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High prevalence of large trematode eggs in schoolchildren in Cambodia

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ABSTRACT

Large trematode eggs (LTE) resembling *Fasciola* spp. eggs were reportedly found in the stools of schoolchildren in Kandal province, Cambodia. This study aimed to assess the prevalence of LTE in the stools of children attending the affected school, identify potential risk factors for infection and ascertain the trematode species. We performed a cross-sectional study involving an in-depth questionnaire administered to schoolchildren at the affected school, and examined cattle droppings in the surrounding area and the livers of slaughtered cattle. Three stool samples were examined per child, using Kato–Katz and formalin–ether concentration techniques. In addition, blood serum enzyme-linked immunosorbent assay (ELISA) and coprological polymerase chain reaction (PCR) was conducted for species clarification. Cattle droppings were examined by cup sedimentation and coprological ELISA. LTE were observed in the stools of 106 schoolchildren (46.5%). Two blood serum samples from schoolchildren were positive for *Fasciola hepatica* in a first ELISA but were negative in a confirmation immunofluorescence antibody test. Out of 221 PCR samples, only one tested positive for *Fasciola* spp. and none for *Fasciolopsis buski*. The consumption of raw aquatic plants (odds ratio (OR) = 3.3) and fermented fish sauce (OR = 2.1) were significantly associated with LTE in the stool. *Fasciola* spp. flukes were observed in 18.3% of 191 cattle livers. The prevalence of fascioliasis in cattle droppings was 88.8%. The low prevalence of schoolchildren that tested positive for *Fasciola* spp. with specific molecular diagnostics and who had no diagnostic evidence of *F. buski* strongly indicates that the majority of microscopically observed LTE are from *Echinostoma* spp. *Fasciola* spp. transmission from cattle to human is possible and public health services need to be alerted accordingly.

1. Introduction

In low- and middle-income countries, intestinal multiparasitism is the rule rather than the exception (McKenzie, 2005; Petney and Andrews, 1998; Steinmann et al., 2010). In Southeast Asia, intestinal helminth and protozoa multiparasitism is well known and has been documented (Lee et al., 2002; Park et al., 2004; Sayasone et al., 2011, 2009; Sinuon et al., 2003; Schär et al., 2014). Several endemic species of food-borne trematodes (FBT) have been identified, such as liver flukes (*Opisthorchis viverrini* and *Fasciola* spp.) and intestinal flukes (*Fasciolopsis buski* and *Echinostoma* spp.) (Hien et al., 2001; Keiser and Utzinger, 2005; Quang et al., 2008; Sayasone et al., 2011; Sohn et al., 2011a,b). Helminthic multiparasitism poses a serious challenge for parasitological diagnosis, as eggs from different species may morphologically resemble one another. For example, the size, oval shape and presence of small and inconspicuous operculum of *Fasciola* spp. eggs are indistinguishable from *F. buski* and some *Echinostoma* spp. eggs. The average egg size of all three parasite genera is similar in terms of length and width: *Fasciola hepatica* 106.5–171.5/63.9–95.4 μm, *Fasciola gigantica* 150.9–182.2/85.1–106.2 μm (Valero et al., 2009), *Echinostoma revolutum* 97–117/61–65 μm (Sohn et al., 2011a); *Echinostoma ilocanum* 89–99/52–58 μm (Sohn et al., 2011b) and *F. buski* 130-140/80-85 μm (Garcia and Bruckner, 1988). They can be characterised as *Fasciola*-like eggs or large trematode eggs (LTE). In 2009, LTE were observed in the stools of 27/150 schoolchildren (18.0%) in the Damrei Chhlang village primary school, Kandal province, Cambodia, during a standard parasitological survey using

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the Kato–Katz technique (Khieu et al., 2013). Given the high prevalence of *Fasciola* spp. infection among cattle in southern Cambodia (Tum et al., 2007), LTE could have been attributable to *Fasciola* spp. infections. However, other trematode species could also have been responsible.

The aim of this study was to assess the prevalence of LTE in the stools of schoolchildren, identify risk factors for infection and ascertain the trematode species, all with a focus on *Fasciola* spp. We performed a cross-sectional study among the schoolchildren, examined cattle droppings in and around Damrei Chhlang village and inspected cattle livers in a local slaughterhouse.

2. Materials and methods

2.1. Ethical considerations

Ethical clearance for this study was obtained from the Ethics Commission of Basel-Stadt and Basel-Land, Switzerland (EKBB; reference no. 159/11) and from the National Ethics Committee for Health Research (NECHR), Ministry of Health (MOH) in Phnom Penh, Cambodia (reference no. 30/NECHR). Before field work started, the provincial and district health authorities, village and school authorities and parents were informed of the study and, in turn, working permission was granted. Prior to enrolling each participant, written informed consent was obtained from the parents, legal guardian or the participant him/herself if they were of legal age. In cases of illiteracy, the informed consent was signed by fingerprint before the village chief, who signed as a witness.

2.2. Study design, population and area

The study was carried out in Central Kandal province, which surrounds the capital Phnom Penh. It is inhabited by 1.2 million people and divided into 11 districts. Between May and June 2011, a cross-sectional study was carried out among schoolchildren at the Damrei Chhlang village primary school, S'ang district (11°21′29.43″ N, 104°59′18.05″ E), a district of 1831 people.

