Parasitology

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Adherence of *Helicobacter pylori* to *Opisthorchis viverrini* gut epithelium and the tegument mediated *via* L-fucose binding adhesin

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Research Article

Cite this article: Thanaphongdecha P, Chamgramol Y, Pairojkul C, Deenonpoe R, Suttiprapa S, Brindley PJ, Sripa B (2022). Adherence of *Helicobacter pylori* to *Opisthorchis viverrini* gut epithelium and the tegument mediated *via* L-fucose binding adhesin. *Parasitology* **149**, 1374–1379. https://doi.org/10.1017/S0031182022000592

Received: 23 February 2022
Revised: 20 April 2022
Accepted: 21 April 2022
First published online: 29 April 2022

Key words:
Glycan receptors; *Helicobacter pylori*; host–pathogen interactions; L-fucose; *Opisthorchis viverrini*

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Abstract

Recent reports implicate both the liver fluke *Opisthorchis viverrini* as a reservoir of *Helicobacter pylori* within the human gastrointestinal tract and *H. pylori* in the pathogenesis of opisthorchiasis-associated cholangiocarcinoma. We postulated that adherence of bacterial ligands to host receptors initiates colonization of the live fluke by *H. pylori* and here we aimed to assess the molecular interaction between *O. viverrini* and *H. pylori* by investigating host receptors for *H. pylori* in the fluke. Several known receptors of *H. pylori* including Lewis B, sialyl-Lewis X, Toll-like receptor 4 and L-fucose were detected immunohistochemically and histochemically by focusing analysis on the gut epithelium and tegument of the adult stage of the fluke. The frequency of detection of Lewis B, sialyl-Lewis X, TLR4 and L-fucose in 100 individual worms was 3, 3, 19 and 70%, respectively. Detection of *H. pylori* by a diagnostic *ureA* gene-based PCR assay revealed the presence of *H. pylori* in individual *O. viverrini* worms in 41 of 49 (79%) worms examined. In addition, numbers of bacteria decreased in a dose- and time-dependent fashion following exposure to fucosidase. These findings suggested that L-fucose represents a tractable receptor for *H. pylori* that can mediate bacterial colonization of the gut of *O. viverrini*.

Introduction

There is increasing evidence that the Southeast Asian liver fluke *Opisthorchis viverrini* may serve as a carrier of *Helicobacter pylori* and implicate this bacterium in carcinogenesis of opisthorchiasis-associated cholangiocarcinoma (CCA) (Boonyanugomol *et al.*, 2012; Deenonpoe *et al.*, 2015, 2017; Sripa et al., 2017). *Helicobacter pylori* and *O. viverrini* may have evolved which facilitates conveyance of the bacillus into human bile duct during the migration of the juvenile fluke, following ingestion of the metacercaria in raw or undercooked cyprinid fish and establishment of infection (Plieskatt *et al.*, 2013; Deenonpoe *et al.*, 2015; Suyapoh *et al.*, 2021). Since *H. pylori* bacteria have been shown to localize on the gut epithelium and the tegument of the fluke (Deenonpoe *et al.*, 2015; Suyapoh *et al.*, 2021), the *H. pylori* bacillus might be passively transported by the migrating parasite *via* these organs (Suyapoh *et al.*, 2021). Once the fluke reaches the biliary tract, the *H. pylori* may further colonize to the cholangiocytes and induce disease in similar ways as described in the gastric epithelium (Boonyanugomol *et al.*, 2013; Hatakeyama, 2014; Backert *et al.*, 2016). However, mechanism(s) by which and how *H. pylori* might colonize the gut epithelium and the tegument of *O. viverrini* have not been described.

