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Formation of non-cholesterol gallstones in populations within clonorchiasis endemic regions is closely related to *Clonorchis sinensis* infection

Xiaobing Luo[1*], Ruihong Ma[1], Hongying Cai[2], Xiangyu Su[1], Sangui Wang[3*] and Tie Qiao[4*]

Abstract

Background Numerous risk factors are linked to gallbladder stone disease (GBSD). Nonetheless, the relationship between *Clonorchis sinensis* (*C. sinensis*) and this condition remains to be clarified.

Methods The antibody against *C. sinensis* in serum and the glucose, triglyceride, and cholesterol levels were investigated in 220 patients with GBSD and 251 controls. Bile components were analysed in patients with gallbladder polyps (GP, $n=18$), gallstones (GS, $n=265$), and GS combined with *C. sinensis* infection (GSI, $n=243$). Additionally, the gallbladder ejection fraction (*%E*), residual gallbladder volume (RGV) at 1 h after a fatty meal, and fasting gallbladder volume (FGV) were compared among the GP ($n=43$), GS ($n=311$), and GSI ($n=277$) groups.

Results The results indicated positive antibody against *C. sinensis* (OR: 1.759, 95% CI: 1.163–2.662) and hyperglycaemia (glucose concentration ≥ 6.10 mmol/L, OR: 2.263, 95% CI: 1.227–4.172) were risk factors for GBSD. There were more non-cholesterol stones in GSI patients (216/241, 89.6%) than in GS patients (137/281, 48.8%) ($P<0.0001$). Microscopic observations revealed that mucus containing glycogen coated the *C. sinensis* eggs and the proportion of dead eggs gradually increased in bile, sediment, and stones alongside rising calcium salt content. Total bile acid and cholesterol concentrations were lower in GSI patients than in GP patients or GS patients ($P<0.05$). Moreover, increased FGV and RGV and decreased *%E* were observed in GSI patients compared with GP patients ($P<0.001$).

Conclusions The formation of non-cholesterol gallstones in populations residing in endemic areas is related to the deposition, death, and calcification of eggs in the gallbladder, changes in bile components, and decreased gallbladder motility caused by *C. sinensis* infection.

Keywords Gallbladder stone disease, *Clonorchis sinensis*, Odds ratio, Bile component, Gallbladder motility

*Correspondence:
Xiaobing Luo
luoxb1976@163.com
Sangui Wang
Wsgwkys@163.com
Tie Qiao
fqj1958@163.com

Full list of author information is available at the end of the article

Background

Gallbladder stone disease (GBSD) is prevalent worldwide. Studies have shown that Age, female sex, obesity, diabetes mellitus, and metabolic abnormalities are risk factors for gallbladder cholesterol stones [1–6]. However, an increasing amount of research has discovered a significant relationship between the condition of the gut or biliary microbiome and the formation of non-cholesterol gallstones [7–11]. According to related research, changes in the composition of the intestinal or biliary microbiome may result in the dysregulation of bile acid metabolism, an increase in the mucus or glycoproteins secreted from the gallbladder, difficulty emptying the gallbladder, and direct participation of microorganisms in the nucleation of stones, eventually leading to gallstone formation [12–16]. It is apparent that the formation process of gallbladder stones varies greatly depending on their composition. Based on our previous studies, gallbladder stones, especially non-cholesterol stones such as pigment stones and calcium carbonate stones, are associated with *Clonorchis sinensis* (*C. sinensis*) infection [17–19]. However, it is unknown whether there is a pathogenic mechanism similar to that of biliary bacteria that causes stone formation, or whether there is an epidemiologic correlation between *C. sinensis* infection and GBSD. The consumption of freshwater fish and shrimp containing *C. sinensis* cysts, either raw or semi-raw, is the primary mechanism by which humans develop clonorchiasis, a zoonotic parasitosis caused by *C. sinensis* parasites in the liver and bile ducts. According to previous reports, this disease affects 15–20 million people globally, primarily in East and Southeast Asian nations, including China, Vietnam, and South Korea. Guangdong Province is one of the main endemic areas in China [20–22]. According to previous studies, adult *C. sinensis* worms can cause damage, create localised inflammation, and cause fibrosis in the human biliary tract. This can result in cholecystitis, cholangitis, bile duct stones, hepatocellular carcinoma or biliary epithelial carcinoma [23–27]. However, the effects of *C. sinensis* infection on bile composition and gallbladder function remain unclear. Thus, this study compared bile component concentration and gallbladder emptying function in patients with and without *C. sinensis* infection, investigated the epidemiological relationship between GBSD and *C. sinensis* infection, and observed the morphology of *C. sinensis* eggs in various samples.

Methods

Participants and specimens

All patients underwent choledochoscopic gallbladder-preserving cholecystolithotomy/cholecystectomy in our general surgery department between June 2016 and August 2023. Patients with symptomatic gallbladder polyps and/or stones met the inclusion criteria. Individuals with malignant gallbladder tumours, combined obstructive jaundice (e.g. intrahepatic choledocholithiasis and choledocholithiasis), an acute gallbladder inflammatory phase, and several underlying disorders not amenable to surgery were excluded. Based on the objective of the study and the completeness and comparability of the clinical data gathered, the patients were divided into five groups for epidemiological investigation, morphological observation, and comparison of bile composition, gallbladder emptying function, and gallstone components. The distribution of each patient group will be discussed separately later and can be referred to in the technical roadmap (Fig. 1).

C. sinensis eggs in the bile and/or stones were observed microscopically to confirm *C. sinensis* infection. However, when conducting risk factor analysis for patients with GBSD, serum antibodies against *C. sinensis* were used to indirectly diagnose *C. sinensis* infections because of the unavailability of bile and stool samples from the control group population.

Participants for the investigation of risk factors for GBSD

The study population comprised two groups: a control group and a patient group with GBSD. Both groups of participants included local permanent residents or residents who had lived locally for more than three years from January 2022 to December 2022. The control group consisted of 251 individuals who underwent medical examinations, all of whom were civil servants employed by the local government. The GBSD group consisted of 220 patients with GBSD who were hospitalised in the Department of General Surgery at our hospital. Participants diagnosed with cholecystitis, intrahepatic bile duct stones, common bile duct stones, gallbladder stones, and/or polyps during medical examination were excluded from the control group. The distribution of the participants by age and sex in the two groups is shown in Table 1.

Fasting venous blood was collected from both groups and used to detect IgG antibodies to *C. sinensis* as well as serum glucose, triglycerides, and cholesterol. If multiple identical results were obtained for the same individual, only one was adopted.

