

RESEARCH ARTICLE

The Carcinogenic Liver Fluke *Opisthorchis viverrini* is a Reservoir for Species of *Helicobacter*

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Abstract

There has been a strong, positive correlation between opisthorchiasis-associated cholangiocarcinoma and infection with *Helicobacter*. Here a rodent model of human infection with *Opisthorchis viverrini* was utilized to further investigate relationships of apparent co-infections with *O. viverrini* and *H. pylori*. A total of 150 hamsters were assigned to five groups: i) Control hamsters not infected with *O. viverrini*; ii) *O. viverrini*-infected hamsters; iii) non-*O. viverrini* infected hamsters treated with antibiotics (ABx); iv) *O. viverrini*-infected hamsters treated with ABx; and v) *O. viverrini*-infected hamsters treated both with ABx and praziquantel (PZQ). Stomach, gallbladder, liver, colonic tissue, colorectal feces and *O. viverrini* worms were collected and the presence of species of *Helicobacter* determined by PCR-based approaches. In addition, *O. viverrini* worms were cultured *in vitro* with and without ABx for four weeks, after which the presence of *Helicobacter* spp. was determined. In situ localization of *H. pylori* and *Helicobacter*-like species was performed using a combination of histochemistry and immunohistochemistry. The prevalence of *H. pylori* infection in *O. viverrini*-infected hamsters was significantly higher than that of *O. viverrini*-uninfected hamsters ($p \leq 0.001$). Interestingly, *O. viverrini*-infected hamsters treated with ABx and PZQ (to remove the flukes) had a significantly lower frequency of *H. pylori* than either *O. viverrini*-infected hamsters treated only with ABx or *O. viverrini*-infected hamsters, respectively ($p \leq 0.001$). Quantitative RT-PCR strongly confirmed the correlation between intensity *H. pylori* infection and the presence of liver fluke infection. *In vitro*, *H. pylori* could be detected in the *O. viverrini* worms cultured with ABx over four weeks. In situ localization revealed *H. pylori* and other *Helicobacter*-like bacteria in worm gut. The findings indicate that the liver fluke *O. viverrini* in the biliary tree of the hamsters harbors *H. pylori* and *Helicobacter*-like bacteria. Accordingly, the association between *O. viverrini* and *H. pylori* may be an obligatory mutualism.

Keywords: *Opisthorchis viverrini* - *Helicobacter* - *H. pylori* - reservoir host - hamster

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Introduction

Cholangiocarcinoma (CCA) is highly prevalent in Asian countries, particularly in Thailand, China, Taiwan and Korea (Shin et al., 2010). The principal risk factor for CCA in this region is infection with the human liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, which are Group 1 carcinogens as classified by the World Health Organization (Sripa et al., 2007; 2012; Bouvard et al., 2009; IARC, 2012). Thailand has the highest incidence of CCA worldwide due to its robust association with opisthorchiasis (Sripa and Pairojkul, 2008; Shin et al., 2010). However, several other risk factors for this cancer have been documented including primary sclerosing cholangitis (see Rizvi and Gores, 2013), inflammatory bowel disease (Huai et al., 2014), metabolic syndromes (Wu et al., 2012), hepatitis virus (Matsumoto et al., 2014)

and infection with *Helicobacter* spp. (Zhou et al., 2013). The latter has attracted increasing research interest in recent years (Mateos-Munoz et al., 2013; Murphy et al., 2014).

Helicobacter pylori infection was the first bacterial infection known to be a causative factor of gastrointestinal diseases including gastric adenocarcinoma (Bouvard et al., 2009). Its virulence factors such as *cagA* and *vacA* are involved in the pathogenesis of the diseases (Cid et al., 2013; Hatakeyama, 2014). Other *Helicobacter* species, specifically *H. hepaticus* and *H. bilis* also are implicated in hepatobiliary disease (Zhou et al., 2013; Mateos-Munoz et al., 2013; Murphy et al., 2014). We observed an association between *H. pylori* and hepatolithiasis or CCA in people in Northeast Thailand, a region endemic for opisthorchiasis (Boonyanugomol et al., 2012b; 2012c). Molecular mechanisms integral to *H. pylori*

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induced hepatobiliary diseases have also been reported (Boonyanugomol et al. 2011; 2012a). However, the underlying mechanisms by which species of *Helicobacter* associate with opisthorchiasis remain unclear. Here we investigated the prospective inter-relationship between infections with *Helicobacter* spp. and liver flukes in a rodent model of this foodborne liver fluke infection.

