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A systematic study on 33 gallbladder stones resembling adult *Clonorchis sinensis* worms

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Abstract

Limited studies provide direct evidence of *Clonorchis sinensis* adults in the early stage of gallbladder stone formation. Our current research systematically studied 33 gallbladder stones resembling adult worms and shed light on the definite connection of *C. sinensis* infection with concomitant cholelithiasis. A total of 33 gallbladder stones resembling adult *C. sinensis* worms were systematically analysed. Fourier transform infrared spectroscopy, scanning electron microscopy and X-ray energy spectrometry were used to analyse the composition and microstructure. Meanwhile, a histopathological examination of the stone was carried out. The 33 gallbladder stones resembling adult *C. sinensis* worms included nine calcium carbonate ($CaCO_3$) stones, 12 bilirubinate stones and 12 mixed stones. *Clonorchis sinensis* eggs were found in 30 cases, including all $CaCO_3$ and mixed stones. Parasite tissues were detected in 12 cases, which were mainly $CaCO_3$ stones or bilirubinate–$CaCO_3$ mixed stones. The outer layer of stones was wrapped with 12.88% calcium salt, as revealed by X-ray energy spectrometry, while surprisingly, many *C. sinensis* eggs were found in the inner part of these stones. Based on our current findings, we concluded that calcification and packaging occurred after *C. sinensis* adult entrance into the gallbladder, subsequently leading to the early formation of $CaCO_3$ or bilirubinate–$CaCO_3$ mixed gallbladder stones. This discovery highlights definite evidence for *C. sinensis* infection causing gallbladder stones.

Introduction

Cholelithiasis, one of the most common causes of hospitalization for gastrointestinal disorders, affects 10–20% of the global population. Nutritional and lifestyle changes in modern life lead to an increase in the diagnosis of cholelithiasis (Lammert *et al.*, 2016; Littlefield & Lenahan, 2019; Stokes & Lammert, 2021), which places a significant burden on healthcare systems worldwide (Everhart & Ruhl, 2009; Peery *et al.*, 2015; Fairfield *et al.*, 2019).

Clonorchiasis is an important food-borne zoonosis caused by the intake of raw or undercooked freshwater fish or shrimp contaminated with living *Clonorchis sinensis* metacercariae (Qian *et al.*, 2016; Tang *et al.*, 2016). When ingested, the metacercariae excyst in the duodenum and ascend to the liver biliary tract through the ampulla of Vater. It takes several days for adult flukes to become mature when they enter the gallbladder. The life span of adult flukes can reach nearly 30 years in humans (Hong & Fang, 2012). It is estimated that one worm produces approximately 2500–3000 eggs per day by sexual reproduction (Hong & Fang, 2012). The eggs flow down to the intestine and gallbladder with bile, which may cause gastrointestinal symptoms due to mechanical stimulation and secretions and metabolites related to inflammation and intermittent obstruction of the bile ducts. In mild cases, symptoms include abdominal pain, nausea, anorexia, and diarrhoea. Severe infections can cause pyogenic cholangitis, which may progress to atrophy of the liver parenchyma and portal fibrosis (Petney *et al.*, 2013; Qian *et al.*, 2013).

Cholecystolithiasis is a common disease associated with *C. sinensis* infection. A few studies have suggested that the formation of intrahepatic bile duct stones is highly correlated with *C. sinensis* infection (Jang *et al.*, 2007; Liu *et al.*, 2019). Moreover, our previous study demonstrated that *C. sinensis* infection was a significant cause of gallbladder stones, especially calcium carbonate ($CaCO_3$) gallbladder stones (Qiao *et al.*, 2012b, 2014; Ma *et al.*, 2017). This study was consistent with another group indicating that calcified parasitic ova of *C. sinensis* might contribute to the formation of gallbladder stones (Huang *et al.*, 1994). However, these studies were based on *C. sinensis* eggs, and there is no definite straightforward evidence from *C. sinensis* adult worms to date. To investigate whether *C. sinensis* adults participate in the early stage of gallbladder stone formation, we recruited patients with cholecystolithiasis and collected stones similar to adult worms. We systematically analysed these stones and confirmed that adult *C. sinensis* tissues were present in some of the stones resembling adult worms characterized by calcium salts wrapped around *C. sinensis* eggs, indicating direct calcification and packaging after *C. sinensis* adults entered the gallbladder, which led to the early formation of $CaCO_3$ or bilirubinate–$CaCO_3$ mixed gallbladder stones.

Materials and methods

Subjects and specimens

Gallbladder stones resembling adult *C. sinensis* worms were obtained from 33 cholecystolithiasis patients who received gallbladder-preserving cholelithotomies (Qiao *et al.*, 2012a) in the Department of General Surgery of The Sixth People's Hospital of Nansha, Guangzhou, China, between July 2017 and June 2019. They consisted of 24 men, ranging in age from 38 to 72 years, with a mean age of 52.8 ± 11.2 (mean ±standard deviation (*SD*)), and nine women, ranging in age from 36 to 69 years, with a mean age of 53.1 ± 11.1 (mean ± *SD*). There was no significant difference when comparing the age of male vs. female groups by an independent sample *t*-test ($P > 0.05$).

In some patients, live *C. sinensis* adult worms can be seen under endoscopy, with internal organs that can be seen vaguely. All the stones were washed twice with distilled water and then dried.

Composition analysis of gallbladder stones by Fourier transform infrared spectroscopy (FTIR)

The main components were analysed using a Bruker (TENSOR27, Germany) FTIR spectrometer. A 2 mg sample of each layer was weighed if the layered structures were distinct. In amorphous stones, 2 mg samples were weighed directly. The samples were mixed with potassium bromide at a ratio of 1:150, ground thoroughly and used to make discs. The main components were analysed using a Bruker (TENSOR27, Germany) FTIR spectrometer in the frequency range of 400–4000 cm^{-1} at 4 cm^{-1} resolution. Control substances (99% pure standard) were obtained from Sigma Chemical Company (St. Louis, MO). Gallstone composition was determined by comparison of the gallbladder stones with standard control spectra.

