Helicobacter

Persistent advanced periductal fibrosis is associated with cagA -positive Helicobacter pylori infection in post-praziquantel treatment of opisthorchiasis

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Persistent advanced periductal fibrosis is associated with *cagA*-positive *Helicobacter pylori* infection in post-praziquantel treatment of opisthorchiasis

Running head: Persistent biliary fibrosis is associated with Helicobacter pylori

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Abstract (278 words)

Background: Liver fluke infection caused by *Opisthorchis viverrini* is associated with several hepatobiliary diseases including advanced periductal fibrosis (APF) and cholangiocarcinoma (CCA). Recently, we demonstrated a persistent APF in over one-third of opisthorchiasis patients after worm removal by praziquantel (PZQ) treatment. However, the underlying mechanism(s) of this phenomena is unclear. Given a co-infection with *Helicobacter pylori* (*H. pylori*) especially *cagA*-positive strain enhances APF, we hypothesized that *H. pylori* with CagA virulent factor contributes to persistent APF.

Materials and Methods: Seventy-five opisthorchiasis patients who underwent ultrasonography and treatment with PZQ were recruited in the 2-year follow-up study. *Helicobacter* and its cagA in the feces were examined by conventional and qPCR. Correlations between prevalence or bacterial loads of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* before and after PZQ treatment were analyzed among resolved, slowly-resolved, relapsed, and persistent APF groups.

Results: Overall, prevalence of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* declined after PZQ treatment. However, only the prevalence and bacterial loads of *cagA*-positive *H. pylori* detected at 2-year post-treatment were significantly lower than those before treatment ($p<0.05$). In addition, both prevalence and bacterial loads of *cagA*-positive *H. pylori* were significantly lower in the resolved APF group after PZQ treatment, while there were no significant changes in the slowly-resolved, relapsed, and persistent APF groups. Among the APF subgroups, *cagA*-positive *H. pylori* prevalence in both relapsed and persistent APF groups were significantly higher than the resolved APF group.

Conclusion: The results support our hypothesis that *H. pylori*, especially *cagA*-positive strain contributes to the relapsed and persistent APF. A supplementary antibiotic treatment for *H. pylori* to reduce persistent APF and eventually CCA is warranted.

Keywords: Opisthorchiasis, *Opisthorchis viverrini*, Praziquantel, Advanced Periductal Fibrosis, *Helicobacter pylori, cagA*

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55 **1. INTRODUCTION**

56 Opisthorchiasis, a fish-borne trematodiasis caused by the carcinogenic liver fluke,
57 *Opisthorchis viverrini*, remains an important health problem in the Lower Mekong Basin
58 including Thailand, Lao People's Democratic Republic (Lao PDR), Cambodia, Myanmar, and
59 Vietnam with over 12 million people infected.[1-3] In Thailand, the highest prevalence was
60 reported in the North and Northeast with over 6 million people infected.[4,5] The infection is
61 associated with several hepatobiliary diseases including cholangitis, gallstones, hyperplasia,
62 dysplasia, advanced periductal fibrosis (APF), and cholangiocarcinoma (CCA), a fatal bile duct
63 cancer.[6] Treatment with praziquantel (PZQ) at a single 40 mg/kg oral dose recommended by
64 the World Health Organization is effective against opisthorchiasis.[7] The treatment not only
65 clears the fluke infection but can also reduce the biliary morbidities. Previous studies reported
66 an improvement of some hepatobiliary abnormalities as observed by ultrasound in
67 opisthorchiasis after PZQ treatment.[8,9] However, APF was still detected in over two-thirds of
68 PZQ-treated patients. Specifically, a 5-year ultrasound follow-up study found that 37.5% of *O.*
69 *viverrini* infected patients showed relapsed or persistent APF post-PZQ treatment.[9] The
70 relapsed and persistent APF is considered as a risk factor or a predisposing lesion for CCA.
71 [6,9-12] However, etiologies and mechanism(s) of this relapsed or persistent APF after removal
72 of the flukes are not yet known.

73 *Helicobacter pylori* has been reported as an etiology for hepatobiliary diseases such as
74 liver cirrhosis, cholangitis, hepatocellular carcinoma, and CCA.[13-16] Pathogenicity of *H. pylori*
75 is strongly associated with its virulence factor, cytotoxin-associated gene A (CagA).[17] During
76 the past decade, there has been increasing evidence from both animal and human studies
77 that *H. pylori,* particularly *cagA*-positive strains, may be involved in the pathogenesis of APF
78 and CCA in opisthorchiasis.[14,18-22] Interestingly, *O. viverrini* has been demonstrated as a
79 reservoir of *Helicobacter* spp., especially *cagA*-positive *H. pylori*.[15,18] The *H. pylori* co-migrates
80 with the liver fluke, colonizes in the biliary epithelium and induces inflammation and APF in
81 chronic opisthorchiasis in animals[22] and humans[15]. We propose that *O. viverrini* may act as a

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carrier for *cagA*-positive *H. pylori* which is the key driver of APF and eventually CCA development. Specifically, 23.6% of chronic opisthorchiasis patients develop APF[23] and *cagA*-positive *H. pylori* rates have been reported in 53.3% and 75% of those with APF at grades 2 and 3, respectively compared to only 25.32% with APF at grade 0.[15] Moreover, the *cagA* genotypes detected in opisthorchiasis are different from those in other pathologies[15,24], suggesting that genetic adaptation may coevolve for their survival in the contrasting harsh environments such as microaerobic, acidic conditions in the gastrointestinal tract or an alkali pH with oxidative stress in the biliary tracts.[6,15,24-26].

Therefore, we hypothesize that *H. pylori*, particularly *cagA* positive strains may be metabolically active and survive in the biliary system. We also hypothesize that relapsed or persistent APF after PZQ treatment is associated with *Helicobacter* and its CagA virulence factor. To test this hypothesis, we measured prevalence and bacterial loads of *Helicobacter* especially the *cagA*-positive strain in resolved, slowly-resolved, relapsed, and persistent APF groups of opisthorchiasis patients before and after PZQ treatment for two consecutive years.

2. MATERIALS AND METHODS

2.1 Study population and design

This study was a 2-year follow up study that recruited adult opisthorchiasis participants who had APF+2 or APF+3 grade at baseline in our cohort study (Ethics # HE591185 and HE480528). After treatment with PZQ (40mg/Kg, in line with WHO recommendations), all participants were annually followed up by ultrasound examination and stool collection for two years to determine their APF-status, and *O. viverrini*- and *Helicobacter*-infection status. Before recruiting, participants were asked to refrain (for up to 14 days) from consumption of fatty foods, antacid medication, antibiotics, anti-parasitic agents, barium, mineral oil, bismuth, or non-absorbable anti-diarrheal agents. Participants with a history of digestive tract diseases (gastritis, gastric ulcer, cholecystitis, cholangitis, cholecystectomy, others), *O. viverrini*-positive stool examination at any follow up visits and pregnant women were excluded from the study. All participants provided written informed consent. Total participants recruited was 75

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110 based on repeated measures design. Sample size calculation is described in Supplementary
111 Figure S1.

112 2.2 Quantitative formalin-ethyl acetate technique for diagnosis of *O. viverrini* infection

113 One gram of fresh stool was mixed with 10 mL of 10% (v/v) formalin solution and filtered
114 through two layers of gauze, vigorously mixed with 3 mL of ethyl acetate for 30-60 seconds,
115 and then centrifuged at 1,300 x g for 5 min. After washing, the pellet was re-suspended in 1
116 mL of 10% formalin solution and examined in duplicate under a light microscope. The number
117 of eggs per gram of feces (EPG) was calculated as follows (number of eggs counts/drop x
118 total drops of fecal solution)/ (gram of feces).

119 2.3 Abdominal ultrasonography to visualize hepatobiliary fibrosis

120 The participants underwent ultrasonography (performed by an experienced radiologist with
121 >30 years experience in field-based ultrasound research) before and after treatment with
122 praziquantel (40 mg/kg). Ultrasonography of the upper abdomen was performed using a
123 mobile, high-resolution ultrasound instrument (LOGIQ E book, GE Healthcare, Chicago,
124 Illinois, USA). APF was graded and recorded as: APF grade 0 when no echoes were observed
125 in any segment of the liver; grade 1+ when echoes were observed in 1 segment of the liver;
126 grade 2+ when echoes were observed in 2 or 3 segments of the liver; grade 3+ when echoes
127 were observed in greater than 3 segments of the liver [11,23]. Participants were then
128 dichotomized into “Non-Advanced Fibrosis” if the ultrasound grade was 0 or 1, and “Advanced
129 Fibrosis” if the ultrasound grade was 2 or 3.

130 2.4 DNA extraction by using phenol- chloroform- isoamyl alcohol

131 Two hundred milligrams of fresh stool were resuspended in 2 mL of normal saline solution
132 (0.9% w/v NaCl solution) by vortexing for 5 min and then centrifuged at 1,300 x g for 5 min
133 and the supernatant collected. Cholestyramine (Sigma) (0.2 g) was added to the supernatant,
134 vortexed well and incubated at room temperature for 10 min to absorb bile salts and PCR
135 inhibitors. The solution was centrifuged at 1,300 x g for 5 min to collect the supernatant, then
136 an equal volume of 20% (v/v) ethanol was added. Recovered supernatants were centrifuged
137 again at 10,000 x g for 3 min to collect microbial pellets. These pellets were resuspended in

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200 µL of distilled water, freeze-thawed 3 times in liquid nitrogen, then heated at 95°C for 10 min. After adding 600 µL of lysis buffer (20mM Tris base, 5 mM EDTA, 10 mM NaCl, pH 8), 8 µL of proteinase K (from 20 mg/ml stock) and 90 µL of SDS (10% w/v) the mixture was incubated at 56°C with gentle shaking for 8 hours, and centrifuged at 10,000 x g for 5 min. The resulting supernatant was collected and mixed with 5 µL of RNase A and incubated at room temperature for 10 min. After adding an equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1), the solution was then subjected to centrifugation at 10,000 x g for 5 min. The aqueous phase was then collected, and meta-genomic DNA was precipitated with 1/10 volume of 3 M sodium acetate and equal volume of isopropanol, and pelleted by centrifugation at 10,000 x g for 5 min. After washing twice with 75% (v/v) ethanol, the resulting DNA was dried and finally dissolved into 50 µL of 1× Tris–EDTA buffer (pH 8.0). All DNA preparations were stored at -20°C until use.