Materials and Methods

Animals

Female Syrian hamsters, *Mesocricetus auratus*, about 8 weeks of age were housed in conventional conditions, fed a commercial diet and given water *ad libitum*. Rodent husbandry conformed to the ethical guidelines of the Animal Laboratory Center, Faculty of Medicine, Khon Kaen University; these studies were approved by the Animal Ethics Committee of Khon Kaen University; approval number AEKKU # 100/2555. Laboratory infection of hamsters with *O. viverrini* was accomplished by oral administration of 50 metacercariae, as described (Lvova et al., 2012). Infected hamsters were maintained up to three months after the infection; control, non-infected hamsters were housed in identical conditions.

Experimental design

The hamsters (n=150) were assigned to five groups of 30 rodents each (Table 1): group 1 - uninfected controls (n=30); group 2 - hamsters treated with antibiotics (ABx) (clarithromycin 7.5 mg/kg BID+ metronidazole 20mg/kg BID + amoxicillin 20 mg/kg BID), omeprazole 0.7

Table 1. Treatment Groups of Hamsters Infected with the Liver Fluke *Opisthorchis viverrini*

Group	Number of hamsters	Infection with <i>O. viverrini</i>	ABx	PZQ
1	30	-	-	-
2	30	+	-	-
3	30	-	+	-
4	30	+	+	-
5	30	+	+	+

*ABx, antibiotics; PZQ, praziquantel

Table 2. Primer Sequences and PCR Conditions for the Detection of Species of *Helicobacter*

Genes	Primer sequences	PCR conditions	Product size (bp)	References
<i>16Sr RNA</i> (nested PCR)	OF-ATTAGTGCGCACGGGTGAGTAA OR-TTTAGCATCCCGACTTAAGGC	94oC 30 sec, 55oC 30 sec, 72oC 1.5 min (35 cycles)	1,300	Pellicano et al. (2004)
	IF-GAACCTTACCTAGGCTTGACATTG IR-GGTGAGTACAAGACCCGGGAA	94oC 30 sec, 60oC 30 sec, 72oC 30 sec (35 cycle)	480	
<i>ureA</i> (nested PCR)	OF-GCTAATGGTAAATTAGTTCCTGG OR-CTCCTTAATGTGTTTTTACATAGTTG	94oC 30 sec, 62oC 30 sec, 72oC 30 sec (40 cycles)	411	Pellicano et al. (2004)
	IF-AGTTCCTGGTGAGTTGTTCTTAA IR-AACCACGCTCTTAGCTCTGTC	94oC 30 sec, 59oC 30 sec, 72oC 30 sec (40 cycles)	350	
<i>H. bilis</i>	OF-CTATGACGGGTATCCGGC OR-CTCACGACACGAGCTGAC	98oC 10 sec, 55oC 30 sec, 72oC 1 minute (35 cycles)	718	Hamada et al. (2009)
	IF- CAGAACTGCATTTGAAACTAC IR-AAGCTCTGGCAAGCCAGC	98oC 15 sec, 57oC 30 sec, 72oC 30 sec (35 cycles)	418	
<i>H. hepaticus</i>	OF-CTATGACGGGTATCCGGC OR-CTCACGACACGAGCTGAC	98oC 10 sec, 55oC 30 sec, 72oC 1 minute (35 cycles)	718	Hamada et al. (2009)
	IF -GAAACTGTTACTCTG IR-TCAAGCTCCCCGAAGGG	98oC 15 sec, 56oC 30 sec, 72oC 30 sec (35 cycles)	405	

mg/kg BID and sucralfate 300 mg/kg) twice a day for 14 days, as described (Khoshnegah et al., 2011); group 3 - *O. viverrini* infected hamsters; group 4 - *O. viverrini*-infected hamsters treated with ABx for 14 days; group 5 - *O. viverrini*-infected hamsters treated with ABx for 14 days and then treated with praziquantel (PZQ) (Biltricide, Bayer, Pittsburgh, PA) suspended in 2% Cremophor EI (Sigma, St. Louis, MO) at 400mg/kg body weight twice, with an interval of two weeks between treatments aiming to eradicate the parasites. Hamsters were euthanized, after which colorectal feces, stomach, gallbladder, liver and worms were collected from each group for DNA extraction. Some worms were cultured for up to four weeks in RPMI 1640 medium supplemented with penicillin and streptomycin. Samples of livers from selected hamsters were fixed in 10% buffered formalin for histochemistry and immunohistochemistry.