Establishment of infection by *H. pylori* requires adhesion and colonization on the gut epithelium as those described in the mammalian stomach (Chmiela and Kupcinskas, 2019; Matos *et al.*, 2021). The colonization involves interaction between the bacterial adhesins and host receptors (Fagoonee and Pellicano, 2019). There are several adhesins in *H. pylori* including blood-antigen binding protein A (BabA) and sialic acid-binding adhesin (SabA), neutrophil-activating protein (NAP), heat-shock protein 60 (Hsp60), adherence-associated proteins (AlpA and AlpB), *H. pylori* outer membrane protein (HopZ) and lacdiNAc-binding adhesin (LabA) (Kao *et al.*, 2016; Matos *et al.*, 2021) that interact with host tissues and cells. BabA mediates binding of the bacteria to Lewis B antigens (Ilver *et al.*, 1998) and related terminal fucose residues found on blood group O (H antigen), A and B antigens (Aspholm-Hurtig *et al.*, 2004). Similar to BabA, the SabA has affinity for sialyl-Lewis X (Mahdavi *et al.*, 2002). Toll-like receptor 4 (TLR4) is a known receptor for bacterial lipopolysaccharide (Pachathundikandi *et al.*, 2013).

Orthologues of the human cell receptors and antigens have yet to be described from the epithelium of the gastrodermis of the gut and/or the tegument of *O. viverrini* or related flukes. Here we examined the expression of host receptors including Lewis B, sialyl-Lewis X, TLR4

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Table 1. Primer sequences for *Helicobacter pylori*-specific *ureA* (*Hp-ureA*).

Genes	Primer sequences	Product sizes (bp)
Hp-ureA (outer) GenBank: CP036399.1	Forward GCTAATGGTAAATTAGTTCCTGG	411
	Reverse CTCCTTAATTGTTTTTACATAGTTG	
Hp-ureA (inner)	Forward GTTCCTGGTGAGTTGTTCTTAA	359
	Reverse CGAGCGTGGTTACAGTTTCA	
Hp-ureA (real time)	*ureA-F*: CGTGGCAAGCATGATCCAT	77
	ureA-R: GGGTATGCACGGTTACGAGTTT	

and L-fucose in the adult stage of *O. viverrini* and investigated role of certain glycans in host–pathogen interaction.

Materials and methods

Liver fluke samples

Opisthorchis viverrini adult worms and infected hamster livers were sourced from an ongoing project (Animal ethics #AEKKU 55/2554). Syrian golden hamsters were infected with 50 *O. viverrini* metacercariae *via* intragastric intubation as described (Suyapoh *et al.*, 2021). Adult worms were recovered from the bile ducts of infected hamster livers 12 weeks post-infection and processed as below. Formalin-fixed tissues of *O. viverrini* were used for the histochemistry and immunolocalization studies.

DNA extraction and PCR amplification for H. pylori

DNA was extracted from 50 individual *O. viverrini* adult worms using Gentra Puregene kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, each worm was homogenized in 300 µL of a lysis solution, the lysates were incubated at 65°C for 60 min after which RNase was added and protein was precipitated. After clarification by centrifugation, the supernatant was transferred to 300 µL isopropanol to precipitate the genomic DNA. The DNA pellet was washed by 70% ethyl alcohol and dissolved in DNA hydration solution, and stored at −20°C until used.

Detection of *H. pylori* in the *O. viverrini* worms was undertaken using nested PCR amplification of the *H. pylori*-specific *ureA* gene using *ureA*-specific primers (Deenonpoe *et al.*, 2017) (Table 1). The reaction mixture contained 5 mM dNTP, 37.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase and 0.2 mM primers. For the outer primer, the analysis settings included 30 s of initial denaturation at 94°C, 40 cycles of 30 s at 62°C and 30 s at 72°C for annealing and elongation steps. For the inner primer, amplification conditions were 30 s of initial denaturation at 94°C, 40 cycles of 30 s at 59°C and 30 s at 72°C for annealing and elongation steps. The PCR was performed in an automated thermocycler GeneAmp® PCR system 9700 (Applied Biosystems CO., LTD, USA). The amplified products were sized by electrophoresis through 1% agarose gel, stained with ethidium bromide and visualized and documented under UV illumination.

