

RESEARCH ARTICLE

Reinfection by *Opisthorchis Viverrini* after Treatment with Praziquantel

Phubet Saengsawang¹, Supanee Promthet^{2*}, Peter Bradshaw²

Abstract

Background: The prevalence of infection by the liver fluke, *Opisthorchis viverrini* (*O. viverrini*), has remained high in Northeast Thailand where it is a major risk factor for the eventual development of cholangiocarcinoma (CCA). The infection is acquired by the consumption of dishes containing unsafely prepared freshwater fish, a dietary tradition which has proved resistant to change. Since many people are aware that dosing with praziquantel (PZQ) is a successful treatment for an episode of the infection, there is a risk that, to avoid the long term consequences, they will engage in a cycle of infection, dosing and reinfection. **Objectives:** There is a dearth of studies of reinfection by *O. viverrini*, and the aims of this study were to assess re-infection rates in a typical province of Northeastern Thailand where *O. viverrini* infection is likely and to investigate factors associated with reinfection. **Materials and Methods:** A total of infected 607 villagers were treated with PZQ, and those found to be no longer infected were followed up at six-monthly intervals over 12 months. **Results:** At the end of this period data on 457 subjects were available for analysis using descriptive statistics and logistic regression, and 50 were found to have become reinfected, giving a cumulative reinfection rate of 10.9%. The results of a multiple logistic regression analysis showed that the only factor found to be associated with reinfection was past use of PZQ. **Conclusions:** Recommendations are made for future larger scale and better designed reinfection studies in the light of limitations of the current study. Further efforts are needed to discourage people from eating fish dishes likely to contain viable metacariae.

Keywords: *Opisthorchis viverrini* - reinfection - Northeastern Thailand.

Asian Pac J Cancer Prev, 17 (2), 857-862

Introduction

Infection by the liver fluke, *Opisthorchis viverrini* (*O. viverrini*), continues to be a serious public problem in Thailand, especially in the northeast region where according to the most recent data the prevalence in 2009 was 16.6%, a rate which had apparently slightly increased over the previous decade (Sithithaworn et al., 2012). Most cases of opisthorchiasis report no symptoms even when heavily infected (Sripa et al., 2007), but the helminth has long been classified as a Group 1 carcinogen (IARC, 1994, 2012) and has been established as the major risk factor for the eventual development of cholangiocarcinoma (CCA), a usually fatal form of cancer (Watanapa and Watanapa, 2002; Poomphakwaen et al., 2009; Songserm et al., 2012; Sithithaworn et al., 2014). On a global level, the incidence of this malignancy in Northeastern Thailand is very high, and in one province of the region, Khon Kaen, the incidence seems to have been higher than in any other part of the world (Khuaprema et al., 2010; Miwa et al., 2014; Sithithaworn et al., 2014).

The primary source of infection by *O. viverrini* is the tradition of eating dishes, such as koi pla (raw fish salad),

which are made with raw, undercooked or improperly fermented freshwater cyprinid fish containing viable metacercariae, an intermediary form of the parasite (Sripa and Pairojkul, 2008; Sripa et al., 2011; Sithithaworn et al., 2012; Prasongwatana et al., 2013). The consumption of these dishes in Northeastern Thailand, indeed throughout the many communities living in the lower Mekong region where freshwater fish are a plentiful source of food, is a well-established cultural practice (Grundy-Warr et al., 2012) which is difficult to break. National control programmes, which ultimately rely on the identification of infection by Kato-Kato smear tests and treatment of infected cases with the antihelmintic, praziquantel, have proved largely unsuccessful largely due to underfunding and limited outreach (Saengsawang et al., 2012). The control programmes also appear to have failed because of an emphasis on a 'top-down' approach which largely ignores the specific socio-cultural and environmental pressures in the endemic areas (Sripa et al., 2014).

Many people are now aware that, if there is any risk of reinfection after an initial infection, they can simply obtain a further dose of praziquantel to eliminate the problem (Wongpa et al., 2015). This can so easily lead to

¹Yasothon Provincial Health Office, Yasothon Province, Yasothon, ²Department of Epidemiology, Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand *For correspondence: supanee@kku.ac.th

an ongoing cycle of reinfection. People continue to eat the customary unsafely prepared freshwater fish dishes as their cultural pressures demand because they believe that the possible serious long consequences of this can be effectively avoided by regularly dosing themselves with praziquantel. The outcome of this can be that a roundabout of infection, treatment and reinfection is perpetuated (Kamsa-ard et al, 2013).

