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.

**Clonorchis**, 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. In China, clonorchiasis prevails mainly in Guangdong, Guangxi, and Heilongjiang provinces. The main reason for this is the consumption of raw fish and crustaceans. 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. 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 CHAO 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 CHAO gallbladder sludge-like stone-absorbing box (patent no. ZL 201110167069.9X) (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 or 3,000 rpm 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 remove 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.

(ii) 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'-GGTTGTGATGATTAGTCACA TTTCG-3') and Cs-R (5'-ACCCACCTACCCAGACAAC-3'), amplify a 121-bp fragment of the COI sequence. The minor-groove-binding TaqMan probe Cs-P (5'-JOE-ACAAAAATGCACAAAGCCCGCC- BHQ1-3' [JOE, 6-carboxy-4-dichloro-2-dimethylfluorescein; BHQ1, black hole quencher 1]) 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 analyzed using the chi-square test, and the partitions of the chi-square method were used for multiple comparisons with SPSS v.11.5 software (SPSS, Inc., Chicago, IL). A P value of <0.05 was regarded as statistically significant.

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 stone samples was 60.9% ([55 + 54]/179), and that in bile sediment and gallbladder stone samples was 74.9% ([80 + 54]/179).

Most of the eggs in the feces were single and “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; rarely, the eggs adhered to bilirubinate granules and/or mucoid matter (Fig. 1a). Most of the eggs in the bile sediments were relatively “old,” were aggregated together, and adhered to or were wrapped by bilirubinate granules and/or mucoid matter; a small portion of them were single and fresh, and their morphology was between that of the eggs in the feces and the

### TABLE 1 Comparison of the detection rates of C. sinensis eggs in the three specimen typesa

<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>0.006d</td>
</tr>
<tr>
<td>Gallbladder bile</td>
<td>80</td>
<td>99</td>
<td>44.7</td>
<td>0.006d</td>
</tr>
<tr>
<td>Gallbladder stones</td>
<td>125</td>
<td>54</td>
<td>69.8</td>
<td>0.006d</td>
</tr>
</tbody>
</table>

aThe total number of the three sample types tested was 179.
bComparison of feces and gallbladder bile.
cComparison of gallbladder bile and gallbladder stones.
dComparison of feces and gallbladder stones.
eggs in the gallbladder stones (Fig. 1b). 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).

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).

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 stone samples. 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
such as anti-
also provide information for determining individualized treatments, not only add new content to research on the etiology of gallstones but above further confirms that eggs were involved in the formation of and miracidium was not visible in this study. Everything discussed may have led to the thickening of the shell; the loss of operculum deficiency, they age and become dehydrated and calcified, which within gallbladder stones for a long time. Because of nutritional present study, most of the eggs in the gallbladder bile were aggre-
37), and eggs adhere to or become wrapped with mucus and/or eggs, the gallbladder secretes much more mucus than usual (36, 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, and eggs adhere to or become wrapped with mucus and/or bilirubinate granules. They also may deposit and aggregate to-
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