The Detection of *Opisthorchis viverrini* DNA in Bile Samples from Obstructive Jaundice Patients by Real-time PCR

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Abstract

Objective: To detect *Opisthorchis viverrini* (*O. viverrini*) DNA in bile samples from obstructive jaundice patients by real-time PCR assay and to compare the results of real-time PCR with conventional PCR.

Methods: Bile samples were collected from 43 patients with obstructive jaundice from the Department of Surgery, Rajavithi Hospital and the Department of Surgery and Department of Diagnostic Radiology, Udon Thani Cancer Hospital. Twenty-eight cholangiocarcinoma (CCA) and 15 non-CCA patients underwent endoscopic retrograde cholangiography (ERCP) and biliary tract surgery. All bile samples were detected for *O. viverrini* DNA by real-time PCR and conventional PCR. The results of positive and negative bile samples for *O. viverrini* DNA detection by real-time PCR and conventional PCR detection were compared.

Results: Thirteen (30.2%) out of 43 bile samples were *O. viverrini* DNA positive. Nine of 13 bile samples were positive by real-time PCR and conventional PCR, while 4 bile samples were positive only by real-time PCR. Thirty bile samples (69.8%) were negative for *O. viverrini* DNA by both methods. The sensitivity, specificity, positive and negative predictive values of the real-time PCR were 100%, 88.2%, 69.2% and 100%, respectively.

Conclusion: Real-time PCR assay is suitable for detection of *O. viverrini* DNA in bile samples especially in obstructive jaundice patients.

Keywords: Bile, *Opisthorchis viverrini*, Obstructive jaundice, Real-time PCR, Conventional PCR

การตรวจหาดีเอ็นเอของพยาธิใบไม้ตับ *Opisthorchis viverrini* ในน้ำดีจากผู้ป่วยที่มีภาวะท่อน้ำดีอุดตันด้วยวิธี real-time PCR

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บทคัดย่อ

วัตถุประสงค์: เพื่อตรวจหาดีเอ็นเอของ *Opisthorchis viverrini* (*O. viverrini*) ในน้ำดีจากผู้ป่วยที่มีภาวะท่อน้ำดีอุดตันด้วยวิธี real-time polymerase chain reaction (PCR) และเปรียบเทียบผลที่ได้กับวิธี conventional PCR

วิธีดำเนินการวิจัย: เก็บน้ำดีระหว่างการทำการส่องกล้องตรวจรักษาท่อน้ำดีและตับอ่อนและการผ่าตัดท่อน้ำดีเพื่อระบายน้ำดีในผู้ป่วยที่มีภาวะท่อน้ำดีอุดตันจำนวน 43 ราย จากกลุ่มงานศัลยศาสตร์ โรงพยาบาลราชวิถี กรุงเทพมหานคร และกลุ่มงานศัลยกรรมและกลุ่มงานรังสีวินิจฉัยและเวชศาสตร์นิวเคลียร์ โรงพยาบาลมะเร็งอุดรธานี จังหวัดอุดรธานี ซึ่งประกอบด้วยผู้ป่วยโรคมะเร็งท่อน้ำดี 28 ราย และผู้ป่วยที่ไม่เป็นโรคมะเร็งท่อน้ำดี 15 ราย นำน้ำดีจากทั้งสองกลุ่มมาตรวจหาดีเอ็นเอของ *O. viverrini* ด้วยวิธี real-time PCR และ conventional PCR เปรียบเทียบผลบวกและผลลบที่ได้จากทั้งสองวิธี

ผลการวิจัย: พบดีเอ็นเอของ *O. viverrini* ในน้ำดีจากผู้ป่วย 13 ราย (ร้อยละ 30.2) ราย โดยผู้ป่วยจำนวน 9 รายให้ผลบวกจากทั้งวิธี real-time PCR และวิธี conventional PCR โดยผู้ป่วย 4 รายให้ผลบวกเฉพาะวิธี real-time PCR และไม่พบดีเอ็นเอของ *O. viverrini* ในน้ำดีจากผู้ป่วย 30 ราย (ร้อยละ 69.8) จากการตรวจด้วยทั้งสองวิธี วิธี real-time PCR มีความไว ความจำเพาะ ค่าทำนายผลบวก และค่าทำนายผลลบเป็นร้อยละ 100, 88.2, 69.2 และ 100 ตามลำดับ

