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ORIGINAL ARTICLE

Role of gallstones in typhoid carriage in Egyptian patients

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ABSTRACT

Objectives: *Salmonella typhi* forms biofilm on the surface of gallstones promoting colonization and carrier state. The aims are to detect its frequency in chronic calcular cholecystitis patients, to find out their antibiotic resistance pattern, and also to examine biofilm on the surface of gallstones and detect its correlation to antibiotic susceptibility pattern.

Materials and methods: The study group included 257 patients with chronic calcular cholecystitis. *S. typhi* was isolated from gall bladder specimens. Antibiotics susceptibility tests of the isolates were done. Biofilm on surface of gallstones were visualized using scanning electron microscopy. Virulence (Vi) antibodies against *S. typhi* were studied in sera of 257 healthy controls.

Results: 10.9% of patients were chronic typhoid carriers compared to only 3.5% of the controls (OR= 3.37, p=0.002). Multidrug resistance was detected in 35.7% of isolates. *S. typhi* biofilms on the surface of the 21/28 (75%) gallstones were detected. Ninety percent of the strains that produced maximum amount of biofilm were multidrug-resistant.

Conclusion: Chronic calcular cholecystitis patients are more prone to typhoid carriage due to biofilm formation of *S. typhi* on the surface of gallstones. *J Microbiol Infect Dis 2012; 2(4): 142-149*

Key words: Gall bladder, Chronic calcular cholecystitis, *Salmonella typhi*, biofilm, antibiotic resistance

Mısırlı hastalarda tifo taşıyıcılığında safra taşlarının rolü

ÖZET

Amaç: *Salmonella typhi* safra taşlarının üzerinde biyofilm oluşturarak kolonizasyonu ve taşıyıcılığı kolaylaştırır. Bu çalışmanın amacı, kronik taşlı kolesistiti olan hastalarda sıklığını saptamak, antibiyotik direnç paternlerini belirlemek, ayrıca safra taşları üzerindeki biyofilmi incelemek ve antibiyotik duyarlılık paterni ile ilişkisini araştırmaktır.

Gereç ve yöntem: Çalışmaya kronik taşlı kolesistiti olan 257 hasta alındı. *S. typhi* safra kesesi örneklerinden izole edildi. İzolatların antibiyotik duyarlılıkları çalışıldı. Safra taşları üzerindeki biyofilm, tarama electron mikroskobu ile gözlendi. *S. typhi*'ye karşı virulans (Vi) antikorları, 257 sağlıklı kontrolde çalışıldı.

Bulgular: Hastaların %10,9'u, kontrollerin ise %3,5'i of kronik tifo taşıyıcısı idi (OR= 3,37, p=0,002). Çoklu ilaca direnç, izolatların %35,7'sinde saptandı. *S. typhi* biyofilmi, 28 safra taşının 21'inde (%75) saptandı. Aşırı miktarda biyofilm üreten kökenlerin %90'ı, çoklu ilaca dirençli idi.

Sonuç: Kronik taşlı kolesistiti olan hastalar, *S. typhi*'nin safra taşı üzerinde biyofilm yapmasına bağlı olarak tifo taşıyıcılığına eğilimlidirler.

Anahtar kelimeler: Safra kesesi, kronik taşlı kolesistit, *Salmonella typhi*, biyofilm, antibiyotik direnci

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INTRODUCTION

Typhoid fever is an important health problem in many developing countries.[1] *Salmonella typhi* causes an estimated 21 million new cases of typhoid fever and 216,000 deaths worldwide every year.[2] Egypt remains a country with intermediate incidence of one to 100 per 100,000 cases of typhoid fever, below nations such as India and Indonesia.[3]

In general, 2-5% of all individuals who develop clinical or subclinical infection with *S. typhi* become chronic gall bladder carriers and thereby serves to maintain endemicity of the disease.[4]

Development of chronic typhoid carriage is frequently associated with the presence of gall bladder abnormalities especially gallstones.[5] About 10% of patients with cholelithiasis and 30% of gallbladder carcinoma in endemic areas are chronic typhoid carriers.[6-8] Gall stones arise from the precipitation of cholesterol and calcium salts in super-saturated bile. They are classified by their content of cholesterol as either cholesterol, pigmented or mixed gallstones.[9]