Participants for the morphological observation of *C. sinensis* eggs

Between July and November 2019, bile, bile sediment, and stone specimens were obtained from 30 patients with GBSD. For microscopic examination of eggs, ten specimens each of gallbladder bile, bile sand-like sediments, and gallstones that tested positive for *C. sinensis* eggs were selected.

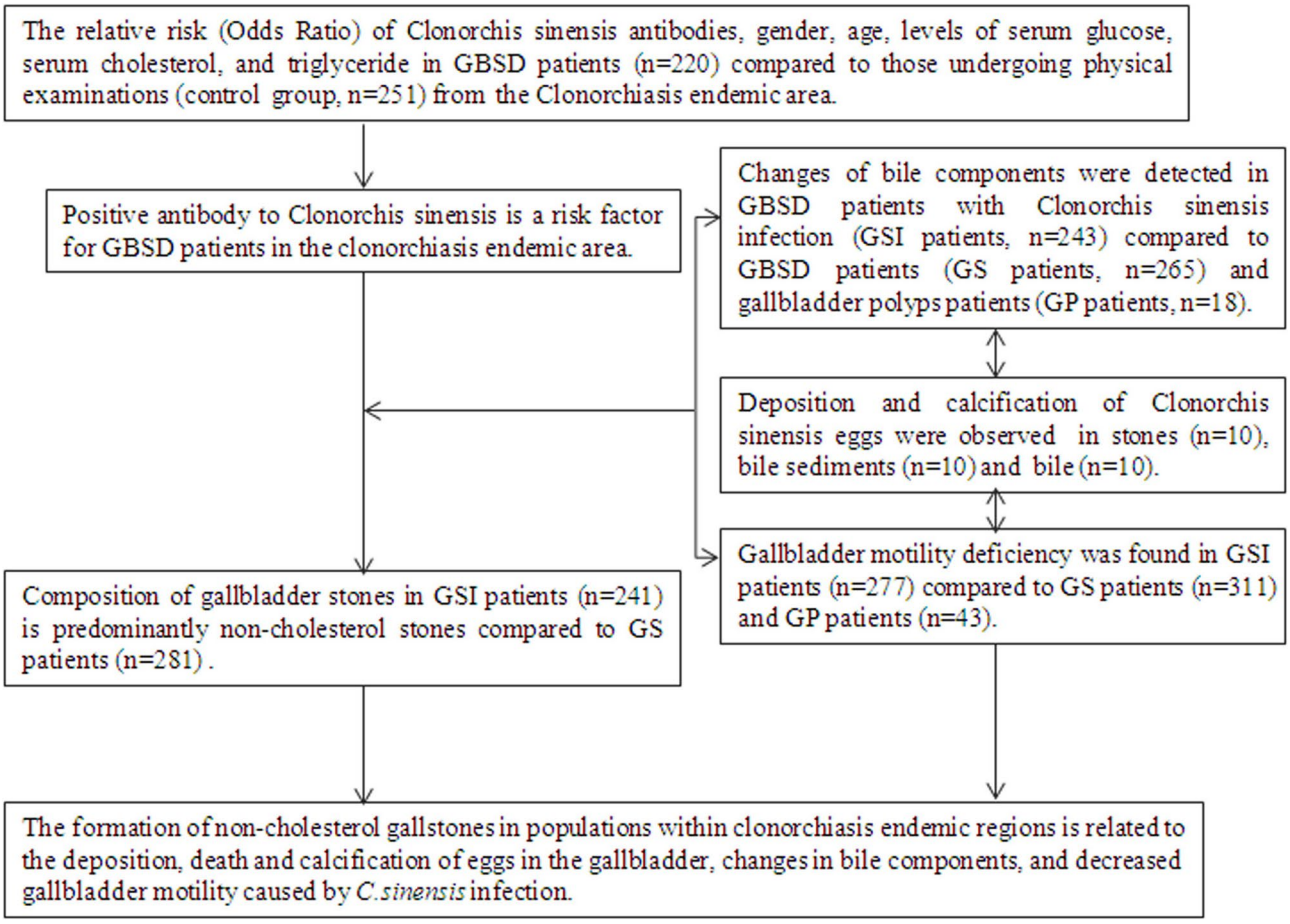

Fig. 1 Study design and analysis idea guide

Table 1 Distribution of the healthy controls and patients with GBSD by age and sex (n)

Age(Years)	GBSD patients			Healthy controls			*P* value
	Male	Female	Total	Male	Female	Total	
~29	2	9	**11**	10	5	**15**	0.014*
30~39	16	11	**27**	21	12	**33**	0.729
40~49	37	24	**61**	42	51	**93**	0.060
50~59	46	26	**72**	39	41	**80**	0.061
60~	35	14	**49**	13	17	**30**	0.013*
Total	**136**	**84**	**220**	**125**	**126**	**251**	**0.009***

Note: * $P < 0.05$ is indicative of a statistically significant difference in the gender distribution ratio of GBSD patients compared to healthy controls across corresponding age groups

Participants for determining the concentration of bile components

Based on the specimen acquisition and completeness of data, the bile components of 526 consecutive patients during the period from March 2017 to March 2019 were retrospectively analyzed, Of these, 18 patients (ten males and eight females, ages 24–58, with a median age of 39) had gallbladder polyps without *C. sinensis* infection (GP); 265 patients (91 males and 174 females, ages 17–80, with a median age of 43) had gallbladder stones (GS); and 243 patients (157 males and 86 females, ages 20–74, with a median age of 48) had gallbladder stones combined with *C. sinensis* infection (GSI).

Participants for the evaluation of gallbladder emptying function

A total of 631 patients were enrolled from June 2016 to December 2019, and their gallbladder emptying function was retrospectively compared under three different conditions. There were 43 GP patients (19 males and 24 females aged 17–60 years, with a median age of 38 years), 311 GS patients (164 males and 147 females aged

10–80 years, with a median age of 44 years), and 277 GSI patients (151 males and 126 females aged 11–78 years, with a median age of 48 years).

Participants for the analysis of gallstone components

A total of 522 patients were enrolled from June 2020 to December 2022, and their gallbladder stones were collected and analysed for stone components. The study population consisted of 281 GS patients and 241 GSI patients. The distribution of patients by age and sex in the two groups is detailed in Table 2.

Specimen collection

A 3–5 mL fasting venous blood sample was drawn 48 h before surgery and sent for testing. Gallbladder bile and stones were aseptically obtained during surgery. Bile specimens were placed in sterile tubes containing 1% heparin and sent for testing or temporarily stored at 4 °C for no more than 6 h if immediate testing was not possible. Stone specimens were placed in sterile dry containers for testing. The blood was tested within 6 h after delivery, bile was tested within 24 h, and the remaining specimens were stored in the refrigerator at -80℃; Stones were tested within 72 h, and excess stones were placed in clean and dry glass containers, and stored under ventilated conditions for a long time.