Immunohistochemistry detection of H. pylori receptors

Formalin-fixed and paraffin-embedded tissue of 100 *O. viverrini* parasites were used (Suyapoh *et al.*, 2021). Histological sections (4 μm) were deparaffinized and rehydrated. For the antigen retrieval, the slides were immersed in a pressure cooker for 5 min in citrate buffer (pH 6) and then allowed to cool for 20 min. After washing with phosphate-buffered saline, pH 7.4 (PBS), the endogenous peroxidase activity was inactivated in solution containing 3% hydrogen peroxide in methanol. Next, the slides were blocked with 5% normal horse serum in PBS. Primary antibody specific for TLR4 (dilution 1:100) (Santa Cruz Biotechnology, Inc., USA), Lewis B (dilution 1:50) (Abcam, UK) and sialyl-Lewis X (dilution 1:50) (Thermo Fisher Scientific, USA) was added and incubated overnight at 4°C. After washing with PBS, the sections were incubated with appropriate secondary antibody conjugated with horse radish peroxidase (HRP) (dilution 1:100) (Sigma, USA) for 30 min at room temperature. The slides were rinsed thoroughly with PBS and then developed with 3-3′-diamino benzidine tetrahydrochloride (DAB) (Sigma, USA), counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene and mounted with Permount®. L-fucose was histochemically stained with biotinylated *Ulex europaeus* agglutinin I (Vector Laboratories, USA) overnight at 4°C, followed by streptavidin-HRP (Vector Laboratories, USA), and developed with DAB, counterstained, cleared and mounted as above. Control experiments with omission of the primary antibody or lectin conjugate were parallelly performed.

Helicobacter pylori strains and growth media

Helicobacter pylori Thai strains (BT112) were cultured in brain heart infusion medium with 5% human blood or sheep blood, at 37°C in a microaerophilic condition for 3–5 days (Suyapoh *et al.*, 2021). Bacterial pellets were washed and labelled with fluorescein isothiocyanate (FITC) as described (Falk *et al.*, 1993).

In vitro assay of H. pylori localization to liver fluke sections

Tissue sections of *O. viverrini* were deparaffinized in xylene, rehydrated in a series of ethanol alcohol, rinsed in tap water followed by PBS for 5 min. The sections were then blocked for non-specific staining with 0.2% BSA/TWEEN for 15–30 min. The FITC-labelled bacterial suspension diluted in blocking buffer (OD_{600} = 0.05) was placed onto the sections and incubated for 60 min at room temperature. After thorough washing in PBS, the slides were viewed and photographed using confocal microscopy (ZEISS, model LSM800). Control experiments with unlabelled *H. pylori* were parallelly done.

Specificity verification of fucose receptor

In order to test the specificity of the putative receptor, we employed the glycosidase, fucosidase, which catalyses the hydrolysis of L-fucose. Individually, 27 adult *O. viverrini* liver flukes were separated into 3 treatment groups of 9 worms each and exposed to increasing concentrations of fucosidase (New England BioLabs, Ltd., UK), specifically 0 (control), 40 and 100 U mL^{-1}. Three worms of each treatment group were incubated at 37°C with the fucosidase for 2, 16 or 24 h, respectively, after which the worms were transferred to DNA extraction buffer (above).

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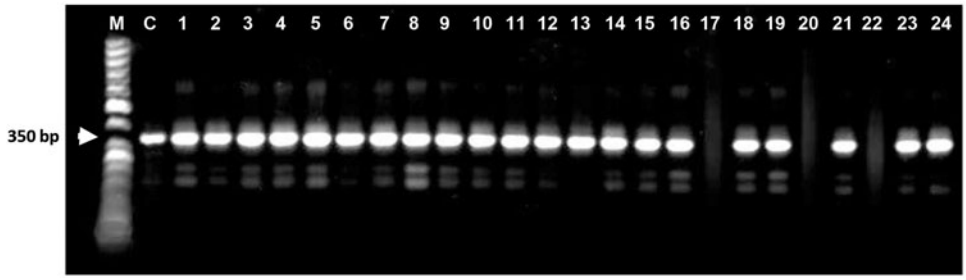

Fig. 1. Representative PCR products amplified by using *Helicobacter pylori ureA gene* (product size = 359 bp). M, marker; C, positive control (*H. pylori*) and lane 1–24 are individual *O. viverrini* worms. Data representative of PCR products amplified from DNA extracted from 49 *O. viverrini* adult worms.