DNA extraction

One gram of stool sample was used for DNA extraction using a QIAamp® DNA Stool Mini Kit (Qiagen, Germany). The worms, liver, stomach, gallbladder and colon were incubated with lysis buffer (10 mM Tris-HCl pH 8.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS), homogenized with tissue grinder, and digested with proteinase K at 55°C overnight. Thereafter, DNAs were recovered using precipitation with ethanol.

Molecular detection of *Helicobacter* species by PCR

Fifty ng DNA from each stool was used in the PCR assay. The reaction mixture consisted of 1x GoTaq® Colorless Master Mix (Promega, USA) containing 0.2 mM dNTP, 1.5 mM MgCl₂, primers at 0.2 mM and 1.25 U of Taq DNA polymerase. Primer sequences for the detection of *Helicobacter* spp. (*16srRNA*), *H. pylori* (*ureA*), *H. bilis*, *H. hepaticus* are provided in Table 2. PCR was performed in a GeneAmp® PCR system 9700 (Applied Biosystems, Life Technologies) thermocycler. Amplified products were sized by electrophoresis through 1.0% agarose, stained with ethidium bromide and visualized under a UV illuminator.

Quantitative real time PCR

H. pylori was quantified by real time PCR in all stool DNA samples and worms from the five groups of hamsters, as described (McDaniels et al., 2005; Linke et al., 2010), with minor modifications. The *H. pylori ureA* gene subunit *ureA* was targeted for real-time PCR, using primers HpyF1: GGGTATTGAAGCGATGTTTCCT and HpyR1: GCTTTTTTGC-CTTCGTTGATAGT. The reaction mixture included 10 μ l SYBR Green Master Mix (Thermo Scientific, USA), 1.0 μ l template DNA; 0.5 μ l of each primer (0.625 μ M) and 9 μ l nuclease-free water. Thermocycling conditions using Roche's Light Cycler 1.5 were: 9 min initial denaturation at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C for the annealing and elongation steps. To quantify number of bacterial cells in samples, a standard curve derived was established using 10-fold serial dilutions of *H. pylori* DNA from 10⁸ cells/ml. Cell numbers of *H. pylori* cell count in one gram of colorectal feces was ascertained by the standard curve at crossing point and log concentration, as described (Linke et al., 2010). The cell concentration/amount of DNA was calculated to obtain the same initial amount for all templates. *Escherichia coli* was used as negative control.

In situ localization of *H. pylori* in *O. viverrini* flukes

Formalin-fixed and paraffin-embedded tissue sections of *O. viverrini* infected hamster livers were histochemically stained using the Warthin-Starry method (Hartman and Owens, 2012). Briefly, sections were deparaffinized, hydrated to water, rinsed in acidic distilled water pH 3.8-4.4, and stained with 1% silver nitrate solution for 45 seconds in a microwave oven. After cooling to room temperature, the slides were immersed in 0.15% hydroquinone, 5% gelatin, 2% silver nitrate (reducing solution) for one minute, rinsed thoroughly in tap water, then dehydrated, cleared and mounted.

Specific localization of *H. pylori* was also undertaken using an immunohistochemistry approach. In brief, hydrated thin tissue sections were subjected to antigen retrieval in 0.01 mol/l citrate buffer using a high temperature pressure cooker; endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Non-specific staining was minimized by incubation in 5% normal horse serum in PBS. Sections were probed with anti *H. pylori* rabbit polyclonal antibody (Dako) overnight (primary antibody). After washing with PBS, sections were incubated in biotinylated goat anti-rabbit IgG-HRP (Dako) for 60 min (secondary antibody), washed and developed with 3,3'-diaminobenzidine (DAB) (Sigma). Subsequently, sections were counterstained in Mayer's hematoxylin, cleared, and mounted. Paraffin-embedded gastric tissue from *H. pylori*-infected patient was used as positive control. Negative control sections were probed only with secondary antibody.

Statistical analyses

Chi-square (X^2) or Fisher's exact test was used to compare the categorical data among treatment groups. One-way ANOVA (Post Hoc test) was used to compare the means of total cell counts per gram feces. p values ≤ 0.05 were considered to be statistically significant.