สรุป: วิธี real-time PCR เหมาะสมสำหรับการตรวจหาดีเอ็นเอของ *O. viverrini* ในน้ำดีจากผู้ป่วยมีภาวะท่อน้ำดีอุดตัน

Introduction

Opisthorchis viverrini (*O. viverrini*) is a human liver fluke that remains an important public health problem in Southeast Asia, particularly in the Mekong River Basin countries of Thailand, Laos, Cambodia, and Vietnam. *O. viverrini* infection is still one of the public health problems in Thailand where an estimated 8 million people are infected with *O. viverrini*. [1] Most commonly, people become infected by eating raw or undercooked freshwater fish which contains the metacercariae such as "koi pla" (fermented fresh fish).[2] *O. viverrini* infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis.[3] Chronic inflammation due to *O. viverrini* is considered to be an underlying cause of carcinogenesis leading to biliary cancer, cholangiocarcinoma (CCA).[1]

Currently, diagnosis of *O. viverrini* infection is based on a standard fecal examination of the fluke's eggs. However, in cases with bile duct obstruction or in mild infection, eggs may not be detected in the feces.[4] Moreover, the closely related *O. viverrini* egg and several species of minute intestinal flukes (MIF) have similar egg features.[5] Therefore, the stool examination of the fluke eggs is not suitable for *O. viverrini* infection diagnosis. Several serology analyses are also used to diagnose *O. viverrini* infection;[6-8] however, cross-reactivity with other helminthes may occur.[9] Molecular diagnosis has been developed as an improvement on conventional diagnostic methods. Polymerase chain reaction (PCR) based techniques have been developed as improvements on conventional diagnostic methods. PCR method has been described for the detection of *O. viverrini* in feces, infected bithynid snails and cyprinoid fishes.[10,11] However, PCR has some disadvantages including the risk of contamination, preparation of agarose gel, time, lack of quantitative capacity, and staining ethidium bromide gel is a carcinogenic agent. Real-time PCR has more advantages than the other diagnostic molecular methods, including elimination of a laborious post-amplification stage, observation of the amplified product and also has many advantages over conventional PCR such as reproducibility, speed, sensitivity, robustness, quantitative and low contamination risk.[12] The real-time PCR method is more widely used for detection of *O. viverrini* DNA in several experimental investigations. But all of those studies were used to detect *O. viverrini* DNA in feces and liver tissues.[13,14] There have been no reports of real-time PCR assay for the detection of *O. viverrini* DNA in bile samples. The use of bile samples for laboratory diagnostic testing was of interest, especially in obstructive jaundice patients such as CCA patients and others with liver diseases. The aim of this study was to detect *O. viverrini* DNA in human bile samples from patients who are symptomatic with obstructive jaundice and to compare the results of real-time PCR and conventional PCR.

Methods

Bile samples collection

Five to ten milliliter bile samples were collected from 43 patients who underwent endoscopic retrograde cholangiography (ERCP) at the Department of Surgery, Rajavithi Hospital, Bangkok and biliary tract surgery at the Department of Surgery and Department of Diagnostic Radiology, Udon Thani Cancer Hospital, Udon Thani province from March 2014 to January 2016. All 43 patients had obstructive jaundice: 28 CCA patients were diagnosed by clinical history, ultrasound, CT scan and liver biopsy, and 15 non-CCA patients, of which 13 were common bile duct stone patients and 2 cholangitis patients, were enrolled in this study. The bile samples were stored at -80 °C until use.