Subsequently, qPCR for *ureA* of *H. pylori* was undertaken by calculation of copy number with the *ureA*-plasmid standard curve. The qPCR reaction was performed in a 96-well microtitre plate using 12.5 μL of Master mix 2× [Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2×)] 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 free nuclease 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) with the following condition; 95°C, 30 s; 55°C, 30 s; 72°C, 30 s × 35 cycles and the melting curve condition with 95°C, 30 s; 55°C, 60 s; 95°C, 15 s. Details of primer sets and product sizes are shown in Table 1. The qPCR output was expressed as number of DNA copies per reaction.

Statistical analysis

Descriptive statistical analysis was carried out using percentages. Specific verification of fucose receptors was analysed by ANOVA followed by Dunnett's test for multiple comparison. Analysis was accomplished using GraphPad Prism version 7 software (GraphPad, San Diego, CA). *P* values <0.05 were considered to be statistically significant.

Results

Detection of H. pylori *ureA in* O. viverrini *adult worms*

To determine the presence of *H. pylori* in *O. viverrini* adult worms, nested PCR for *ureA* was employed. The results revealed that the *ureA* with approximately 359 bp was identified in 41 out of 49 (79%) genomic DNAs, each from individual *O. viverrini*. Representative nested PCR amplification of *ureA* gene products in 1% agarose gel is shown in Fig. 1.

Expression of H. pylori*-specific receptors in* O. viverrini *gut epithelium*

Immunohistochemical analysis of 94 adult worm sections revealed certain specific receptors for *H. pylori* presented along the gut epithelium of *O. viverrini*. Lewis B, sialyl-Lewis X and TLR4 were detected in 3.2% (3/93), 3.1% (6/97) and 18.2% (16/88) of the samples, respectively. Notably, L-fucose residue was detected along the gut epithelium of *O. viverrini* in 70.2% (66/94) of the sections. Figure 2A–D provides representative micrographs of the expression of these receptors in the epithelium of gut and in the tegument of the liver fluke.

Colocalization between H. pylori *and fucose residues along the gut epithelium of* O. viverrini

Given that L-fucose expression showed the highest frequency mainly in the gut epithelium of the worms (70.2%), which was similar in frequency to the positive rates of *H. pylori* detection in *O. viverrini* DNA samples (79%), we proceed to investigate the co-localization of the bacillus and host cell fucose using an *in vitro* adhesion assay, which involved probing thin sections of worms in the hepatobiliary tract of hamsters with FITC-labelled *H. pylori*. The investigation revealed that *H. pylori* adhered to the epithelium of the *O. viverrini* gut where the L-fucose was presented (Fig. 3A and D). Adherence was not observed by *H. pylori* on the gut epithelium in L-fucose-negative *O. viverrini* worms (Fig. 3B and E). Intense adhesion of *H. pylori* was also observed in regions of the tegument where L-fucose-staining also was present (Fig. 3C and F).

Specificity of fucose as a receptor for H. pylori *in* O. viverrini

The specificity of L-fucose was tested by using fucosidase enzyme that cleaves fucose residues and thus prevents *H. pylori* adhesion in the gut and tegument of the worm. After incubating *O. viverrini* parasites with increased concentrations of the fucosidase and for increasing intervals, 40 and 100 U mL^{-1} at 2, 16 and 24 h, the worms were harvested and copy numbers of *ureA* determined by qPCR. We observed a significantly decreased adhesion of *H. pylori* in both enzyme concentration- and time-dependent fashion (Fig. 4).