Results

Prevalence of *Helicobacter* in hamster feces

Figure 1 presents the prevalence of infection with species of *Helicobacter* as determined by fecal PCRs targeting the 16S rRNA, *ureA*, *H. bilis*, and *H. hepaticus*-specific genes in *O. viverrini*-infected hamster with and without treatments and controls. Baseline prevalence of species of *Helicobacter* was significantly higher in hamsters with *O. viverrini* infection (90%) than in non-*O. viverrini* infected hamsters (43.3%) ($p \leq 0.001$). Similarly, the prevalence of species of *Helicobacter* in the *O. viverrini* infected group was significantly higher than both the *O. viverrini*-infected group treated with ABx (46.7%) and the *O. viverrini*-infected group treated with ABx and PZQ (30%) ($p \leq 0.05$). Notably, the prevalence of *H. pylori* infection (*ureA* gene positive) was significantly higher in hamsters without *O. viverrini* infection (33.3%) than that in non-*O. viverrini* infected hamsters treated with ABx (16.7%) ($p \leq 0.001$).

Moreover, the prevalence of *H. pylori* in hamster infected with *O. viverrini* (73.3%) was significantly higher than in hamsters infected with *O. viverrini* and treated with ABx (36.7%) and in those treated with both ABx and PZQ (16.7%) ($p \leq 0.001$). Likewise, the prevalence of *H. bilis* but not *H. hepaticus* infection was higher in the *O. viverrini* infected hamsters (40%) than the non-*O. viverrini* infected rodents (26.7%) ($p \leq 0.001$). In addition, *H. bilis* infection was more prevalent in hamsters with *O. viverrini* infection (26.7%) than in hamsters with *O. viverrini* treated with ABx (13.3%) and the *O. viverrini* infected group treated with ABx and PZQ (13.3%) ($p \leq 0.05$). Last, and notable, the prevalence of *H. pylori* and *H. bilis* was lower in hamsters infected with *O. viverrini* and treated with ABx and PZQ than in *O. viverrini*-infected hamsters treated only with ABx, and was similar to that in the non-*O. viverrini* infected hamsters.

Prevalence of *Helicobacter pylori* in hamster organs

The prevalence of *H. pylori* in diverse hamster organs-stomach, liver, colon, gallbladder including feces from

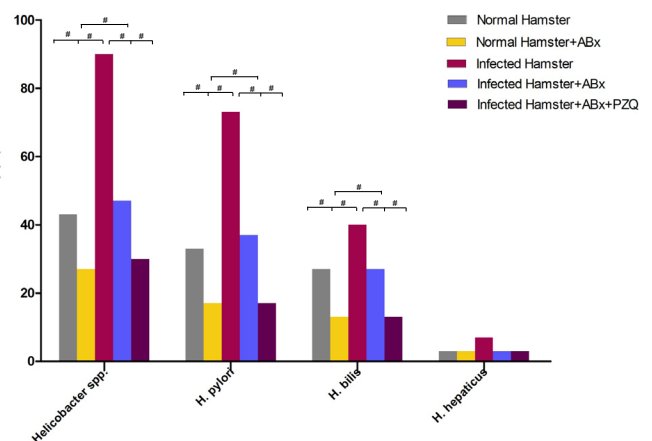


Figure 1. Prevalence of *Helicobacter* spp. Infection in feces from 5 groups of hamsters. X-axis represents PCR positivity (%) of *Helicobacter* 16SrRNA, *UreA*, *H. bilis*, and *H. hepaticus* among groups. *Significant difference ($p \leq 0.001$)

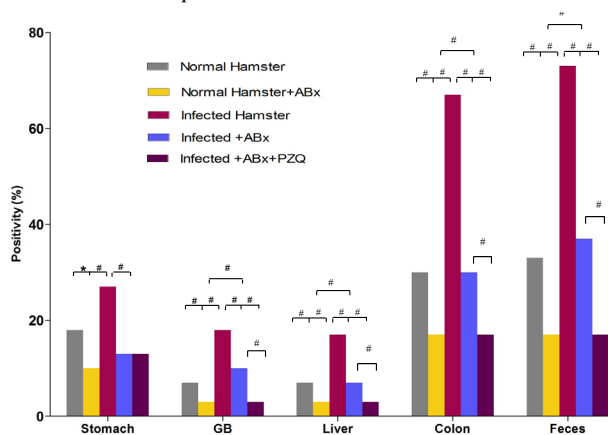


Figure 2. Prevalence of *Helicobacter pylori* Infection in Various Hamster Organs by PCR. Data (%) for *H. pylori* #($p \leq 0.001$) and *($p \leq 0.05$)