Instruments and methods

Study design

This study involved five participant groups and a complex technical approach, including epidemiologic investigations, clinical specimen testing, and imaging assays, for which an experimental idea guide was provided (Fig. 1) to facilitate a better understanding of the content.

Assays for blood samples

Serum glucose, triglyceride, and cholesterol levels were determined using commercially available kits (Shenzhen Mindray Biotechnology Company, China) as follows: glucose (glucokinase-colorimetric assay), triglycerides (phosphoglycerol oxidase-colorimetric assay), and cholesterol (cholesterol oxidase [COD-PAP] assay). A BS-2000 automated biochemistry analyser (Mindray, China) was used. Serum *C. sinensis* IgG antibody detection reagents were provided by Shenzhen Huakang Biomedical Engineering Co., Ltd., and enzyme-linked immunosorbent assays (ELISA) were used.

Examination of positive *C. sinensis* egg specimens under a microscope

Observation of eggs under an optical microscope

Specimen preparation For analysis, 3–5 mL of bile was centrifuged for 10 min at 380×*g*, and the upper layer of bile (reserved for the determination of bile components) was aspirated. Approximately 400 μL of the centrifugal precipitate was retained and mixed via sufficient shaking to make it usable. If any sludge or sand-like precipitate was found in the process described above, it was kept as gallbladder sediment for subsequent use. The stones for use were first cut into pieces around the size of a soybean (or a whole grain if they were not big enough), crushed and evenly ground in an agate mortar, and then placed into a sterile test tube with 400 μL of physiological saline to create the suspension of stone particles. All bile precipitates, bile sediments, and suspensions containing gallbladder stones obtained in the above steps were smeared on three slides measuring 26 × 76 mm, with an area spanning nearly half of each slide. An optical microscope was used for observations. Between July and November 2019, all smears of bile, bile sediment, and stone specimens containing *C. sinensis* eggs were tagged, dried naturally, and preserved for subsequent use.

Specimen staining From smears containing *C. sinensis* eggs kept in the sample preparation stage mentioned above, 30 smears (10 pieces of each of bile centrifugal precipitate, bile sediment, and stones) from 30 patients with GBSD were selected and subsequently dried at 37 °C for 8 h. The smears were fixed using gradient dehydration with 50%, 70%, and 95% anhydrous ethanol for 3 min each. They were then dried at 37 °C for 2 h for subsequent use. Finally, smears from the three specimen types were equally divided into two groups and hydrated for 1 min

Table 2 Distribution of GS patients and GSI patients for the analysis of gallstone components (n)

Age(Years)	GS patients			GSI patients			*P* value
	Male	Female	Total	Male	Female	Total	
~29	15	16	**31**	5	6	**11**	0.867
30~39	23	50	**73**	28	15	**43**	< 0.001*
40~49	25	43	**68**	58	18	**76**	< 0.001*
50~59	39	33	**72**	35	21	**56**	0.344
60~	13	24	**37**	30	25	**55**	0.067
Total	**115**	**166**	**281**	**156**	**85**	**241**	**< 0.001***

Note: * $P < 0.05$ is indicative of a statistically significant difference in the gender distribution ratio of GS patients compared to GSI patients across corresponding age groups

before staining. The staining solution was obtained from Guangdong Zhuhai Baso Bio-Technology Co., Ltd. Periodic acid-Schiff staining [28] and von Kossa staining [29] were performed according to the manufacturer's instructions and references, respectively. After staining, the slides were clarified with xylene, sealed with neutral gum, and examined under a microscope before being tagged.

For trypan blue staining [30], 10 specimens each of bile centrifugal precipitate, bile sediment, and a suspension of stone particles that were positive for eggs during the sample preparation stage were chosen for staining. Subsequently, 100 μL of these aforementioned specimens was taken and placed in a clean test tube. Next, 100 μL of 4% trypan blue staining solution (Sigma, USA) was added, mixed well, allowed to stand for 3–5 min, blowed, and mixed again. The mixture was then aspirated and smeared onto a clean slide. A cover slip was placed on the slide, and a microscopic examination was performed to document and observe the staining of the larvae inside the eggs.

Optical microscope observation

All smears and stained samples were examined under a light microscope (BX51, Olympus Corporation, Japan), and an attached microcamera system (DP-25, Olympus Corporation, Japan) was used to capture images of the areas of interest. Each smear was examined under high magnification in at least 50 fields of view.

Observation of eggs using a scanning electron microscope (SEM)

Three types of samples, totalling 15 (5 of each), containing *C. sinensis* eggs were observed. The stones were thoroughly cleaned, and bile precipitates and sediments were prepared according to the sample preparation procedure. After drying for 8 h, a conductive adhesive was placed on the sample stage for all three specimens, and gold was sprayed onto them. The SEM (LIS10, *ZEISS*, Germany) was utilised for observation, and an electron gun accelerating voltage of 20 kV was used.

Detection of bile component concentration

Before the experiment, all bile samples were pretreated using an approach developed in our laboratory to minimise bile pigment interference. An automatic biochemical analyser (BS-800, Shenzhen Mindray Medical Bioelectronics Co., Ltd.) was used to determine the following gallbladder bile components: potassium (K^+), sodium (Na^+), chloride (Cl^-), calcium (Ca^{2+}), carbon dioxide (CO_2), total bilirubin (TBIL), total bile acids (TBA), and cholesterol. Commercial kits from Shenzhen Mindray Biotechnology Co., Ltd. were used to measure Ca^{2+}, TBIL, cholesterol, CO_2, and TBA. Meanwhile, the ion-selective electrode method was used to determine K^+, Na^+, and Cl^-. A specialised pH assessor (Thermo Fisher Scientific) was used to determine the pH value.