Recent evidence indicates that *S. typhi* forms biofilms on the surface of cholesterol gallstones. Biofilms are communities of microorganisms that adhere to each other and to inert or live substrates and are encased in an extracellular matrix.[10] Biofilm formation is dependent on the presence of bile because organisms cultured without bile do not readily form biofilm.[11,12]

Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in systemic infection or release of the organism into the environment. Bacteria shed by asymptomatic carriers contaminate food and water and account for much of the person-to-person transmission of *S. typhi* in underdeveloped countries.[13] One of the most important properties of the biofilm-associated bacteria in clinical medicine is the markedly enhanced resistance to antimicrobial agents, through protection by the extracellular polymeric substance (EPS), leading to multidrug resistance and therapeutic failure.[14]

Cholecystectomy increases the cure rate, but it does not guarantee elimination of the carrier state because additional foci of infection can persist in the biliary tree, mesenteric lymph nodes or liver. Hence, the most effective treatment is a combination of surgery and antibiotics.[15] The aim of this work is to detect the frequency of *S. typhi* in Egyptian calcular cholecystitis patients, to find out the antibiotic resistance pattern of isolates, and also to examine biofilm formation on the surface of the gallstones and detect its possible correlation to antibiotic susceptibility pattern.

Research question

Does patient with gallstones is more prone to chronic typhoid carriage?

Research hypothesis

Patients with chronic calcular cholecystitis are more prone to typhoid carriage due to biofilm formation of *S. typhi* on the surface of gallstones.

Objectives

1. To compare the frequency of chronic typhoid carriage in patients complained of chronic calcular cholecystitis versus healthy controls.

2. To assess antibiotic resistance pattern of *S. typhi* isolates.

3. To ascertain the presence of biofilm on surface of the gallstones.

4. To detect possible correlation between biofilm grading and antibiotic susceptibility pattern.

MATERIALS AND METHODS

The present study was carried out in Fakous and Minia El-Kamh, Sharkia Governorate, Egypt during the period from February 2009 to March 2012. Fakous and Minia El-Kamh are rural districts with about 500,000 and 600,000 populations; respectively.

Study design: Case-control study.

Sample size determination

Up to our knowledge, this study was the first one to discuss this topic in Middle-East countries. Hence, we depend on other developing countries with nearly the same predisposing factors like that of Egypt. 257 of each arm using the following equation.[16]

$$n = \frac{K[(P1(1-P1) + P2(1-P_2)}{(P1-P2)^2}$$

K = 10.5 assuming significance of 5% and power of 90%.

P1 = 0.1 as about 10% of patients with gallstones in endemic areas are typhoid carriers (known from previous studies).

P2=0.03 as about 3% of general population in endemic areas are typhoid carriers (known from previous studies).

Ethical considerations: The study was reviewed and approved by the review boards of the participating institutes and informed consent was obtained from all participants.

This study was conducted in health insurance, general or private hospitals in Fakous and Minia El-kamh. It included two groups; the study group included patients presented by chronic cholecystitis (178/257) and acute on chronic cholecystitis (79/257). Patients were subjected to either laparoscopic (201 patients) or open cholecystectomy (56 patients; 40 of which were acute on chronic cholecystitis patients). Admission to hospital 24 hours before operation for preoperative assessment and investigations was provided. No antibiotics were prescribed before surgery while third generation cephalosporins were given intra-operatively. The healthy control group included individuals presented without calcular cholecystitis or acute fever.

Due to expected low prevalence of typhoid carriage; the gall bladder specimens from cases as well as serum samples from controls were taken from those with past history suggestive of typhoid fever; fever associated with headache, abdominal discomfort, weight loss, cough, anorexia, vomiting or constipation.[17]

Sample collection: Gallbladder tissues, gallstone sections (Figure 1) and bile obtained from each patient after cholecystectomy were used for cultivation and immediate freezing at -20°C for DNA extraction. Blood samples from healthy controls were centrifuged, and sera were stored at -20°C.

