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Research article

Molecular detection of *Salmonella typhi* from gallbladder tissue of cholecystitis patients and its relation to gallstone formation

Running title: *Salmonella typhi* and its relation to gallstone formation

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Abstract: The gallbladder is often colonized by *Salmonella* during typhoid fever, and cholelithiasis contributes to many factors one of them is cholecystitis, which results from bacterial infections. This study aims to detect *Salmonella typhi* in the tissue of the gallbladder and find out its role in cholelithiasis. A total of 55 patients undergoing clinical and ultrasound examination were enrolled in this study, 43 with cholelithiasis and 12 without cholelithiasis, in which the gallbladder was taken as part of the surgical treatment for morbid obesity. DNA from tissue was extracted using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations; primer sets were used in this study that targeted *fliC* and *SopB* genes. A considerable number of cholelithiasis patients, 35 (81%), presented with multiple stone formation, while 8 (18.6%) presented with single stone formation. As many as 43 (78.1%) of cases presented with chronic cholecystitis, while only 12 (21.8%) of cases presented with acute cholecystitis by sonographic gallbladder; out of 55 samples, 10 (18.1%) were positive for the *fliC* gene in amplification of 367 bp. All 10 positive samples for *Salmonella typhi* showed gallbladder stone formation. However, out of 10 positive tissue samples for *Salmonella typhi* 10 (100%), of them were positive for the *SopB* gene. The findings in this study add to the body of knowledge around the occurrence rate of *Salmonella typhi* in gallbladder tissue samples obtained from patients with cholecystitis.

Keywords: *Salmonella*; cholelithiasis; cholecystitis; *fliC* gene; *SopB* gene

1. Introduction

Salmonella is a huge genus and is a member of the Enterobacteriaceae family. *Salmonella* is important for global public health as it is thought to be a major cause of foodborne illnesses that have claimed thousands of lives worldwide [1]. Contaminated food or water after consumption is the primary route of infection with *S. enterica* serovar. Typhi, because it can penetrate the intestinal wall and subsequently move to other systematic areas such as the liver, spleen, gallbladder, and pancreas, is known to be a systemic infection; after 10 to 14 days of intake, typhoid fever symptoms can appear, and symptoms include an initial high fever, malaise, headache, muscle aches, abdominal discomfort, and constipation or diarrhea; the infection is solved using antibiotics with most cases, although about 5% of infected patients with *S. Enteric* Ser. Typhi are unsuccessful in the elimination of the bacteria, then becoming a chronic carrier with the ability to spread the disease [2].

Infection with *Salmonella typhi* can lead to a chronic comparatively asymptomatic infection of the human gallbladder, and *S. typhi* carriers account for much of the human-to-human spread of typhoid fever; when some people are exposed to *S. typhi*, they become carriers. Bile is an antibacterial with detergent-like characteristics kept in storage in the gallbladder [3]. Through the epithelial microfold (M) cells, *Salmonella typhi* invade the intestinal mucosa and disseminate to the lymphatics and blood stream via phagocytes and ultimately spread to the spleen and liver [4].

Numerous studies have demonstrated that bile influences the expression of various *Salmonella* proteins, including those linked to virulence-associated phenotypes (such as motility, invasion of epithelial cells, and resistance to antibiotics and bile). Thus, the capacity to detect and react to bile is probably a crucial characteristic of *Salmonella* required to create a persistent carrier state. Furthermore, there is a strong association between the emergence of the *Salmonella* carrier state and problems related to the human gallbladder, particularly gallstones [5].

The *fliC* gene, encoding the flagellin protein, has been used as a target gene in assays to test the genetic diversity in *Salmonella* [6]. The *fliC* gene has a conserved terminal region and a variable central region, which determines antigenic specificity [7]. *Salmonella* outer protein B (*SopB*) is a SPI-1 encoded protein and its synthesis is tightly regulated. The long half-life in cells allows *SopB* to exert multiple roles during *Salmonella* infection; in addition to its function in invasion, its inositol phosphatase activity has been linked to a number of some biological functions [8]. The combination of the *fliC* and *SopB* genes as targets for the detection of *Salmonella typhi* has been reported in the literature as they provide a high degree of specificity and sensitivity for the identification of this important human pathogen. The use of molecular targets has significantly improved the accuracy and reliability of typhoid fever diagnosis [9,10].