2.5 Detection of *Helicobacter* species and their virulence genes by PCR technique

The primer sequences and PCR conditions for *16S rRNA (*for *Helicobacter* spp.*)*, *ureaA* (for *H. pylori*) genes were designed based as previously described [33, 27,28], with slight modifications (as shown in Supplementary Table S1). The PCR reaction was performed in a total volume of 20 µL containing 1x PCR buffer S (Vivantis Technologies), 0.2 mM dNTP, 1 µM of each primer, 1U of *Taq* DNA polymerase (Vivantis Technologies) and 1 µL of DNA template using a GeneAmp PCR system 9700 (Applied Biosystem, Life Technologies) thermocycler. PCR products were sized by electrophoresis through 1.5% agarose, stained with ethidium bromide, and visualized under UV light.

2.6 Quantitative real-time PCR for detection of *ureaA* gene and *cagA* gene

The presence of *H. pylori* and *cagA*-positive *H. pylori* was established and quantified by qPCR, performed by calculation of gene samples with a *ureaA*-plasmid standard curve and *cagA*-plasmid standard curve, respectively. The qPCR reaction was performed in a 96-well microtiter plate using 12.5 µL of Master mix 2X (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X)) containing Maxima® Hot Start Taq DNA polymerase and dNTPs (dATP, dCTP, dGTP, and dTTP) in an optimized PCR buffer, 0.5 µM forward/reverse primer mix, and

10 ng of DNA template, in nuclease-free water to a final volume of 25 μL.The PCR was performed in duplicate in the Applied Biosystems® QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies, Singapore). Details of primer sets, qPCR conditions, melting curve conditions, and product sizes are shown in Supplementary Table S2. The qPCR output was expressed as number of DNA copies per reaction. *H. pylori*- specific DNA loads (infection intensity) were calculated in 1 g of stool samples according to the formula: $\left(\frac{A \times B \times 50}{10} \times \frac{1000}{200}\right)$ where A = DNA copies from qPCR data; B = 10 ng input DNA. 50 μL DNA stool sample (extracted from 200 mg of stool).

2.7 Statistical analyses

All analyses were conducted using STATA version 17 software (StataCorp, LLC). Descriptive statistics including frequency, percentage, minimum value, maximum value, mean ± standard deviation **(**SD**)**, median, and interquartile range **(**IQR**)** were calculated for the demographic database **(**age, gender**)**, incidence of APF status, prevalence of *H. pylori* and *cagA*-positive *H. pylori* and bacterial load of *H. pylori/ cagA-positive H. pylori* infection according to sample time **(**baseline, follow up at 1 year and follow up at 2 years**)** and in subgroups of APF status**.**

Loads of *H. pylori* and *cagA*-positive *H. pylori* per 1 gram of stool of all infected participants were log-transformed for scatter plots to display distribution of infection. Generalized estimating equation (GEE) logistic regression was performed to identify correlation of *Helicobacter* spp., *H. pylori*, *cagA*-positive *H. pylori* infection before and after PZQ-treatment as well as correlation of *Helicobacter* spp., *H. pylori*, *cagA*-positive *H. pylori* infection among APF subgroups. The analysis was adjusted for age, sex and EPG. Results of GEE logistic regression analysis were expressed as odds ratios (OR) for the prevalence and β coefficient for intensity of infection with 95% confidence intervals (CI). P values less than 0.05 are considered as statistically-significant.

2.8 Ethics statement

This study was specifically approved by the Khon Kaen University Ethics Committee for Human Research (HE641332)**.** All methods were performed in accordance with the relevant

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guidelines and regulations of the committee**.** Written informed consents were obtained from all participants in the study**.**

3. RESULTS

3.1 Characteristics and study samples

A total 75 participants were recruited to this study: 53.3% females and 47.7% males, with a median age of 49 years old (IQR from 43 to 53). Most of the participants (70.7%) had light infection (<50 EPG) of *O. viverrini* and ultrasound results showed 54.7% with grade 2 APF and 45.3% with grade 3 APF at baseline. The prevalence of infection with *Helicobacter* spp*.,* *H. pylori* and *cagA*- positive *H. pylori* were 90.7%, 77.3%, and 74.7%, respectively (Table 1).

3.2 *Helicobacter* spp. infection in opisthorchiasis participants after PZQ treatment.

Overall, the prevalence of *Helicobacter* spp., *H. pylori,* and *cagA-positive H. pylori* in the participants declined after PZQ treatment. Prevalence of *Helicobacter* spp. was slightly decreased from 90.7% at baseline to 86.7% after 1year post-PZQ treatment, and further declined to 76.0% 2 years after PZQ treatment with an odds ratio (OR) of 0.39 (p= 0.05) (Table 2). Similarly, infection with *H. pylori* declined after PZQ treatment at 1 year (from 77.3% to 64.0%, OR=0.52) and 2 year (63.1%, OR=0.51). However, the decrease did not reach statistical significance (p>0.05). On the other hand, while the frequency of *cagA*-positive *H. pylori* infection was slightly decreased from 74.7% at baseline to 61.3% after 1 year (OR=0.54, p=0.081), it was significantly decreased to 44.0% after 2 years (OR=0.27, p<0.001) (Table 2).

The bacterial loads of *H. pylori* and *cagA*- positive *H. pylori* in 1 gram of stool were determined by quantitative real- time PCR (qPCR). The median of bacterial load of *H. pylori* before PZQ-treatment was 2.44 x 10^5 (IQR= 1.24 x10^5 – 5.20 x 10^5). While it decreased after 1 year PZQ-treatment (Median= 1.59 x 10^5, IQR= 8.34 x 10^4 – 5.16 x 10^5), it was slightly increased (Median= 3.06 x 10^5, IQR= 1.62 x10^5 – 6.16 x 10^5) at year 2; however, the difference was not statistically-significant (p>0.05) (Figure 1A and Supplementary Table S3). The quantity of *cagA*-positive *H. pylori* was slightly decreased from 9.64 x10^4 (IQR= 5.70 x 10^4-

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3 220 2.03×10^5) to 7.98×10^4 (IQR=4.47×10^4 to 1.27×10^5) after 1 year. However, it was significantly
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5 221 decreased to 7.61×10^4 (IQR= 3.21×10^4 to 1.44×10^5) at 2 years after treatment (β coefficient
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7 222 = -103793.6, 95%CI = -184540.5 to -23046.64, p= 0.012) (Figure 1B and Supplementary
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9 223 Table S3).
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12 224 **3.3 APF status in opisthorchiasis after PZQ treatment**
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14 225 APF status differed amongst participants at 1 year and 2 year after PZQ treatment. More than
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16 226 half of the participants showed no APF at the 1st year follow-up (41/75 participants) and at the
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18 227 2nd year follow-up (43/75 participants). However, APF persisted in some individuals, and some
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20 228 showed an absence of APF at 1-year follow-up but the APF relapsed by 2 years. Based on
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22 229 the presence or absence of APF in each follow-up visit, the participants were divided into 4
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24 230 groups: 1) resolved APF group accounting for 34.7% (participants who were APF- negative in
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26 231 both of 1st and 2nd follow up visits); slowly-resolved APF group accounting for 22.7%
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28 232 (participants who were APF positive in 1st year but APF negative in 2nd year); relapsed APF
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30 233 accounting for 20.0% (participants who were APF negative in 1st year and APF positive in 2nd
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32 234 year); persistent APF accounting for 22.7% (participants who were APF positive in both 1st
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34 235 year and 2nd year follow up visits) (Table 3).
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38 236 **3.4 Correlation between *Helicobacter* prevalence and APF in opisthorchiasis after PZQ**
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40 237 **treatment**
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42 238 Overall, prevalence within groups of *Helicobacter* spp., *H. pylori,* and *cagA*-positive *H. pylori*
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44 239 gradually declined after PZQ treatment in all APF subgroups**.** The prevalence of *Helicobacter*
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46 240 spp. was over 82.0% at baseline that gradually declined after PZQ treatment. However, this
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48 241 correlation did not reach statistical-significance (p>0.05). The respective prevalence of
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50 242 *Helicobacter* spp. at baseline, 1st follow up and 2nd year follow up were 92.3%, 84.6%, and
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52 243 79.6% in resolved APF group; 82.4%, 82.4%, and 82.4% in slowly resolved group; 100%,
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54 244 93.3%, and 66.7% in relapsed APF group; 88.2%, 88.2%, and 82.4% in persistent APF group,
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56 245 respectively (Figure 2A).
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H. pylori infection was detected more frequently in relapsed APF and persistent APF groups than in the resolved APF and slowly-resolved groups (Figure 2B). The prevalence of *H. pylori* at baseline, 1st follow up and 2nd year follow up, respectively, were 69.3%, 46.2%, and 50.0% in resolved APF group; 70.8%, 58.8%, and 64.7% in slowly-resolved group; 93.3%, 86.7%, and 60.0% in relapsed APF group; and 82.4%, 76.57%, and 82.4% in persistent APF group. The prevalence of *H. pylori* in both 1 year and 2 years after PZQ treatment was lower than that at baseline in all APF subgroups, but these decreases were not statistically-significant (Figure 2B).

There was a significant decrease of *cagA*-positive *H. pylori* prevalence in the resolved APF group after PZQ treatment (Figure 2C). The prevalence declined from 65.4% at baseline to 42.3% at 1st year (OR=0.34 (95%CI= 0.13 to 0.89, p=0.028) and 34.6% at 2nd year follow-up (OR=0.25 (95%CI= 0.09 to 0.72, p=0.01). Similar decreases in *cagA*-positive *H. pylori* infection were observed in all other APF groups, but these decreases did not reach statistical-significance (p>0.05).

We further investigated the frequency of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* infection after PZQ treatment among slowly-resolved, relapsed, and persistent APF groups compared to the resolved group as a reference. The *H. pylori* prevalence in the persistent APF group was significantly higher than that of the resolved APF group with OR= 4.28 (95%CI=1.33 to 13.08, p=0.015). Interestingly, the *cagA*-positive *H. pylori* prevalence was significantly higher in both relapsed APF and persistent APF groups when compared to the resolved APF group with OR=3.52 (95%CI=1.17 to 10.69, p=0.025) and OR=3.11 (95%CI=1.21 to 7.97, p=0.018), respectively (Table 4).