Determination of gallbladder emptying function

Each patient completed the test within 24 h before surgery. Three indices were employed to evaluate gallbladder emptying—namely, the fasting gallbladder volume (FGV), residual gallbladder volume (RGV) at 1 h after a fatty meal, and relative gallbladder emptying fraction (*%E*) [31]. The ultrasonic inspection apparatus was a colour ultrasound diagnostic device, the Aloka-α7 (Japan) or PHILIPS M2540A (USA), with a probe frequency of 3.5–5 MHz. It was performed by two of our hospital's permanent physicians specialising in ultrasonic examination of the liver and gallbladder. The participants were placed in the supine position after fasting for >8 h. The maximum longitudinal section of the gallbladder was first displayed along its long axis and its maximum longitudinal diameter (L) was measured. Next, the probe was rotated 90 °to display the gallbladder's maximum cross-sectional area, and its maximum width (W) and height (H) were measured to determine the gallbladder volume using Formula 1. Subsequently, the individuals had a high-fat breakfast (two fried eggs), which was finished within 5 min. At 60 min after each meal, the maximum dimensions of the gallbladder, including length, width, and height, were measured. The gallbladder volume and ejection fraction were computed. The gallbladder ejection fraction was computed using Formula 2:

$$\text{Gallbladder volume (V)} = \frac{\pi}{6} \times (\text{L} \times \text{W} \times \text{H}) \quad (1)$$

V:gallbladder volume(mLor cm^3); L:gallbladder length(cm);

W: gallbladder width(cm); H:gallbladder height(cm)

$$\text{Ejection fraction}(\%\text{E}) = \frac{(\text{Vo} - \text{Vt})}{\text{Vo}} \times 100\% \quad (2)$$

%E:gallbladder emptying rate; Vo:fasting gallbladder volume (mL or cm^3)

Vt:residual gallbladder volume at 1 hour after a fatty meal (mL or cm^3)

Stone component analysis

Component analysis of gallbladder stones was performed using Fourier-transform infrared spectroscopy (FTIR). A 2 mg sample of each stone layer was weighed if the layered structures were distinct. For amorphous stones, 2 mg of the sample was directly weighed. The samples were mixed with potassium bromide (KBr) at a 1:150 ratio, ground thoroughly, and pressed into 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.

Statistical analysis and processing of data

Statistical analyses were performed using SPSS version 16.0. The chi-square test was initially used to assess qualitative data, such as comparisons of rates between the two groups. If the differences were statistically significant (two-tailed, $P<0.05$), binary logistic regression (forward LR method) was used to further determine whether it was a risk factor. One-way ANOVA variance was used to compare differences in the quantitative data among the three groups ($P<0.05$), indicating that the differences were statistically significant. The bile component concentration, gallbladder volume, and gallbladder ejection fraction were normally distributed quantitative data, and their distribution results did not show statistically significant differences across different ages and sexes.

Results

Positive IgG antibody against *C. sinensis* and hyperglycaemia were risk factors for GBSD

The results from the binary logistic regression analysis model indicated that a positive antibody against *C. sinensis* (OR: 1.759, 95% CI: 1.163–2.662, $P=0.008$) and hyperglycaemia (fasting serum glucose concentration ≥ 6.10 mmol/L) (OR: 2.263, 95% CI: 1.227–4.172, $P=0.009$) were independent risk factors for GBSD. However, sex (OR: 1.298, 95% CI: 0.855–1.970, $P=0.220$), age ($P=0.308$), high serum cholesterol concentration ≥ 5.20 mmol/L (OR: 0.943, 95% CI: 0.631–1.409, $P=0.773$) and high triglyceride concentration ≥ 1.70 mmol/L (OR: 0.637, 95% CI: 0.403–1.006, $P=0.053$) were not associated with GBSD ($P>0.05$) (Fig. 2a).

Morphologic alterations in *C. sinensis* eggs in bile, bile sediment, and stones

Using light and scanning electron microscopy, *C. sinensis* eggs were observed in bile centrifugal precipitates, bile sediments, and stone specimens. The surfaces and surrounding regions of the eggs in the stones were observed under SEM to be attached to mucus, bilirubin-like particles, and/or calcium carbonate crystals, which appeared to be entrenched in the crystals and/or mucus-like substances. Additionally, some eggs showed signs of losing their egg caps, and as the rough structure on their surface vanished, the egg surfaces smoothed out (Fig. 3). Furthermore, the calcium salt content progressively increased from the gallbladder bile and sediment to the gallstones (Fig. 4). Moreover, the survival or mortality of eggs may also, in part, represent the stage at which eggs deposit. Trypan blue staining indicated a progressive increase in egg death in gallbladder bile, sediment, and stones, with egg mortality rates of 13.4%, 57.9%, and 88.5%, respectively ($P<0.0001$; Fig. 4).

Changes in the component concentration of gallbladder bile in GSI patients

The results indicated that bile from GSI patients had lower TBA and cholesterol levels than that of GP patients ($P<0.05$); however, no statistically significant differences in the other indicators of bile composition were observed. Compared with GS patients, GSI patients had lower concentrations of TBA, TBIL, and cholesterol in their bile ($P<0.05$) but higher concentrations of CO_2 and pH values ($P<0.01$; Fig. 5).

Changes in the gallbladder motility of GSI patients

Since there were no statistically significant differences in FGV, RGV, and *%E* among all patients with GBSD across different age groups (< 30 years, 30–50 years and > 50 years) by One-way ANOVA ($P=0.618$ for FGV, $P=0.644$ for RGV, and $P=0.532$ for *%E*, respectively; data not shown), the three indicators were not refined for stratified comparisons among the three groups of patients. The results indicated that the GSI patients had higher FGV and RGV and decreased gallbladder ejection fraction (*%E*) compared to the GP patients ($P<0.001$). Furthermore, the GSI patients had higher FGV ($P<0.001$) and RGV ($P<0.05$) than GS patients; however, no statistically significant difference in the ejection fraction (*%E*) was found ($P>0.05$; Fig. 5).

Comparison of stone components between GSI patients and GS patients

The results showed that, compared with GS patients, the proportion of cholesterol stones was significantly lower in the GSI patients (10.4% [25/241] vs. 51.2% [144/281], $P<0.0001$), and the proportion of non-cholesterol stones (including pigment stones, mixed bilirubin-cholesterol stones, calcium carbonate stones, and other types) was significantly higher (89.6% [216/241] vs. 48.8% [137/281], $P<0.0001$; Fig. 2b).

Discussion

Numerous studies have revealed a correlation between *C. sinensis* infection and gallbladder formation [18, 19, 32–34]. Nonetheless, whether *C. sinensis* infection is a risk factor for GBSD and the underlying pathogenic mechanisms remain to be clarified. The results of this study indicated that a positive serum antibody against *C. sinensis* was a risk factor for GBSD (Fig. 2a). Although the current clinical diagnostic standard for *C. sinensis* infection is the detection of eggs in bile and faeces, detecting antibodies against *C. sinensis* in serum still holds diagnostic and epidemiological value when bile and faeces specimens are unavailable. Our previous studies

