Microscopic Examination of Gallbladder Stones Improves Rate of Detection of Clonorchis sinensis Infection

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To improve the rate of detection of Clonorchis sinensis infection, we compared different specimens from patients with cholecystolithiasis. Feces, gallbladder bile, and gallbladder stones collected from 179 consecutive patients with cholecystolithiasis underwent microscopic examination, and according to the results, 30 egg-positive and 30 egg-negative fecal, gallbladder bile, and gallbladder stone specimens, respectively, underwent real-time fluorescent PCR. The detection rates of eggs in feces, bile, and gallbladder stones were 30.7%, 44.7%, and 69.8%, respectively, and the differences were statistically significant ($P < 0.01$). The PCR results confirmed that the eggs in the specimens were C. sinensis eggs. Eggs in the feces were “fresh” and in the gallbladder stones were “old.” Microscopic examination of gallbladder stones may improve the detection rates of C. sinensis infection, which is important for developing individualized treatments to prevent the recurrence of gallbladder stones and to prevent the occurrence of severe liver damage and cholangiocarcinoma.

Clonorchiasis, also known as liver fluke disease, is an important amphixenosis which can be transmitted between people and other vertebrates. It is distributed mainly in east and southeast Asia, including China, the Democratic People’s Republic of Korea, the Republic of Korea, Vietnam, and the Philippines (1). In China, clonorchiasis prevails mainly in Guangdong, Guangxi, and Heilongjiang provinces. The main reason for this is the consumption of raw fish and crustaceans (1–4). In the province of Guangdong, epidemiological surveys were carried out in 62 of 95 counties from 1997 to 2003. The results showed that about 18% of the 862,393 people were infected with Clonorchis sinensis (5–8). Some surveys also revealed that 78.5% to 85% of individuals were infected in areas where the parasite is endemic, such as Guangyuan, Dongyong, Sanshui, and Shunde, where the consumption of raw fish is very common (7, 8). Low-grade infection with adult C. sinensis flukes would not produce obvious clinical symptoms, but the metabolites produced by these adults and the mechanical stimulation from them can cause cholangitis, cholangiohepatitis, liver cirrhosis, and even cholangiocarcinoma (9–16). Eggs, dead worms, debris, and tissues shed from the bile duct may become stone cores and lead to the formation of gallstones (17).

Cholecystolithiasis is a common and frequently encountered disease. Epidemiological studies have indicated that the incidence is approximately 10% in the world (18, 19). Our previous data indicated that gallbladder stones were associated with Clonorchis sinensis infection (20). Therefore, improving the detection rate of C. sinensis infection may be important in the treatment of and postoperative recovery from this disease. At present, the diagnostic method for C. sinensis infection in the laboratory involves mainly the immunological detection of antigens or antibodies and the detection of eggs (21–25). Although immunological detection is simple and fast, there is always the potential for false-positive or -negative results because of the inherent characteristics of the immunological responses. Detection of eggs includes performance of direct fecal smears and microscopic examination of the sediment of duodenal drainage fluid and bile. Immunological detection is used for the preliminary diagnosis and the general investigation of C. sinensis infection, while detection of pathogens is used for the final diagnosis. To compare the detection of C. sinensis eggs among different specimens, feces, bile, and gallbladder stones from patients with cholecystolithiasis underwent microscopic examination. The morphologies of C. sinensis eggs were also compared among different specimens.

MATERIALS AND METHODS

Ethics statement. Written informed consent was obtained from each subject. This research was approved by the Medical Ethics Committee of The Second People’s Hospital of Panyu, Guangzhou, People’s Republic of China.

Subjects and specimen collection. The 179 patients with cholecystolithiasis who underwent endoscopic gallbladder-preserving cholecystectomy in the department of general surgery of our hospital from January 2010 to June 2010 were from Guangdong Province. This study included 98 males and 81 females with a mean age (± standard deviation [SD]) of 46.6 years (±12.8 years). Feces, gallbladder bile, and gallbladder stones were collected from each patient. Feces were collected for the examination of eggs before endoscopy. The approach for obtaining bile and stones during the operation was as follows. With the patient under laryngeal mask general anesthesia, the bottom of the gallbladder was cut off (<6 mm) laparoscopically. The bile was drained with a sterile ventricular drainage tube into a sterile injector and transferred to sterile tubes. When the bile had been drained, the gallbladder was explored with a CHIAO cholecystoscope (Chinese national patent no. ZL200810026985.X), and stones were collected with a stone extractor (when they were ≥5 mm in diameter) or with a CHIAO gallbladder sludge-like stone-absorbing box (patent no. ZL 201110167069.X) (when they were <5 mm in diameter or sludge-like) (26). The operation involved seven manipulations: pushing, squeezing, pressuring, tearing, bracing, flushing, and sucking (27). After these, the cut at the bottom of the gallbladder was sutured using a double
interlocking technique with 3-0 absorbable catgut, and the abdominal wall was sutured layer by layer.