Microscopic analysis with scanning electron microscopy and X-ray energy spectrometry

In the present study, all gallbladder stones resembling adult *C. sinensis* worms were analysed by scanning electron microscopy (SEM). The stones were split, and 1–2 pieces (3–5 mm in size) were sampled from each layer if the layered structures were distinct. In amorphous stones, 1–2 pieces, 3–5 mm in size, were sampled and fixed on the sample table using an electroconductive adhesive. One piece was secured to the surface while the other was placed facing the opposite direction. The surface to be analysed was polished, thus making the surface and the bottom surface parallel. The samples were then dried at 40°C overnight. The dried samples were subsequently sputter-coated with gold (ETD-2000, Beijing Elaborate Technology Development, China), observed using a ZEISS (EVO LS10, Cambridge, Germany) SEM and photographed. The extra high tension was 20 kV. The microstructure was then analysed through amplification of 400, 1000, 3000, 6000, 10,000 and 20,000.

The region of interest was amplified to 100 and 3000 to analyse element composition and distribution by X-ray energy spectrometry

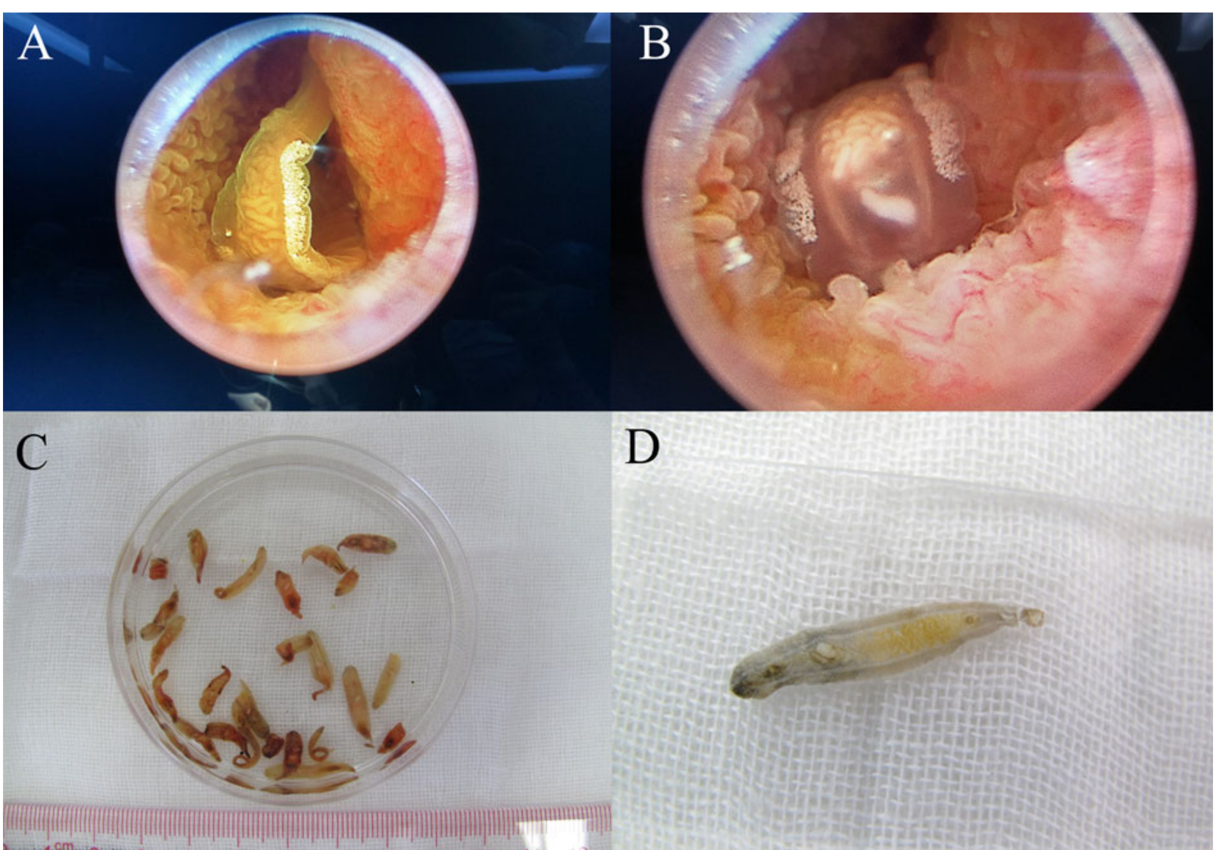

Fig. 1. Adult worms of *Clonorchis sinensis*: (A, B) adult worms under endoscopy; and (C, D) adult worms collected from infected patients.