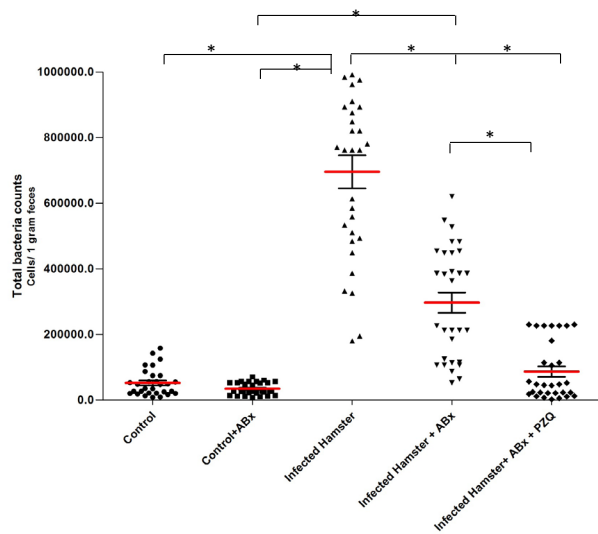


Figure 3. Quantification of *Helicobacter pylori* in Colorectal Feces of Hamsters Using Quantitative RT-PCR. Total bacterial cell counts among the following 5 groups of hamsters are compared; 1. Control (Group 1), 2. Control+ ABx (Group 2), 3. Infected hamster (Group 3), 4. Infected hamster + ABx (Group 4), and 5. Infected hamster + ABx+ PZQ (Group 5). *Significant difference among groups ($p \leq 0.001$)

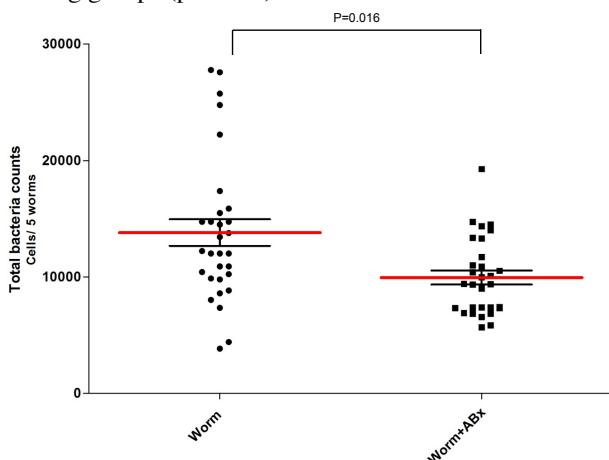


Figure 4. Quantification of *Helicobacter pylori* in *O. viverrini* from Infected Hamsters (Group 2) and *O. viverrini* from Infected Hamsters Treated with ABx (Group 3). Data are representative of bacterial counts from 5 worms/hamster recovered from each group

colon, varied among the treatment groups (Figure 2). *H. pylori* was more common (>70%) in colon and feces of *O. viverrini*-infected compared to <40% in control hamsters not infected with *O. viverrini* ($p \leq 0.001$). After treatment with ABx, the prevalence of *H. pylori* in both colon and colorectal feces declined significantly. The prevalence of *H. pylori* was significantly lower in *O. viverrini*-infected hamsters treated with ABx and PZQ - 16.7% vs 66.7% and 16.6% vs. 73.9% for the colon tissue and feces, respectively ($p \leq 0.001$), levels about half those from the ABx treated hamsters.

For the liver and gallbladder, in like fashion, significantly higher prevalence of *H. pylori* was seen in hamsters infected with *O. viverrini* compared to non-infected controls (16.6% vs 6.7% and 18.0% vs 6.7%, respectively). Treatments with ABx and PZQ of *O. viverrini*-infected hamsters significantly reduced the prevalence of *H. pylori* to less than half of their ABx baselines in both the liver and gallbladder (6.7% vs. 3.3% and 10.0% vs 3.3%, respectively). Similar to other organs, infection prevalence of *H. pylori* in the stomach was significantly higher in *O. viverrini* infected (26.7%) compared to uninfected hamsters (18.0%) ($p \leq 0.05$). However, the prevalence of *H. pylori* in *O. viverrini*-infected hamsters treated with ABx and PZQ (13.3%) was similar to those treated with ABx alone (13.3%), unlike those organs/ tissues reported above. In overview, the prevalence of *H. pylori* in organs of the ABx and PZQ treated hamsters declined to about their ABx-treated baselines.