This potential cycling of infection and reinfection requires much further research because of its additional risk to the eventual development of a fatal CCA. Evidence from animal studies has indicated that repeated infections by *O. viverrini* increase the risk of CCA (Pinlaor et al., 2004) and that treatment with praziquantel may in itself can lead to a greater risk of CCA due an increase in liver damage by free radicals released from inflammatory cells (Sudsarn et al., 2015) and possibly by retarding the rate of resorption of periductal fibrosis if treatment with praziquantel is delayed (Pinlaor et al., 2009). In human studies, there is some evidence that, while a combination of repeated infection and dosing with praziquantel increases the risk of CCA (Chernrungraj, 2000; Kamsa-ard et al., 2015), the relative contribution of each of these factors remains unclear. Nevertheless, there is sufficient evidence to raise concern about reinfection and the apparent cycling of infection and reinfection after dosing with praziquantel.

To our knowledge (and perhaps surprisingly), there have been only two studies of the rates of reinfection by *O. viverrini* in the lower Mekong region where high infection rates are regularly found (Sornmani et al., 1984; Upatham et al., 1988). Both happened to be conducted in the same northeastern province of Thailand, but the data were collected over 25 years ago, and no attempt was made to investigate the factors associated with reinfection. The aims of the present study were to assess reinfection rates in a typical province of Northeastern Thailand where *O. viverrini* infection is likely and to investigate factors associated with reinfection.

Materials and Methods

A prospective cohort study was conducted during January-December, 2012, in Hua Mueang, a subdistrict of Maha Chana Chai, and in Kut Kung, a subdistrict of Kham Khuean Kaew, which are both rural wetland areas in the southern part of Yasothon Province, Northeast Thailand, and are considered to be places where *O. viverrini* infection is endemic. The terrain in this part of the province includes the lowland of the Chi River and contains swamps and numerous ponds. The two sub-districts were selected by multistage sampling from the nine provincial districts. Further details about the two sub-districts are available in Saengsawang et al., 2012.

All villagers aged 20-65 years who had resided in either of the study areas for at least six months were invited to participate and provide stool samples which were analysed using a single Kato-Katz thick smear examination (WHO, 1991) within two days of collection. Those who had reported taking PZQ in the week prior to stool collection (N=5) were excluded. Information about socio-demographic characteristics, beliefs and

knowledge about PZQ, and previous use of the medication was obtained using a structured interview questionnaire. Knowledge about PZQ were assessed by responses ('agree', 'disagree', 'don't know') to (1) three statements about the correct use of PZQ (for example, 'PZQ should only be taken if you are infected with liver flukes'), (2) three statements about its side-effects/contraindications (for example, 'PZQ can make you dizzy', 'You should not take PZQ if you are pregnant or breast-feeding'), and (3) five statements about its benefits (for example, 'PZQ kills liver flukes'). Correct responses scored 1-point, and incorrect or 'don't know' responses scored zero. Three of the five statements about benefits were decoy terms, and the correct response was to disagree. For the purpose of analysis subjects in the reinfected and not reinfected groups were classified as having higher or lower knowledge in each of the three knowledge areas on the basis of individual scores being above or below the mean scores for the combined group.

The process of selecting study subjects for the analyses is summarised in Figure 1. The subjects for the present study were the 607 cases of OV infection found among the 1,569 participants. All were treated with praziquantel in a single dose of 40 mg per kg body weight after meals and were re-tested for OV infection after two weeks by the formalin ether concentration technique (FECT) according to the procedure described by Stensvold (Stensvold et al., 2006). Subjects found to be infected with OV by FECT (N=30) were then dropped from the study. The remaining 577 were followed up with the intention of retesting uninfected subjects by FECT at six and twelve months after praziquantel treatment. Of the 554 subjects who had remained uninfected at the sixth month stage, only 434 provided a stool sample at the 12 month stage due to deaths (N=3) and loss to follow-up (N=117). The analyses were therefore based on 50 cases of reinfection over the 12 month follow up period and 407 subjects who had remained uninfected. All subjects found to be reinfected