Acute cholecystitis (AC) can be accurately diagnosed with a modern ultrasonography (US) exam of the gallbladder, especially when patients required surgical removal of the gallbladder (cholecystectomy) [11]. Acute cholecystitis typically arises as a result of gallbladder neck obstruction, followed by infection. As a result, the gallbladder looks unnaturally enlarged and spherical, and the wall surface of the gallbladder is thick and edematous [12]. It is important to note that the study of *Salmonella typhi* from gallbladder tissue of cholecystitis patients and its relation to gallstone formation can lead to improved disease prognosis and management and can assist in the development of strategies that can lead to reduce the complication *Salmonella typhi* infection. So, the aims of this study were to detect the *Salmonella typhi* in tissue of gallbladder and its relation with gallbladder stone formation.

2. Patients and methods

2.1. Patients

A total of 55 patients undergoing clinical and ultrasound examination were enrolled in this cross-sectional study, of them 33 were female and 22 were male, the mean age was 44.3 ± 17.4 years, and 43 were with cholelithiasis and 12 were without. The patients were referred to the Imamein Kadhimein Medical City; every one of them had undergone an abdominal ultrasound to confirm the presence of gallbladder stone and cholecystitis before the cholecystectomy. Of the 12 patients who did not have cholelithiasis, 9 had their gallbladder removed during surgery to treat their morbid obesity and 3 patients had gallbladder inflammation (cholecystitis). According to the ultrasonography findings, gallbladder wall thickness was categorized as normal for up to 2 mm, mild thickness for 3–4 mm, and severe wall thickness for more than 6 mm [12].

2.2. Preparation of tissue homogenate and DNA isolation

DNA from tissue was extracted according to Jonsson Y et al. 2002 [13]. Briefly, 25 g of the gallbladder tissue was homogenized with 180 μL of lysis buffer (buffer ATL) using tissue homogenizer (Srijan Scientific India) for five cycles, the cell suspension was exposed to liquid nitrogen for three minutes, and was then boiled for one minute and fifteen seconds. DNA was purified from the lysate following the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations.

2.3. Molecular detection of fliC and Sop genes

Two sets of primers were used for the detection of the *fliC* gene according to Haque A et al. [14], which were F 5′-ACT GCT AAA ACC ACT ACT-3′ and R 5′-TGG AGACTT CGG TCGCGTAG-3′. The primer's specificity was ascertained using the Basic Local Alignment Search Tool (BLAST). The PCR conditions were as follows: 1 minute at 94℃; 36 cycles of 1 minute at 94℃, 1 minute at 55℃, and 2 minutes at 72℃; and 10 minutes at 72℃. The reactions were carried out in a 20 μL volume containing PCR buffer 1X, dNTPs 0.5 mM, 0.5 U Taq polymerase, and 0.05 mM forward and reverse primers.

In each experiment, negative controls were used, and to amplify the collected DNA, a NYX Technik PCR thermal cycler was employed. Furthermore, the positive samples for the *fliC* gene were used for the detection of the *SopB* gene using two primers were F5′-GATGTGATTAATGAAGAAATGCC-3′ and R 5′-GCAAACCATAAAAACTACACTCA3′ [15]. One cycle of 94℃ for 5 min, then 30 cycles of 94℃ for 1 min, 60℃ for 1 minute, and 72℃ for 2 min, ending with a final extension step of 72℃ for 5 min, the reactions were carried out in a 20 μL volume containing PCR buffer 1X, dNTPs 0.5 mM, 0.5 U Taq polymerase, and 0.05 mM forward and reverse primers. After 45 minutes of running the PCR, products were detected in a 1.5% agarose gel with 150 mA current, the desired band of 367 bp for *fliC* gene and 1170 bp *SopB* gene were visible on a UV illuminator (Thermo Scientific, USA). The PCR products were analyzed on ethidium bromide stain.