3.5 Correlation of the bacterial loads of *H. pylori*, *cagA*-positive *H. pylori* before and after PZQ treatment

There were no significant differences in *H. pylori* loads among APF subgroups before and after PZQ treatment (p>0.05) (Figure 3A and Supplementary Table S3). There were, however, significantly lower loads of *cagA*-positive *H. pylori* in resolved APF and slowly-resolved APF

group at 2nd year when compared to that at baseline (β coefficient= -136996.3, p=0.03 and β coefficient= -303090, p=0.028, respectively) (Figure 3B and Supplementary Table S4).

The *cagA*-positive *H. pylori* loads in slowly-resolved, relapsed, and persistent APF group were higher than those of resolved APF. However, these differences did not reach statistical significance (p>0.05) (Supplementary Table S5).

4. DISCUSSION

Despite worm removal by PZQ treatment, over 40% of opisthorchiasis patients showed APF after 2 a year follow up. Factors that drive this phenomenon remain unknown. Here, we explored the role of *H. pylori* and its virulence factor in the pathogenesis of APF after PZQ treatment in chronic opisthorchiasis. By comparing the rates and bacterial loads of *Helicobacter* bacteria among different APF groups, we found that *H. pylori*, especially cagA-positive *H. pylori*, played a significant role in the APF outcomes. Specifically, the presence and amount of *cagA*-positive *H. pylori* in the resolved APF group were significantly-decreased after PZQ treatment; however, these reductions were not significant in slowly-resolved, relapsed, and persistent APF groups.

Mairiang et al reported that the liver fluke-associated hepatobiliary abnormalities were improved after PZQ treatment.[8,9] Specifically, the incidence of mild to moderate fibrosis persisted in opisthorchiasis participants at 10 months following PZQ treatment was 26.4% compared to 41.7% at baseline. A 5-year follow up study showed that more than 38% of the recruited opisthorchiasis participants presented with persistent APF during the five years following PZQ treatment, and that only 30.8% of the study participants had resolved APF. Overall, our study findings have shown more than 50% of opisthorchiasis participants who had APF+2/APF+3 at baseline showed different degree of APF at 2 years post-PZQ treatment, and only 34.7% of participants had completely resolved APF during the study period. The presence of relapsing and persistent APF in the participants implies that hepatobiliary pathological processes continued even after worm removal by PZQ treatment [9]. Following worm removal, APF may be driven by at least three mechanisms.[9] First, APF may be due to

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the *O. viverrini*-induced inflammation in response to excretory–secretory *O. viverrini* antigens. Even after PZQ treatment, the biliary epithelium may remain activated and continue to release mediators of fibrosis. Second, persistent APF may be driven by a pro-inflammatory cytokine, such as interleukin-6 (IL-6), released from the activated fibroblasts in APF. Third, *O. viverrini* is a reservoir for *H. pylori* in the liver fluke-infected individuals and coinfection may orchestrate the pathogenesis of liver fluke–induced hepatobiliary diseases, including CCA. [18] Our study highlights the role of *H. pylori* and its virulence factor in the pathogenesis of relapsed/persistent advanced periductal fibrosis post-PZQ treatment.

The association between *O. viverrini* and *Helicobacter* infection has been reported in animal and human studies.[14,18-22] The majority of *O. viverrini*-infected residents in liver fluke endemic areas in northeastern Thailand are co-infected with *H. pylori.*[15] Moreover, APF, which is the major pathologic characteristic of chronic opisthorchiasis, is associated with *cag*A-positive *H. pylori.*[15] Recently, we demonstrated that *O. viverrini* acts as a carrier of *cag*A-positive *H. pylori* and co-migrates to the bile ducts, and that *O. viverrini* facilitates *H. pylori* colonization and enhances biliary pathogenesis in a hamster model.[22] This evidence suggests that *H. pylori* depends on the liver fluke to be able to survive and colonize the bile ducts. Therefore, in theory, *H. pylori* could also be eliminated by worm removal. However, in this study, we demonstrated that *H. pylori* could persist without *O. viverrini* in most PZQ-treated opisthorchiasis patients and, particularly, the *cagA*-positive bacteria, continue to induce pathology, specifically persistent fibrosis.

The mechanism(s) by which the *Helicobacter* remains in the hepatoduodenal system is unknown. In an animal model of experimental co-infection with the liver fluke and cagA+ *H. pylori*, we detected these bacteria in the bile 3 months after infection.[22] We have also amplified cagA+ *H. pylori* by PCR from bile samples from CCA patients (manuscript in preparation). We hypothesize that there may be two populations of *cagA*-positive *H. pylori*; adaptive and non-adaptive. Morphological and genetic adaptations are commonly observed in *H. pylori* under suboptimal environmental conditions, such as aerobiosis, temperature or pH changes, prolonged culture, or exposure to antibiotics or proton pump inhibitors and this can lead to

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3 330 increase pathogenicity of the bacteria.[29,30] The opisthorchiasis patients with resolved APF
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5 331 might be infected by non-adaptive *H. pylori* that can survive only in the gut of the flukes. When
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7 332 the flukes are killed by PZQ, the released bacteria cannot survive in the hepatobiliary
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9 333 environment. In contrast, the persistent APF sub-group may be infected by an adaptive strain
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11 334 of bacteria which can survive in the harsh bile environment. These phenotypes might be
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13 335 determined by the cagPAI genotypes, specifically *cagA*. According to Deenonpoe et al. (2017),
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15 336 there are 3 predominant CagA types; EPIYA-AB type, EPIYA-ABC type and EPIYA-AB'C type
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17 337 (B' = EPIYT) in opisthorchiasis that is endemic Northeast Thailand.[15,31-33] Interestingly, the
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19 338 Western type CagA with EPIYA-AB'C showed a higher frequency in the liver fluke-infected
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21 339 cases in this region, whereas participants who were not infected with *O. viverrini* showed a
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23 340 higher frequency of EPIYA-AB type than the liver fluke infected participants. Additionally, all
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25 341 cases of CagA with EPIYA-AB'C genotypes contained a CagA multimerization (CM) motif
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27 342 which is comprised of 16 amino acids and highly conserved for Western and Eastern
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29 343 CagA.[32,33] However, the prevalence of CagA with CM in EPIYA-AB type was only 30.8 Vs.
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31 344 36.2% in non liver fluke-infected and liver fluke-infected patients. The CM motif is a
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33 345 membrane-targeting signal, which interacts with PAR1b, thus inducing junctional and polarity
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35 346 defects. Furthermore, structural polymorphism in the CM reflects the degree of virulence of
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37 347 CagA.[34] This evidence suggests that both the EPIYA-C/D motif and CM sequences increase
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39 348 phosphorylation motifs capable for disease pathogenesis.[34] Thus, CagA types in the
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41 349 opisthorchiasis group, especially the CagA with EPIYA-AB'C type bearing CM sequences may
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43 350 be associated with biliary periductal fibrosis with an odds ratio as high as 38.[15] Accordingly,
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45 351 we hypothesize that the group of persistent APF individuals may infected by an adaptive strain
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47 352 of *H. pylori* with EPIYA-AB'C type CagA bearing CM sequence. In addition, *O. viverrini* may
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49 353 act as a selector for virulent *cagA*-positive *H. pylori* strains.[15] Given this strong association
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51 354 between these two carcinogenic pathogens, further studies on their molecular interactions are
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53 355 needed to fully explain the pathogenesis of liver disease in opisthorchiasis.
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CONCLUSION

This study is the first report the association between *Helicobacter* spp., its virulence genes with APF status in opisthorchiasis after PZQ treatment. We detected significant decreases of *cagA*-positive *H. pylori* infection after *O. viverrini* eradication by treatment with PZQ. We report the association of *H. pylori,* and in particular *cagA*-positive *H. pylori* with persistent of hepatobiliary APF after PZQ treatment. The results suggest that *H. pylori* and *cag*A may be involved in pathogenesis of persistent of APF in opisthorchiasis participants post-treatment with PZQ. Thus, the eradication of *Helicobacter* spp., and, in particular, *H. pylori*, may be useful for the prevention of CCA development.

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CONFLICT OF INTEREST

All other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RD, ST, EM, SWE, and BS conceived and designed this study. HTTP, EM, and BS collected the data and performed the analysis. HTTP, SS, RD wrote the draft manuscript. BS and SS,

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384 critically-revised the manuscript for intellectual content. RD, SWE and BS obtained funding.
385 All authors have been involved in reviewing and approving the final manuscript.

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Tables

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Table 1. Demographics and baseline characteristics of the study participants.

Characteristics	Frequency (n (%) N=75
Sex	
Male	35 (46.7%)
Female	40 (53.3%)
Age groups (years)	
<50	42 (56.0%)
≥50	33 (44.0%)
Mean ± SD	48±7.5
Median (IQR)	49 (43 to 53)
Intensity of *O. viverrini* infection	
Low (< 50 EPG)	53 (70.7%)
High (≥ 50 EPG)	22 (29.3%)
Mean ± SD	62.9 ± 109.6
Median (IQR)	16.5 (11 to 56)
Periductal fibrosis status	
APF+2	41 (54.7%)
APF+3	34 (45.3%)
Helicobacter spp. infection	68 (90. 7%)
H. pylori infection	58 (77.3%)
cagA- positive *H. pylori* infection	56 (74.7%)

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Table 2. Prevalence of *Helicobacter* spp., *H. pylori* and *cagA*- positive *H. pylori* in recruited participants before and after PZQ treatment.

Characteristics	Baseline	Follow up 1 year	Follow up 2 years
***Helicobacter* spp. infection**			
Frequency, n (%)	68 (90.7%)	65 (86.7%)	57 (76.0%)
OR	1	0.78	0.39
95% CI		0.29-2.05	0.15-1
P- value		0.618	0.05
***H. pylori* infection**			
Frequency, n (%)	58 (77.3%)	48 (64.0%)	46 (61.3%)
OR	1	0.56	0.54
95% CI		0.29-1.07	0.27-1.08
P- value		0.081	0.083
***cagA*-positive *H. pylori* infection**			
Frequency, n (%)	56 (74.7%)	46 (61.3%)	33 (44.0%)
OR	1	0.57	0.29
95% CI		0.31-1.07	0.15- 0.56
P- value		0.081	<0.001

Table 3. Four subgroups of participants classified according to their advanced periductal fibrosis (APF) status during the study period.