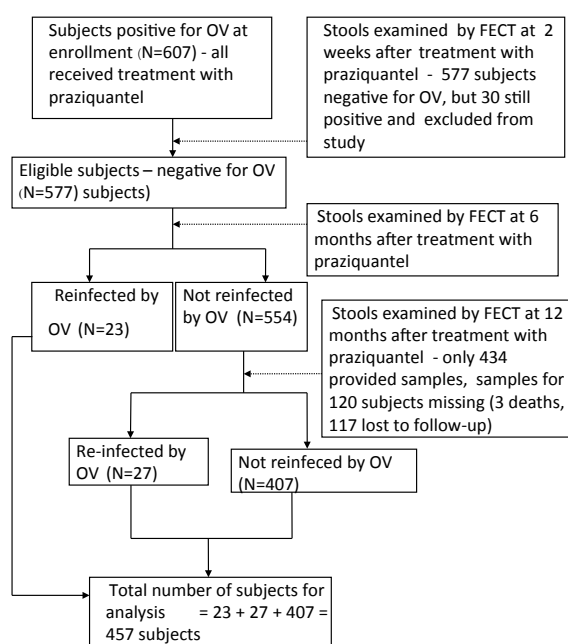


Figure 1. Flow Chart to Show Subjects Included in Final Analysis

Table 1. Characteristics of Participants and Associations with Reinfection

Characteristic (n=457)	Reinfection						Associations with reinfection (OR, 95%CI)			
	Positive (n=50)	%	Negative (n=407)	%	Total	%	Crude OR	p-value	Adjust OR	p-value
Gender										
Female	23	9.9	210	90.1	233	50.9	1		1	
Male	27	12.1	197	87.9	224	49.1	1.25 (0.69-2.25)	0.456	1.26 (0.69-2.30)	0.436
Age (years)										
<50	22	10.3	191	89.7	213	46.6	1			
50+	28	11.5	216	88.5	244	53.4	1.23 (0.68-2.23)	0.489		
Education										
Primary education only	42	11.5	324	88.5	366	80.1	1		1	
Secondary education or higher	8	8.8	83	91.2	91	19.9	0.63 (0.27-1.44)	0.271	0.7 (0.30-1.64)	0.418
Occupation										
Other	1	3.6	27	96.4	28	6.1	1		1	
Agriculture	49	11.4	380	88.6	429	93.9	3.48 (0.46-26.19)	0.226	2.73 (0.35-21.13)	0.335
Consumption of raw fish in past year										
No	1	14.3	6	85.7	7	1.5	1			
Yes	49	10.8	401	89.1	450	98.5	0.75 (0.09-6.36)	0.792		
Frequency of past use of PZQ										
never used	24	8.2	267	91.8	291	63.8	1		1	
at least once	26	15.7	140	84.3	166	36.2	2.07 (1.14-3.73)	0.016	2.04 (1.13-3.69)	0.019
Belief that PZQ can prevent infection										
yes, it can	22	14.9	125	85.1	147	32.2	1			
no, it cannot	15	9.9	137	90.1	152	33.3	0.57 (0.28-1.17)	0.13		
do not know	13	8.2	145	91.7	158	34.6	0.55 (0.27-1.12)	0.102		
Knowledge of correct use of PZQ										
lower	22	11.1	177	88.9	199	43.5	1			
higher	28	10.9	230	89.1	258	56.5	0.98 (0.54-1.77)	0.945		
Knowledge of side-effects of PZQ										
lower	19	11.2	150	88.8	169	36.9	1			
higher	31	10.8	257	89.2	288	63.1	0.95 (0.52-1.74)	0.874		
Knowledge of benefits of PZQ										
lower	30	11.7	226	88.3	256	56.1	1			
higher	20	9.9	181	90.1	201	43.9	0.83 (0.46-1.51)	0.548		

were given a further dose of PZQ.

The data were summarised using descriptive statistics, and associations with reinfection were analysed by simple and multiple logistic regression. Backward elimination of the variables was used in the multivariate analysis, and the candidate variables were those found to have a statistically significant ($p < 0.25$) association with reinfection of OV in the crude analysis and those with no significant association but likely to be related on the basis of previous research into factors related to OV infection. In the final model

statistical significance was set at $p < 0.05$.

The research was approved by the Khon Kaen University Ethics Committee for Human Research (reference number HE542213).

Results

All 457 subjects included in the analysis had responded to all the interview questionnaire items and provided the requisite stool samples. Their characteristics are

Table 2. Rates of Reinfection by *O. viverrini* of Subjects at 6 and 12 Month Follow-ups

	Number providing stool samples	Number reinfected	% reinfected
At the first 6 months	511	23	4.5
At the second 6 months	434	27	6.22

summarised in Table 1. There were approximately equal numbers of males and females, and most (53.4%) were over 50 years of age. A large majority (80.1%) were not educated beyond primary school level, and almost all (93.9%) were employed in some aspect of agriculture. Nearly everyone (98.5%) had reported consuming unsafely prepared raw freshwater fish in the 12 months prior to the study. Table 2 shows the reinfection rates over the first and second six month periods (4.5% and 6.2% respectively) and the cumulative rate over the entire 12 months (10.9%). The results of the logistic regression analyses are also presented in Table 1. The only factor found to be significantly associated with reinfection in the final model was reported past use of PZQ (OR_{adj}=2.04, 95%CI=1.13-3.69, p-value=0.019).