Quantification H. pylori by quantitative real-time PCR (qPCR)

Levels of *H. pylori* (*H. pylori* cell counts) in the five groups of hamsters, as established from the qRT-PCR standard curve, are presented in Figure 3. Significantly divergent intensities of infection with *H. pylori* were

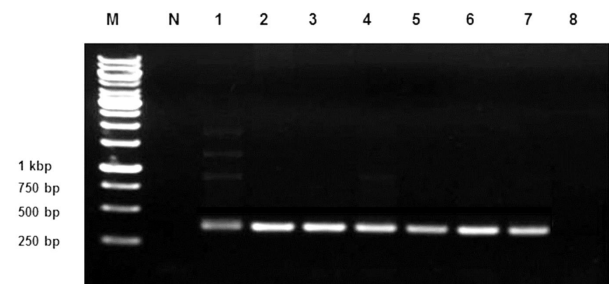


Figure 5. Representative PCR Products Amplified by using *Helicobacter pylori* Specific Primer (Product Size=350 bp) from *O. viverrini* Worms (Recovered from Group 2) Cultured with Antibiotics upto 4 Weeks, Excretory-secretory (ES) Products, Fresh Worms and Ov metacercariae. M=Maker, N=Negative control, Lane 1=Positive control (*H. pylori*), Lane 2=Fresh Ov, Lane 3=Ov cultured 1 week, Lane 4=Ov cultured 2 weeks, Lane5=Ov cultured 3 weeks Lane6=Ov cultured 4 weeks, Lane7=ES products and Lane 8=Metacercariae, respectively. Data are representative of PCR products amplified from DNA extracted from 10 worms per duration or DNA from 500 *O. viverrini* metacercariae. Ov=*Opisthorchis viverrini*

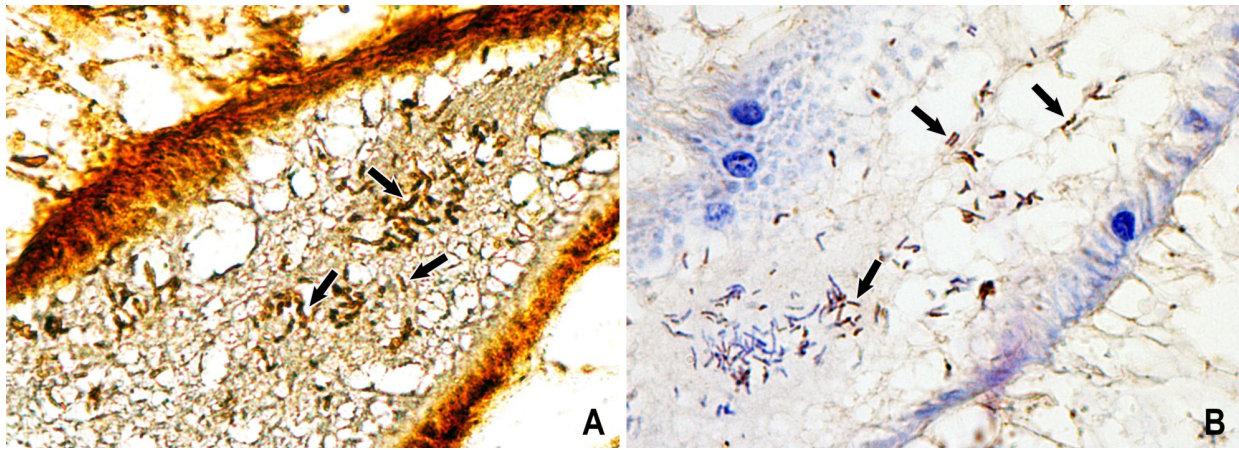


Figure 6. Identification of *Helicobacter pylori* in the *O. viverrini* Gut. (A) Cluster of *H. pylori*-like (short arrow) stained with the Warthin-Starry method shown as dark brown curved rod-like bacteria in the lumen. (B) Specific detection of *H. pylori* with anti-*H. pylori* antibody immunostained as a dark brown color (short arrow). Original magnification, x400

evident: *O. viverrini*-infected hamsters, $695,712 \pm 270,962$ cells (mean \pm SD/five worms); *O. viverrini* infected hamsters treated with ABx, $297,033 \pm 167,158$; *O. viverrini* infected hamsters treated with ABx and PZQ, $86,763 \pm 86,585$; uninfected hamsters, $52,105 \pm 40,004$; and uninfected hamsters treated with ABx, $34,497 \pm 18,360$ ($p \leq 0.001$). Overall, *H. pylori* infection intensity in *O. viverrini* infected hamsters was significantly greater than that of uninfected hamsters ($p \leq 0.001$). Of note, *O. viverrini* infected hamsters treated with ABx and PZQ showed significant reduction of *H. pylori* infection intensity ($p \leq 0.001$), similar to levels detected in the non-*O. viverrini*-infected hamsters treated or not treated with ABx.