Isolation and identification of S. typhi: Gallbladder tissue and gallstone sections were injected into a Stewart transport medium (Oxoid, Basingstoke, UK). One gram of gallbladder tissue was crushed in a sterile mortar and pestle in one ml saline and the tissue homogenate was used for culture. Gallstones were washed with water, dried and grinded to a fine powder with a mortar and pestle. They were homogenized in a 50 mL conical tube containing 1 ml of 1 x PBS. A portion of tissue and a loopfull of gallstone homogenate were cultured on selenite F medium (Oxoid, Basingstoke, UK) and after 12 to 24 hours incubation in 37°C, it was subcultured on selective Salmonella-Shigella agar (SS) medium (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. On the other hand, approximately 1 mL of bile was obtained, homogenized and plated.[18]

If growth was present, individual colonies were identified by colonial morphology, Gram staining and biochemical testing; triple sugar iron test, IMViC (Indole, Methyl Red, Voges' Proskauer, Citrate utilization) tests, motility and urease and lysine decarboxylase activities detection.[19]

Serotyping was performed using somatic, flagellar and virulence (Vi) Salmonella antisera (Difco, Detroit, Michigan) to determine corresponding antigens of *S. typhi* by the slide agglutination method according to the manufacturer's instructions.

Antimicrobial susceptibility testing: It was determined by the disc diffusion method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2008).[20] The following antimicrobial agents (Oxoid, Basingstoke, UK) were tested: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg) and ceftriaxone (30 µg). *S. typhi* isolate was classified as multidrug resistant (MDR), if it was resistant to all three first-line antibiotics used to treat typhoid fever: ampicillin, chloramphenicol, and co-trimoxazole.[21]

Molecular studies: To confirm the identification of *S. typhi*, nested PCR was done for genomic DNA from culture positive samples, as well as gallbladder tissue, gallstone and bile samples.

DNA extraction was done using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracts were then kept at -20°C until they were used in PCR reactions. The nested PCR described by Song and others [22] with a modified forward primer for the first PCR as suggested by Frankel and co-workers [23] to increase its specificity. It is based on the amplification of unique sequences in the VI region of the flagellin gene. The first reaction amplified a 458-basepair (bp) fragment corresponding to nucleotides 1063-1530 of the gene and a nested 343-bp fragment corresponding to nucleotides 1072-1435 was amplified in the second reaction.

The oligonucleotide primers (Sigma-Aldrich Chemie, Munich, Germany) for the first round of PCR were forward - ST 1 5'- ACT GCT AAA ACC ACT ACT-3' and reverse - ST2 5'- TTA ACG CAG TAA AGA GAG-3'. For the nested PCR, these were forward- ST3 5'- AGA TGG TAC TGG CGT TGC TC-3' and reverse -ST4 5'- TGG AGA CTT CGG TCG CGT AG-3' primers. A PCR reaction using PCR-gold master-mix beads (Bioron, Germany); contain (2.5U taq DNA polymerase, 250mM each dNTP, 10mM tris Hcl (pH 9.0), 30mM kcl, 1.5mM Mgcl2) to which 70 ng template DNA, 20 pmole for each primer and distilled water was added to a total volume 50 µl. They were mixed well by vortexing. The reaction was performed in thermal cycler (Biometra, Goettingen, Germany) under the following conditions: 40 cycles for 1 min denaturation at 94°C, 1 min 15 s annealing at 57°C, and 1 min elongation at 72°C, with a final elongation step extended to 7 min. The nested PCR master mix was the same as that of the first-round PCR, except it contained 21 pmol of each primer ST3 and ST4 and 4 µl of DNA template (1:6-diluted product of the primary cycle). Thermal cycling was carried out as described for first-round PCR, except that the annealing temperature was set to 63°C. Each run of PCR amplification included both positive and negative controls where the positive control included DNA extracted from *S. typhi* (National research center, Cairo, Egypt) while the negative control included no template DNA to avoid false positive results caused by suspected contamination. The amplified DNA was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

Scanning Electron Microscopy (SEM): SEM processing and examination were carried out in the Anatomy Department, Faculty of Medicine, Ain Shams University, Egypt. Gallstones recovered from positive and negative patients for *S. typhi* were rinsed with sterile sodium phosphate buffer, fixed in 2% glutaraldehyde, and air dried in a Laminar flow hood. The specimens were dehydrated in ascending grades of ethanol, dried with a Baltec 030 critical point dryer (Natick, Massachusetts, USA) and coated with gold using Baltec 030 sputter coater. Examination was carried out using Philips XL30 SEM (Amsterdam, Netherlands) under a high tension of 25 kV. According to Crawford et al.,[24] biofilms on gallbladder stone surfaces were graded quantitatively. They were graded as ++: biofilm cover an estimated 80-95% of gallstone surface, +: little biofilm present (covering ≈5-20% of gallstone surface), -: absence of biofilm on gallstone surface.