Sub-groups	APF status			Frequency (n (%)
	Baseline	Follow up 1 year	Follow up 2 years	
Resolved APF	+	-	-	26 (34.7%)
Slowly resolved APF	+	+	-	17 (22.7%)
Relapsed APF	+	-	+	15 (20%)
Persistent APF	+	+	+	17 (22.7%)
Total				75 (100%)

(+) APF positive; (-) APF negative

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497 **Table 4.** Correlation of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* prevalence

498 among APF subgroups of study participants after PZQ treatment.

Characteristics	Resolved APF (n=26)	Slowly resolved APF (n=17)	Relapsed APF (n=15)	Persistent APF (n=17)
***Helicobacter* spp. infection**				
OR (95% CI)	reference	1.27 (0.37 - 4.41)	1.10 (0.33 - 3.66)	1.56 (0.37 - 6.46)
P-value	reference	0.704	0.877	0.542
***H. pylori* infection**				
OR (95% CI)	reference	1.85 (0.69 - 4.99)	3.11 (0.95 - 10.19)	4.28 (1.33 - 13.80)
P-value	reference	0.219	0.062	0.015
***cagA*-positive *H. pylori* infection**				
OR (95% CI)	reference	1.80 (0.66 - 4.90)	3.52 (1.17 - 10.69)	3.11 (1.21 - 7.97)
P-value	reference	0.249	0.025	0.018

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501 **Figure legends**

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503 **Figure 1**. *Helicobacter pylori* and *cagA*-positive *H. pylori* loads before and after PZQ**-**

504 treatment.

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506 **Figure 2.** *Helicobacter* spp., *H. pylori,* and *cagA*-positive *H. pylori* prevalence in APF

507 subgroups of study participants before and after PZQ treatment.

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509 **Figure 3.** *Helicobacter pylori* (A) and *cagA***-**positive *H. pylori* (B) loads in different APF

510 subgroups before and after PZQ**-** treatment. FU1 = Year 1 follow up; FU2 = Year 2 follow up.

511 * $p<0.05$ compared to baseline

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1 **Persistent advanced periductal fibrosis is associated with *cagA*-positive *Helicobacter***

2 ***pylori* infection in post-praziquantel treatment of opisthorchiasis**

3 *Running head: Persistent biliary fibrosis is associated with Helicobacter pylori*

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Abstract (278 words)

Background: Liver fluke infection caused by *Opisthorchis viverrini* is associated with several hepatobiliary diseases including advanced periductal fibrosis (APF) and cholangiocarcinoma (CCA). Recently, we demonstrated a persistent APF in over one-third of opisthorchiasis patients after worm removal by praziquantel (PZQ) treatment. However, the underlying mechanism(s) of this phenomena is unclear. Given a co-infection with *Helicobacter pylori* (*H. pylori*) especially *cagA*-positive strain enhances APF, we hypothesized that *H. pylori* with CagA virulent factor contributes to persistent APF.

Materials and Methods: Seventy-five opisthorchiasis patients who underwent ultrasonography and treatment with PZQ were recruited in the 2-year follow-up study. *Helicobacter* and its cagA in the feces were examined by conventional and qPCR. Correlations between ~~infection rates~~prevalence or bacterial loads of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* before and after PZQ treatment were analyzed among resolved, slowly-resolved, relapsed, and persistent APF groups.

Results: Overall, ~~infection rates~~prevalence of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* declined after PZQ treatment. However, only the ~~infection rates~~prevalence and bacterial loads of *cagA*-positive *H. pylori* detected at 2-year post-treatment were significantly lower than those before treatment ($p<0.05$). In addition, both ~~infection rates~~prevalence and bacterial loads of *cagA*-positive *H. pylori* were significantly lower in the resolved APF group after PZQ treatment, while there were no significant changes in the slowly-resolved, relapsed, and persistent APF groups. Among the APF subgroups, only *cagA*-positive *H. pylori* ~~infection rates~~prevalence in both relapsed and persistent APF groups were significantly higher than the resolved APF group.

Conclusion: The results support our hypothesis that *H. pylori*, especially *cagA*-positive strain contributes to the relapsed and persistent APF. A supplementary antibiotic treatment for *H. pylori* to reduce persistent APF and eventually CCA is warranted.

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Keywords: Opisthorchiasis, *Opisthorchis viverrini*, Praziquantel, Advanced Periductal Fibrosis, *Helicobacter pylori, cagA*

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1. INTRODUCTION

Opisthorchiasis, a fish-borne trematodiasis caused by the carcinogenic liver fluke, *Opisthorchis viverrini*, remains an important health problem in the Lower Mekong Basin including Thailand, Lao People's Democratic Republic (Lao PDR), Cambodia, Myanmar, and Vietnam with over 12 million people infected.[1-3] In Thailand, the highest prevalence was reported in the North and Northeast with over 6 million people infected.[4,5] The infection is associated with several hepatobiliary diseases including cholangitis, gallstones, hyperplasia, dysplasia, advanced periductal fibrosis (APF), and cholangiocarcinoma (CCA), a fatal bile duct cancer.[6] Treatment with praziquantel (PZQ) at a single 40 mg/kg oral dose recommended by the World Health Organization is effective against opisthorchiasis.[7] The treatment not only clears the fluke infection but can also reduce the biliary morbidities. Previous studies reported an improvement of some hepatobiliary abnormalities as observed by ultrasound in opisthorchiasis after PZQ treatment.[8,9] However, APF was still detected in over two-thirds of PZQ-treated patients. Specifically, a 5-year ultrasound follow-up study found that 37.5% of *O. viverrini* infected patients showed relapsed or persistent APF post-PZQ treatment.[9] The relapsed and persistent APF is considered as a risk factor or a predisposing lesion for CCA. [6,9-12] However, etiologies and mechanism(s) of this relapsed or persistent APF after removal of the flukes are not yet known.

Helicobacter pylori has been reported as an etiology for hepatobiliary diseases such as liver cirrhosis, cholangitis, hepatocellular carcinoma, and CCA.[13-16] Pathogenicity of *H. pylori* is strongly associated with its virulence factor, cytotoxin-associated gene A (CagA).[17] During the past decade, there has been increasing evidence from both animal and human studies that *H. pylori,* particularly *cagA*-positive strains, may be involved in the pathogenesis of APF and CCA in opisthorchiasis.[14,18-22] Interestingly, *O. viverrini* has been demonstrated as a reservoir of *Helicobacter* spp., especially *cagA*-positive *H. pylori*.[15,18] The *H. pylori* co-migrates with the liver fluke, colonizes in the biliary epithelium and induces inflammation and APF in chronic opisthorchiasis in animals[22] and humans[15]. We propose that *O. viverrini* may act as a

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carrier for *cagA*-positive *H. pylori* which is the key driver of APF and eventually CCA development. Specifically, 23.6% of chronic opisthorchiasis patients develop APF[23] and *cagA*-positive *H. pylori* rates have been reported in 53.3% and 75% of those with APF at grades 2 and 3, respectively compared to only 25.32% with APF at grade 0.[15] Moreover, the *cagA* genotypes detected in opisthorchiasis are different from those in other pathologies[15,24], suggesting that genetic adaptation may coevolve for their survival in the contrasting harsh environments such as microaerobic, acidic conditions in the gastrointestinal tract or an alkali pH with oxidative stress in the biliary tracts.[6,15,24-26].

Therefore, we hypothesize that *H. pylori*, particularly *cagA* positive strains may be metabolically active and survive in the biliary system. We also hypothesize that relapsed or persistent APF after PZQ treatment is associated with *Helicobacter* and its CagA virulence factor. To test this hypothesis, we measured the ~~infection rate~~prevalence and bacterial loads of *Helicobacter* especially the *cagA*-positive strain in resolved, slowly-resolved, relapsed, and persistent APF groups of opisthorchiasis patients before and after PZQ treatment for two consecutive years.

2. MATERIALS AND METHODS

2.1 Study population and design

This study was a 2-year follow up study that recruited adult opisthorchiasis participants who had APF+2 or APF+3 grade at baseline in our cohort study (Ethics # HE591185 and HE480528). After treatment with PZQ (40mg/kg, in line with WHO recommendation), all participants were annually followed up by ultrasound examination and stool collection for two years to determine their APF-status, *O. viverrini*- and *Helicobacter*-infection status. Before recruiting, participants were asked to refrain (for up to 14 days) from consumption of fatty foods, antacid medication, antibiotics, anti-parasitic agents, barium, mineral oil, bismuth, or non-absorbable anti-diarrheal agents. Participants with a history of digestive tract diseases (gastritis, gastric ulcer, cholecystitis, cholangitis, cholecystectomy, others), *O. viverrini*-positive stool examination at any follow up visits and pregnant women were excluded from the

study. All participants provided written informed consent. Total participants recruited was 75 based on repeated measures design. Sample size calculation is described in Supplementary Figure S1.

~~The required sample size was estimated at 75 participants, using F test for repeated measures, within factors; partial eta squared equal 0.03, effected size factor value is 0.1758; number of groups is one; number of measurements are 3; correlation among repeated measure is 0.5; non-sphericity correction was 1, to give 92% power at the 5% significance level. This calculation was performed by using G*Power version 3.1.9.2.~~

2.2 Quantitative formalin-ethyl acetate technique for diagnosis of *O. viverrini* infection

One gram of fresh stool was mixed with 10 mL of 10% (v/v) formalin solution and filtered through two layers of gauze, vigorously mixed with 3 mL of ethyl acetate for 30-60 seconds, and then centrifuged at 1,300 x g for 5 min. After washing, the pellet was re-suspended in 1 mL of 10% formalin solution and examined in duplicate under a light microscope. The number of eggs per gram of feces (EPG) was calculated as follows (number of eggs counts/drop x total drops of fecal solution)/ (gram of feces).