The study was approved by the Ethics Committee on Research Involving Human Subjects, Rajavithi Hospital (N0.051/2557) and the Ethics Committee on Human Research, Udon Thani Cancer Hospital (N0.4/2557). All subjects provided prior written informed consent.

DNA extraction

All DNA samples were extracted from bile samples using the QIAamp DNA mini kit (Qiagen, Hilden, North Rhine-Westphalia, Germany),

according to manufacturer's recommendations. Five hundred microliters of each bile sample was firstly centrifuged, and the supernatant was discarded before the pellet was washed three times with 1X Phosphate Buffered Saline to dissolve the bilirubin from the samples. Subsequently, the pellet was used for DNA extraction. Then, the pellet was incubated with proteinase K at 56°C on an agitator until total pellet digestion. The mixture was centrifuged at 14,000xg at room temperature, and 1 volume of phenol/chloroform (1:1 ratio, equilibrated with 0.1 M phosphate buffered saline, pH 7.5) was added to the supernatant. After being vortexed, they were centrifuged at 14,000xg; the aqueous phase was precipitated with 2 volumes of 100% ethanol molecular grade (Merck Millipore, Darmstadt, Hesse, Germany). After two washes with 100% ethanol molecular grade, the pellet was dried and resuspended with 200 µl distilled water for further use. DNA concentration was determined by measuring the optical density at 260 nm using NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). DNA extraction samples were collected and aliquoted, and stored at -20°C until use.

Primer design

The amplification of genomic DNA of *O. viverrini* was carried out using the primers: forward primer 5'-CAT AAG GTT GAC TAG GAA ACC GGG-3' (position 97-120) and reverse primer 5'-TGT TCT CAG GCA AGT GAG TGT GCT-3' (position 288-310). The primers were designed to amplify a repetitive DNA fragment specific for *O. viverrini,* which showed no significant homology to other parasites. Nucleotide sequence data reported are available in the GenBank, EMBL, and DDJB databases under the accession numbers S80278 version S80278.1 gi: 244506. The complementary nucleotide sequence was also 100% (215/215) homologous to *O. viverrini* with GenBank accession number of S80278. The amplicon size was 215 bp.

Real-time PCR for RPLO gene detection

In order to confirm the DNA presentation in each bile sample after DNA extraction, RPLO (reference gene), housekeeping gene, was utilized for gene normalization and target gene quantification. All bile extracted DNA samples were detected for RPLO gene by real-time PCR using Chromo 4™ System and MJ Opticon Monitor™ Version 3.1 (Bio-Rad, Hercules, California, USA). The reaction was carried out in a 20 µl final reaction volume which consisted of 10 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.2 µM concentration of each forward and reverse primer, forward primer 5'GGC GAC CTG GAA GTC CAA CT 3' and reverse primer 5'CCA TCA GCA CCA CAG CCT TC 3', 2 µl of template DNA and 7.2 µl of distilled water. The reaction was performed with preliminary denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 95°C for 15s, annealing at 57.8°C for 30s. A final cooling step was performed at 4°C for 1 min.

Real-time PCR for *O. viverrini* DNA detection

The detection of *O. viverrini* DNA was performed using Chromo 4TM System and MJ Opticon Monitor™ Version 3.1(Bio-Rad). The reaction was carried out in a 20 µl final reaction volume consisting of 10 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.2 µM concentration of each forward and reverse primer, 2 µl of template DNA and 7.2 µl of distilled water. The reaction was performed with preliminary denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 61.7°C for 30 seconds. A final cooling step was performed at 4°C for 1 minute. For each run, ultrapure water was the negative control and *O. viverrini* DNA was the positive control. The melting temperature (Tm) values between 75°C and 81.5°C indicated with *O. viverrini* infection (Figure 1A). In order to confirm the finding, real-time PCR amplification products were separated by 2% agarose gel electrophoresis and stained with RedSafe™ nucleic acid staining solution (Intron Biotechnology Inc., Seongnam, Gyeonggi-do, South Korea). The band could be visualized with Gel Doc™ XR+ Gel Documentation System (Bio-Rad) in comparison with DNA molecular weight. The expected DNA

band's size of 215-bp product was considered infection with *O. viverrini* (Figure 1B). In every run, the non-template was the negative control and *O. viverrini* extracted DNA from adult worms was the positive control. Positive and negative controls were tested along with the unknown samples.