Another cross-sectional study aimed to estimate the prevalence of *Fasciola* spp. eggs in cattle droppings in the villages surrounding the Damrei Chhlang primary school (S'ang district). These villages included Damrei Chhlang and Preaek Khmer (11°21′21.70″ N, 104°58′59.33″ E), both located in S'ang Phnom commune; and Preaek Run (11°21′16.30″ N, 104°59′46.54″ E), located in Preaek Koy commune. Additionally, an abattoir study of a small facility that is slaughtering local cattle was conducted in Preaek Koy commune.

2.3. Procedures in the field

On day 1, enrolled study participants assembled at the community hall next to the school. A trained interviewer questioned each child about potential risk factors for infection and about experiences of ill-health. Subsequently, a physician assessed participants' general health status and clinical symptoms by using a standardized assessment form. Additionally, a nurse drew a venous blood sample of 5 ml from each child. The blood samples were stored in a cooling box at about 5–10 °C until they reached the laboratory of the National Center for Parasitology, Entomology and Malaria Control (CNM), Ministry of Health in Phnom Penh.

A pre-labeled stool container was given to each child, along with instructions on how to fill it. Children were asked to collect morning stools to ensure freshness of the stool samples. The following morning, the filled container was collected and a new pre-labeled container was provided. Three stool samples were collected from each child over 4 consecutive days. Each day after collection, containers were directly transported to the laboratory of the CNM for analysis.

The stool containers for cattle droppings were distributed among randomly selected cattle owners, each of whom collected one fresh stool sample from every one of their cattle. Containers were collected in the morning, the day after distribution, and directly transported to the laboratory of the CNM.

The butcher at the study abattoir examined the livers of slaughtered cattle on a continuous basis for meat quality assurance. For study purposes, he recorded in a diary the total number of slaughtered cattle and of *Fasciola* spp. infected livers.

2.4. Laboratory procedures

For each of the three stool samples per child, one Kato–Katz thick smear (Katz et al., 1972) was prepared directly after the samples arrived in the laboratory. After a half an hour clearance time, the smears were examined under a light microscope (magnification 400×) for intestinal helminth eggs. The number of eggs per parasite species was counted and recorded.

From each of the three stool samples per child, approximately 1 g was preserved in 15 ml sodium acetate–acetic acid–formalin (SAF) fixative for analysis by formalin–ether concentration technique (FECT) (Marti and Escher, 1990). Four weeks after collection, the fixed samples were processed and examined under a light microscope (magnification 1000×) for intestinal helminths and protozoa.

From the first stool sample per child, approximately 0.5 g of fresh stool was preserved in 70% ethanol for later molecular analysis using real-time quantitative polymerase chain reaction (PCR) to diagnose *Fasciola* spp. (Alasaad et al., 2011) and *F. buski* infections (unpublished, developed by Dr. med. Stefanie Kramme, Swiss TPH). The preserved samples were sent to the Swiss TPH laboratory in Basel, Switzerland, where molecular analysis was carried-out about 2 months later. First, from each preserved stool sample, 200 μg was washed twice with phosphate buffered saline (PBS) to eliminate the ethanol. Subsequently, DNA was extracted with the QIAamp DNA Stool Mini Kit (QIAGEN, USA). The extracted DNA was frozen at −20 °C until PCR was performed, 1 week later. On 0.5 g of the first stool samples from each child, a commercially available *F. hepatica* coproantigen enzyme-linked immunosorbent assay (ELISA) (Bio-X, Belgium) was performed at the CNM laboratory.

Upon arrival, blood samples were stored at room temperature to clot. Subsequently, the blood samples were centrifuged and the serum was frozen and stored at −20 °C at the CNM laboratory. The frozen samples were sent to the Diagnostic Center of the Swiss TPH, Switzerland, where an ELISA for *F. hepatica*-antibodies was performed 2 months after sample collection. Sera samples with a positive result were retested with an immunofluorescence antibody test (IFAT) for confirmation. All sera samples with a positive or critical (close to positive) result were retested with a helminth screening ELISA (Speiser, 1982), which included *F. hepatica*, *Strongyloides stercoralis*, *Schistosoma* spp., *Brugia malayi*, *Wuchereria bancrofti*, *Echinococcus* spp., *Toxocara* spp. and *Trichinella* spp.

Cattle droppings were stored at 5 °C in a fridge at the CNM laboratory until analysis. The next day, cattle droppings were examined by the same *F. hepatica* coproantigen ELISA test (Bio-X, Belgium) that had been used to examine the stool samples from children. Identical procedures were used, only the weight of stool was increased from 0.5 g to 2 g. Within 48 h of collection, cattle droppings were examined a second time by cup sedimentation. Approximately 10 g of faecal material was mixed with NaCl 0.9% and filtered through a double layer of gauze. The cup was filled with NaCl 0.9% and left to stand for 1 h. Three slides of the sediment were examined under a light microscope (magnification 400×) for *Fasciola* spp. eggs. The left over sediment was centrifuged, mixed with SAF-solution and ether, vigorously shaken, centrifuged