2.3 Abdominal ultrasonography to visualize hepatobiliary fibrosis

The participants underwent ultrasonography (performed by an experienced radiologist with >30 years experience in field-based ultrasound research) before and after treatment with praziquantel (40 mg/kg). Ultrasonography of the upper abdomen was performed using a mobile, high-resolution ultrasound instrument (LOGIQ E book, GE Healthcare, Chicago, Illinois, USA). APF was graded and recorded as: APF grade 0 when no echoes were observed in any segment of the liver; grade 1+ when echoes were observed in 1 segment of the liver; grade 2+ when echoes were observed in 2 or 3 segments of the liver; grade 3+ when echoes were observed in greater than 3 segments of the liver [11,23]. Participants were then dichotomized into “Non-Advanced Fibrosis” if the ultrasound grade was 0 or 1, and “Advanced Fibrosis” if the ultrasound grade was 2 or 3.

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3 137 **2.4 DNA extraction by using phenol- chloroform- isoamyl alcohol**
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5 138 Two hundred milligrams of fresh stool were resuspended in 2 mL of normal saline solution
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7 139 (0.9% w/v NaCl solution) by vortexing for 5 min and then centrifuged at 1,300 x g for 5 min
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9 140 and the supernatant collected. Cholestyramine (Sigma) (0.2 g) was added to the supernatant,
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11 141 vortexed well and incubated at room temperature for 10 min to absorb bile salts and PCR
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13 142 inhibitors. The solution was centrifuged at 1,300 x g for 5 min to collect the supernatant, then
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15 143 an equal volume of 20% (v/v) ethanol was added. Recovered supernatants were centrifuged
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17 144 again at 10,000 x g for 3 min to collect microbial pellets. These pellets were resuspended in
18
19 145 200 μL of distilled water, freeze-thawed 3 times in liquid nitrogen, then heated at 95°C for 10
20
21 146 min. After adding 600 μL of lysis buffer (20mM Tris base, 5 mM EDTA, 10 mM NaCl, pH 8), 8
22
23 147 μL of proteinase K (from 20 mg/ml stock) and 90 μL of SDS (10% w/v) the mixture was
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25 148 incubated at 56°C with gentle shaking for 8 hours, and centrifuged at 10,000 x g for 5 min.
26
27 149 The resulting supernatant was collected and mixed with 5 μL of RNase A and incubated at
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29 150 room temperature for 10 min. After adding an equal volume of saturated phenol: chloroform:
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31 151 isoamyl alcohol (25:24:1), the solution was then subjected to centrifugation at 10,000 x g for
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33 152 5 min. The aqueous phase was then collected, and meta-genomic DNA was precipitated with
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35 153 1/10 volume of 3 M sodium acetate and equal volume of isopropanol, and pelleted by
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37 154 centrifugation at 10,000 x g for 5 min. After washing twice with 75% (v/v) ethanol, the resulting
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39 155 DNA was dried and finally dissolved into 50 μL of 1× Tris–EDTA buffer (pH 8.0). All DNA
40
41 156 preparations were stored at -20°C until use.
42
43 157 **2.5 Detection of *Helicobacter* species and their virulence genes by PCR technique**
44
45 158 The primer sequences and PCR conditions for *16S rRNA (*for *Helicobacter* spp.*)*, *ureaA* (for
46
47 159 *H. pylori*) genes were designed based as previously described [33, 27,28], with slight modifications
48
49 160 (as shown in Supplementary Table S1). The PCR reaction was performed in a total volume of
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51 161 20 μL containing 1x PCR buffer S (Vivantis Technologies), 0.2 mM dNTP, 1 μM of each primer,
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53 162 1U of *Taq* DNA polymerase (Vivantis Technologies) and 1 μL of DNA template using a
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55 163 GeneAmp PCR system 9700 (Applied Biosystem, Life Technologies) thermocycler. PCR
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164 products were sized by electrophoresis through 1.5% agarose, stained with ethidium bromide,
165 and visualized under UV light.

166 **2.6 Quantitative real-time PCR for detection of *ureaA* gene and *cagA* gene**

167 The presence of *H. pylori* and *cagA*-positive *H. pylori* was established and quantified by qPCR,
168 performed by calculation of gene samples with a *ureaA*-plasmid standard curve and *cagA*-
169 plasmid standard curve, respectively. The qPCR reaction was performed in a 96-well microtiter
170 plate using 12.5 μL of Master mix 2X (Thermo Scientific Maxima SYBR Green/ROX qPCR
171 Master Mix (2X)) containing Maxima® Hot Start Taq DNA polymerase and dNTPs (dATP,
172 dCTP, dGTP, and dTTP) in an optimized PCR buffer, 0.5 μM forward/reverse primer mix, and
173 10 ng of DNA template, in nuclease-free water to a final volume of 25 μL.The PCR was
174 performed in duplicate in the Applied Biosystems® QuantStudio™ 6 Flex Real-Time PCR
175 System (Life Technologies, Singapore). Details of primer sets, qPCR conditions, melting curve
176 conditions, and product sizes are shown in Supplementary Table S2. The qPCR output was
177 expressed as number of DNA copies per reaction. *H. pylori*- specific DNA loads (infection
178 intensity) were calculated in 1 g of stool samples according to the formula: $\left(\frac{A \times B \times 50}{10} \times \frac{1000}{200}\right)$
179 where A = DNA copies from qPCR data; B = 10 ng input DNA. 50 μL DNA stool sample
180 (extracted from 200 mg of stool).

181 **2.7 Statistical analyses**

182 All analyses were conducted using STATA version 17 software (StataCorp, LLC). Descriptive
183 statistics including frequency, percentage, minimum value, maximum value, mean ± standard
184 deviation **(**SD**)**, median, and interquartile range **(**IQR**)** were calculated for the demographic
185 database **(**age, gender**)**, incidence of APF status, ~~infection rates~~prevalence of *H. pylori* and
186 *cagA*-positive *H. pylori* and bacterial load of *H. pylori/ cagA***-***positive H. pylori* infection
187 according to sample time **(**baseline, follow up at 1 year and follow up at 2 years**)** and in
188 subgroups of APF status**.**

189 Loads of *H. pylori* and *cagA*-positive *H. pylori* per 1 gram of stool of all infected
190 participants were log-transformed for scatter plots to display distribution of infection.

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Generalized estimating equation (GEE) logistic regression was performed to identify correlation of *Helicobacter* spp., *H. pylori*, *cagA*-positive *H. pylori* infection before and after PZQ-treatment as well as correlation of *Helicobacter* spp., *H. pylori*, *cagA*-positive *H. pylori* infection among APF subgroups. The analysis was adjusted for age, sex and EPG. Results of GEE logistic regression analysis were expressed as odds ratios (OR) for the ~~infection rate~~prevalence and β coefficient for intensity of infection with 95% confidence intervals (CI). P values less than 0.05 are considered as statistically-significant.

2.8 Ethics statement

This study was specifically approved by the Khon Kaen University Ethics Committee for Human Research (HE641332)**.** All methods were performed in accordance with the relevant guidelines and regulations of the committee**.** Written informed consents were obtained from all participants in the study**.**

3. RESULTS

3.1 Characteristics and study samples

A total 75 participants were recruited to this study: 53.3% females and 47.7% males, with a median age of 49 years old (IQR from 43 to 53). Most of the participants (70.7%) had light infection (<50 EPG) of *O. viverrini* and ultrasound results showed 54.7% with grade 2 APF and 45.3% with grade 3 APF at baseline. The prevalence of infection with *Helicobacter* spp., *H. pylori* and *cagA*- positive *H. pylori* were 90.7%, 77.3%, and 74.7%, respectively (Table 1).

3.2 *Helicobacter* spp. infection in opisthorchiasis participants after PZQ treatment.

Overall, the prevalence ~~infection rates~~ of *Helicobacter* spp., *H. pylori,* and *cagA-positive H. pylori* in the participants declined after PZQ treatment. Prevalence ~~Infection rates~~ of *Helicobacter* spp. were slightly decreased from 90.7% at baseline to 86.7% after 1year post-PZQ treatment, and further declined to 76.0% 2 years after PZQ treatment with an odds ratio (OR) of 0.39~~; however, the decrease was borderline significant~~ (p=0.05) (Table 2). Similarly, infection with *H. pylori* declined after PZQ treatment at 1 year (from 77.3% to 64.0%, OR=0.52)

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and 2 year (63.1%, OR=0.51). However, the decrease did not reach statistical significance ($p>0.05$). On the other hand, while the frequency of *cagA*-positive *H. pylori* infection was slightly decreased from 74.7% at baseline to 61.3% after 1 year (OR=0.54, $p=0.081$), it was significantly decreased to 44.0% after 2 years (OR=0.27, $p<0.001$) (Table 2).

The bacterial loads of *H. pylori* and *cagA*- positive *H. pylori* in 1 gram of stool were determined by quantitative real- time PCR (qPCR). The median of bacterial load of *H. pylori* before PZQ-treatment was 2.44×10^5 (IQR= $1.24 \times 10^5 – 5.20 \times 10^5$). While it decreased after 1 year PZQ-treatment (Median= 1.59×10^5, IQR= $8.34 \times 10^4 – 5.16 \times 10^5$), it was slightly increased (Median= 3.06×10^5, IQR= $1.62 \times 10^5 – 6.16 \times 10^5$) at year 2; however, the difference was not statistically-significant ($p>0.05$) (Figure 1A and Supplementary Table S3). The quantity of *cagA*-positive *H. pylori* was slightly decreased from 9.64×10^4 (IQR= 5.70×10^4- 2.03×10^5) to 7.98×10^4 (IQR=4.47×10^4 to 1.27×10^5) after 1 year. However, it was significantly decreased to 7.61×10^4 (IQR= 3.21×10^4 to 1.44×10^5) at 2 years after treatment (β coefficient = -103793.6, 95%CI = -184540.5 to -23046.64, p= 0.012) (Figure 1B and Supplementary Table S3).

3.3 APF status in opisthorchiasis after PZQ treatment

APF status differed amongst participants at 1 year and 2 year after PZQ treatment. More than half of the participants showed no APF at the 1st year follow-up (41/75 participants) and at the 2nd year follow-up (43/75 participants). However, APF persisted in some individuals, and some showed an absence of APF at 1-year follow-up but the APF relapsed by 2 years. Based on the presence or absence of APF in each follow-up visit, the participants were divided into 4 groups: 1) resolved APF group accounting for 34.7% (participants who were APF- negative in both of 1st and 2nd follow up visits); slowly-resolved APF group accounting for 22.7% (participants who were APF positive in 1st year but APF negative in 2nd year); relapsed APF accounting for 20.0% (participants who were APF negative in 1st year and APF positive in 2nd year); persistent APF accounting for 22.7% (participants who were APF positive in both 1st year and 2nd year follow up visits) (~~Supplement~~ Table ~~S4~~3).