Detection limit of the real-time PCR for *O. viverrini* detection

The detection limit was determined on a 10-fold serial dilution of DNA extracted from adult *O. viverrini*. A final concentration of positive control for *O. viverrini* DNA was 7 ng/µl. The highest dilution with a positive signal indicated the detection limit.

Conventional PCR for *O. viverrini* DNA detection

We used conventional PCR as a reference method for *O. viverrini* detection. The conventional PCR for *O. viverrini* DNA amplification was performed using Thermal Cycler machine (Little Genius, Bioer Technology, Minato, Tokyo, Japan). The reaction was carried out in a 20 µl solution. The final reaction volume consisted of 10 µl of Taq 2X Master Mix (M0270S, New England Biolabs, Ipswich, Massachusetts, USA), 0.2 µM concentration of each forward and reverse primer, 2 µl of template DNA and 7.2 µl of distilled water. The reaction was performed with an initial denaturation step at 95°C for 5 min, denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 20 seconds. A final cooling step was performed at 4°C for 1 minute. DNA samples were amplified for 35 cycles. For each run, ultrapure water was the negative control and *O. viverrini* DNA was the positive control. The PCR products were separated by 2% agarose gel electrophoresis and stained with RedSafe™ nucleic acid staining solution (Intron Biotechnology Inc.). The band could be visualized with Gel Doc™ XR+ Gel Documentation System (Bio-Rad) in comparison with DNA molecular weight. The expected DNA band size of 215-bp product was considered to indicate infection with *O. viverrini* (Figure 1B). The non-template was the negative control and the extracted *O. viverrini* DNA from adult worm was the positive control. The results of positive and negative bile samples for *O. viverrini* DNA detection by real-time PCR and conventional PCR detection were compared.

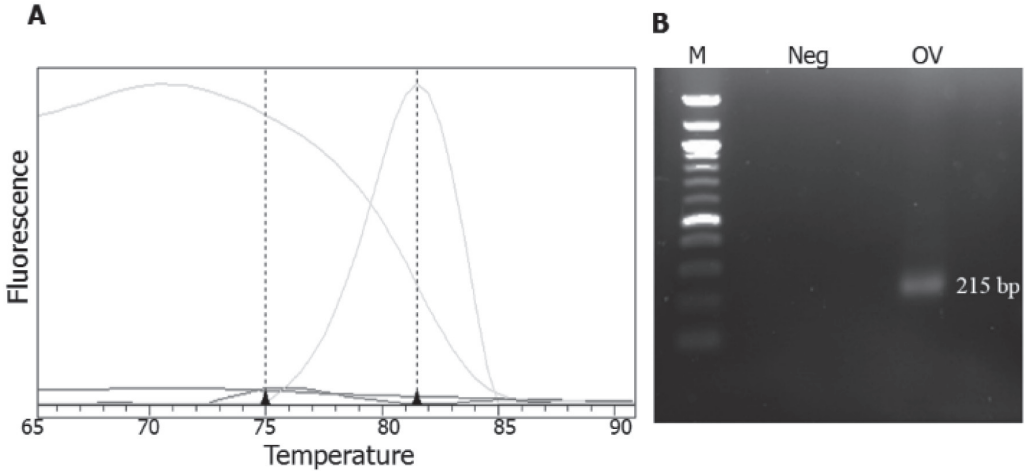

Figure 1: An example of results from real-time PCR and conventional PCR in this study. (A) Melting curve analysis of PCR product of *O. viverrini*. The melting temperature (Tm) value was approximately 81.5°C indicating *O. viverrini* infection. Negative control had no peak of amplification products. (B) Amplified Products' sizes by gel electrophoresis: lane 1 was DNA marker (100 bp), lane 2 was negative control and lane 3 was *O. viverrini* DNA (215 bp).