Seropositivity: Virulence (Vi) antibodies were measured using ELISA (VaccZyme™ Binding Site Group Ltd, Birmingham, UK) to detect chronic typhoid carriage in the control group according to the manufacturer's instructions.

Statistical analysis

Data was checked, entered and analyzed using SPSS version 15 for data processing and statistic. Data was expressed as number and percentage for qualitative variables and mean ±standard deviation for quantitative one. P value of < 0.05 indicates significant results. Odds ratio and 95% confidence interval (CI) were calculated.

RESULTS

Demographic, clinical and laboratory data of calcular cholecystitis patients are given in Table 1. Patients included 155 females and 102 males with mean age of 42.17±13.47 years (range 20-66 years). The controls included 140 females and 117 males with mean age of 40.22±13.11 years (range 20-63 years).

Twenty-eight out of 257 (10.9%) patients were chronic typhoid carriers compared to only 9/257 (3.5%) of the controls (Odds ratio=3.37, 95% Confidence Interval = 1.56-7.29, P=0.002). Hence, the total percentage of typhoid carriage in both groups was 37/514 (7.2%). Twenty-eight, 12 and 4 *S. typhi* isolates were from gallstones, gall bladder epithelial tissues and bile; respectively. Nested PCR amplification products of *S. typhi* isolates are demonstrated in Figure 2. Varying percentages of antibiotic resistance patterns of isolates were observed (Table 2).

S. typhi biofilm on the surface of the 21/28 (75%) gallstones were detected. Correlation between biofilm grading and antibiotic susceptibility pattern was presented in Figure 3.

Table 1. Demographic and clinical data of calcular cholecystitis patients.

Demographic data	Calcular cholecystitis patients No. (%)
Age (years)	
20-40	92 (35.8%)
40-60	135 (52.5%)
>60	30 (11.7)
Sex	
Male	102 (39.7%)
Female	155 (60.3%)
Clinical data	
BMI (≥ 25%)	171 (66.5%)
Dyspepsia	240 (93.4%)

Table 2. The percentage of antibiotic resistance pattern of isolates

Antibiotics	Resistance n (%)
Ampicillin	13 (46.4)
Chloramphenicol	12 (42.9)
Co-trimoxazole	11(39.2)
Ciprofloxacin	1 (3.6)
Ceftriaxone	1 (3.6)
SDR	14 (50.0)
MDR	10 (35.7)