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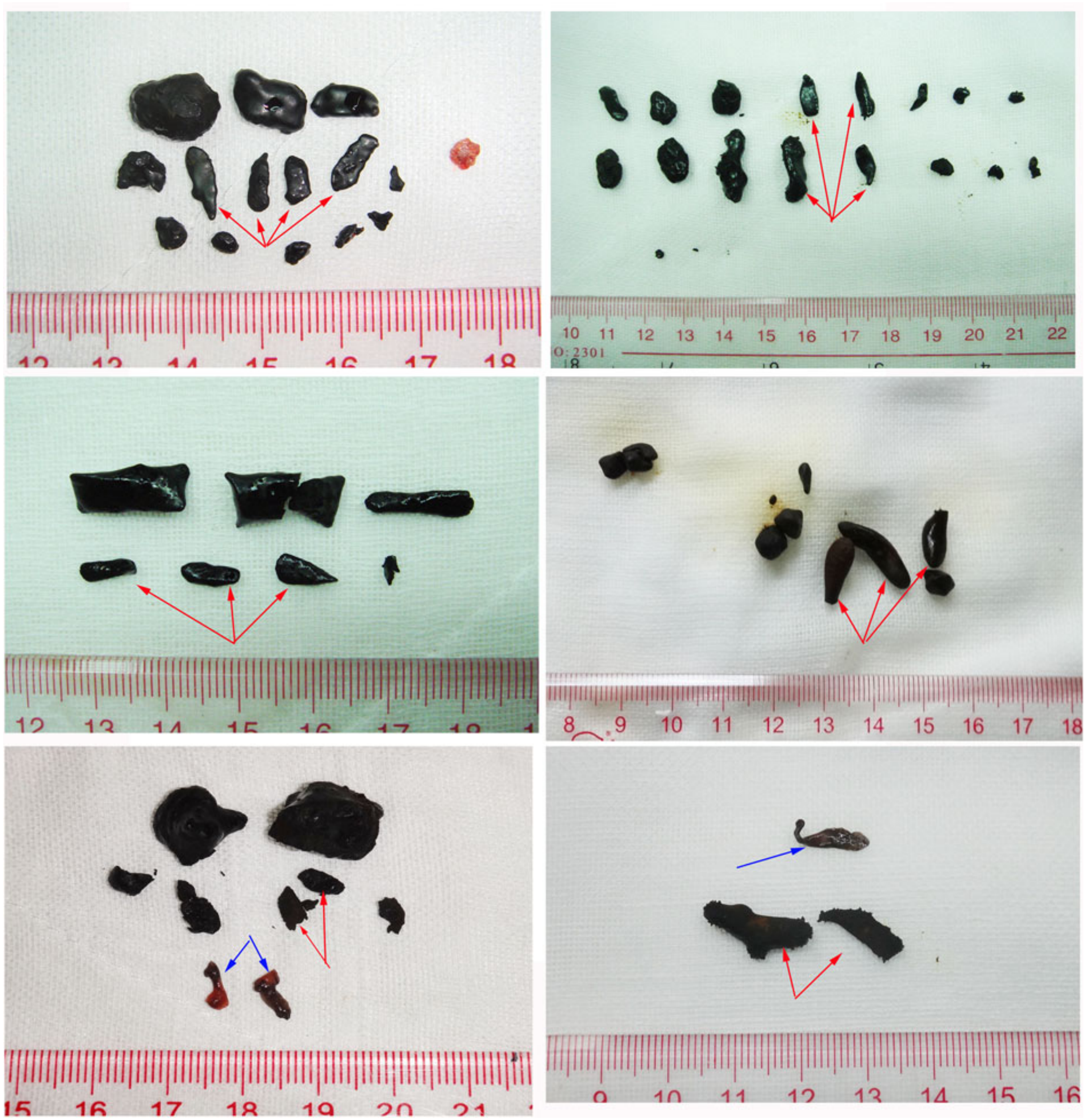

Fig. 2. *Clonorchis sinensis* adult worms and gallbladder stones resembling adult worms: red arrowhead indicates the stones resembling adult *C. sinensis* worms, and blue arrowhead indicates the adult worms collected from patients.

(X-Max, Oxford, United Kingdom). The elements from B5 to U92 can be detected only if the weight percentage was more than 0.01%.

Microscopic examination of bile sediments

Two-millilitre bile samples were centrifuged at 1450×g for 10 min. The supernatant of each sample was transferred to another clean tube for analysis of the chemical composition of the bile (data not shown), and approximately 0.5 ml of sediment was kept. The sediment was then smeared onto labelled slides and observed with a BX51 system microscope (Olympus, Japan).

Histopathological examination of gallstones

All gallbladder stones resembling adult *C. sinensis* worms were analysed by histopathological examination. Gallbladder stone samples fixed in formalin were taken to the pathology laboratory, decalcified in 10% nitric acid solution, dehydrated in gradient alcohol, embedded in paraffin and sectioned into 3- to 5-µm-thick slices (HM315, Thermo, USA). Microscopic examination was performed on all slides of the sample after haematoxylin and eosin staining (Bessot, China). We used an Olympus microscope (model BX51) equipped with a digital camera to examine the slides as well as to take pictures. To reduce bias, the microscopic examination of pathology slides was performed in single blinded conditions.

Statistical analysis

The experimental data analysis was executed by SPSS v.16.0 software. Ages are presented as the means ± *SDs*. The detection rate

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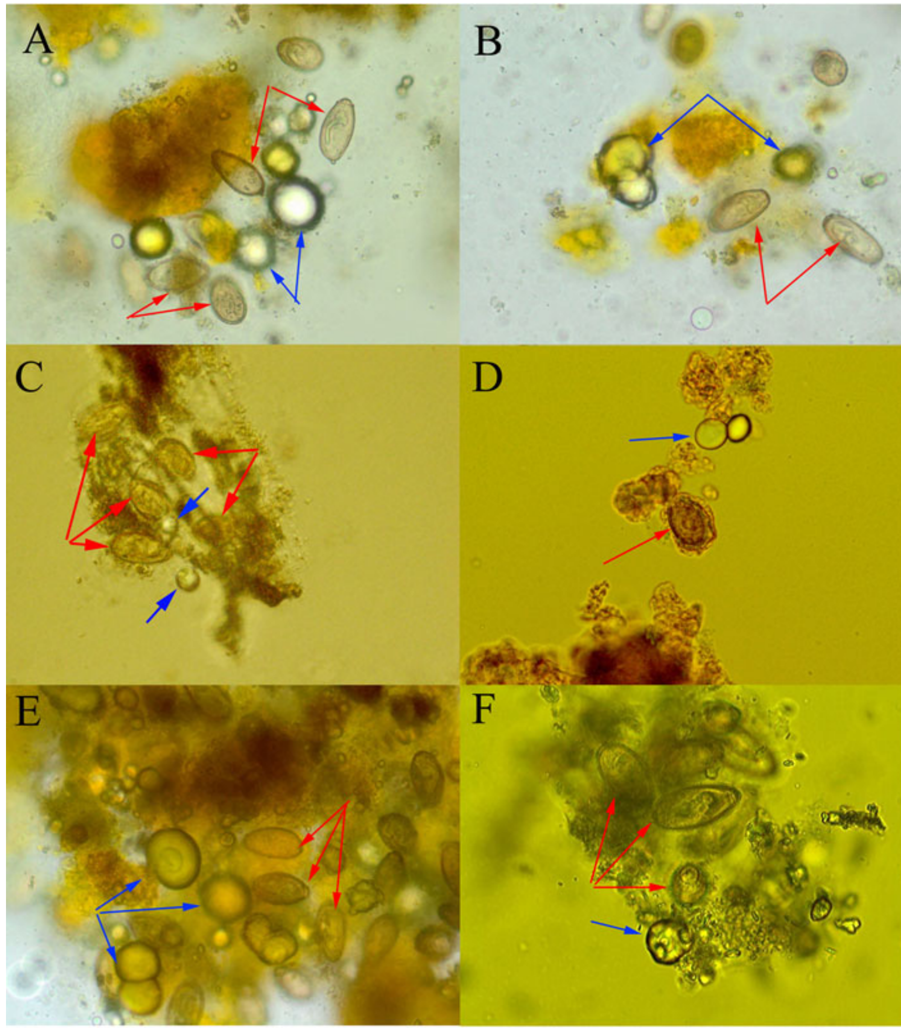