Discussion

Helicobacter pylori and *O. viverrini* co-infections orchestrate pathogenesis of opisthorchiasis, specifically periductal fibrosis both in experimental rodent models (Dangtakot *et al.*, 2017; Suyapoh *et al.*, 2021) and in naturally infected people (Boonyanugomol *et al.*, 2012; Deenonpoe *et al.*, 2017), and are implicated in malignant transformation (Sripa et al., 2017; Dangtakot *et al.*, 2021). Given that the liver fluke is known to harbour *H. pylori* in its gut and tegument (Deenonpoe *et al.*, 2017; Suyapoh *et al.*, 2021), *H. pylori* has, *sine qua non*, to bind to these helminth tissues *via* appropriate receptors. Although major *H. pylori* adhesins essential for colonization and establishment of infection have been described in human gastric epithelia (Chmiela and Kupcinskas, 2019; Fagoonee and Pellicano, 2019; Matos *et al.*, 2021), orthologues of the human cell receptors have yet to be reported in the gastrodermis of *O. viverrini*. Here, we report the adhesion of *H. pylori* to *O. viverrini* gut epithelium and the tegument *via* L-fucose binding adhesin.

In this study, we investigated the expression of 4 well-investigated receptors of *H. pylori* including Lewis B, sialyl-Lewis X, TLR4 and fucose residues in *O. viverrini* adult worms. Among these 4, L-fucose residue was detected in the gut in over 70% of the worms. The rate of fucose detection was close to the number of *O. viverrini* that carried *H. pylori* (79%). We have reported the expression of L-fucose in the gut of *O. viverrini* in an earlier study (Talabnin *et al.*, 2013). Thus, we

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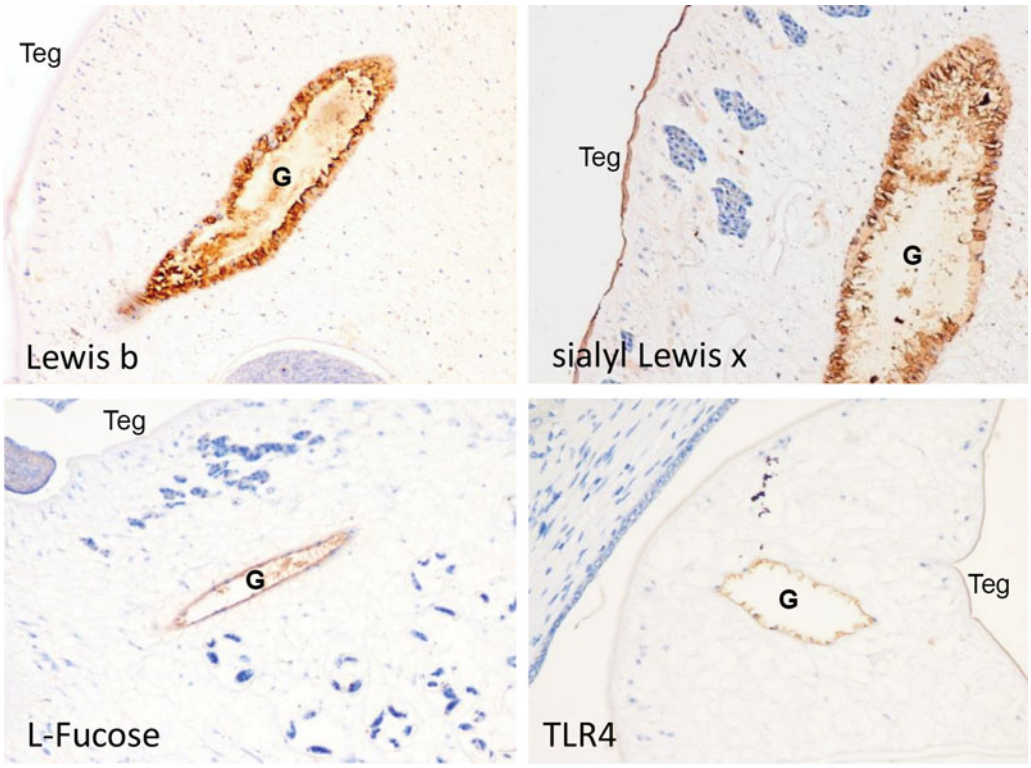

Fig. 2. Immunohistochemistry of adult *O. viverrini* tissue sections using antibodies raised against *Helicobacter pylori*-specific receptors. Lewis B (upper left), sialyl-Lewis X (upper right), L-fucose (lower left) and TLR4 (lower right) along the gut epithelium (G) and tegument (Teg) of *Opisthorchis viverrini*. G, gut; Teg, tegument; original magnification, 20×.