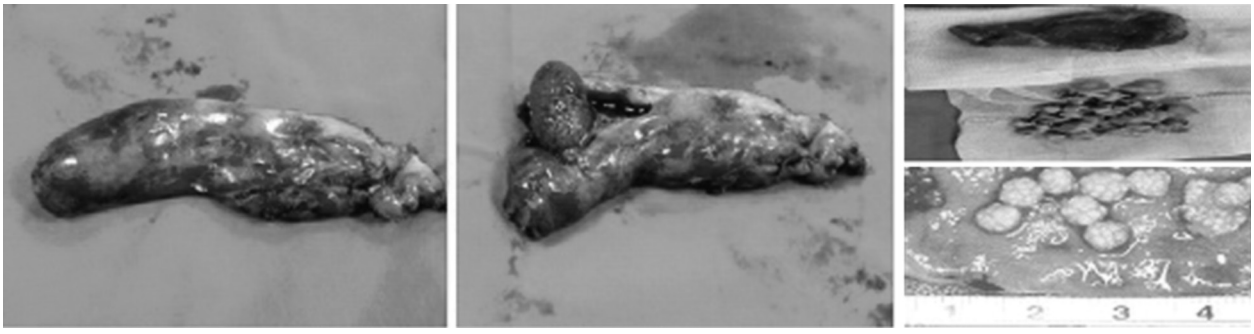

Figure 1. Chronic calcular cholecystitis affected gallbladder and gallstones

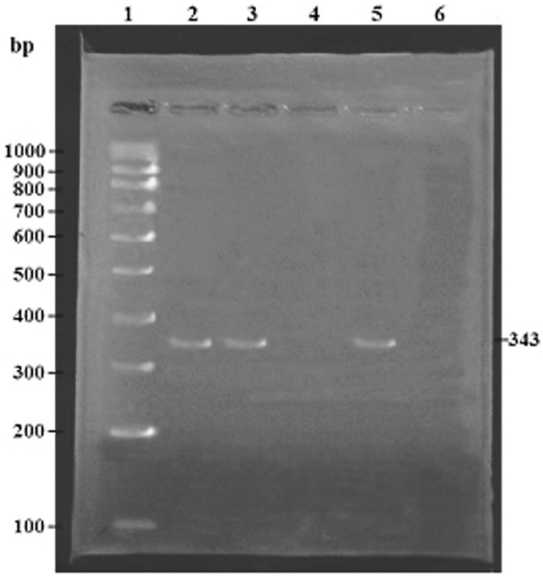

Figure 2. Ethidium bromide stained agarose gel showing results of nested PCR technique for detection of the flagellin gene of *S. typhi*; Lane 1: Molecular weight marker (100-1000bp), lanes 2, 3: Amplification products of 343 bp from the nested PCR, lane 4: No band, lane 5: Positive control, and lane 6: Negative control

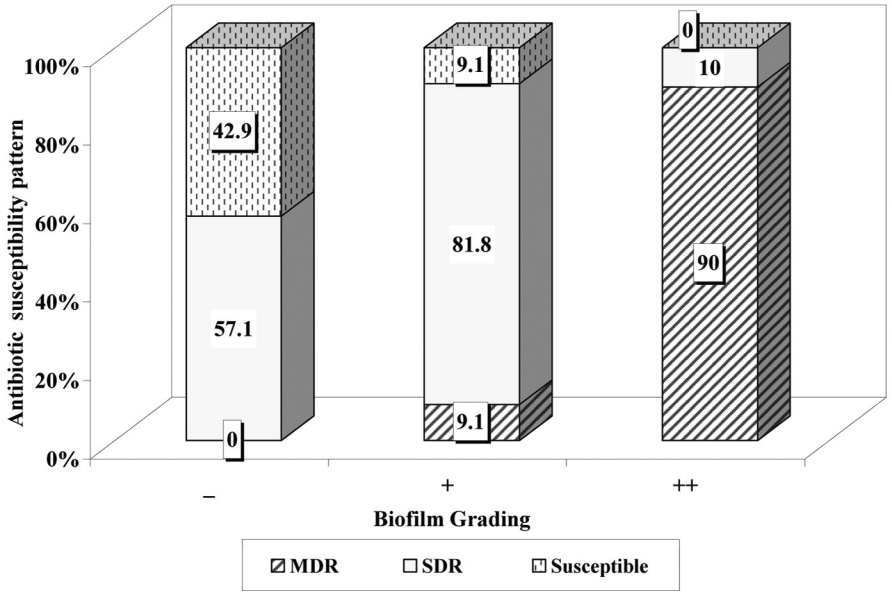

Figure 3. Correlation between biofilm grading and antibiotic susceptibility pattern

DISCUSSION

Individuals with gallstones are more likely to become typhoid carriers in whom antibiotic treatment is often ineffective.[25] In this study, we tried to find the role of gallstones in typhoid carriage in Egypt that has intermediate incidence of typhoid.

In this study, the patients' ages ranged from 20-66 years and the peak incidence was in the fourth decades of life. This finding is in agreement with the statement that chronic cholecystitis is common among the age of forty.[18,26] In addition, the high incidence of females (60.3%) affected by chronic cholecystitis is attributed to estrogens which tend to make hepatic bile more lithogenic.[27]

The current study showed that 66.5% of our patients were obese. This was in accordance with Montasser and El-Sayed who detected this in 70% of their patients. They explained this by increased cholesterol synthesis that predisposes to cholesterol cholelithiasis.[17]

For detection of chronic typhoid carriage, we used conventional methods of identification confirmed by nested PCR for DNA from culture positive isolates as well as gallbladder samples from patients and Vi antibodies in sera of controls. This choice was in accordance to Nath et al.[27] who found that for detection of typhoid carriers, Vi antibodies in serum are comparable to PCR in biliary specimens as no significant difference between *S. typhi* DNA extracted from hepatobiliary specimens from 424 autopsies free from gall bladder pathology on postmortem examination and Vi antibodies in 508 healthy volunteers.