Discussion

The 12-month cumulative reinfection rate found in the present study is lower than the corresponding rates reported in the two studies previously conducted in Northeastern Thailand. The subjects for the first of these (Sornmani et al., 1984) were 594 infected residents in three villages treated with 40 mg/kg PZQ and negative for infection one month later. All were regularly tested for reinfection over a 12 month follow-up period. In two of the villages, PZQ treatment was combined with an intervention programme involving health education and improved sanitation. The cumulative reinfection rates at 12 months were 55.5% in the control village where there was no intervention programme and 21.6% in the two intervention villages. In the second study (Upatham et al., 1988), 622 infected residents of one village were found to test negative one month after being treated with 40 mg/kg PZQ. At the 12 month follow-up, 51.5% were found to have been reinfected to an at least moderate degree of intensity. As far as we can determine, there is only one other relevant study of *O. viverrini* reinfection rates (Pungpak et al., 1994). This was conducted in Central Thailand and involved 329 infected villagers who were given the same PZQ dose as in the present and previous two other studies, but the infection status of those retested at the various followed-up stages and retested is unclear, and some subjects who were found to have been reinfected were given a further dose of PZQ. While the authors claim a reinfection rate of 10% over two years, exact rates cannot be calculated from the data provided, and any valid comparison of the results of this study with

the present findings is not possible.

However, the results of the two other Thai studies do indicate an improvement in reinfection rates over the past 25 years or so, and one of these (Sornmani et al., 1984) suggests that this may have occurred due to a progressive improvement in sanitation and awareness of the problem of *O. viverrini* infection. Certainly, there has been a major reduction in national prevalence rates during the period 1981-2001 (Saengsawang et al., 2012). Nevertheless, our data confirm that, at least in our study area, many people continue to eat unsafely prepared and probably infected freshwater fish dishes; furthermore, whatever improvements are made to human sanitation, humans are not only the definitive hosts with, for example, high levels of infection occurring in reservoir hosts such as cats (Aunpromma et al., 2012). Our own and other studies point to the core issue being a longstanding and persistent Thai cultural attachment to freshwater fish dishes. Breaking this traditional habit of eating fish containing viable metacercariae should be the primary focus for any infection control programme.

An important limitation of the present study is the reliance on single stool samples analysed by FECT. This method of detecting *O. viverrini* infection can have poor sensitivity (Sithithaworn et al., 1991; Lovis et al., 2009) and may therefore lead to the infection rate being underestimated. This appears to be particular risk where an only light infection is involved (Sayasone et al., 2015). A further problem with the diagnostic efficiency of FECT is that its specificity can be reduced because of the potential failure to distinguish between *O. viverrini* eggs and those released by minute intestinal flukes which have a similar microscopic appearance (Lovis et al., 2009; Sripa et al., 2011). Similar problems arise in the diagnostic use of a single Kato-Katz stick smear examination, but the collection of multiple stool samples and analysis by both the Kato-Katz and FECT techniques has been shown to improve diagnostic accuracy (Lovis et al., 2009; Sayasone et al., 2015). However, the logistics and costs of this approach make it difficult to apply in rural field surveys such as the present study, and the use of serological or molecular diagnostic techniques can be even harder to resource in these settings. One promising new diagnostic method may overcome many of these difficulties, and this involves the detection of *O. viverrini* infection by the analysis of *O. viverrini* antigens in urine samples. This technique has been found to have considerably higher sensitivity and specificity than FECT, only single 5 ml urine samples are needed, and there is the prospect of developing a simple strip test kit for routine use in the community to avoid the problems associated with sample collection, storage and costly 'high tech' analysis (Worasith et al., 2015).

The extremely high reported consumption of unsafely prepared freshwater fish and the association of previous use of PZQ with reinfection are findings consistent with the notion of a cycling of infection, treatment and reinfection, but the relevant data were collected at baseline. More information is needed about exposure to infection, PZQ use and other potentially important independent variables during the follow-up period when reinfection is being

monitored. In addition, the lack of statistically significant associations of factors with reinfection may be a function of the underpowering effect of a small sample size. Larger scale studies are needed with estimated required sample sizes computed at the planning stage.

In the light of the evidence now suggesting that a cycling of repeated infection and treatment with PZQ increases the risk of CCA, the dearth of studies on OV reinfection and the lack of information about factors leading to reinfection are clearly matters requiring more attention. The present study has a number of shortcomings, but it does highlight the importance of the topic and various major issues about the design of future studies. The over-riding concern is that, so long as Thai people believe that they can continue to eat unsafely prepared fish dishes and avoid the consequent risk of CCA by regularly dosing themselves with PZQ, then the strong, culturally engrained habit of consuming these dishes will survive. This belief should therefore be the principal focus for public education campaigns

Acknowledgements

This study was supported by Khon Kaen University (KKU) Graduate School, the KKU Faculty of Nursing Research and Training Centre for Enhancing Quality of Life of Working-Age People, the National Research University Project of Thailand through the Center of Excellence in Specific Health Problems in the Greater Mekong Sub-region (SHeP-GMS), and a KKU research grant (no. NRU542005). Thanks are due to Dr Siriporn Kamsa-ard and Khun Kornthip Jeephet for their advice on statistical power analysis.

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