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3.4 Correlation between *Helicobacter* ~~infection rates~~prevalence and APF in opisthorchiasis after PZQ treatment

Overall, prevalence ~~infection rates~~ within groups of *Helicobacter* spp., *H. pylori,* and *cagA*-positive *H. pylori* gradually declined after PZQ treatment in all APF subgroups**.** The prevalence~~infection rate~~ of *Helicobacter* spp. was over 82.0% at baseline that gradually declined after PZQ treatment. However, this correlation did not reach statistical-significance (p>0.05). The respective prevalence ~~infection rates~~ of *Helicobacter* spp. at baseline, 1st follow up and 2nd year follow up were 92.3%, 84.6%, and 79.6% in resolved APF group; 82.4%, 82.4%, and 82.4% in slowly resolved group; 100%, 93.3%, and 66.7% in relapsed APF group; 88.2%, 88.2%, and 82.4% in persistent APF group, respectively (Figure 2A).

H. pylori infection was detected more frequently in relapsed APF and persistent APF groups than in the resolved APF and slowly-resolved groups (Figure 2B). The prevalence ~~infection rates~~ of *H. pylori* at baseline, 1st follow up and 2nd year follow up, respectively, were 69.3%, 46.2%, and 50.0% in resolved APF group; 70.8%, 58.8%, and 64.7% in slowly-resolved group; 93.3%, 86.7%, and 60.0% in relapsed APF group; and 82.4%, 76.57%, and 82.4% in persistent APF group. The prevalence ~~infection rates~~ of *H. pylori* in both 1 year and 2 years after PZQ treatment were lower than that at baseline in all APF subgroups, but these decreases were not statistically-significant (Figure 2B).

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There was a significant decrease of *cagA*-positive *H. pylori* prevalence ~~infection rate~~ in the resolved APF group after PZQ treatment (Figure 2C). The prevalence ~~infection rate~~ declined from 65.4% at baseline to 42.3% at 1st year (OR=0.34 (95%CI= 0.13 to 0.89, p=0.028) and 34.6% at 2nd year follow-up (OR=0.25 (95%CI= 0.09 to 0.72, p=0.01). Similar decreases in *cagA*-positive *H. pylori* infection were observed in all other APF groups, but these decreases did not reach statistical-significance (p>0.05).

We further investigated the frequency of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* infection after PZQ treatment among slowly-resolved, relapsed, and persistent APF groups compared to the resolved group as a reference. The *H. pylori* prevalence ~~infection rate~~ in the persistent APF group was significantly higher than that of the resolved APF group with

OR= 4.28 (95%CI=1.33 to 13.08, p=0.015). Interestingly, the *cagA*-positive *H. pylori* prevalenceinfection rate was significantly higher in both relapsed APF and persistent APF groups when compared to the resolved APF group with OR=3.52 (95%CI=1.17 to 10.69, p=0.025) and OR=3.11 (95%CI=1.21 to 7.97, p=0.018), respectively (Table 43).

3.5 Correlation of the bacterial loads of *H. pylori*, *cagA*-positive *H. pylori* before and after PZQ treatment

There was no significant different in *H. pylori* loads among APF subgroups before and after PZQ treatment (p>0.05) (Figure 3A and Supplementary Table S32). There were, however, significantly lower loads of *cagA*-positive *H. pylori* in resolved APF and slowly-resolved APF group at 2nd year when compared to that at baseline (β coefficient= -136996.3, p=0.03 and β coefficient= -303090, p=0.028, respectively) (Figure 3B and Supplementary Table S45).

The *cagA*-positive *H. pylori* loads in slowly-resolved, relapsed, and persistent APF group were higher than those of resolved APF. However, these differences did not reach statistical significance (p>0.05) (Supplementary Table S56).

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4. DISCUSSION

Despite worm removal by PZQ treatment, over 40% of opisthorchiasis patients showed APF after 2 a year follow up. Factors that drive this phenomenon remain unknown. Here, we explored the role of *H. pylori* and its virulence factor in the pathogenesis of APF after PZQ treatment in chronic opisthorchiasis. By comparing the rates and bacterial loads of *Helicobacter* bacteria among different APF groups, we found that *H. pylori*, especially cagA-positive *H. pylori*, played a significant role in the APF outcomes. Specifically, the presence and amount of *cagA*-positive *H. pylori* in the resolved APF group were significantly-decreased after PZQ treatment; however, these reductions were not significant in slowly-resolved, relapsed, and persistent APF groups.

Mairiang et al reported that the liver fluke-associated hepatobiliary abnormalities were improved after PZQ treatment.[8,9] Specifically, the incidence of mild to moderate fibrosis

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3 301 persisted in opisthorchiasis participants at 10 months following PZQ treatment was 26.4%
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5 302 compared to 41.7% at baseline. A 5-year follow up study showed that more than 38% of the
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7 303 recruited opisthorchiasis participants presented with persistent APF during the five years
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9 304 following PZQ treatment, and that only 30.8% of the study participants had resolved APF.
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11 305 Overall, our study findings have shown more than 50% of opisthorchiasis participants who had
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13 306 APF+2/APF+3 at baseline showed different degree of APF at 2 years post-PZQ treatment,
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15 307 and only 34.7% of participants had completely resolved APF during the study period. The
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17 308 presence of relapsing and persistent APF in the participants implies that hepatobiliary
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19 309 pathological processes continued even after worm removal by PZQ treatment [9]. Following
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21 310 worm removal, APF may be driven by at least three mechanisms.[9] First, APF may be due to
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23 311 the *O. viverrini*-induced inflammation in response to excretory–secretory *O. viverrini* antigens.
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25 312 Even after PZQ treatment, the biliary epithelium may remain activated and continue to release
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27 313 mediators of fibrosis. Second, persistent APF may be driven by a pro-inflammatory cytokine,
28
29 314 such as interleukin-6 (IL-6), released from the activated fibroblasts in APF. Third, *O. viverrini*
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31 315 is a reservoir for *H. pylori* in the liver fluke-infected individuals and coinfection may orchestrate
32
33 316 the pathogenesis of liver fluke–induced hepatobiliary diseases, including CCA. [18] Our study
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35 317 highlights the role of *H. pylori* and its virulence factor in the pathogenesis of relapsed/persistent
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37 318 advanced periductal fibrosis post-PZQ treatment.
38
39 319 The association between *O. viverrini* and *Helicobacter* infection has been reported in
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41 320 animal and human studies.[14,18-22] The majority of *O. viverrini*-infected residents in liver fluke
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43 321 endemic areas in northeastern Thailand are co-infected with *H. pylori.*[15] Moreover, APF, which
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45 322 is the major pathologic characteristic of chronic opisthorchiasis, is associated with *cag*A-
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47 323 positive *H. pylori.*[15] Recently, we demonstrated that *O. viverrini* acts as a carrier of *cag*A-
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49 324 positive *H. pylori* and co-migrates to the bile ducts, and that *O. viverrini* facilitates *H. pylori*
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51 325 colonization and enhances biliary pathogenesis in a hamster model.[22] This evidence suggests
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53 326 that *H. pylori* depends on the liver fluke to be able to survive and colonize the bile ducts.
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55 327 Therefore, in theory, *H. pylori* could also be eliminated by worm removal. However, in this
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57 328 study, we demonstrated that *H. pylori* could persist without *O. viverrini* in most PZQ-treated
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329 opisthorchiasis patients and, particularly, the *cagA*-positive bacteria, continue to induce
330 pathology, specifically persistent fibrosis.

331 The mechanism(s) by which the *Helicobacter* remains in the hepatoduodenal system is
332 unknown. In an animal model of experimental co-infection with the liver fluke and cagA+ *H.*
333 *pylori*, we detected these bacteria in the bile 3 months after infection.[22] We have also amplified
334 cagA+ *H. pylori* by PCR from bile samples from CCA patients (manuscript in preparation). We
335 hypothesize that there may be two populations of *cagA*-positive *H. pylori*; adaptive and non-
336 adaptive. Morphological and genetic adaptations are commonly observed in *H. pylori* under
337 suboptimal environmental conditions, such as aerobiosis, temperature or pH changes,
338 prolonged culture, or exposure to antibiotics or proton pump inhibitors and this can lead to
339 increase pathogenicity of the bacteria.[29,30] The opisthorchiasis patients with resolved APF
340 might be infected by non-adaptive *H. pylori* that can survive only in the gut of the flukes. When
341 the flukes are killed by PZQ, the released bacteria cannot survive in the hepatobiliary
342 environment. In contrast, the persistent APF sub-group may be infected by an adaptive strain
343 of bacteria which can survive in the harsh bile environment. These phenotypes might be
344 determined by the cagPAI genotypes, specifically *cagA*. According to Deenonpoe et al. (2017),
345 there are 3 predominant CagA types; EPIYA-AB type, EPIYA-ABC type and EPIYA-AB'C type
346 (B' = EPIYT) in opisthorchiasis that is endemic Northeast Thailand.[15,31-33] Interestingly, the
347 Western type CagA with EPIYA-AB'C showed a higher frequency in the liver fluke-infected
348 cases in this region, whereas participants who were not infected with *O. viverrini* showed a
349 higher frequency of EPIYA-AB type than the liver fluke infected participants. Additionally, all
350 cases of CagA with EPIYA-AB'C genotypes contained a CagA multimerization (CM) motif
351 which is comprised of 16 amino acids and highly conserved for Western and Eastern
352 CagA.[32,33] However, the prevalence of CagA with CM in EPIYA-AB type was only 30.8 Vs.
353 36.2% in non liver fluke-infected and liver fluke-infected patients. The CM motif is a
354 membrane-targeting signal, which interacts with PAR1b, thus inducing junctional and polarity
355 defects. Furthermore, structural polymorphism in the CM reflects the degree of virulence of
356 CagA.[34] This evidence suggests that both the EPIYA-C/D motif and CM sequences increase

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phosphorylation motifs capable for disease pathogenesis.[34] Thus, CagA types in the opisthorchiasis group, especially the CagA with EPIYA-AB'C type bearing CM sequences may associated with biliary periductal fibrosis with an odds ratio as high as 38.[15] Accordingly, we hypothesize that the group of persistent APF individuals may infected by an adaptive strain of *H. pylori* with EPIYA-AB'C type CagA bearing CM sequence. In addition, *O. viverrini* may act as a selector for virulent *cagA*-positive *H. pylori* strains.[15] Given this strong association between these two carcinogenic pathogens, further studies on their molecular interactions are needed to fully explain the pathogenesis of liver disease in opisthorchiasis.