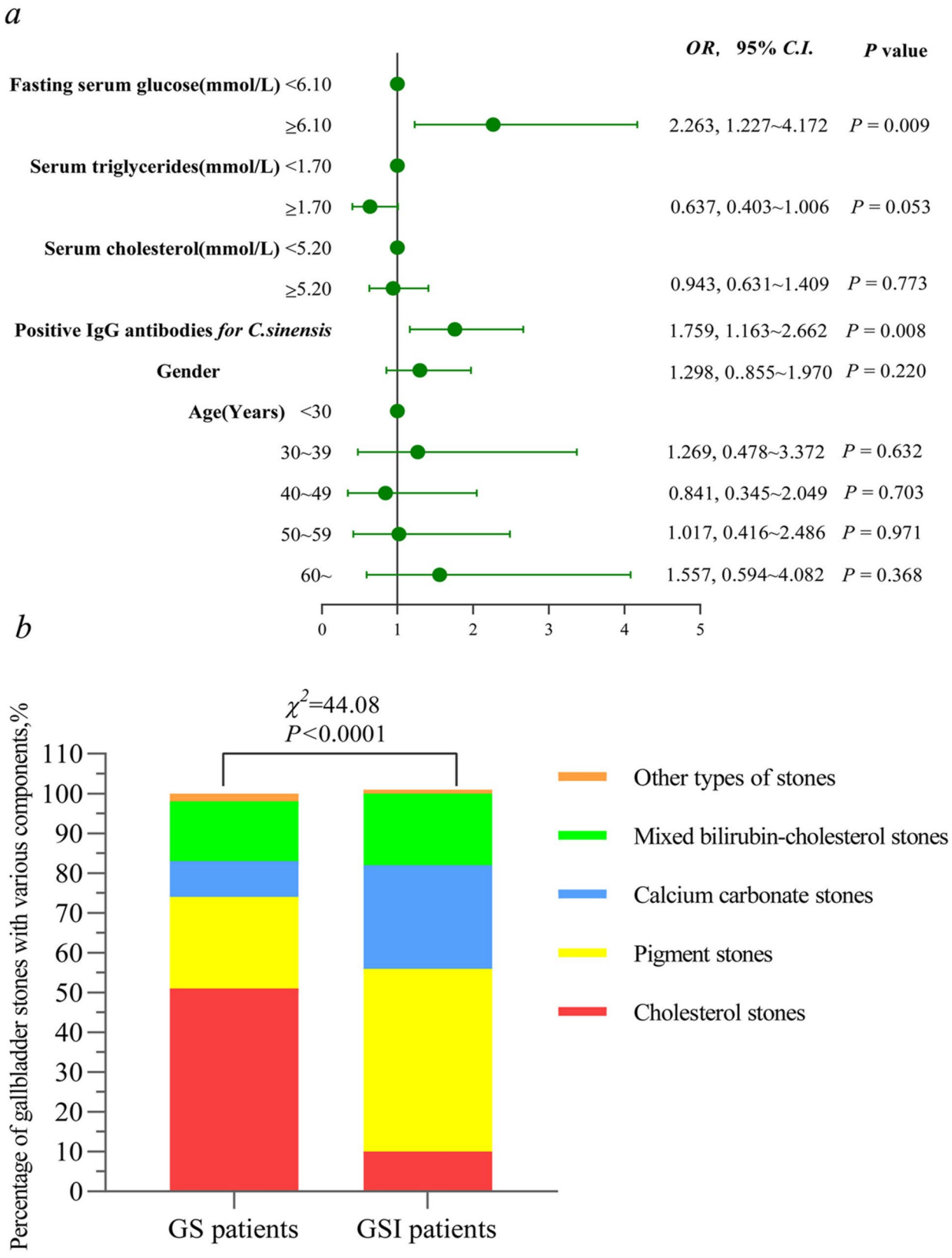

Fig. 2 (**a**) The association between patients with gallbladder stones and *Clonorchis sinensis* (*C. sinensis*) infection (indicated by positive IgG antibody), sex, age, and serum glucose, triglyceride, and cholesterol levels. OR, odds ratio; CI, confidence interval. (**b**) Comparison of the percentage of gallbladder stones with various components (%) in gallbladder stone patients (GS patients) and gallbladder stone patients with *C. sinensis* infection (GSI patients)