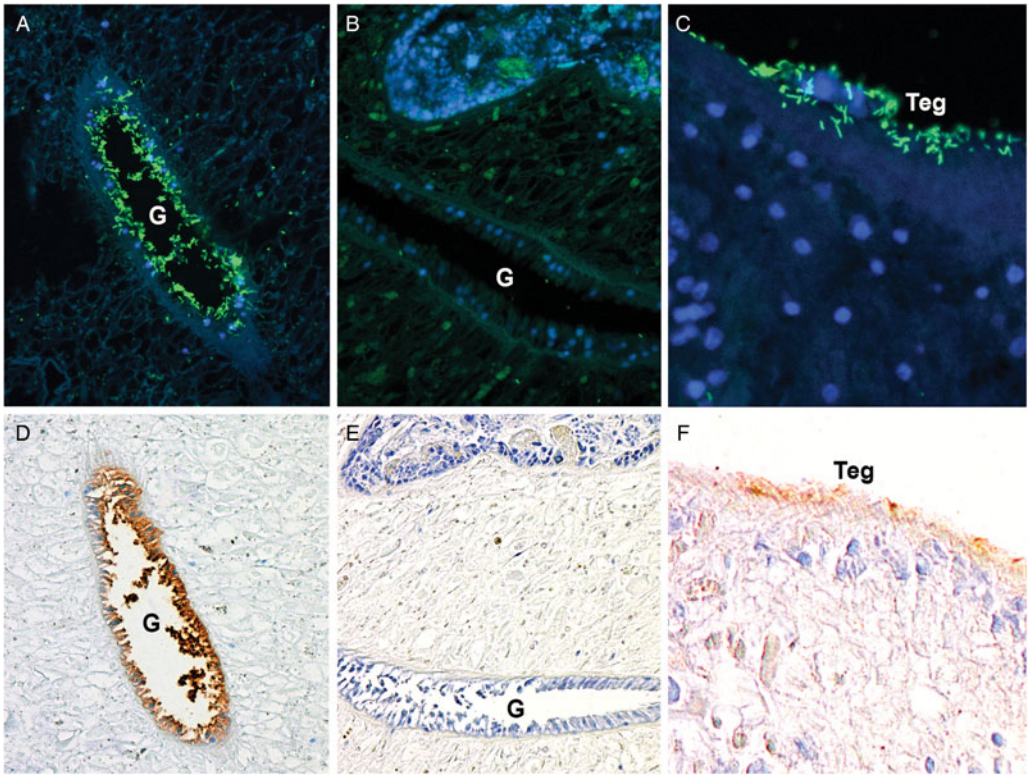

Fig. 3. Co-localization between FITC-labelled *Helicobacter pylori* and L-fucose. Adherence of *H. pylori* was apparent along the gut epithelium of the liver fluke in regions positive for L-fucose (A, D). Adhesion by *H. pylori* was not observed in L-fucose-negative worms (B, E). Certain sites on the tegument showed intense *H. pylori* adhesion where L-fucose is positive (C, F). Upper panel (A, B, C) = adhesion assay using FITC-labelled *H. pylori*. Lower panel (D, E, F) = L-fucose histochemistry. G, gut epithelium; Teg, tegument; original magnification, 20×.

hypothesized that this fucose residue might play a part in the *H. pylori* adhesion in *O. viverrini* adult worm, and accordingly we further investigated the relationship between *H. pylori* and fucose expression in *O. viverrini* tissues. An *in vitro* assay for adhesion revealed that *H. pylori* localized to the gut epithelium and tegument of *O. viverrini* where the fucose expression had also been

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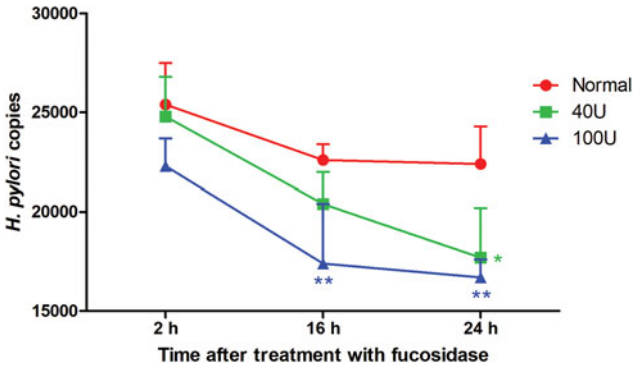

