December 10, 2021

Editor
World Journal of Gastroenterology
Dear Editor:

We wish to re-submit the manuscript titled "**Microbiologic Risk Factors of Recurrent Choledocholithiasis Post-Endoscopic Sphincterotomy.**" The manuscript ID is 72007.

We thank you and the reviewers for the valuable comments and suggestions. Accordingly, the manuscript has been rechecked and the necessary changes have been made. The responses to all the comments are provided below. We look forward to working with you to move this manuscript closer to publication in *World Journal of Gastroenterology.*

Thank you for your consideration. We look forward to hearing from you.

Sincerely,
Qingping Wu
Guangdong Provincial Key Laboratory of Microbial Safety and Health
State Key Laboratory of Applied Microbiology Southern China
Guangdong Institute of Microbiology
Guangdong Academy of Sciences
Guangzhou 510070, China
Email: wuqp203@163.com

Wenhui Tan
Digestive Endoscopy Center
Guangdong Second Provincial General Hospital
Guangzhou, 510317, China
Email: winnytam@163.com

Science editor:

This manuscript uses sequencing technology to explore the role of the bile microbiota in the recurrence of cholelithiasis after endoscopic sphincterotomy. The difference in the microbiological composition of bile samples obtained from postoperatively stable patients and patients with recurrent cholelithiasis provides information on possible biomarkers that may predict disease recurrence. But as mentioned in the manuscript, the number of recurrence was only four, and it is too small to draw definite conclusions. So this study is more likely to have selection bias. Please add the following to the discussion:

Response: Thank you for your comment. We acknowledge that the number of recurrence cases was small and that it is the major limitation of this study. We have included this limitation at the end of the DISCUSSION section.

Among the recurrent cases, verification of microbiome might be useful at the time of re-intervention. Cholecystecmy is preferred after EST or lithotomy of choledocolithiasis. The most frequent cause of choledocolithiasis is spilled gallstones. How many cases cholecystectomy after EST was performed? The influences of cholecystecmy into the microbiome should be included in consideration.

Response: Thank you for your comment pertaining to the number of cases of cholecystectomy. All the patients in this study underwent laparoscopic cholecystectomy three days after the EST or EPBD treatment. Therefore, spilled stones from the gallbladder could be excluded as a risk of choledocholithiasis recurrence. We have included the detailed procedures of the treatment in the revised manuscript. We believe that this revision would clarify the potential influencing factors in this study.

Reviewer #1

The authors tried to identify the microbiome factors for CBD stone recurrence. The study idea is very intriguing and manuscript is relatively well-written. However, the method especially in bile sampling and clinical outcome measurement (stone recurrence) is somewhat ambiguous and not clear.

1 The authors mentioned that the patients who were diagnosed with cholelithiasis using CT or MRCP were included. In general, "cholelithiasis" means GB stones, and "choledocholithiasis" means CBD stones. Thereby, did the authors mean to say that the patients with GB stones were enrolled? Not the patients with CBD stones?

Response: We apologize for the ambiguity in the use of “cholelithiasis (GB)”; we were describing patients with choledocholithiasis (CBD). We have rechecked the medical records and confirmed that all 43 participants in our research were diagnosed

as choledocholithiasis and in 13 of them there was a co-occurrence of cholelithiasis. We have revised our manuscript; we have included the detailed clinical features in the RESULTS section, in Table 1.

2 How many patients who had acute cholangitis were included in this study? Or what is the number of just GB stone patients who were not accompanied by acute cholangitis? As you know, the presence of gallstones does not always mean that patients have got inflammatory conditions such as acute cholangitis or cholecystitis.
Response: Thank you for your question. We have rechecked the medical records of all 43 participants and the co-occurrence of acute cholangitis was reported in 5 participants. We have included the detailed clinical information in the RESULTS section, in Table 1.

3 Furthermore, I think the term of "cholelithiasis" in this manuscript was not used appropriately, and a bit confusingly used. So, please check the word usages once again in entire manuscript.
Response: We apologize for the misuse of “cholelithiasis”. We have re-checked the manuscript and corrected the term to “choledocholithiasis”.

4. I am wondering about the timing of bile juice sampling. Did the authors do sample firstly from the beginning of ERCP procedure? or the bile sample was done at the last time of ERCP procedures after EST and CBD stone extraction had been done?
Response: Thank you for your questions about the timing of bile juice sampling. Obstructions in the biliary tract could increase the risk of surgical complications if bile juice suction was performed at the beginning of the ERCP procedure; therefore, we chose to obtain the bile juice sample after EST or EPBD. We could guarantee a bile sample volume of 3 mL, while lowering the risk of surgical complication such as bleeding. We have included the detailed description of the sampling procedures in the MATERIALS AND METHODS section. We hope the revisions provide a clear description of our sampling procedures.