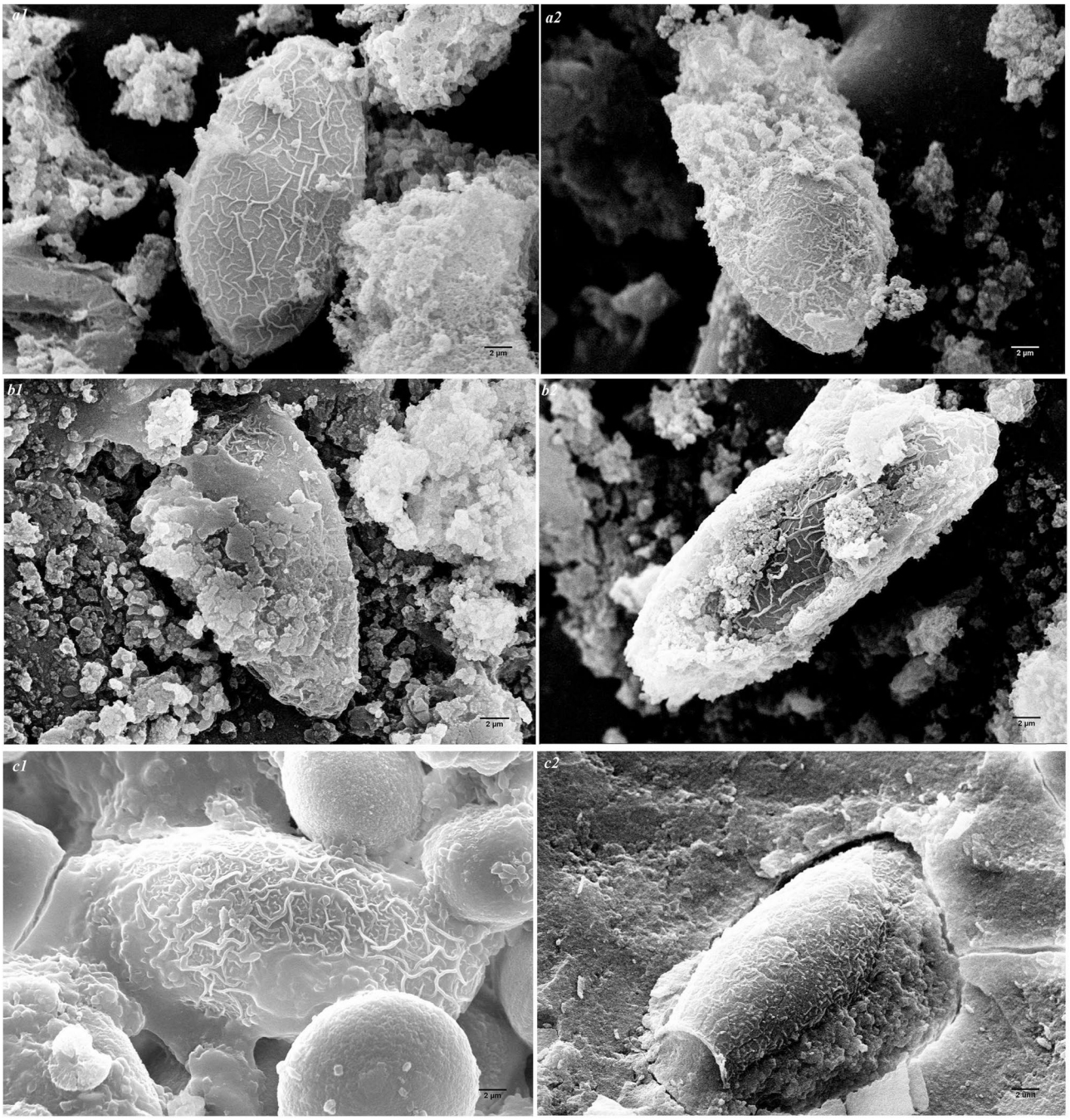

Fig. 3 Morphology of *Clonorchis sinensis* eggs and their surrounding material in gallbladder bile, bile sediment, and gallbladder stones under SEM. (**a1**, **a2**) Morphology of eggs in bile. (**b1**, **b2**) Eggs in bile sediment, mostly encapsulated by mucus material. (**c1**, **c2**) Eggs in gallbladder stones, embedded in board-shaped structure or bile pigment granular material (**c2**) or adhered to by mucus-like material and rounded calcium carbonate crystals (**c1**)

demonstrated that the ELISA method for detecting IgG antibodies in patients with GBSD had a higher sensitivity (93.9% vs. 63.3%), coincidence rate (90.2% vs. 80.4%), and Youden index (0.80 vs. 0.63) than the Kato-Katz method for detecting faecal eggs; however, it showed poorer specificity (86.0% vs. 99.9%, more false positives) [35]. As this study failed to obtain the faeces and bile of healthy individuals undergoing routine medical examinations in the control group and only obtained serum samples, this study utilized the serum antibody against *C. sinensis* to indirectly indicate *C. sinensis* infection in order to maintain the consistency of the overall research samples and detection data. The results showed that *C. sinensis* infection was an independent risk factor for GBSD, suggesting a close association between gallstone formation and *C. sinensis* infection. However, the endemic population is

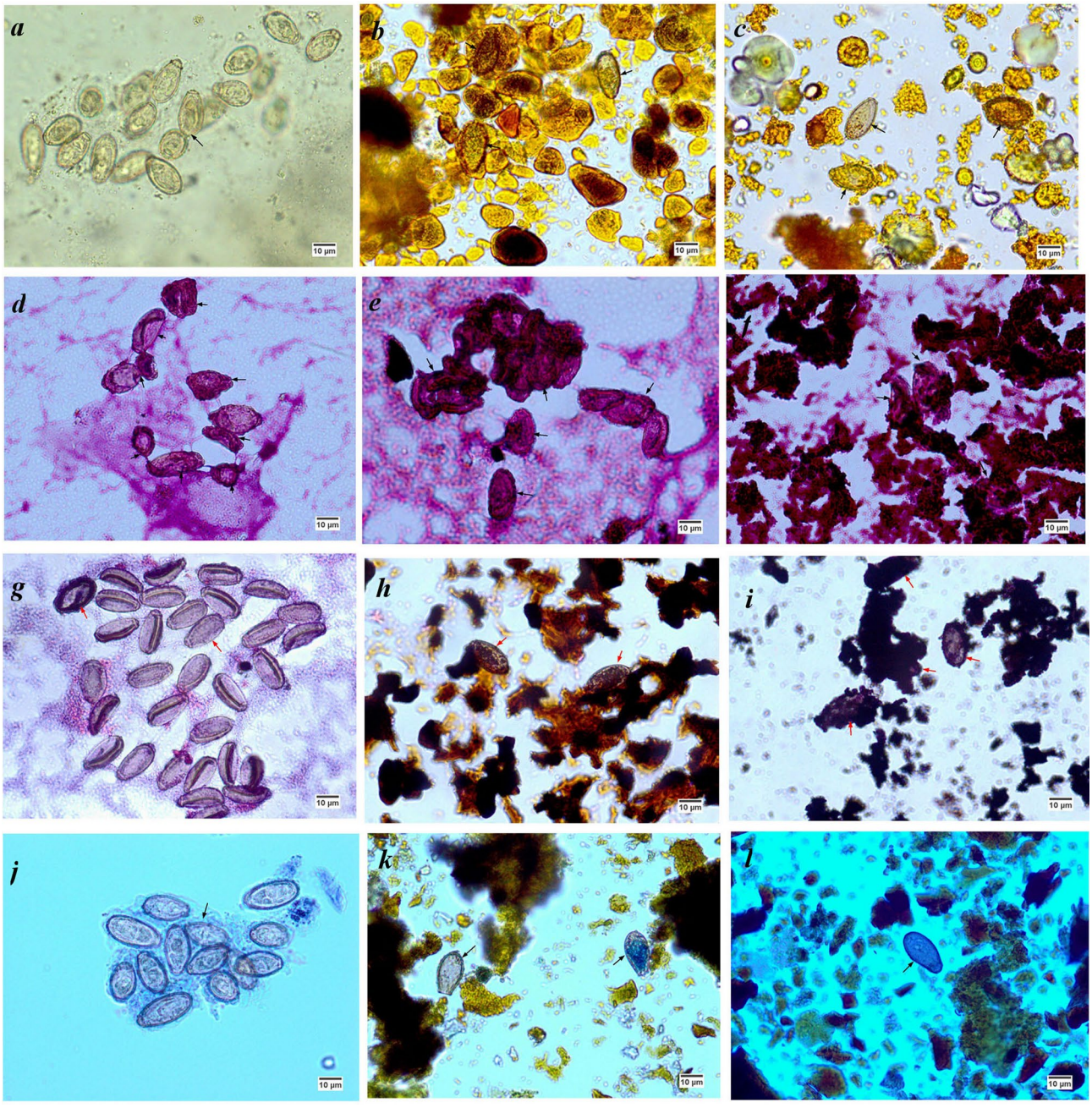

Fig. 4 Morphology and staining of *Clonorchis sinensis* eggs and their surroundings in gallbladder bile (**a**, **d**, **g**, **j**), bile sediment (**b**, **e**, **h**, **k**), and gallbladder stones (**c**, **f**, **i**, **l**) under an optical microscope, with the arrow pointing to the eggs. (**a**, **b**, **c**) Eggs in three specimens examined under a microscope without staining. (**d**, **e**, **f**) Results of periodic acid-Schiff staining with red coloration. (**g**, **h**, **i**) Von Kossa staining: the brown to dark-brown colour indicates positivity for calcium salts. (**j**, **k**, **l**) Trypan blue staining: blue colour indicates non-viable or dead eggs

geographically restricted because infection is acquired by eating raw fish or shrimp with *C. sinensis* metacercariae, and the participants of this study included local people who had resided in the region for more than three years. Thus, the importance of the results is restricted to *C. sinensis* endemic regions. Furthermore, the use of antibodies as evidence of infection in this study had certain limitations. Under feasible conditions, egg detection may be more reliable for diagnosis and grouping.