Fig. 4. Hydrolysis by fucosidase diminishes *H. pylori* adherence and colonization. Copy numbers of *H. pylori* were significantly decreased in a dose- and time-dependent fashion after exposure of *O. viverrini* worms to the fucosidase. Each time point represents copy numbers ± S.E. from 3 biological replicates (*$P < 0.05$; **$P < 0.005$).

detected. In parallel, the copy numbers of *H. pylori*-specific *ureA* gene were decreased in a dose- and time-dependent manner upon removal of fucose by fucosidase enzyme ($P < 0.01$). Altogether, these data provide increased support for our conjecture that the L-fucose is a candidate receptor for the adherence of *H. pylori* within the liver fluke, *O. viverrini*.

Despite the result from this study suggesting that the L-fucose was essential for *H. pylori* adhesion, the source of the fucosylated oligosaccharides found in parasite tissues is still unclear. There is a possibility that these fucosylated oligosaccharides are the remains of host biomolecules ingested by worms, which could be acquired by the gut epithelium of the fluke. Our previous study showed that only the cagA-positive strain was detected in the gut of the worm and co-infection between cagA-positive *H. pylori* and *O. viverrini* resulted in a more severe biliary pathology and decreased E-cadherin expression *in vivo* and *in vitro* than those of the cagA-negative strain (Suyapoh *et al.*, 2021). The present study demonstrated that the binding of *Helicobacter* to the gut of *O. viverrini* is fucose-dependent. Taking these results together, we hypothesize that fucose is a selective molecule for cagA-positive *H. pylori* binding in the worm's gut, which can promote *H. pylori* colonization and enhance the biliary pathogenesis and carcinogenesis.

To conclude, this report presents novel evidence that L-fucose, which is detected in the gut epithelium and tegument of the *O. viverrini* liver fluke, plays a role in adhesion of *H. pylori* to this liver fluke. These findings provide experimental support for our previously proffered hypothesis that co-migration of the 2 carcinogenic pathogens to the bile ducts orchestrates and exacerbates hepatobiliary disease including CCA (Deenonpoe *et al.*, 2017). Indeed, barriers to malignant transformation of the biliary tract epithelium may be compromised by this relationship involving helminth parasite and a bacterial pathogen (Dheilly *et al.*, 2017). One of the limitations of this study is that we cannot directly quantify the copy numbers of *H. pylori* and correlate with L-fucose expression in each individual worm.

Acknowledgements. We are grateful to Suwit Balthaisong for assistance with histological works, Wachiraporn Donsa for adhesion assay and Chalida Chuenchom for bacterial culture and maintenance.

Author contributions. P. T., S. S., C. P., Y. C., B. S. and P. J. B. conceived and designed the study. P. T. and B. S. performed the experiments. P. T., R. D. and B. S. analysed and interpreted the findings. P. T., R. D., S. S. and B. S. wrote the draft manuscript. B. S. and P. J. B. revised final draft manuscript. B. S. obtained funding. All authors read and approved the final version of the paper.

Financial support. This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health Cluster (SHeP-GMS), Khon Kaen University, Thailand (award number I56110) (B. S.), Khon Kaen University (grant# RP64018) and partly by award R01CA164719 from National Cancer Institute, National Institutes of Health, USA (P. J. B.). P. T. acknowledges the support from the Royal Golden Jubilee PhD Program (grant number PHD/0013/2555), the Thailand Research Fund. B. S. is supported by KKU Senior Research Scholar Fund.

Conflict of interest. None.

Ethical standards. Animal procedures complied with guidelines of the Guide for the Care and Use of Laboratory Animals by National Research Council, USA, 8th edition and the Thai Institutional Animal Care and Use Committee.

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https://doi.org/10.1017/S0031182022000592 Published online by Cambridge University Press