CONCLUSION

This study is the first report the association between *Helicobacter* spp., its virulence genes with APF status in opisthorchiasis after PZQ treatment. We detected significant decreases of *cagA*-positive *H. pylori* infection after *O. viverrini* eradication by treatment with PZQ. We report the association of *H. pylori,* and in particular *cagA*-positive *H. pylori* with persistent of hepatobiliary APF after PZQ treatment. The results suggest that *H. pylori* and *cag*A may be involved in pathogenesis of persistent of APF in opisthorchiasis participants post-treatment with PZQ. Thus, the eradication of *Helicobacter* spp., and, in particular, *H. pylori*, may be useful for the prevention of CCA development.

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CONFLICT OF INTEREST

All other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RD, ST, EM, SWE, and BS conceived and designed this study. HTTP, EM, and BS collected the data and performed the analysis. HTTP, SS, RD wrote the draft manuscript. BS and SS, critically-revised the manuscript for intellectual content. RD, SWE and BS obtained funding. All authors have been involved in reviewing and approving the final manuscript.

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Tables

Table 1. Demographics and baseline characteristics of the study participants.

Characteristics	Frequency (n (%) N=75
Sex	
Male	35 (46.7%)
Female	40 (53.3%)
Age groups (years)	
<50	42 (56.0%)
≥50	33 (44.0%)
Mean ± SD	48±7.5
Median (IQR)	49 (43 to 53)
Intensity of *O. viverrini* infection	
Low (< 50 EPG)	53 (70.7%)
High (≥ 50 EPG)	22 (29.3%)
Mean ± SD	62.9 ± 109.6
Median (IQR)	16.5 (11 to 56)
Periductal fibrosis status	
APF+2	41 (54.7%)
APF+3	34 (45.3%)
Helicobacter spp. infection	68 (90. 7%)
H. pylori infection	58 (77.3%)
cagA- positive *H. pylori* infection	56 (74.7%)

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507 **Table 2.** Prevalence ~~Infection rates~~ of *Helicobacter* spp., *H. pylori* and *cagA*- positive *H. pylori*

508 in recruited participants before and after PZQ treatment.

Characteristics	Baseline	Follow up 1 year	Follow up 2 years
***Helicobacter* spp. infection**			
Frequency, n (%)	68 (90.7%)	65 (86.7%)	57 (76.0%)
OR	1	0.78	0.39
95% CI		0.29-2.05	0.15-1
P- value		0.618	0.05
***H. pylori* infection**			
Frequency, n (%)	58 (77.3%)	48 (64.0%)	46 (61.3%)
OR	1	0.56	0.54
95% CI		0.29-1.07	0.27-1.08
P- value		0.081	0.083
***cagA*-positive *H. pylori* infection**			
Frequency, n (%)	56 (74.7%)	46 (61.3%)	33 (44.0%)
OR	1	0.57	0.29
95% CI		0.31-1.07	0.15- 0.56
P- value		0.081	<0.001

509

510 **Table 3.** Four subgroups of participants classified according to their advanced periductal

511 fibrosis (APF) status during the study period.

Sub-groups	APF status			Frequency (n (%)
	Baseline	Follow up 1 year	Follow up 2 years	
Resolved APF	+	-	-	26 (34.7%)
Slowly resolved APF	+	+	-	17 (22.7%)
Relapsed APF	+	-	+	15 (20%)
Persistent APF	+	+	+	17 (22.7%)
Total				75 (100%)

512 (+) APF positive; (-) APF negative

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515 **Table 34.** Correlation of *Helicobacter* spp., *H. pylori*, and *cagA*-positive *H. pylori* infection

516 rates among APF subgroups of study participants after PZQ treatment.

Characteristics	Resolved APF (n=26)	Slowly resolved APF (n=17)	Relapsed APF (n=15)	Persistent APF (n=17)
***Helicobacter* spp. infection**				
OR (95% CI)	reference	1.27 (0.37 - 4.41)	1.10 (0.33 - 3.66)	1.56 (0.37 - 6.46)
P-value	reference	0.704	0.877	0.542
***H. pylori* infection**				
OR (95% CI)	reference	1.85 (0.69 - 4.99)	3.11 (0.95 - 10.19)	4.28 (1.33 - 13.80)
P-value	reference	0.219	0.062	0.015
***cagA*-positive *H. pylori* infection**				
OR (95% CI)	reference	1.80 (0.66 - 4.90)	3.52 (1.17 - 10.69)	3.11 (1.21 - 7.97)
P-value	reference	0.249	0.025	0.018

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519 **Figure legends**

520

521 **Figure 1***.* *Helicobacter pylori* and *cagA*-positive *H. pylori* loads before and after PZQ**-**

522 treatment.

523

524 **Figure 2.** *Helicobacter* spp.*, H. pylori,* and *cagA*-positive *H. pylori* infection rates in APF

525 subgroups of study participants before and after PZQ treatment.

526

527 **Figure 3.** *Helicobacter pylori* (A) and *cagA***-**positive *H. pylori* (B) loads in different APF

528 subgroups before and after PZQ**-** treatment. FU1 = Year 1 follow up; FU2 = Year 2 follow up.

529 * $p<0.05$ compared to baseline

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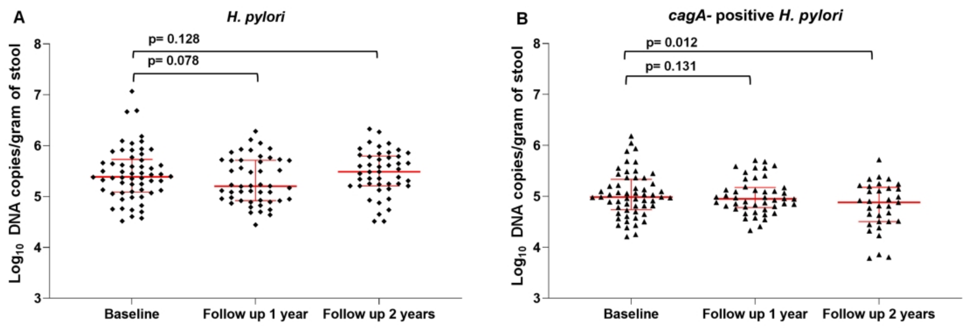

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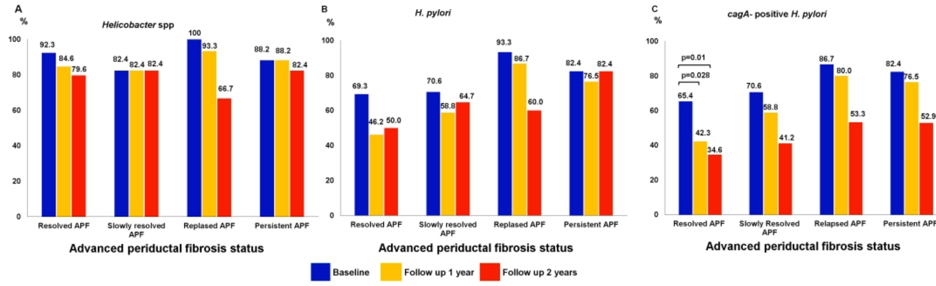

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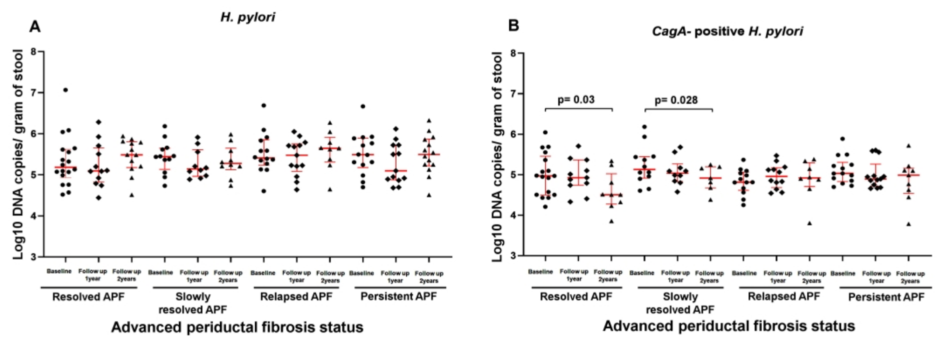

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Supplementary Tables and Figures

Table S1. Primer sequences and PCR conditions for amplification of *Helicobacter spp*.

