.0% (68/80), respectively. The specificity and positive predictive value (PPV) for diagnosing *O. viverrini* eggs were 100.0% (92/92), regardless of the method. The specificity and PPV of FECT for MIF diagnosis were 70.6% (12/17) and 93.2% (68/73), respectively. For both parasites, the negative predictive value of FECT was low; 60.0% (3/5) in the case of *O. viverrini* and 50.0% (12/24) for MIFs.

### 3.5. Diagnostic performance of FECT

Data obtained from 232 Lao individuals aged ≥15 years who complained of hepatobiliary or intestinal symptoms confirm that multiparasitism is the norm rather than the exception. In fact, more than three-quarter of the participants harboured at least two helminth species concurrently, with *O. viverrini* and MIFs being the most common trematodes encountered. A rigorous diagnostic approach was employed, with three Kato-Katz thick smears performed on consecutive stool specimens, supplemented with FECT on one of the specimens. Previous studies have shown that multiple Kato-Katz thick smears plus other diagnostic methods are mandatory to achieve a high diagnostic sensitivity.21—23 In the present study, only 15 individuals (6.5%) were diagnosed as free of any intestinal parasite. It is conceivable that the actual number of parasite-free individuals is even lower, as some light infections might have been missed despite the rigorous diagnostic approach. Besides the liver fluke *O. viverrini* and various kinds of intestinal flukes, hookworm infections were also highly prevalent. Interestingly, *S. stercoralis*, arguably the most neglected of the soil-transmitted helminths,23,24 was more prevalent than *T. trichuris* and *A. lumbricoides*. Purgation of a subsample of individuals allowed identification of parasites at species level, with MIFs and *H. taichui* found at the highest frequency.

Figure 5 Logarithmic transformation of the association between the number of *Opisthorchis viverrini* flukes recovered after purgation (n = 97) and eggs per worm by formalin ethyl-acetate concentration technique.

### 4. Discussion

Our data underscore that multiparasitism is very common in Lao PDR and these findings support observations from neighbouring countries such as Vietnam25—27 and from other parts of the developing world.21,22,28–30 Hence, our results and those from other groups who worked in Lao PDR7,8 call for concerted action to remedy the issue of multiparasitism. Of particular public health relevance are our results in relation to the diversity of trematodes identified. In the purgation of 97 patients, as many as seven different trematode species were identified. *O. viverrini* was the most abundant fluke, followed by *H. taichui*. Another five intestinal trematodes were recorded (P. bonnei, Haplorchis yokogawai, P. molenkampi, Haplorchis pumilio and Echinococcus japonicus) although at significantly lower prevalences, which is consistent with previous reports from other parts of Lao
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A number of the identified human trematodes have eggs of similar size and shape. Species differentiation is therefore difficult with simple coprologic diagnostic tools such as direct faecal smears or the widely used Kato-Katz thick smear examination. However, differentiation of MIFs from O. viverrini is of considerable public health importance as the latter fluke provokes a range of hepatobiliary diseases and is a major risk factor for cholangiocarcinoma. Hence, monitoring of interventions that are targeted against O. viverrini require diagnostic techniques that are capable of differentiating between O. viverrini and MIFs.

Our analysis showed that O. viverrini worm burden is highly significantly associated with the egg counts in stool samples. Very similar linear regression lines were described in Thailand. Hence, we can confirm that egg counts are a valid proxy measure for intensity of infection with O. viverrini. In addition, similar to Elkins et al., we also found a decreasing fecundity of O. viverrini with increasing worm burden, a phenomenon that may reflect density-dependent constraints on fecundity.

Our validation of a commonly employed faecal concentration technique with results of the purging examination, the latter serving as a diagnostic "gold standard", showed high sensitivity and specificity for diagnosis both of O. viverrini and MIFs. Our findings therefore support the use of FECT in future studies emphasising parasite species-specific diagnosis. Repeated stool examinations have been shown to improve significantly the diagnostic accuracy for parasitic infections. It follows that FECT performed on multiple stool samples holds promise for accurate and species-specific diagnosis. However, such an approach is time consuming and is likely to compromise compliance, as study participants are reluctant to provide multiple stool specimens. Alternatively, species-specific PCR-based techniques have been developed. However, PCR methods are less suitable for large-scale community-based investigations as they are costly and still of limited direct applicability under field conditions.

It is important to note that dishes based on raw or insufficiently cooked fish, other aquatic products and meat are frequently consumed in Lao PDR and other Southeast Asian countries. The common habit of raw or undercooked fish consumption is a key factor in the transmission of trematode infection, which in turn explains the high prevalence and infection intensity of O. viverrini; indeed, over one-half of the subjects examined harboured a moderate or heavy infection with O. viverrini.

Interestingly, we also found two MIF species that are transmitted by consumption of raw naiads, i.e. P. bonnei and P. molenkampi. Both species were actually diagnosed quite frequently (22.7% and 14.4%, respectively). In the 1970s, the first cases of human infections with these trematodes were described in the Udon Thani area of northeastern Thailand, where communities have similar alimentary habits as in Lao PDR. Furthermore, in three patients living in Khamsida village, a rare Echinostomatidae fluke was diagnosed, namely E. japonicus. To our knowledge, this is the first report of E. japonicus from Lao PDR and details will be presented elsewhere.

Of clinical and public health importance is the fact that virtually all patients included in the study were infected with trematodes, with more than three-quarters harbouring O. viverrini and MIFs concurrently. Conversely, only a few patients had a single-species infection (O. viverrini 16.5%, MIF 2.1%). Although it is acknowledged that poly-parasitism may negatively impact on health and well-being, new research is needed to deepen our understanding of the underlying mechanisms and how to measure improvements following control measures at the individual and population level. Further investigations coupled with rigorous monitoring of control interventions are urgently needed to further our knowledge and to provide a rationale for evidence-based interventions.

Authors' contributions: SS and PO designed the study; SS, YV, MV and PO collected field data; SS and OR identified parasites in the laboratory; SS analysed data and drafted the manuscript; PO, JU and KA contributed to data analysis and revised the manuscript; KA held overall responsibility for data collection. All authors read and approved the final manuscript. SS and PO are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the institutional review board of the Swiss Tropical Institute (Basel, Switzerland) and the Ethics Committee of the University and the State of Basel, Switzerland (EKBB; reference no. 255/06). Ethical clearance was obtained from the National Ethics Committee, Ministry of Health in Vientiane, Lao PDR (reference no. 027/NECHR).

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