Results

All DNA from bile samples in CCA and non-CCA patients were tested for RPLO gene by real-time PCR. RPLO gene, a 15 kDa basic protein, plays a central role in the latter stages of 50 S subunit assembly during in vitro reconstitution of *Escherichia coli* subunits, where it promotes the assembly of more than ten other 50 S subunit proteins. All DNA bile samples were positive for RPLO (See Figure 2).

The sensitivity of quantitative real-time PCR for detection of the *O. viverrini* DNA was determined. A final concentration of positive control for *O. viverrini* DNA was 7 ng/µl. Ten-fold serial dilution was prepared ranging from undiluted (a final concentration) to 10^{-10} ng/µl. The series of undiluted (a final concentration) to 10^{-6} ng/µl were seen on the quantitative curves, while the sensitivity of this method, and/or this equipment mode, l could not detect above 10^{-6} ng/µl (10^{-7} ng/µl to 10^{-10} ng/ µl) dilution, meaning real-time PCR was able to detect *O. viverrini* DNA dilutions as low as 7 fg/µl (Figure 3).

A total of 43 patients with obstructive jaundice had bile specimens collected during ERCP and biliary tract surgery: 28 (65.1%) from CCA patients and 15 (34.9%) from non-CCA patients, *O. viverrini* DNA was detected by conventional PCR and real-time PCR methods. The amplified 215 bp bands from conventional PCR products were observed in the *O. viverrini* DNA positive cases. With real-time PCR, the melting curve analysis of *O. viverrini* DNA was approximately 81.5 °C, indicating *O. viverrini* DNA positive. Thirteen (30.2%) out of 43 bile samples were *O. viverrini* DNA positive. Nine of 13 bile samples were positive by real-time PCR and conventional PCR, while 4 bile samples were positive by only real-time PCR. Thirty (69.8%) bile samples were negative for *O. viverrini* DNA by both methods. The sensitivity, specificity, positive and negative predictive values of the real-time PCR method were 100% (9/9+0), 88.2% (30/30+4), 69.2% (9/9+4) and 100% (30/30+0), respectively (Table 1).

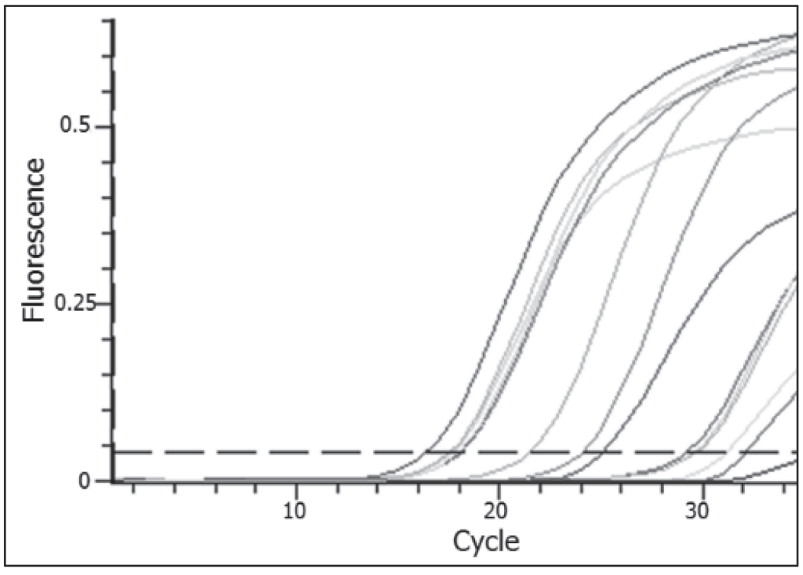

Figure 2: Amplification curves of real-time PCR analysis for RPLO gene detection. An example of extracted DNA from bile samples showed RPLO gene positive.