Fig. 3. *Clonorchis sinensis* eggs in the bile sediment: blue arrowhead indicates the calcium carbonate ($CaCO_3$) crystals; and red arrowhead indicates the *C. sinensis* eggs. Most of the eggs have a complete structure with a size of (26–33) µm × (15–17) µm, with a classic sesame-like shape and opercular shoulders, a small operculum on the front end, a visible abopercular knob on the posterior end and a miracidium inside. *Clonorchis sinensis* eggs were adhered and packaged with $CaCO_3$ crystals and bilirubinate particles.

was analysed using a Chi-square test. $P < 0.05$ was regarded as statistically significant.

Results

Thirty-three gallbladder stones resembling adult C. sinensis *worms were obtained*

Clonorchis sinensis adults were obtained from patients, and the adult worms and internal organs could be clearly seen under endoscopy (fig. 1A, B). The size of the fresh worm is approximately 10–25 mm × 3–5 mm, and organs such as mouth suckers, abdominal suckers, ovaries, testes, seminal vesicles, etc., were visible to the naked eye (fig. 1C, D). All 33 gallbladder stones were black melon seed-like in appearance, with a similar shape and size to *C. sinensis* adult worms (fig. 2). Some of the stones had a hard texture, and others had a brittle texture. There was no obvious layered structure in the section of the stones.

C. sinensis eggs were found in the corresponding bile sediment

Mostly, the bile was dark yellow and clear with low viscosity. Microscopic examination revealed that 28 of the 33 cases of bile sediments were positive for *C. sinensis* eggs, which were adhered and packaged with $CaCO_3$ crystals and bilirubinate particles. Most of the eggs had a complete structure with a size of (26–33) µm × (15–17) µm, plus a classic sesame-like shape and opercular shoulders, a small operculum on the front end, a visible abopercular knob on the posterior end and a miracidium inside, as shown in fig. 3.

Gallbladder stones resembling adult C. sinensis *worms were mostly $CaCO_3$, bilirubinate and bilirubinate–$CaCO_3$ mixed stones*

Of the 33 gallbladder stones resembling adult *C. sinensis* worms, nine cases were $CaCO_3$ stones and mainly calcite, 12 cases were bilirubinate stones and 12 cases were mixed stones, including nine cases of bilirubinate–$CaCO_3$ mixed stones, two cases of bilirubinate–calcium phosphate mixed stones and one case of bilirubinate–$CaCO_3$–calcium phosphate mixed stones. The infrared spectrograms of the $CaCO_3$ and bilirubin standard control as well as $CaCO_3$ and bilirubinate–$CaCO_3$ mixed gallbladder stones are shown in fig. 4. Cholesterol and calcium stearate stones were not found in the present study. Under SEM, *C. sinensis* eggs were found in 30 of the 33 gallbladder stones resembling adult *C. sinensis* worms, including all the $CaCO_3$ and mixed stones.

Mapping analysis of element distribution in general stones using an X-ray energy spectrometer with a magnification of ×100 showed that in gallbladder stones resembling adult *C. sinensis* worms,