2.4. Statistical analysis

All data were presented with the Statistical Package for Social Sciences (SPSS) software (version 26). Binominal data were expressed as frequency and percentage of categorical data, and the Chi-square test was used to compare the results. The significance was set at *P*-value < 0.05.

3. Results

A total of 55 patients were enrolled in this study, of them 33 (60%) were females and 22 (40%) males; the mean age was 44.3 ± 17.4 years. However, no significant effect was observed with regard to sex (P = 0.138). Wall thickness by Ultrasound revealed that 39 (70.9%) had severe thickness gallbladder wall, with the remaining range between normal to mild thickness. As many as 43 (78.1%) cases presented with chronic cholecystitis, while only 12 (21.8%) cases presented with acute cholecystitis by sonographic gallbladder. A considerable number of cholelithiasis patients, 35 (81%), presented with multiple stone formation, while 8 (18.6%) presented with single stone formation. Out of 43 with cholelithiasis and 12 without cholelithiasis, 30 (69.7%) was female, and the rest, 13 (30.2%), were male with highly statistically significance (P = 0.0001) (Table 1).

On the other hand, a total of 55 tissue samples were tested to detect *S. typhi* targeting the *fliC* gene by monoplex PCR assay, where 10 (18.1%) were positive for this gene in amplification of (367) bp (Figure 1). All 10 positive samples for *S. typhi* showed gallbladder stone formation. However, out of 10 positive tissue samples for *S. typhi*, 10 (100%) of them were positive for the *SopB* gene in amplification of (1170) bp (Figure 2).

Table 1. Distribution of clinical, demographic, and genetic characteristics of *Salmonella* analysis.

Attributes		Statistical value	*P* value
Age/years (mean ± SD)		44.3 ± 17.4	0.003 *
Sex	Male	22 (40%)	0.138 ^{NS}
	Female	33 (60%)	
Wall thickness by Ultrasound	Mild thickness	4 (7.2%)	0.0001 *
	Normal	12 (21.8%)	
	Severe thickness	39 (70.9%)	
Cholecystitis	Acute	12 (21.8%)	0.0001 *
	Chronic	43 (78.1%)	
Number of stone	Single	8 (18.6%)	0.0001 *
	multiple	35 (81%)	
Sex with cholelithiasis	Male	13 (30.2%)	0.0001 *
	Female	30 (69.7%)	
PCR results for *fliC gene*	Positive	10 (18.18%)	0.0001 *
	Negative	45 (81.81%)	
PCR results for *SopB gene*	Positive	10 (100%)	0.0001 *
	Negative	0 (0%)	

Note: ^{NS}: Non-significant *P* value more than 0.05; *: Significant *P* value < 0.05. SD: Standard deviation.

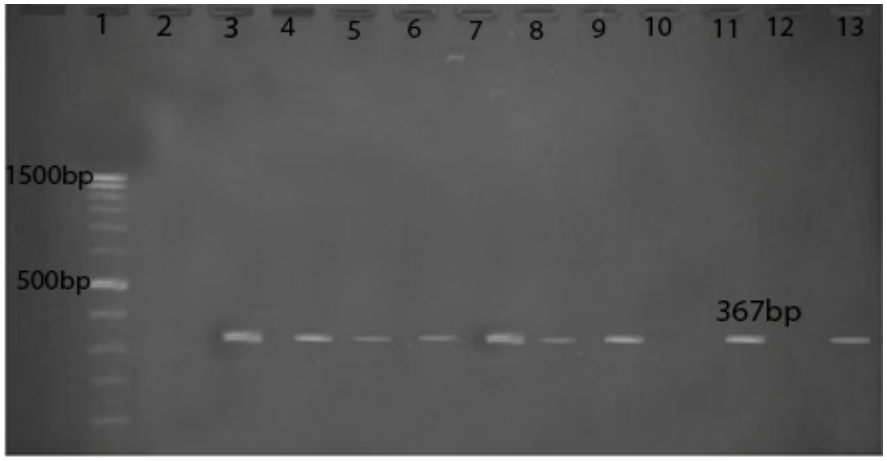

Figure 1. Results of the amplification of the *fliC* gene (367 bp) *Salmonella typhi* fractionated on 1.5% agarose gel electrophoresis line 2 (negative control), while lines 3–9, 11, and 13 were positive samples and lines 10 and 12 were negative.