**Microscopic examination of fecal, bile, and gallbladder stone samples.** (i) **Microscopic examination of feces.** Small amounts of fresh feces were smeared onto labeled slides and observed with a BX51 system microscope (Olympus, Tokyo, Japan). Each specimen was smeared onto two or three slides.

(ii) **Microscopic examination of bile sediments.** Two-milliliter bile samples were centrifuged at 1,450 × g for 10 min. The supernatant of each sample was transferred to another clean tube for analysis of the bile’s chemical composition (data not shown), and approximately 0.5 ml of sediment was kept. The sediment was then smeared onto labeled slides and observed with a BX51 system microscope (Olympus).

(iii) **Microscopic examination of gallbladder stones (Chinese national inventive patent no. for the whole process, ZL201010123552.3).** Gallbladder stones were washed twice with distilled water and dried at 60°C for 12 h. The stones were then split, and about 10 mg of each layer was weighed if the layered structures were distinct; otherwise, about 10 mg was weighed directly. The stones were placed in a mortar, 200 μl of 0.9% NaCl was added, and the stones were thoroughly ground and filtered with 260-mesh nylon yarn (the pore diameter was about 55 μm). The filtrate was smeared onto labeled slides and observed with a BX51 system microscope (Olympus).

**Detection of C. sinensis DNA by real-time fluorescent PCR.** Thirty egg-positive and 30 egg-negative fecal, gallbladder bile, and gallbladder stone specimens were selected randomly to undergo real-time fluorescent PCR testing. Each selected specimen was examined for **C. sinensis DNA.**

The purpose was to detect **C. sinensis DNA** in the egg-positive and egg-negative specimens and to confirm whether the eggs found were **C. sinensis** eggs. The adult worm was used as the positive control, and distilled water was used as the negative control.

(i) **Extraction of DNA.** (a) **Adult C. sinensis worm.** An adult worm of **C. sinensis** obtained from a clinical patient was first washed twice with 0.9% NaCl, dried on filter paper, and ground thoroughly in a mortar by constantly adding liquid nitrogen. Only a single specimen was used, and DNA was extracted using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s instructions with no variations. Briefly, the grinding powder was suspended into 180 μl of a lysis solution (ATL buffer; Qiagen), and then 20 μl proteinase K was added and incubated at 58°C for 4 h with brief vortexing every 30 min. Thereafter, 200 μl buffer AL (Qiagen) containing guanidine hydrochloride and 200 μl absolute alcohol were added successively and mixed by vortexing for 15 to 20 s. Finally, the genomic DNA was collected using a DNeasy mini spin column, eluted in 100 μl elution buffer (AE; Qiagen), and stored at −20°C until use.

Genomic DNA of **C. sinensis** eggs in human feces was extracted following a protocol described previously (28). Briefly, 1 g of feces was added to 5 ml of phosphate-buffered saline (PBS) and filtered, 1.5 ml of ethyl acetate was added, and the suspension was centrifuged at 2,000 × g for 10 min. The supernatant was discarded, and the pellet was washed three times with 5 ml of PBS and recentrifuged at 3,000 × g for 10 min. The pellet was added to 200 μl of PBS and stored at 4°C for 2 days. Finally, the DNA of **C. sinensis** eggs was extracted using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s instructions with no variations.

(b) **Gallbladder bile.** First, 1 ml of bile was centrifuged, and the supernatant was discarded. Then, the pellet was washed three times with 1 ml 80% ethanol to resolve some bilirubin. Finally, the pellet was washed once with 1 ml of 0.9% NaCl to remove residual ethanol. The supernatant was removed, and the pellet was used for DNA extraction as described above. All centrifugation steps were carried out at 15,000 × g for 10 min.

(c) **Gallbladder stones.** For mechanical breaking, 10 mg of the stones was weighed, frozen in liquid nitrogen gas for 10 min, and then ground thoroughly in a mortar. After the addition of 1 ml 0.9% NaCl, the suspension was transferred to a new 2.0-ml EP tube and centrifuged at 15,000 × g for 10 min. The pellet was treated and used for DNA extraction with the same procedure used for the bile.