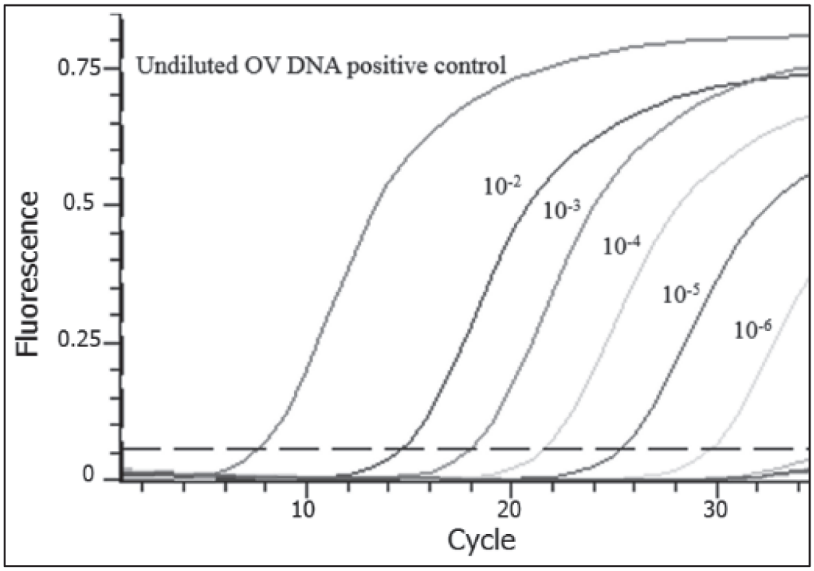

Figure 3: The limitation of real-time PCR for *O. viverrini* detection. Detection of 10-fold serial dilution of DNA extracted from adult *O. viverrini*. The series of undiluted extracts (a final concentration) to 10^{-6} ng/µl is seen on the quantitative curves.

Table 1:

Comparison of total number of O. viverrini DNA detected in bile samples by real-time PCR and conventional PCR

Real-time PCR	Conventional PCR		Total no. (%)
	Positive	Negative	
Positive	9	4	13 (30.2)
Negative	0	30	30 (69.8)
Total	9 (20.9)	34 (79.1)	43 (100)

Discussion

Stool is widely used for detection *O. viverrini* infection. However, in cases with bile duct obstruction or in a light infection, eggs may not be detected in the feces. The fluke's egg cannot be recovered in the feces when there is biliary obstruction. [4] Although bile samples involve an invasive collection method, they were collected from 28 CCA and 15 non-CCA patients with obstructive jaundice during endoscopic retrograde cholangiography (ERCP) and biliary tract surgery for biliary drainage. We detected *O. viverrini* DNA in bile samples by real-time PCR and compared the results of real-time PCR and conventional PCR.

Bile duct obstruction is the major complication of patients with *O. viverrini* infection. The flukes reside in the peripheral small bile ducts of the liver. Chronic *O. viverrini* infection results in chronic inflammation of the bile duct, bile duct dilatation, mechanical obstruction, and bile duct wall

thickening. The peripheral bile ducts are obstructed in a heavy infection because aggregates of flukes pack and block the small peripheral intrahepatic bile ducts.[15] In patients with opisthorchiasis, the flukes reside mainly in the medium sized and large bile ducts and these bile ducts are dilated.[16] The adult worms gather to cause obstruction of biliary tract and inhibit the entry of eggs into the intestine. Therefore, in cases with bile duct obstruction or in a light infection, eggs may not be detected in the feces. In the present study, *Clonorchis sinensis* (*C. sinensis*) DNA in bile samples. In the CCA group, 7 out of 28 cases were positive by real-time PCR and conventional PCR (data not shown). In agreement with a previous study, *Clonorchis sinensis* (*C. sinensis*) DNA in gall bladder bile and stone samples from patients with cholecystolithiasis were detected by TaqMan-based real-time PCR assay. All of the egg-positive samples determined by microscopy yielded positive results by real-time PCR.[17] Chronic inflammation and cellular injury, together with partial obstruction of bile flow, results in bile-stasis and in chronic exposure of biliary cells to the carcinogenetic action of bile components.[18]