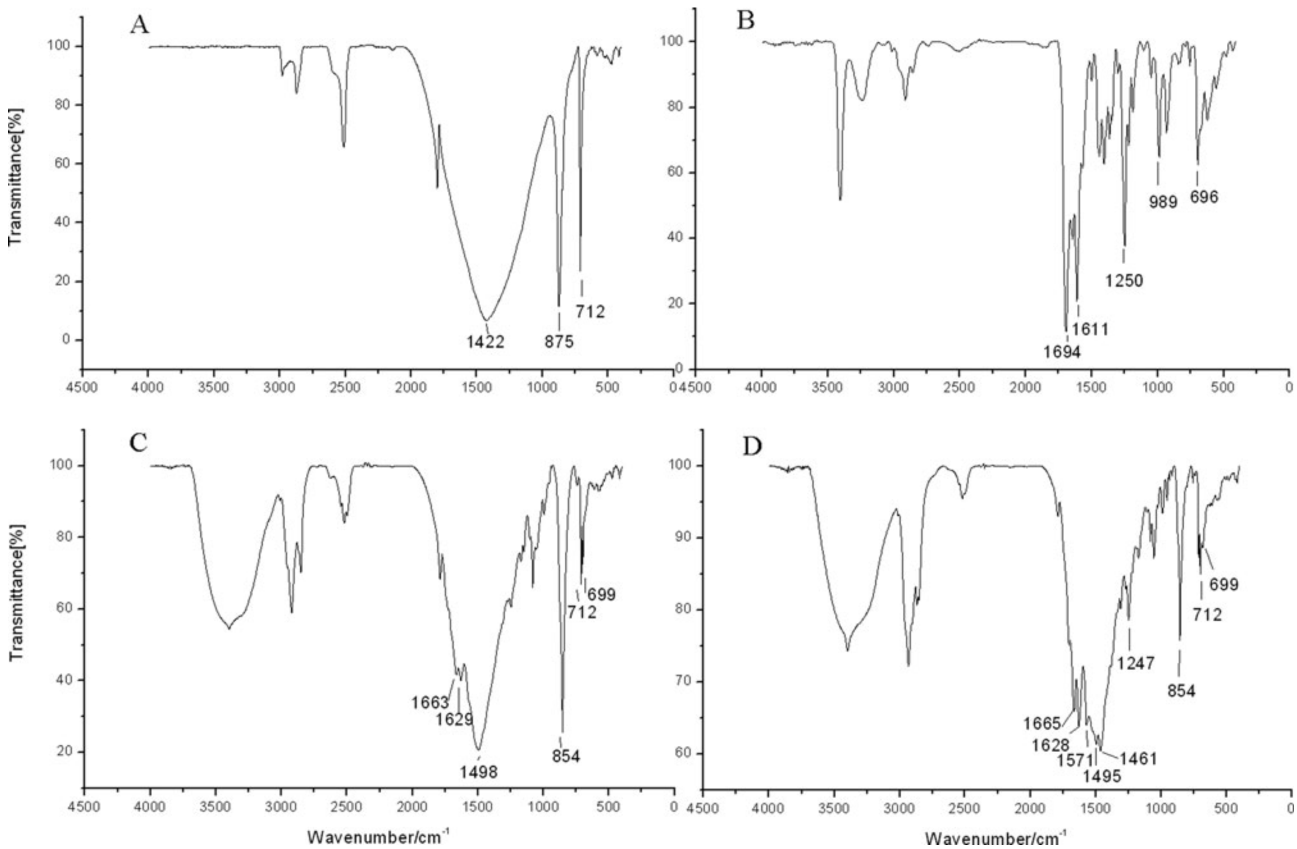

Fig. 4. Infrared spectrogram of the calcium carbonate ($CaCO_3$) and bilirubin standard control as well as $CaCO_3$ and bilirubinate–$CaCO_3$ mixed gallbladder stones: (A) infrared spectrogram of the $CaCO_3$ standard control; (B) infrared spectrogram of the bilirubin standard control; (C) infrared spectrogram of $CaCO_3$ gallbladder stones; and (D) infrared spectrogram of bilirubinate–$CaCO_3$ mixed gallbladder stones.

calcium ions, with an amount of 12.88% (table 1), were mainly distributed in the outer layer (fig. 5A). When the stones were magnified to × 400, a large number of *C. sinensis* eggs in clusters and surrounding calcified particles could be seen in some stones (fig. 6A); when magnified to × 3000, we found that *C. sinensis* eggs were packaged by mucoid matter and adhered to each other with $CaCO_3$ crystals and bilirubinate granules (fig. 6B). Mapping analysis of element distribution in the micro field showed that a large amount of calcium ions with an amount of 18.87% can be detected on the surface and surroundings of *C. sinensis* eggs, suggesting that *C. sinensis* eggs adsorbed a large amount of calcium salts, and the eggs as well as their surroundings were wrapped by calcium salt particles (fig. 5B). The elemental composition in the general stone and in the micro field is shown in table 1.

Parasite tissues were found in 12 gallbladder stones resembling adult C. sinensis *worms*

According to the histopathological examination, parasite tissues were found in 12 gallbladder stones resembling adult *C. sinensis* worms, including seven $CaCO_3$ stones, three bilirubinate–$CaCO_3$ mixed stones and two bilirubinate stones. Under the optical microscope, we found that parasite tissues were surrounded with stone particles or crystals when the slide was magnified × 40 or × 100, and a large number of *C. sinensis* eggs in clusters could be seen in the parasite tissues when the slide was magnified × 200 (fig. 7).

C. sinensis adult worms may be more likely to calcify and form $CaCO_3$ stones or bilirubinate–$CaCO_3$ mixed stones

Of the nine $CaCO_3$ gallbladder stones resembling adult *C. sinensis* worms, seven cases contained parasite tissue and three of the nine bilirubinate–$CaCO_3$ mixed gallbladder stones resembling adult *C. sinensis* worms contained parasite tissue, while only two of the 12 bilirubinate stones embraced it. The detection rate of parasite tissues was highest in $CaCO_3$ stones, followed by bilirubinate–$CaCO_3$ mixed stones and bilirubinate stones, with a statistically significant difference ($P < 0.05$, table 2). A large number of *C. sinensis* eggs in clusters were observed under optical microscopy

Table 1. Elemental composition in general stone and in the micro field.

Interest region/elements	Carbon	Oxygen	Sodium	Magnesium	Chlorine	Calcium	Copper	Manganese	Gold*
In general stones	49.36	27.7	0.72	0.14	0.19	12.88	0.58	none	8.44
In micro field	45.36	26.92	1.47	0.24	0.32	18.87	none	0.16	6.67

*Gold was from the coating layer.