### Table 1: Comparison of the detection rates of **C. sinensis** eggs in the three specimen types

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>No. of positive samples</th>
<th>No. of negative samples</th>
<th>Detection rate (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>55</td>
<td>124</td>
<td>30.7</td>
<td>0.006²</td>
</tr>
<tr>
<td>Gallbladder bile</td>
<td>80</td>
<td>99</td>
<td>44.7</td>
<td>0.000²</td>
</tr>
<tr>
<td>Gallbladder stones</td>
<td>125</td>
<td>54</td>
<td>69.8</td>
<td>0.000⁴</td>
</tr>
</tbody>
</table>

*²The total number of the three sample types tested was 179.

*²Comparison of feces and gallbladder bile.

*²Comparison of gallbladder bile and gallbladder stones.

*²Comparison of feces and gallbladder stones.

(iii) **Fluorescent PCR.** The primers and TaqMan probe were designed to detect the cytochrome c oxidase subunit I (COI) gene of **C. sinensis** (GenBank accession no. FJ965388.1) using Beacon Designer v7.51 software. It was then submitted to the BLAST program of the NCBI for specific analysis. These primers, Cs-F (5'-GTTTTGGTGATGATTAGTCACA TTGG-3') and Cs-R (5'-ACACACCTACCCAGAACAC-3'), amplify a 121-bp segment of the COI sequence. The minor-groove-binding TaqMan probe Cs-P (5'-JOE-AGGAAATTACGCCAACCACAGCGGCGC BHQ1-3' [JOE, 6-carboxy-4-dichloro-2-dimethoxyfluorescin; BHQ1, black hole quencher]) was used to detect the **C. sinensis**-specific product. The real-time PCR assay was run in a total reaction volume of 50 μl. The final concentration of the reaction solution included 0.2 μM concentrations of both forward and reverse primers, 0.2 μM TaqMan probe; 25 μl of Premix Ex Taq (Takara), 1 μl ROX buffer (Takara, Dalian, China), and 2 μl of a single template. The real-time PCR cycling parameters were an initial step at 95°C for 30 s and then 45 cycles of 15 s at 95°C, and 31 s at 60°C. The PCR amplification, detection, and data analysis were performed with an ABI 7300 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA).

The sensitivity and specificity of the PCR detection were certified in previous research, which showed that there was no cross-reactivity with other trematodes, and the detection limit of this assay was 0.1 pg of adult **C. sinensis** genomic DNA (29).

**Statistical analysis.** Ages are presented as means (±SD). Detection rates in these samples were 30.7%, 44.7%, and 69.8%, respectively, and were statistically significant (Table 1).

**RESULTS**

Among all specimens from the 179 patients, 55 fecal samples, 80 bile sediment samples, and 125 gallbladder stones were egg positive. The detection rates in these samples were 30.7%, 44.7%, and 69.8%, respectively, and were statistically significant (Table 1).

Among the 125 patients with egg-positive gallbladder stones, 55 and 80 corresponding fecal and bile samples, respectively, were egg positive. Among the 54 patients with egg-negative gallbladder stones, eggs were not found in the corresponding fecal or bile samples. The coincidence of egg detection in fecal and gallbladder bile samples, and 125 gallbladder stones were egg positive. The detection rates in these samples were 30.7%, 44.7%, and 69.8%, respectively, and were statistically significant (Table 1).
eggs in the gallbladder stones (Fig. 1b). Most of the eggs in the gallbladder stones were old; they had a thickened shell because of calcification or no operculum, or the miracidium was not visible. Eggs in the gallbladder stones adhered to or were wrapped by bilirubinate granules, mucoid matter, and/or calcium carbonate crystals (Fig. 1c). Eggs were preliminarily judged to be *C. sinensis* eggs according to their morphological characteristics.

The results of real-time fluorescent PCR showed a positive amplification curve emerging in the DNA of the positive control and the egg-positive fecal, bile, and gallbladder stone samples and no positive amplification curve (a straight line) in the DNA of the negative control and the egg-negative fecal, bile, and gallbladder stone samples. The 30 egg-positive fecal, bile, and gallbladder stone samples were also positive by PCR, while the 30 egg-negative fecal, bile, and gallbladder stone samples were also negative by PCR. This confirmed that the eggs in the fecal, bile, and gallbladder stone samples were *C. sinensis* eggs.