The results of this study revealed that hyperglycaemia was an independent risk factor for GBSD, which is consistent with relevant reports [36–38]. However, the present study also showed that sex, age, hypercholesterolaemia, and hypertriglyceridaemia were not associated with GBSD, which differs from the findings of

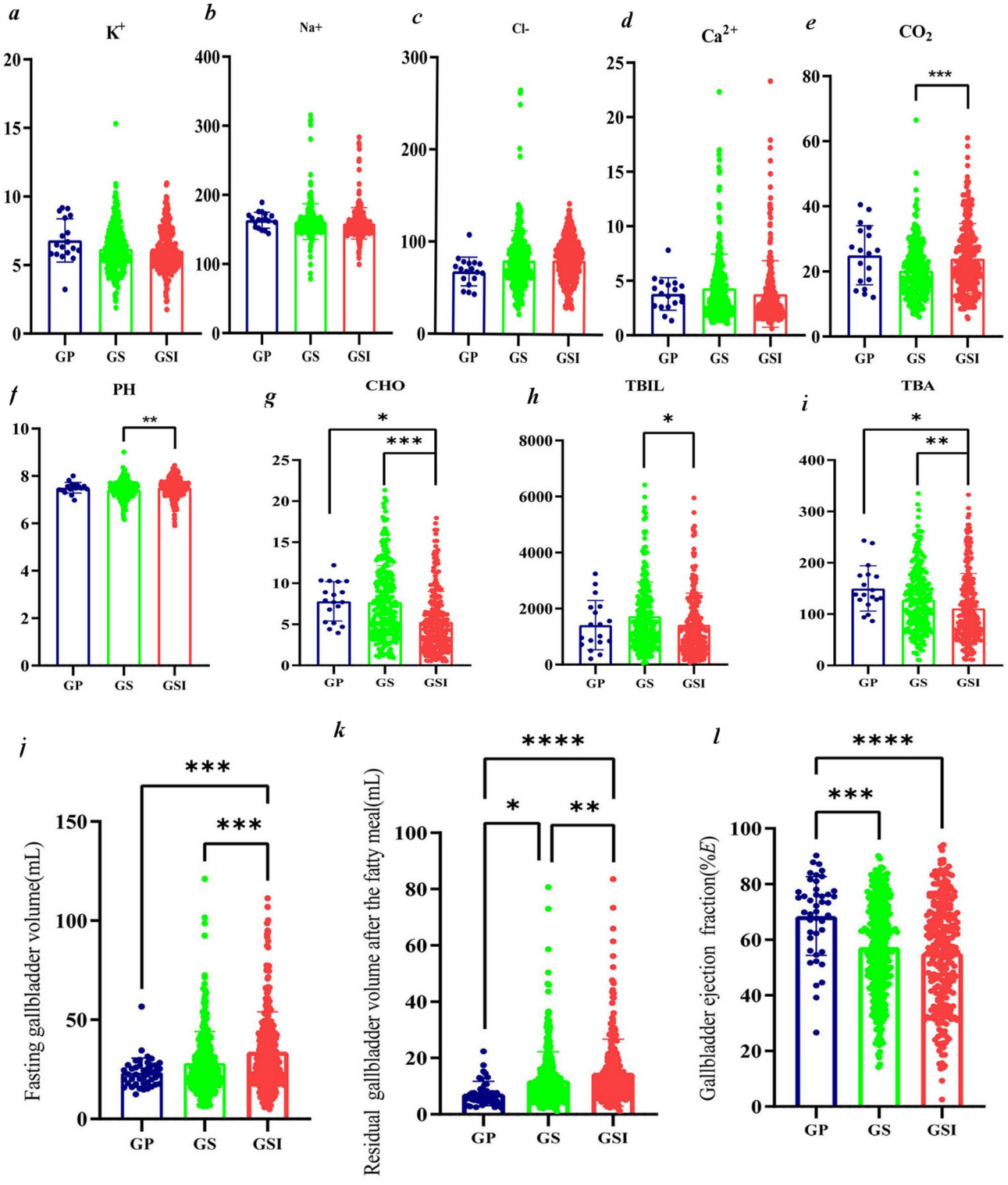

Fig. 5 Analysis and comparison of bile component concentration and three indicators of gallbladder emptying function in patients with gallbladder polyps (GP), patients with gallbladder stones (GS), and patients with gallbladder stones with *Clonorchis sinensis* infection (GSI). (a–i) Bile component indicators and their units: (**a**) potassium (K^+, mmol/L), (**b**) sodium (Na^+, mmol/L), (**c**) chloride (Cl^-, mmol/L), (**d**) calcium (Ca^{2+}, mmol/L), (**e**) carbon dioxide (CO_2, mmol/L), (**f**) total bile acids (TBA, μmol/L), (**g**) total cholesterol (mmol/L), (**h**) total bilirubin (TBIL, μmol/L), (**i**) pH value. (**j**) Fasting gallbladder volume (FGV, mL). (**k**) Residual gallbladder volume after a fatty meal (RGV, mL). (**l**) Gallbladder ejection fraction (%*E*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

previous reports [1–6, 36–39]. This discrepancy may be attributed to the following two factors: First, men in *C. sinensis* endemic regions are more likely to consume raw fish and to dine out, thereby increasing their exposure risk and resulting in a significantly higher infection rate among male patients and a notably higher prevalence of gallstones in men than in women [40]. Second, as our hospital is located in a *C. sinensis* endemic area and performs gallbladder-preserving cholecystolithotomy, male and younger patients may prefer this surgical option, as shown in Tables 1 and 2, respectively. The two aforementioned factors may overshadow the influence of female sex and age on gallstone disease. This could be attributed to the amplified effect of *C. sinensis* infection, which negated the significance of these demographic factors.