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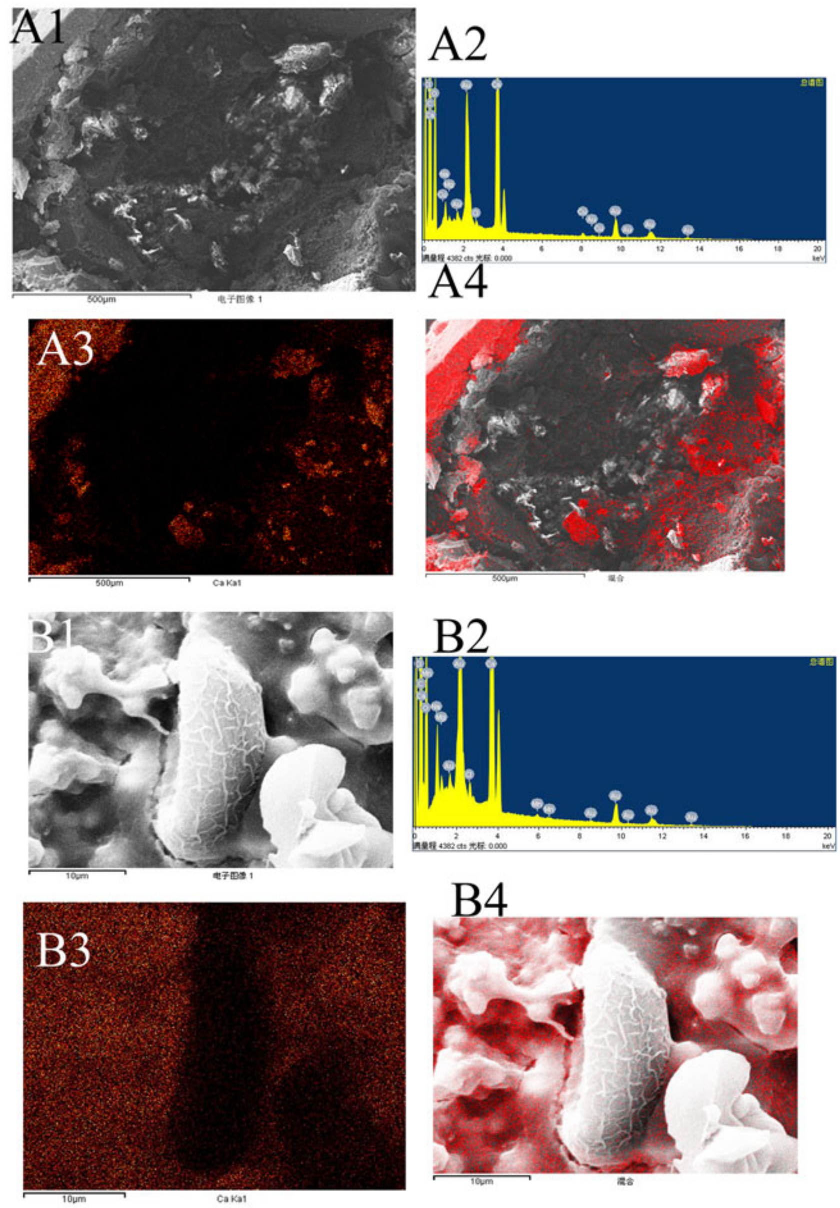

Fig. 5. Mapping analysis of element distribution in general stones and in the micro field (red dot represents calcium distribution): (A1) general stones with a magnification of × 100; (A2) energy spectrum of general stone; (A3, A4) calcium distribution in general stones, mainly in the outer layer; (B1) micro field with a magnification of × 3000; (B2) energy spectrum of the micro field; and (B3, B4) calcium distribution in the micro field, mainly in the surroundings of *Clonorchis sinensis* eggs.

and SEM. Parasite tissues were surrounded by $CaCO_3$ crystals and other particles, suggesting that *C. sinensis* adults may more easily absorb calcium salts or calcify to form $CaCO_3$ stones or bilirubin–$CaCO_3$ mixed stones.

Discussion

Gallbladder stones, as a highly prevalent disease in general populations with substantial hospital admission (Peery *et al.*, 2015; Pape *et al.*, 2019; Joshi *et al.*, 2020), were deemed to be formed in a complex process involving multiple factors (Bonfrate *et al.*, 2014; Lin *et al.*, 2014; Shabanzadeh *et al.*, 2016; Shabanzadeh, 2018; Di Ciaula *et al.*, 2019; Wang *et al.*, 2020). Analysis of the chemical components of gallstones contributes extremely great value to analyzing disease progression. They were traditionally divided into cholesterol and pigment (bilirubin) stones (Schafmayer *et al.*, 2006; Cariati, 2015); however, our previous research overturned this view, as we found that gallbladder stones could be classified into eight types and more than ten subtypes, including cholesterol stones, pigment stones, $CaCO_3$ stones, phosphate stones, calcium stearate stones, protein stones, cystine stones and mixed stones (Qiao *et al.*, 2013b). Further research implied that different types of stones may expose a distinct formation mechanism (Qiao *et al.*, 2013a, 2014; Ma *et al.*, 2017).

According to previous research, gallbladder stones, especially $CaCO_3$ and bilirubinate stones, are associated with *C. sinensis* infection, but the role of *C. sinensis* adult worms has not been clarified. In the present study, 33 gallbladder stones resembling adult *C. sinensis* worms were obtained from cholecystolithiasis patients. The morphology of these stones exhibited a shape and size similar to those of *C. sinensis* adult worms. These stones aroused our interest, and we intended to explore whether they were stones, worms or even calcified worms. Based on the clinical experience of hepatobiliary surgeons, live *C. sinensis* adult worms could enter the cystic duct or gallbladder cavity, which is a normal