Usually flukes reside in the medium sized or small intrahepatic bile ducts. Intraductal flukes cause mechanical obstruction, inflammation, adenomatous hyperplasia, and periductal fibrosis. In a heavy infection, flukes may reside in the extrahepatic ducts and in the gallbladder. In the present study, we found *O. viverrini* DNA in bile from non-CCA patients. Bile samples of non-CCA were collected from common bile stone and cholangitis patients. Recurrent pyogenic cholangitis is an important and frequent complication of opisthorchiasis.[16] Mucin produced from adenomatous hyperplasia with goblet-cell metaplasia combined with parasites and eggs causes bile stasis that provides a favorable environment for bacterial infection. The eggs and bacteria serve as nidus for stone formation.[16,19,20] Bile-stasis-caused obstruction induces stagnation of the bile components leading to stone formation.[21] Sripa et al.[22] demonstrated *O. viverrini* eggs in the sludge from gallbladder bile by scanning electron microscopic (SEM) study. Bile examination was positive for *O. viverrini* eggs in 50% of 113 consecutive cholecystectomies, gallstones and/or common bile duct stones. Therefore, there was a possibility that we found *O. viverrini* DNA in bile samples from non-CCA patients.

In our study, real-time PCR were compared with conventional PCR method for identification of *O. viverrini* DNA in bile samples. Nine of 13 samples were positive by real-time PCR and conventional PCR, while 4 of 13 bile samples were positive by only real-time PCR. The real-time PCR assay is more sensitive for the detection of *O. viverrini* DNA in bile samples from obstructive jaundice patients. The real-time PCR assay showed a higher percentage of *O. viverrini* positive bile samples than conventional PCR. The limited detection of this assay was 7 fg/µl of adult *O. viverrini* genomic. Intapan et al.[13] reported the development of a fluorescence resonance energy transfer (FRET) real-time PCR for molecular detection of *O. viverrini* in human fecal samples which was compared with QFECT as the gold standard. The diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values of the real-time FRET PCR were 97.5%, 100%, 98.9%, 100%, and 98.2%, respectively. The conventional PCR assays provided high sensitivity and specificity but they are time consuming, labor-intensive with a high risk of contamination and sometimes include non-specific products of the same size, while real-time PCR assay is quicker, has high sensitivity, and low risk of contamination. Reaction results can instead be observed in real-time and clearly interpreted.[23] In the present study, the real-time PCR assay had a higher percentage of *O. viverrini* positive bile samples than conventional PCR. Therefore, the real-time PCR assay is suitable for the detection of *O. viverrini* DNA in bile samples.

Since chronic inflammation due to *O. viverrini* is considered to be an underlying cause of carcinogenesis leading to biliary cancer, CCA, the incidence of CCA in Thailand is highest in the region with the highest prevalence of *O. viverrini* infection.[24] There is a significant need for a diagnostic method

to provide early detection. Early identification of *O. viverrini* DNA in obstructive jaundice patients is important in order to decrease the incidence of CCA and controlling the distribution of *O. viverrini* infection. We concluded that bile can serve as an alternative sample for the detection of *O. viverrini* DNA, especially in obstructive jaundice patients. Our results demonstrate that the real-time PCR assay is suitable for detection of *O. viverrini* DNA in bile samples in patients with obstruction jaundice.

One limitation of this study was that early-stage CCA patients did not present in the study group. Because CCA is clinically silent at early stages, severe symptoms of most CCA patients are revealed only at the later stages of the disease. In future studies, it should be feasible to develop specific and sensitive techniques for *O. viverrini* detection in non-invasive samples including stool, serum, and urine for early cancer screening of at-risk populations.

Conflict of interest statement

We declare that we have no conflict of interest.

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