5. Please show whether the enrolled patients have GB stone or not because CBD stone can be developed by migration of GB stones (secondary CBD stone). How do you define the CBD stone recurrence in this study? How do you discriminate the residual CBD stone or secondary CBD stone due to migration of GB stone from true CBD stone recurrence?
Response: We thank you for your questions pertaining to the diagnosis of the 43 participants. We have made the following revisions to clarify them:
(1) Among the enrolled participants, 13 patients exhibited co-occurrence of GB. We have included the detailed clinical features in the RESULTS section, Table 1. We did not find any correlation between CBD recurrences and the co-occurrence of GB.
(2) We have defined the CBD stone recurrence as the discovery of newly formed stones in the common bile duct using CT/ MRCP or recurrent symptoms of cholangitis during the one-year follow-up period. All 43 patients in this study

underwent laparoscopic cholecystectomy three days after the EST or EPBD treatment. CT or MRCP examinations were performed to confirm complete stone removal one week after the LC treatment; no stone was observed in the bile duct in all these patients. This enabled exclusion of patients with residual CBD stones and secondary migrated CBD stones and confirmed our diagnosis of the CBDS recurrence as true recurrence. We have included the detailed description of the treatment and the follow-up strategies along with the diagnostic criteria of recurrence in the MATERIALS AND METHODS section of the revised manuscript.
Our criteria for CBD stone recurrence relied mainly on the imaging examinations; therefore, we might have missed some stones which were invisible in these examinations. We have included this limitation in the last paragraph of the DISCUSSION section, and we believe we have clarified the shortcomings of our study.

6. Please, show the results of stone characteristics of enrolled patients. For example, cholesterol stone, mixed stone, black pigmented stone or brown pigmented stone. And is there any differences of bile microbiome composition according to the stone nature?

Response: Thank you for your comment. In order to reduce the surgical injury during the EST treatment, we left the stones in the intestinal tract and let them evacuate with feces instead of taking them out for composition analysis. However, in response to your comment, we reviewed the image data from the endoscopic surgeries and concluded the potential stone composition based on their image features. We have included the details of the stone characteristics in both the MATERIALS AND METHODS section and the RESULTS section, Table 1. The accurate stone characteristics could not be detected using infrared spectroscopy or through chemical analysis; therefore, there is a high chance of misdiagnosis. It was unreliable to conclude the bile microbial characteristics based on the stone characteristics. However, we believe such analysis is of great significance in the diagnosis and recurrence prediction in choledocholithiasis and therefore, we have included the points in the DISCUSSION section and have included this in our future research plans.

Reviewer #2

This manuscript revealed the significance of the analysis of microbiomes using NGS. *Lactobacillales* is very promising risk factor of recurrent choledocholithiasis. It was very interesting that the results of culture might be different from those of NGS. According to one explanation, EST breaks the barrier of invasion of intestinal (duodenal) bacterium into biliary tract and might increase retrograde biliary infection.

1. In this study, the source of analysis was taken at the time of EST. So, alteration of microbiomes might occur. It would be more appreciated that these considerations might add into discussion.

Response: Thank you for your comment. We agree with you that the timing of sampling might influence the analysis of bile microbiome, which could have caused

the differences in the results, compared to that from other studies. We have included this point in the second paragraph of the DISCUSSION section. We hope the revisions provide a more precise description of the study design.

2. Among the recurrent cases, verification of microbiome might be useful at the time of re-intervention.

Response: Thank you for your suggestion. Unfortunately, we did not perform the microbiome verification experiments at the time of re-intervention in this study. However, we agree with you on the importance of this verification; therefore, we have included them in our further study. We have included a brief note in this regard, in the last paragraph of the DISCUSSION section, in the revised manuscript.

3.Cholecystecmy is preferred after EST or lithotomy of choledocolithiasis. The most frequent cause of choledocolithiasis is spilled gallstones. In the population of this study, how many cases cholecystectomy after EST was performed? The influences of cholecystecmy into the microbiome should be included in consideration.

Response: Thank you for your comment. All the patients in this study underwent the laparoscopic cholecystectomy three days after the EST or EPBD treatment. Therefore, spilled stones from the gallbladder could be excluded as a risk of choledocholithiasis recurrence. We have included the detailed procedures of the treatment in the revised manuscript. We believe that this revision would clarify the potential influencing factors in this study.

4. Other cause is infection in biliary tract. The infection rate varies depending on regions. The incidence is assumed to be low in areas with good hygiene. So, the differences between regions might be taken into consideration.

Response: Thank you for your comment. We agree with you regarding the importance of biliary infection in choledocholithiasis recurrence. Therefore, we have included the pertinent points regarding the possible cause of infection in the DISCUSSION section in the revised manuscript.

4. In the abstract, “RA” lacks annotation.

Response: Thank you for your suggestion. We apologize for the oversight. We have included the annotation of “RA” in the abstract.