The quantification of *H. pylori* in *O. viverrini* worms

The *H. pylori* infection intensity in *O. viverrini* recovered from hamsters (mean = $13,806 \pm 6,162$ cells/5 worms) was significantly greater than that in *O. viverrini* from infected hamsters treated with ABx ($9,951 \pm 3,220$) (Figure 4).

Detection of *H. pylori* in cultured *O. viverrini* worms

To investigate the presence of *H. pylori* in long-term culture with ABx, we did examine by PCR up to 4 weeks culture as well as in fresh worms, ES products and metacercariae. The results showed that *H. pylori* could be observed in fresh recovered adults, all cultured worms, and ES products but not in the metacercariae of *O. viverrini* (Figure 5).

In situ localization of *H. pylori* in *O. viverrini*

To directly investigate the existence/presence of *H. pylori* in the *O. viverrini* flukes, in situ localization using the Warthin-Starry technique and immunohistochemistry was undertaken. Warthin-Starry staining revealed dark brown curved rod-like structures that clearly resembled *H. pylori* within the lumen of the gut of the flukes (Figure 6A). In like fashion, immunohistochemical staining with antibody specific for *H. pylori* showed strong, positive signals for the bacteria in the lumen of the gut of the flukes (Figure 6B).

Discussion

Cholangiocarcinoma is highly prevalent in Asian countries; particularly in Thailand (Shin et al., 2010) and, besides dietary carcinogens, the main risk factor is infection with the human liver flukes, *O. viverrini* and *Clonorchis sinensis* (Sripa et al., 2007; Sripa and Pairojkul, 2008; Petney et al., 2013; Sithithaworn et al., 2014). Other risk factors associated with the infection such as microbiota in the biliary system may also be involved (Plieskatt et al., 2013). We recently found a significant association between *H. pylori* or mixed *H. pylori* and *H. bilis* but not *H. hepaticus* infection and CCA in patients from Northeast Thailand, a region highly endemic for opisthorchiasis (Boonyanugomol et al., 2012b; 2012c). However, which species of *Helicobacter* associate with opisthorchiasis, and why, remain unclear. We show here, for the first time in a rodent model a human opisthorchiasis, significantly higher prevalence of co-infection with *H. pylori* and *H. bilis* but not *H. hepaticus* in *O. viverrini*-infected and uninfected hamsters. Interestingly, clearance of the liver fluke infection following treatment with PZQ significantly reduced the prevalence and intensity of *H. pylori* in *O. viverrini*-infected hamsters suggested a strong inter-relationship between the two pathogens. In addition, in situ localization revealed *H. pylori* in the gut of the worm and the presence of *H. pylori* in worms cultured with lengthy periods in antibiotics supports that adult *O. viverrini* may act as a reservoir for *H. pylori*.

Species of *Helicobacter* including *H. pylori*, *H. hepaticus* and *H. bilis* have been described from several other mammals as well as from people (Fox et al., 1995; Pellicano et al., 2008; Goldman et al., 2010). Fox et al. (2009) reported hamsters naturally infected with *H. bilis* and that aged animals showed chronic hepatitis, hepatic dysplasia, fibrosis, and biliary hyperplasia. During other liver fluke infections, *H. bilis*-like bacteria has been identified in the intrahepatic bile ducts of rats experimentally infected with *Fasciola hepatica* (Foster, 1984). Our study reported a significant higher prevalence of *H. pylori* and *H. bilis* but not *H. hepaticus* in the colon feces of *O. viverrini* infected hamsters