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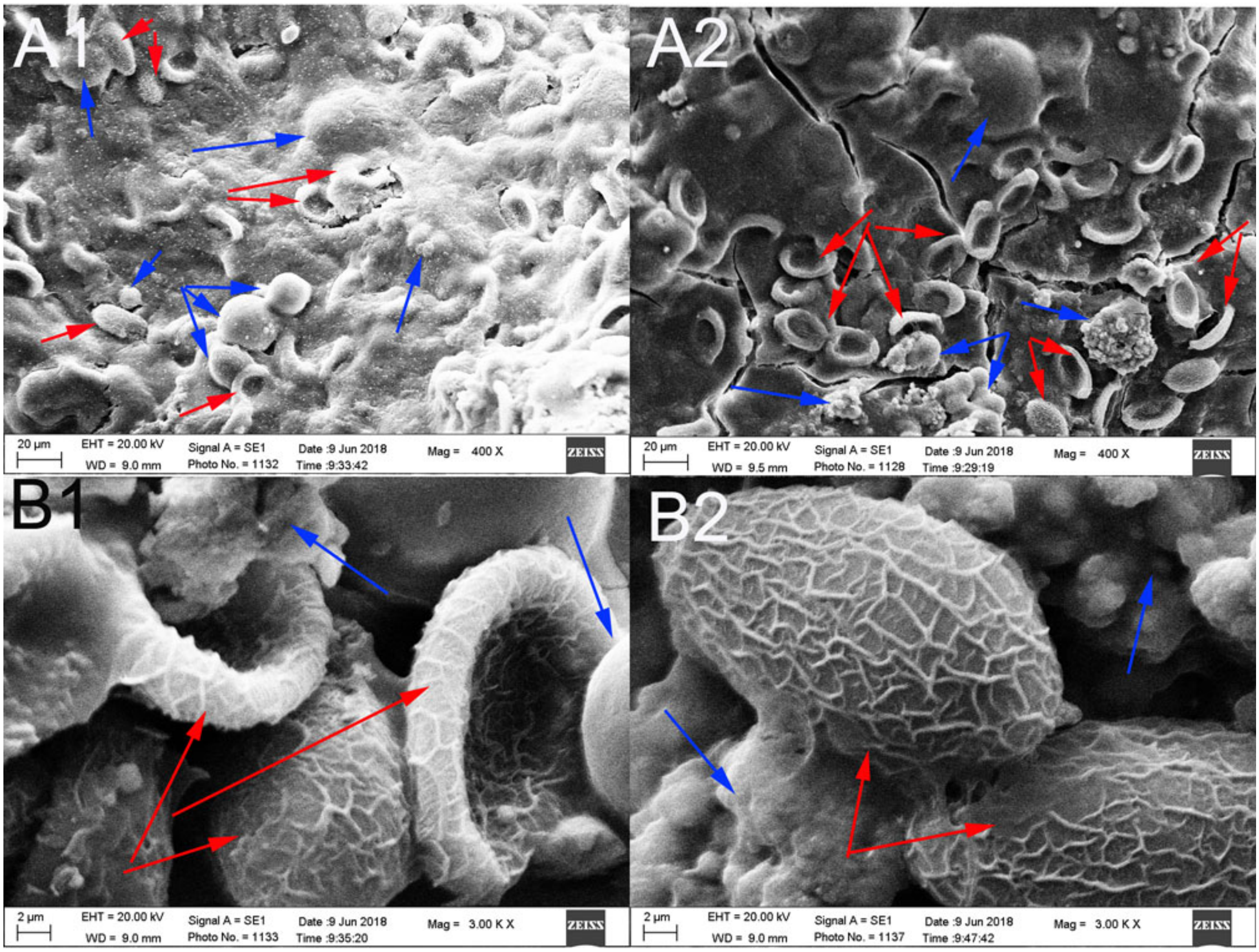

Fig. 6. *Clonorchis sinensis* eggs and calcified particles or calcium carbonate ($CaCO_3$) crystals were seen in the stones: blue arrowhead indicates the $CaCO_3$ crystals; and red arrowhead indicates the *C. sinensis* eggs. (A) *Clonorchis sinensis* eggs in clusters and surrounding calcified particles were seen (original magnification, × 400); and (B) *C. sinensis* eggs were packaged by mucoid matter or adhered to $CaCO_3$ crystals and bilirubinate granules (original magnification, ×3000).

phenomenon under endoscopy, implying that *C. sinensis* adult worms enter the gallbladder with bile.

Histopathological examination revealed parasite tissues in 12 gallbladder stones resembling *C. sinensis* adult worms, the main component of which was $CaCO_3$ and bilirubinate according to FTIR analysis. Parasite tissues surrounded with stone particles or $CaCO_3$ crystals could be seen under the optical microscope with a lower magnification, while a large number of *C. sinensis* eggs in clusters could be seen in the parasite with a higher magnification. Mapping analysis of element distribution in general stones revealed that calcium ions wrapping the core (parasite tissue) were mainly distributed in the outer layer of the stones. Furthermore, SEM revealed a large number of *C. sinensis* eggs in clusters surrounded by calcified particles. These data suggested that *C. sinensis* adults may be prone to absorb calcium salts to form $CaCO_3$ stones or bilirubinate–$CaCO_3$ mixed stones.

Tissue analysis studies showed that samples with parasite tissue are derived from early calcification of dead worms, providing strong evidence that calcification and packaging occurred after *C. sinensis* adults entered the gallbladder, which led to the formation of $CaCO_3$ or bilirubinate–$CaCO_3$ mixed gallbladder stones. On the other hand, those without parasite tissue may result from calcification of *C. sinensis* eggs or dead worms during long-term formation, while the parasite tissues may be dehydrated and further calcified and degraded due to nutritional deficiency in the stone (Qiao *et al.*, 2013c).

We have developed a sensitive and specific real-time polymerase chain reaction assay for the detection of *C. sinensis* DNA in adult worms, gallbladder bile and stone samples of patients with cholecystolithiasis (Qiao *et al.*, 2012c). Our previous research confirmed that *C. sinensis* DNA genes could be detected in all egg-positive samples and some egg-negative stones (Qiao *et al.*, 2012c, 2013c), which provides strong evidence for our current finding.

A systematic analysis of the stones demonstrated that the main components were $CaCO_3$ and bilirubinate. *Clonorchis sinensis* eggs were found in 30 of these stones, including all $CaCO_3$ and mixed stones. Moreover, *C. sinensis* eggs have also been found in the corresponding bile sediment, with a relatively fresh appearance, but the detection rate in bile sediment was lower than that in stones, which was consistent with our previous research (Qiao *et al.*, 2013c). Adult worms parasitize the hepatobiliary duct and ovulate eggs in an intermittent process, most of which enter the gallbladder along with the bile and are not easily eliminated because of hindrance by the valves of Heister. Stone formation is a long process from adherence or wrapping with mucus