**DISCUSSION**

It has been proven that bile duct stones are associated with *C. sinensis* infection, and our previous data showed that *C. sinensis* infection was involved in the formation of gallbladder stones (30, 31). Thus, improving the detection rates of *C. sinensis* infection may be important in the treatment of and postoperative recovery from gallstones (17, 25). Detection of eggs is the main method of diagnosis of *C. sinensis* infection. In the present study, we examined three kinds of specimens, feces, gallbladder bile, and gallbladder stones. The direct smear method, a routine method for clinical examination, was used for fecal samples. Although it was reported that concentration techniques were more sensitive than direct examination for feces, they were still less sensitive than bile examination (32), and our previous data also indicated that concentration techniques for feces were less sensitive than bile examination (data not shown). Furthermore, the procedures for concentration techniques were time-consuming and labor-intensive and might not be acceptable for processing large numbers of specimens. Therefore, direct examination of feces was used in this study. However, using direct examination instead of a concentration technique for feces is a shortcoming of this study. The data showed that egg detection rates were the lowest in feces and the highest in gallbladder stones. Because of the subjectivity of morphological

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**FIG 1** *C. sinensis* eggs in different specimens. (a) *C. sinensis* eggs in feces. Most of the eggs in the feces were fresh with a classic urn-like shape and opercular shoulders, a small operculum on the front end, a visible abopercular knob on the posterior end, and a miracidium inside (a1 and a2). The eggs rarely adhered to bilirubinate granules and/or mucoid matter (a2). (b) *C. sinensis* eggs in bile. Most of the eggs in bile sediments were relatively old; some eggs often adhered to bilirubinate granules (b1), and some eggs adhered to or were wrapped with bilirubinate granules, mucoid matter, and/or calcium carbonate crystals (b2). (c) *C. sinensis* eggs in gallbladder stones. Most of the eggs in the gallbladder stones were old; they had a thickened shell or no operculum, or the miracidium was not visible. Some eggs adhered to or were wrapped by bilirubinate granules (c1), and some eggs adhered to bilirubinate granules and/or calcium carbonate crystals (c2).
assessment, we adopted real-time fluorescent PCR to detect the DNA in the specimens and confirmed that the eggs in the specimens were *C. sinensis* eggs (33, 34). A possible reason that the egg detection rate was the highest in gallbladder stones is that gallbladder stone formation is a continuous process, and oviposition of adult worms is an intermittent process. Eggs eliminated into bile are apt to aggregate together and adhere to or be wrapped by mucus, sloughed tissue, and bilirubinate granules, becoming the core of stones. The process is circular, which leads to the accumulation of large amounts of eggs from nucleation to the consolidation, and promotes stone formation.

The adult parasitizes the human hepatic bile duct (35), and the oviposition of the adult worm is an intermittent process. Most of the eggs ovulated by the adult enter the gallbladder along with the bile and are not easy to eliminate because of hindrance by the valves of Heister. A small portion of the eggs go directly into the intestine and are eliminated with feces; therefore, most of the eggs in the feces are from the hepatic bile duct, explaining the fact that in our study, they were single and fresh with a typical appearance and rarely adhered to bilirubinate granules or mucoid matter. This also explains why the detection rate of eggs in the feces was lower than that in the bile. Perhaps because of stimulation by the eggs, the gallbladder secretes much more mucus than usual (36, 37), and eggs adhere to or become wrapped with mucus and/or bilirubinate granules. They also may deposit and aggregate together more easily than they enter into the intestinal tract. In the present study, most of the eggs in the gallbladder bile were aggregated together and adhered to or became wrapped with mucoid matter or bilirubinate granules, which further promoted stone formation. Because stone formation is a long process, eggs exist within gallbladder stones for a long time. Because of nutritional deficiency, they age and become dehydrated and calcified, which may have led to the thickening of the shell; the loss of operculum and miracidium was not visible in this study. Everything discussed above further confirms that eggs were involved in the formation of gallbladder stones.

The comparative analysis of *C. sinensis* eggs in three types of specimens provides a new line of thought for research on the genesis of gallbladder stones. The microscopic examination of gallstones was highly sensitive. Although patients were in the advanced stages of clonorchiasis, as evidenced by the presence of gallbladder stones, which are a detriment to the early discovery and treatment of the clonorchiasis, more severe damage to the liver may be prevented. Moreover, this method revealed another etiological factor, *C. sinensis* eggs, in the formation of gallbladder stones. Furthermore, this research reminds us that postoperative analysis of gallstones may be important for the diagnosis of *C. sinensis* infection and for the prevention of gallstones as well as cholangitis, cholangiohepatitis, liver cirrhosis, and even cholangiocarcinoma (9–16). Our results not only add new content to research on the etiology of gallstones but also provide information for determining individualized treatments, such as anti-*C. sinensis* treatment with praziquantel and changes in the eating habits of patients with cholecystolithiasis and *C. sinensis* infection. Furthermore, our method promotes healing and prevents recurrence in thousands of patients who undergo gallbladder-preserving cholecystectomy (38–40).

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