Although *C. sinensis* infection is associated with GBSD, gallbladder stone formation is not a certainty if an infection occurs. In light of the findings of our previous work linking *C. sinensis* eggs to the development of gallstones [17, 18, 36], *C. sinensis* eggs, similar to other microbes, are directly involved in stone development [13, 41, 42]. Microscopic examination of *C. sinensis* eggs and the surrounding material in the gallbladder stones, bile sediments, and bile revealed their involvement in stone formation (Fig. 3). Their retention in the gallbladder was due to the highly viscous mucus substance abundant in glycogen that covered the egg surface and surroundings and bound them (Fig. 4). The increased mucus production in the gallbladder has been hypothesised to facilitate gallbladder stone formation [43–45]. Concurrently, the calcium salt content around the eggs and the proportion of dead eggs increased progressively from the bile and bile sediments to the stones. These observations suggest a correlation between egg deposition, death, gallbladder calcification, and stone formation. Furthermore, the results of this study showed that among patients with GSI, those whose bile and/or stones contained *C. sinensis* eggs had stone compositions consisting mainly of non-cholesterol stones (Fig. 2b), which is consistent with the findings of other studies [10, 11, 13, 16], indicating that stones caused by microbial infections are mostly non-cholesterol stones. These results suggest that the pathogenesis of non-cholesterol stones, primarily caused by *C. sinensis* infection, may differ from that of cholesterol stone formation. However, further studies are required to confirm these hypotheses.

Damage to bile duct epithelial cells, bile duct obstruction, and cholestasis resulting from *C. sinensis* infection can trigger further alterations in bile components and feedback regulation [46–48]. The results revealed that the bile of GSI patients had lower TBA and cholesterol levels than that of GP patients ($P<0.05$; Fig. 5), which is consistent with our previous research [49]. These changes may be attributed to adult worms of *C. sinensis* parasitising the intrahepatic bile ducts, which damage cholangiocytes, impair bile acid secretion and excretion, and cause cholestasis to some extent [50–52]. In addition, comparative analysis revealed that, in contrast to patients with GS, the concentrations of TBIL, TBA, and cholesterol in the bile of patients with GSI were lower, whereas the CO_2 and pH values were higher. These results suggest that patients with GSI may have a different mechanism of gallbladder stone formation than patients with GS, as suggested by the different proportions of stone component types in the two groups of patients [17, 18] and as shown in Fig. 2b. However, there was no statistically significant difference in bile components between patients with GS and those with GP. This could be attributed to the limited number of GP patient cases in the current study, the unavailability of normal human bile, and the fact that some component indices did not cover the indices linked to gallbladder cholesterol stones, such as phospholipid indices and bile acid subtypes [53–55]. Consequently, the findings of this study require further validation in additional controlled studies.

A decline in gallbladder motility, precipitated by reduced bile acid concentrations and increased gallbladder mucus or mucin production, promotes gallbladder stone formation [56–60]. The results of this study showed that the gallbladder ejection fraction (*%E*) in both GS and GSI patients was significantly lower than that in GP patients (Fig. 5), suggesting a correlation between reduced gallbladder emptying function and gallbladder stone formation. However, further research is required to determine whether a causal connection exists between the two. Furthermore, the FGV and RGV were both higher in patients with GSI than in those with GP. These results suggest a possible link between *C. sinensis* infection and impaired gallbladder emptying function, characterised by a significant increase in FGV, which increases the RGV and decreases the gallbladder ejection fraction (*%E*). Elevated mucus levels in the gallbladder, biliary obstruction [61], and reduced bile acid excretion [62] due to *C. sinensis* infection may be associated with the significant increase in FGV observed in patients with GSI. However, further studies are required to provide definitive evidence.

Conclusions

In summary, as a risk factor for GBSD, the potential mechanism by which *C. sinensis* infection induces gallstone formation is as follows: adult *C. sinensis* worms parasitise the intrahepatic bile ducts of infected individuals, damaging the bile duct cells, inducing bile duct inflammation [63], and increasing mucus secretion [64]. Adult worms continuously reproduce and lay eggs, which are discharged into the bile ducts and gallbladder along with the bile. Some eggs are encapsulated by glycan-rich mucous substances and remain trapped within the gallbladder, aggregating into small masses. Subsequently, this phenomenon can potentially act as a trigger point, coinciding with an infection-induced reduction in the TBA concentration, elevation in

CO_2 levels, and increased bile pH. Continuous accumulation and sedimentation of eggs within the gallbladder leads to their death and calcification. Dead or calcified eggs can stimulate the gallbladder wall, and induce inflammation and mucus secretion. Concomitant changes in bile composition may result in reduced gallbladder motility, primarily characterised by an increase in FGV. Reduced gallbladder activity may further exacerbate egg retention and aggregation. Consequently, these interacting factors may contribute to gallstone development.

Despite the inherent limitations of this study, the conclusions provide a reference for interdisciplinary research on the relationship between foodborne parasitic infections and gallstones as well as the diagnosis, treatment, prevention, and control of common diseases, such as parasitic infections and gallstones.

Abbreviations

GBSD	Gallbladder stone disease
C. sinensis	Clonorchis sinensis
GP	Gallbladder polyps
GS	Gallstones
GSI	GS combined with C. sinensis infection
%E	Gallbladder ejection fraction
RGV	residual gallbladder volume
FGV	Fasting gallbladder volume
TBA	Total bile acid
K^+	Potassium
Na^+	Sodium
Cl^-	Chloride
Ca^{2+}	Calcium
CO_2	Carbon dioxide
TBIL	Total bilirubin
CHO	Cholesterol

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Author contributions
Luo Xiaobing was the major contributor in experiment design, data analysis and manuscript writing. Ma Ruihong performed the scanning electron microscopy examination of the specimens and provided the images. Cai Hongying performed the chemical staining of samples and provided related images. Su Xiangyu performed the collection and arrangement of all patients data. Wang Sangui and Qiao Tie provided guidance in experiment design, manuscript writing and results interpretation. All authors read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
The operation and sample collection procedures were explained to all patients. The principles outlined in the 1975 Declaration of Helsinki (as revised in 1983) were followed throughout this study. This study was approved by the Ethics Committee of Sixth People's Hospital of Nansha, Guangzhou, China. All methods were performed in accordance with the relevant guidelines and regulations of Guangdong Clinical Laboratory Center. Informed consent was obtained from all patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Clinical Laboratory, Guangzhou Nansha People's Hospital, No. 7 Xingye Road, Dagang Town, Nansha District, Guangzhou, Guangdong, China
[2]Department of Pathology, Guangzhou Nansha People's Hospital, No. 7 Xingye Road, Dagang Town, Nansha District, Guangzhou, Guangdong, China
[3]Dongguan Nancheng Hospital, No. 55 Nancheng Avenue, Guantai Road, Dongguan, Guangdong, China
[4]The Second People's Hospital of Guangzhou Panyu District, No. 88 Gangdong Road, Dashi Street, Panyu District, Guangzhou, Guangdong, China

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