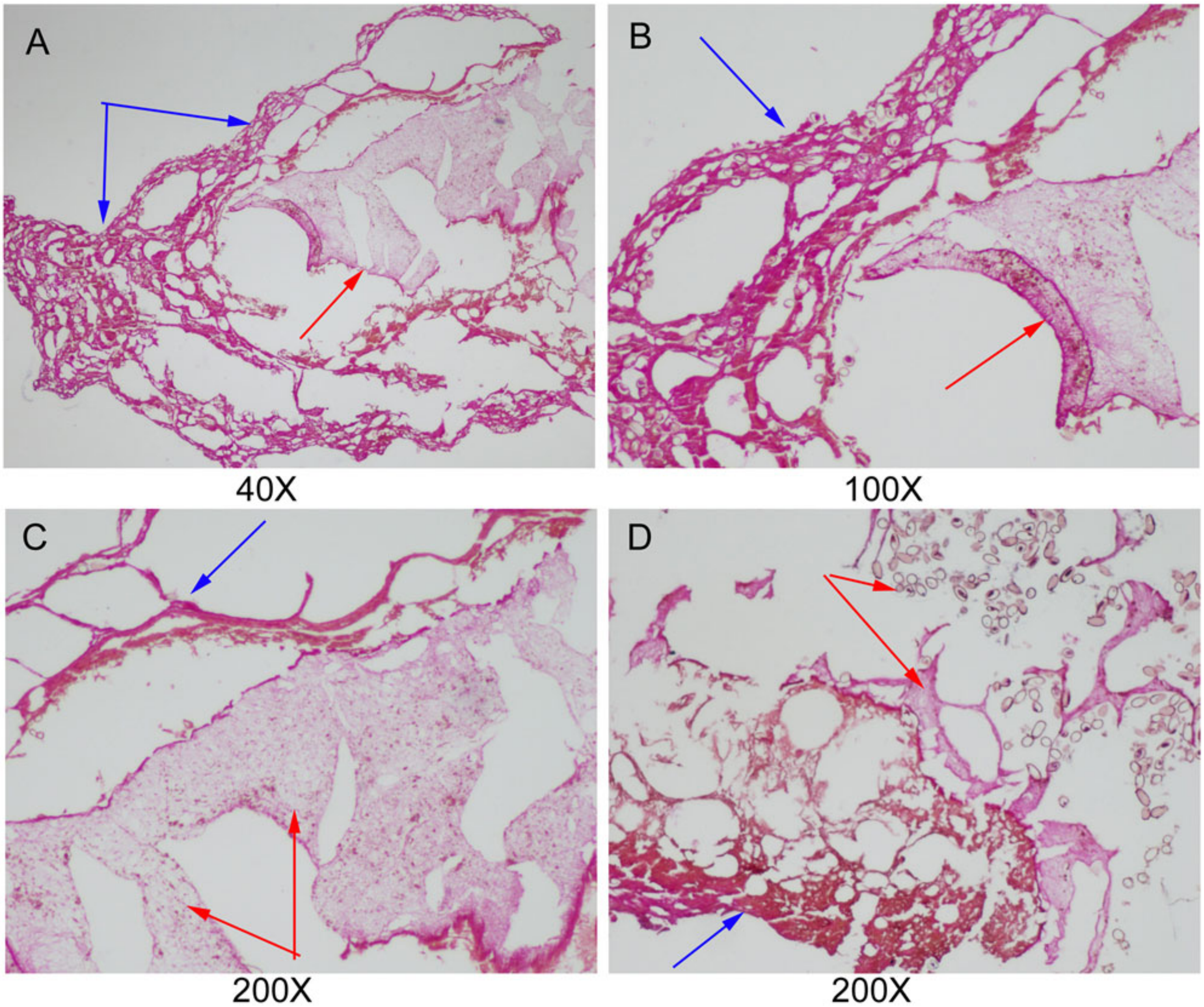

Fig. 7. Parasite tissues were found in gallbladder stones resembling adult *Clonorchis sinensis* worms: blue arrowhead indicates the stone particles or crystals; and red arrowhead indicates parasite tissues. (A–C) parasite tissues were surrounded with stone particles or crystals (original magnification, ×40×100×200); and (D) a large number of *C. sinensis* eggs in clusters could be seen in the parasite tissues (original magnification, ×200).

and granules to dehydration and calcification. Consequently, the detection rate of *C. sinensis* eggs in stones was higher than that in bile sediments; additionally, the eggs were morphologically 'older' in the stone than those found in the bile.

Further analysis demonstrated that $CaCO_3$ was mainly calcite, which was consistent with our previous research. $CaCO_3$ can form three homogeneous polycrystals in nature, including calcite, aragonite and vaterite, among which calcite and aragonite are the main types found in $CaCO_3$ gallstone (Ma *et al.*, 2017). It was supposed that *C. sinensis* eggs could stimulate mucin secretion, which contributes to the specific appearance of these stones. The released mucin and the muskmelon wrinkles on the surface of eggs easily adhere to $CaCO_3$ crystals and bilirubinate granules, which are mainly calcite $CaCO_3$ (Ma *et al.*, 2017). In addition, *C. sinensis* adult worms stimulate much more mucin metabolite production, which could absorb bilirubinate particles and calcium salts, thus leading to calcification and the formation of $CaCO_3$ stones or bilirubinate–$CaCO_3$ mixed stones morphologically similar to worms.

In general, we provided straightforward evidence here that entrance of *C. sinensis* adult worms into the gallbladder would

Table 2. The detection of parasite tissues in different types of gallbladder stones resembling adult *Clonorchis sinensis* worms.

Stone type	With parasite tissues	Without parasite tissues	Total	Detection rate (%)	*P* value
bilirubinate	2	10	12	16.7	0.022
calcium carbonate ($CaCO_3$)	7	2	9	77.8	
bilirubinate–$CaCO_3$ mixed	3	6	9	33.3	
Total	12	18	30	40.0	

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lead to the formation of stones, during which they went through aging and dead, being wrapped with stone particles or crystals enriched in calcium ions. During a long-term process of stone formation, some of the parasite tissues might be destroyed and degraded; thus, only some of the stones in the early stage of formation could be detected by histopathological examination. We further presumed that stones with diverse appearances, such as black melon seed-like or coralliform, were derived from wrapped *C. sinensis* eggs and other nucleation factors.

Conclusion

Our current data provide evidence of direct calcification and packaging occurring after the entrance of *C. sinensis* adults into the gallbladder, which leads to the formation of $CaCO_3$ or bilirubinate–$CaCO_3$ mixed gallbladder stones resembling adult worms in the early stage. This finding highlights strong evidence for *C. sinensis* infection causing gallbladder stones.

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Conflicts of interest. None.

Ethics statement. The operation and sample collection procedures were explained to all patients. Informed consent was signed by each patient. The principles outlined in the Declaration of Helsinki of 1975 (revised in 1983) were followed throughout the study period. This research was approved by the Ethics Committee of The Sixth People's Hospital of Nansha, Guangzhou, China.

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