Gene specific for	Genes	Primer sequences (5'- 3')	PCR condition	PCR product size (bp)	Reference
Helicobacter spp.	16SrRNA (Nested PCR)	OF-ATTAGTGGCGCACGGGTGAGTAA OR-TTTAGCATCCCGACTTAAGGC	94°C 30 sec, 55°C 30 sec, 72°C 1.5 min (35 cycles)	*1300*	1
		IF-GAACCTTACCTAGGCTTGACATTG IR-GGTGAGTACAAGACCCGGGAA	94°C 30 sec, 60°C 30 sec, 72°C 30 sec (35 cycle)	*480*	
H. pylori	UreA (Nested PCR)	*ureA*-aF - ATGAAACTCACCCCAAAAGA *ureA*-bR-CCGAAAGTTTTTTCTCTGTCAAAGTCTA	95°C 30 sec, 55°C 30 sec, 72°C 30 sec (35 cycles)	*488*	2
		ureA-bF-AAACGCAAAGAAAAAGGCATTAA *ureA*-aR-TTCACTTCAAAGAAATGGAAGTGTGA	95°C 30 sec, 55°C 30 sec, 72°C 30 sec (35 cycles)	*389*	

Table S2. Primer sequences and PCR- condition of qPCR for detection of *ureA and cagA* gene

Gene	Primer sequences (5'- 3')	PCR condition	Melting curve condition	PCR product (bp)	References
ureA	*ureA-F*: CGTGGCAAGCATGATCCAT *ureA-R*: GGGTATGCACGGTTACGAGTTT	(95˚C-30s, 55 ˚C-30s, 72 ˚C - 30s) x 35 cycles	95 ˚C-30s, 55 ˚C-60s, 95 ˚C- 15s	77	3
cagA	*cagA-F*: GACCGACTCGATCAAATAGCA *cagA-R*: TTAGCTGAAAGCCCTACCTTAC	(95˚C-30s, 55 ˚C-30s, 72 ˚C - 40s) x 35 cycles	95 ˚C-30s, 55 ˚C-60s, 95 ˚C- 15s	113	4

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Table S3. *H. pylori* and *cagA*- positive *H. pylori* load before and after PZQ- treatment

Characteristics	Baseline	Follow up 1 year	Follow up 2 years
***H. pylori* infection**			
Min	3.301×10^4	2.78×10^4	3.261×10^4
Max	1.2×10^7	1.9×10^6	2.1×10^6
Median (IQR)	2.44×10^5 (1.24×10^5– 5.20×10^5)	1.59×10^5 (8.34×10^4 – 5.16×10^5)	3.06×10^5 (1.62×10^5 – 6.16×10^5)
β coefficient	reference	-386880.3	-339315
95% CI	reference	-816676 to 42915.33	-776183.6 to 97553.64
p-value	reference	0.078	0.128
***cagA*- positive-*H. pylori* infection**			
Min	16202	21427	6077
Max	1.5×10^6	5.1×10^5	5.2×10^5
Median (IQR)	9.64×10^4 (5.70×10^4- 2.03×10^5)	8.91×10^4 (6.18×10^4 – 1.48×10^5)	7.62x 104 (3.21×10^4 - 1.44×10^5)
β coefficient	Reference	-56300.74	-103793.6
95% CI	Reference	-129397.5 to 16796.05	-184540.5 to -23046.64
p-value	reference	0.131	0.012

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Table S4. *H. pylori* and *cagA*- positive *H. pylori* load in subgroups before and after PZQ-treatment

Characteristics	Baseline	FU1	FU2
H. pylori			
Resolved APF			
Min	3.3 x 10^4	2.8 x 10^4	3.3 x 10^4
Max	1.2x 10^7	1.9x 10^6	8.7x 10^5
Median (IQR)	1.55x 10^5 (9.82x 10^4– 4.11x 10^5)	1.25x 10^5 (7.28x 10^4 – 4.15x 10^5)	3.07x 10^5 (1.61x 10^5 – 6.16 x 10^5)
β coefficient	ref	-438756.8	-587631
95% CI	ref	-1721441 to 843927.5	-1831541 to 656279.5
p-value	ref	0.503	0.354
Slowly reduced APF			
Min	5.4 x 10^4	7.8 x 10^4	5.5 x 10^4
Max	1.5x 10^6	8.2x 10^5	9.7x 10^5
Median (IQR)	2.78x 10^5 (1.73x 10^5 – 4.37x 10^5)	1.39x 10^5 (9.54x 10^4 – 3.64x 10^5)	1.91x 10^5 (1.62x 10^5- 3.85x 10^5)
β coefficient	ref	-224904.7	-159698.9
95% CI	ref	-506481.5 to 56672.05	-443208.8 to 123810.9
p-value	ref	0.117	0.27
Relapsed APF			
Min	4.0x 10^4	4.4x 10^4	4.5x 10^4
Max	4.9x 10^6	1.1x 10^6	1.8x 10^6
Median (IQR)	2.58x 10^5 (1.87x 10^5 – 6.84x 10^5)	3.01x 10^5 (1.62x 10^5- 5.19x 10^5)	4.44x 10^5 (2.40x 10^5– 6.08x 10^5)
β coefficient	ref	-452980.4	-320959.5
95% CI	ref	-1058059 to 152098.6	-989807.7 to 347888.6
p-value	ref	0.142	0.347
Persistent APF			
Min	5.0x 10^4	4.8x 10^4	3.3x 10^4
Max	4.7x 10^6	1.3x 10^6	2.1x 10^6
Median (IQR)	3.10x 10^5 (1.76x 10^5 – 7.71x 10^5)	1.25x 10^5 (7.84 x 10^4- 5.29x 10^5)	3.15x 10^5 (1.78x 10^5 – 7.20x 10^5)
β coefficient	ref	-234271.2	-146569.3
95% CI	ref	-801187.6 to 332645.2	-707478.6 to 346345.9
p-value	ref	0.418	0.609
cagA*- positive *H. pylori			
Resolved APF			
Min	1.6x 10^4	2.1x 10^4	7.2 x 10^3
Max	1.1x 10^6	5.1x 10^5	306887

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Median (IQR)	$9.35x\ 10^4$ (3.20x 10^4- 2.31x 10^5)	$8.40x\ 10^4$ (5.51x 10^4 – 2.32 x 10^5)	$3.21x\ 10^4$ (2.10x 10^4 – 6.42x 10^4)
β coefficient	ref	-61026.71	-136996.3
95% CI	ref	-175393 to 53339.62	-260600.9 to -13391.72
p-value	ref	0.296	0.03
Slowly reduced APF			
Min	4.0 x 10^5	3.8x 10^4	2.4x 10^4
Max	1.5x 10^6	4.8x 10^5	177182
Median (IQR)	1.37x 10^5 (8.52x 10^4 – 2.78x 10^5)	1.09x 10^5 (6.97x 10^4 – 1.48 x 10^5)	8.29x 10^4 (4.75x 10^4 – 1.57x 10^4)
β coefficient	ref	-227806.5	-303090
95% CI	ref	-474881.9 to 19268.8	-573584.8.2 to -32595.28
p-value	ref	0.071	0.028
Relapsed APF			
Min	1.8x 10^4	3.5x 10^4	6.4x 10^3
Max	2.3x 10^5	2.93x 10^5	240469
Median (IQR)	6.54x 10^4 (4.64x 10^4- 1.08x 10^5)	9.35x 10^4 (5.41x 10^4- 1.44 x 10^5)	8.31x 10^4 (6.04x 10^4- 1.79x 10^5)
β coefficient	ref	59976.62	62694.18
95% CI	ref	-1178.119 to 121131.4	-3512.311 to 128900.7
p-value	ref	0.055	0.063
Persistent APF			
Min	5.0x 10^4	4.6x 10^4	6.1x 10^3
Max	7.7x 10^5	4.0x 10^5	524844
Median (IQR)	1.07 x 10^5 (7.10x 10^4- 1.76 x 10^5)	7.84x 10^4 (5.64x 10^4- 9.33x 10^4)	9.76x 10^4 (4.22x 10^4 – 1.27x 10^5)
β coefficient	ref	-107576.7	-118346
95% CI	ref	-234122.7 to 18969.43	-255246.3 to 18554.26
p-value	ref	0.096	0.09

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Table S5. The correlation of *H. pylori, cagA*-positive *H. pylori* loads among subgroups after PZQ-treatment.

Characteristic	Resolved APF	Slowly reduced APF	Relapsed APF	Persistent APF
H. pylori				
β coefficient	reference	-124007.1	85882.41	40158.35
95% CI	reference	-360704.8 to 112690.5	-142060.5 to 313825.3	-174120.5 to 254437.2
P-value	reference	0.304	0.46	0.713
cagA-* positive *H. pylori				
β coefficient	reference	32607.6	12690.14	32503.37
95% CI	reference	-45430.3 to 110645.5	-62532.91 to 87913.19	-39835.71 to 104842.4
P-value	reference	0.413	0.741	0.379

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Figures

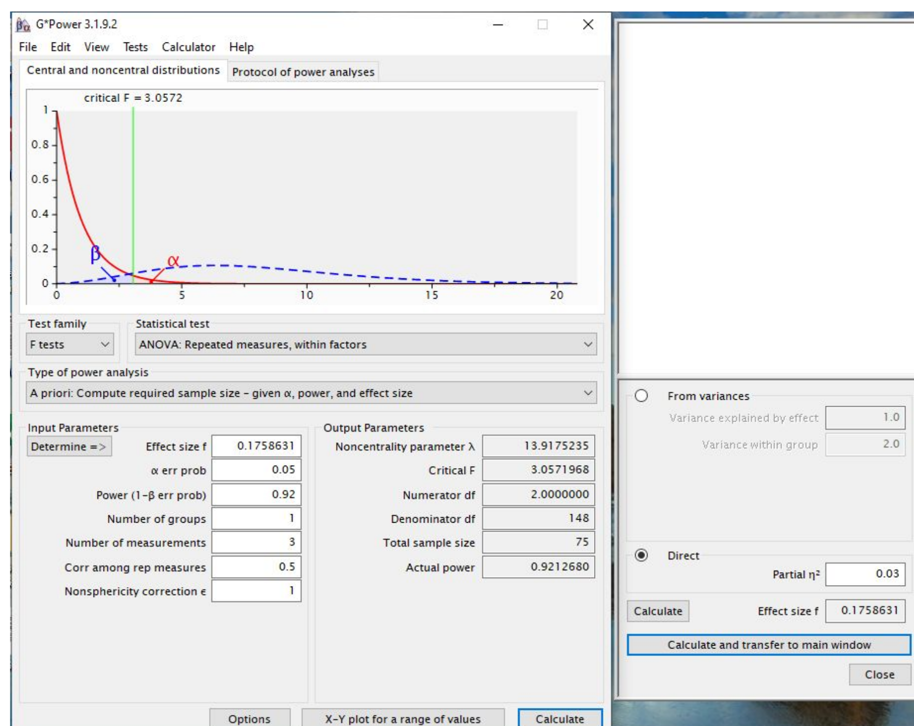

Figure S1 Sample size calculation. The sample size was estimated using F test for repeated measures, within factors; partial eta squared equal 0.03, effected size factor value is 0.1758; number of groups is one; number of measurements are 3; correlation among repeated measure is 0.5; non-sphericity correction was 1, to give 92% power at the 5% significance level. This gave 75 participants to be included in the study. This calculation was performed by using G*Power version 3.1.9.2.[5]

Reference

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