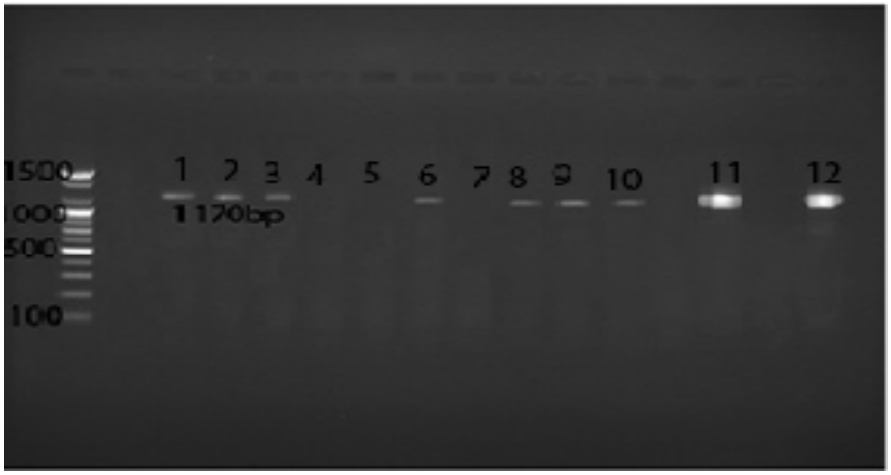

Figure 2. Results of the amplification of the *SopB* gene (1170 bp) of *Salmonella typhi* fractionated on 1.5% agarose gel electrophoresis lines 4, 5 (negative control) while lines 1–3, 6, and 8–12 were positive samples and line 7 was negative sample.

4. Discussion and conclusions

Salmonella typhi has the ability to colonize the gallbladder and continue in an asymptomatic carrier condition, which is often linked to gallstones. When *Salmonella typhi* enters the gallbladder in humans, it can cause an acute, active infection with inflammation (cholecystitis) or stay there for a long time after symptoms go away, indicating the bacterium's special mechanisms for mediating colonization in an environment rich in bile [16].

Gallstone formation (cholelithogenesis) is attributed to a combination of environmental and genetic causes, and most chronic gallbladder carriers have *S. typhi* embedded within biofilms present on the surface of infected cholesterol gallstones [17]. Findings in the present study showed that gallbladder disease occurs more in females, consistent with the findings of Dua et al. [18] who reported

that they accounted for 65% of admissions for cholecystitis, consistent with the study of Bailey KS et al. [19], who mentioned that there is a higher incidence of gallbladder disease in women.

Consistent with previous publications that mentioned, the wall thickness of the gallbladder is an indicator of cholecystitis in patients who have presented with symptoms of gallstone disease [20]. The gallbladder wall is normally measured up to 2 mm by ultrasonography (USG) and a thickness of more than 2 mm is suggestive of cholecystitis and the diameter of the gallbladder wall is directly part of the underlying pathology [21].

In line with these expectations, results from current data demonstrate that 39 (70.9%) respondents presented with thick gallbladder walls, which indicates a positive correlation between gallbladder wall thickness and gallbladder disease. In this study, out of 43 cholelithiasis patients, females were more prone to have cholelithiasis (69.7%) compared to males (30.2%). This analysis supports the finding of a study by Al-Zuharri OAR [22]. And, concordant with a broad study by Sattar et al. [23], who noted that endogenous and exogenous estrogen, progesterone, or both, which lower the chenodeoxy cholic acid and total bile acid pools and increase bile cholesterol secretion and saturation, are responsible for the increased incidence of cholelithiasis in females.

The current analysis indicates that the majority of patients (81%) presented with multiple stone formation. The formation of gallstones is a complex process that involves a combination of factors, including an imbalance in bile composition or the gallbladder not normally contracting and releasing bile in response to food intake; if the gallbladder doesn't empty properly or if there is a sluggish movement of bile, it can lead to the accumulation of bile and increase the risk of stone formation and addition to some genetic related factors [24].