compared to uninfected hamsters both before and after ABx treatment. We further investigated the source of the higher fecal infection by examining the prevalence of *H. pylori* in the stomach, liver, gallbladder, and colon mucosa. *Helicobacter* spp. have been observed previously in these sites (Fox et al., 1995). The findings presented here demonstrated infection of liver and gallbladder with *H. pylori*, and indicated that the *H. pylori* in these organs originated from the liver fluke in the bile ducts rather than from microbiome of the hamster stomach. The findings indicated that infection with *O. viverrini* influenced the higher positivity and intensity in the feces since levels of *H. pylori* significantly declined following treatments with ABx and PZQ (Figures 2 and Figure 3). The source of *Helicobacter* infection of the liver and biliary system may be ascending infection through the small intestine (Pellicano et al., 2008). Partial bile duct obstruction by the flukes as well as host inflammatory responses cause bile stasis and facilitate ascending infection and cholangitis (Carpenter, 1998; Sripa, 2003). We recently reported higher number of bacterial species in *O. viverrini* infected hamster bile and also colorectal feces (Plieskatt et al., 2013). Although *Helicobacter* is bile-sensitive, the mechanism by which colonization by this microbe of the biliary system is accomplished is uncertain. It has been suggested that the 'acidification' of bile through reflux into the low pH of the stomach precipitates inhibitory bile acids, thereby allowing colonization by *H. pylori* (Hynes et al., 2003; Shao et al., 2008). In case of opisthorchiasis, the infection by the flukes can disturb bile acid composition (Wongpaitoon et al., 1988; Wonkchalee et al., 2012) that may reduce alkalinity of the bile, leading to damage to biliary epithelia and inflammation (Strazzabosco et al. 2000). All these phenomena may facilitate conditions that favor colonization by *Helicobacter* during concurrent infection with liver flukes.

Whether the presence of *H. pylori* in the biliary system during opisthorchiasis occurs only in the bile and biliary mucosa or also within the fluke had until now remained unresolved. Here we demonstrated in situ localization of *H. pylori* histochemically and immunohistochemically in the gut or gastrodermal of the fluke. Accidental contamination during specimen preparation cannot account for these new findings since *H. pylori* was qualitatively and quantitatively detected in the thoroughly washed worms and cultured in the presence of ABx for more than a month. However, the origin of infection of the liver fluke with *Helicobacter* spp. is unclear. The fluke likely ingests bile contents and biliary epithelium for nutrition (Sripa, 2003; Sripa et al., 2007), and *H. pylori* is likely ingested in the bile by the fluke. We postulate that *H. pylori* can colonize and propagate in the gut epithelium of *O. viverrini* and, in turn, continue to be released back into bile with the excretions and secretions (ES) of the flukes. DNA from *H. pylori* can be detected by PCR in the ES products of the cultured worms (Figure 5). The epithelia of the gut of *O. viverrini* may provide conditions favorable to *Helicobacter* i.e. acidic to neutral pH, mucus as well as specific adhesion molecules (Sachs et al, 2011; Dunne et al., 2014). Together, these new findings suggest that *O. viverrini* within the mammalian hepatobiliary system may

be a reservoir of *H. pylori*.

The elevated prevalence of *Helicobacter* spp. in *O. viverrini*-infected hamsters raises the question of whether infection with *Helicobacter* contributes to pathogenesis of hepatobiliary diseases as well as CCA. Hepatobiliary diseases caused by species of *Helicobacter* in naturally infected hamsters (Fox et al., 2009; Boonyanugomol et al. 2012b; 2012c) are similar to those of *O. viverrini* (Lvova et al., 2012; Mairiang et al., 2012). Indeed, the chronic lesions ascribed to liver fluke infection in both hamsters and humans, i.e., cholangitis, biliary hyperplasia and metaplasia, and periductal fibrosis or even CCA may be due, in part, to *Helicobacter*-associated hepatobiliary disease. *H. pylori* and *H. bilis* DNA has been isolated from liver tissue of humans with CCA and cholecystitis/cholelithiasis from Thai patients living in regions endemic for opisthorchiasis (Boonyanugomol et al. 2012b; 2012c). Moreover, serological assays indicate active infection with *H. pylori* and *H. bilis* in Thais at high risk for CCA (Pisani et al., 2008). *H. bilis* was also associated with biliary cancers in two high-risk populations in Japan and Thailand (Matsukura et al., 2002). The strong association between liver fluke and *H. pylori* or *H. bilis* infection reported in this study supports a role of these *Helicobacter* species in the hepatobiliary diseases known during opisthorchiasis.

In conclusion, this study in a well-controlled hamster model of infection revealed a significant relationship between *O. viverrini* and *Helicobacter* spp., specifically *H. pylori* and *H. bilis*. The in situ localization of *H. pylori* in the gut of the fluke supports the hypothesis that *O. viverrini* may be a reservoir of this carcinogenic bacterium. The co-infection may orchestrate in the pathogenesis of liver fluke-induced hepatobiliary diseases including CCA.

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