In this study, 10.9% of patients with chronic calcular cholecystitis were typhoid carriers compared to only 3.6% of the controls with a total percentage of typhoid carriers of 7.2% in both groups. Twenty-eight, 12 and 4 gallstones, gall bladder epithelial tissues and bile samples; respectively were positive for *S. typhi*.

This frequency was higher than that of Crawford et al.[28] who investigated this condition in typhoid endemic Mexico City. They reported the presence of *S. typhi* in five of the 103 patients with cholelithiasis (~5% of patient samples). Five, two and one gallstones, gall bladder epithelial tissues and bile; respectively were positive for *S. typhi*.

Moreover, Vaishnavi and co-workers found that 6.9% of patients with gall bladder/common bile duct stones compared to 3.9% of patients with miscellaneous gastrointestinal disorders had Salmonellae in their bile samples after bacteriological investigations.[24] The high prevalence of typhoid fever in Egypt may explain this difference. In addition, it may be as a result of our selection of the studied groups with past history suggestive of typhoid fever due to the expected low prevalence of typhoid carriage.

On the contrary, Montasser and El-Sayed found that bile and gall bladder tissue cultures gave negative results for Salmonella in fifty Egyp-

tian cases with chronic cholecystitis subjected to cholecystectomy[17]. This may be explained by our use of the more sensitive method; nested PCR for detection of *S. typhi*.[29] Also, there was a low prevalence; 4/977 (0.41%) of chronic typhoid infection in case-control study in Shanghai, China.[30]

The highest antibiotic resistance was to ampicillin (46.4%), chloramphenicol (42.9%) and co-trimoxazole (39.2%) which are conventionally used to treat typhoid fever and least resistant to ciprofloxacin (3.6%) and ceftriaxone (3.6%). Similar results where some isolates showed the resistances to these antibiotics were reported.[14,31,32]

Fluoroquinolones (ciprofloxacin) and broad spectrum cephalosporin (cefotaxime) are the most commonly used antimicrobial agents for the treatment of invasive Salmonella infections in man. However, treatment failures due to invasive strains of Salmonella displaying reduced susceptibility have been reported where resistance to ciprofloxacin was found in <2%.[33] In this study out of various *S. typhi* isolates, 35.7% were multidrug resistant and 50.0% were single drug resistant. This was higher than Bhattacharya and others[34] who reported that MDR was found in 12.75% of isolates. However, it was lower than that was reported by Raza et al. 16/30 (53.33%).[35]

Twenty-one out of 28 (75%) gall bladder typhoid carrier patients in our study had *S. typhi* on the surface of gallstones. This was in agreement with Prouty et al. who demonstrated that Salmonella can form biofilms on human gallstones in vitro and that biofilm formation is markedly enhanced in the presence of bile.[12] Also, Crawford et al.[28] performed SEM for *S. typhi* biofilm on the surface of the gallstones revealed that gallstones from three out of four of these patients exhibited a dense bacterial biofilm. The gallstone from the patient who lacked biofilm formation was thought to be a pigment stone, with calcium bilirubinate and not cholesterol as the main constituent.

In this current study, 90% of the strains that produced maximum amount of biofilm were MDR. This in accordance with that of Raza et al. [35] who detected that in 81.82% of MDR isolates.

Conclusion: These data correlate with previous in vivo observations and support the hypothesis that chronic carriage of *S. typhi* is mediated by biofilm formation on gallstones. In addition, they demonstrate the presence of a subset of healthy carriers in an endemic population and highlight the importance of the development of methods to identify and successfully treat such patients.

Investigation of cholesterol biofilm inhibitors could lead to the development of promising therapies to eliminate typhoid carriage, especially in areas of high endemicity. In addition, meticulous use of antibiotic may contribute to the efforts of limiting the spread of MDR isolates.

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