Cholelithiasis is typically seen in the context of chronic cholecystitis. Recurrent episodes of acute cholecystitis or chronic gallstone irritation causing an inflammatory reaction in the gallbladder wall are the suggested etiologies [25]. According to numerous researchers, cholelithiasis is frequently linked to the majority of cases of chronic cholecystitis [26,27].

The observation in this study showed that *Salmonella typhi* was detected in 10 (18.1%) of 55 samples by using molecular method targeting the *fliC* gene, which is in disaggrement with the study of Sadeq et al. [28], who reported that *S. typhi* was found in 6.6% out of fifty tissue samples of gallbladder by means of culture methods, while Mansour and his colleagues recorded the ratio of *S. typhi* isolates as 7.2% [29]. The proportion of Salmonella *typhi* positive samples obtained by Udin et al. (2022) was 21.2% (53 out of 250 samples) [30]. However, the proportion obtained in the present study is not significantly different. Consequently, it can be concluded that the results of the two studies are concordant. That may be explained by sample size differences between both studies and the prevalence of *Salmonella typhi* infection in Pakistan, which exhibits a much higher prevalence of *Salmonella typhi* compared to Iraq [31,32].

In this study, the results of PCR of the *SopB* virulence gene clarify that the gene was seen frequently (100%) in all *Salmonella typhi* positive samples which indicates that this gene may play a significant role in the cholecystitis process caused by *Salmonella typhi*. Many studies have generally highlighted the significance of those genes in the pathogenesis that *Salmonella typhi* produces, and the majority of the genes necessary for *Salmonella* virulence are grouped within five *Salmonella* pathogenicity islands (SPI-1–SPI-5), which supports the pathogen's ability to survive inside cells [33,34].

Raffatellu M et al. reported a significant correlation between the expressions of the gene and cholecystitis, suggesting that the gene is involved in the process of cholecystitis. Furthermore, they

found that in vitro induction of the gene increased the invasiveness of the epithelia cells (human colon carcinoma cell line T84) by *Salmonella enterica* [35]. Moreover, the Boyle EC study reported there to be an important role of the *SopB* gene in the process of biofilm aggragation [36].

Additionally, an important matter that emerged from findings of this result is that all *S. typhi* positive patients for the *SopB* gene were suffering from cholilethiasis, and the explanation of such data may be coming from that the *SopB* gene is associated with biofilm formation in *S. typhi* and many studies have considered the relationship between biofilm formation and cholilethiasis [36,37]. Alternatively, a hypothesis that suggests the gallbladder's motility and the expression of mucin genes (MUC1, Muc3, MUC4) may be one of the promoters of gallbladder stone formation through mechanisms of immunomodulation that may be aided by certain enterobacteriace virulence genes [38]. The capacity of *S. typhi* to manufacture beta-glucuronidase (B-glucuronidase) in bile salt provides an additional intriguing explanation for the association between the two conditions. This component, together with the bile's inactivity, is crucial in the development of gallbladder stones [39].

The finding in this study adds to the body of knowledge around the occurrence rate of *Salmonella typhi* in gallbladder tissue samples obtained from patients with cholecystitis. Furthermore, there is a significant role for the *SopB* gene of *S. typhi* with cholelithiasis.

The obtaining of gallbladder tissue, particularly the availability of well-characterized and representative tissue samples and diagnosis of cholecystitis in a clinical setting as well as detecting of *Salmonella typhi* in gallbladder tissue can be a challenge which can be considered a major limitation of this study.

Author contributions

Bashar A. Abdul Hassan: sample and data collection; Jabbar S. Hassan: methodology, resources, data curation and formal analysis; Ali Nayyef Umayra: investigation and writing—original draft preparation; Thanaa R. Abdulrahman: original draft conceptualization and supervision.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Ethics approval of research and informed consent

The current study was approved by the Institutional Review Board (I.R.B.) in Al-Nahrain University College of Medicine" (in: Jan.-10th-2023/No.96), and we confirm that we have obtained the patients' informed consent and Ethical approval prior to study.

Data availability

Further data is available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflict of interest.

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