Dongol, S; Thompson, CN; Clare, S; Nga, TV; Duy, PT; Karkey, A; Arjyal, A; Koirala, S; Khatri, NS; Maskey, P; Poudel, S; Jaiswal, VK; Vaidya, S; Dougan, G; Farrar, JJ; Dolecek, C; Basnyat, B; Baker, S (2012) The microbiological and clinical characteristics of invasive salmonella in gallbladders from cholecystectomy patients in kathmandu, Nepal. PloS one, 7 (10). e47342. ISSN 1932-6203 DOI: https://doi.org/10.1371/journal.pone.0047342

Downloaded from: http://researchonline.lshtm.ac.uk/1878100/

DOI: 10.1371/journal.pone.0047342

Usage Guidelines

Please refer to usage guidelines at http://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/
The Microbiological and Clinical Characteristics of Invasive *Salmonella* in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal

Sabina Dongol¹, Corinne N. Thompson²,³, Simon Clare⁴, Tran Vu Thieu Nga⁵, Pham Thanh Duy⁶, Abhilasha Karkey¹, Amit Arjyal¹, Samir Koirala¹, Nely Shrestha Khatri¹, Pukar Maskey⁶, Sanjay Poudel⁵, Vijay Kumar Jaiswal⁵, Sujan Vaidya⁶, Gordon Dougan⁴, Jeremy J. Farrar²,³, Christiane Dolecek²,³, Buddha Basnyat¹, Stephen Baker²,³*

¹ Oxford University Clinical Research Unit, Patan Academy of Health Sciences, Kathmandu, Nepal, ² The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam, ³ Centre for Tropical Medicine, Oxford University, Oxford, United Kingdom, ⁴ The Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom, ⁵ Patan Hospital, Kathmandu, Nepal

Abstract

Gallbladder carriage of invasive *Salmonella* is considered fundamental in sustaining typhoid fever transmission. Bile and tissue was obtained from 1,377 individuals undergoing cholecystectomy in Kathmandu to investigate the prevalence, characteristics and relevance of invasive *Salmonella* in the gallbladder in an endemic area. Twenty percent of bile samples contained a Gram-negative organism, with *Salmonella Typhi* and *Salmonella Paratyphi A* isolated from 24 and 22 individuals, respectively. Gallbladders that contained *Salmonella* were more likely to show evidence of acute inflammation with extensive neutrophil infiltrate than those without *Salmonella*, corresponding with higher neutrophil and lower lymphocyte counts in the blood of *Salmonella* positive individuals. Antimicrobial resistance in the invasive *Salmonella* isolates was limited, indicating that gallbladder colonization is unlikely to be driven by antimicrobial resistance. The overall role of invasive *Salmonella* carriage in the gallbladder is not understood; here we show that 3.5% of individuals undergoing cholecystectomy in this setting have a high concentration of antimicrobial sensitive, invasive *Salmonella* in their bile. We predict that such individuals will become increasingly important if current transmission mechanisms are disturbed; prospectively identifying these individuals is, therefore, paramount for rapid local and regional elimination.

Introduction

Enteric fever is a systemic infection caused by the invasive bacteria *Salmonella Typhi* (S. Typhi) and *Salmonella Paratyphi A* (S. Paratyphi A). The disease is contracted by the ingestion of fecal material containing the pathogens [1]. The disease remains common in regions with poor standards of hygiene and sanitation, with global estimates suggesting that 27 million people are affected annually, of which 200,000 people die [2]. With adequate treatment >95% of patients recover completely from typhoid [1]. However, an estimated 2-5% of individuals infected with S. Typhi develop a sustained infection of the gallbladder [3]. These individuals are referred to as ‘carriers’, and like the infamous ‘typhoid Mary’ [4], are outwardly asymptomatic, continue to intermittently shed organisms for a prolonged period and often have no recollection of an acute episodes of typhoid [1].

During acute typhoid, invasive *Salmonella* cross the intestinal epithelial barrier, invade and survive within macrophages, eventually reaching the bone marrow, liver, pancreas and spleen [5]. Invasion of the gallbladder occurs either directly from the blood or by retrograde spread from the bile [1]. In a subset of individuals infected with S. Typhi, the organisms chronically colonize the gallbladder and carriers shed these organisms intermittently into the intestinal lumen and thus in the feces. It is gallbladder colonization and fecal shedding that form a central dogma for the transmission and persistence of typhoid fever. As a consequence of the internal localization of organisms, this dogma is difficult to challenge in humans and the host-restricted nature of the relevant pathogens make carriage difficult to replicate precisely in non-mutant mouse models [6]. As a result, data regarding the prevalence, bacteriology and mechanisms of carriage are sparse. The only population-based study estimating chronic *Salmonella* carriage in an endemic setting is from Chile where investigators gathered data from autopsies, calculating a carriage rate of 694 per 100,000 [3].

Investigations of *Salmonella* carriage suggest that the propensity to become a chronic carrier follow the typical epidemiology of gallbladder disease. Thus, the likelihood of carriage increases with age and is more common in females [7]. Existing data also imply that individuals with gallstones or other gallbladder abnormalities are at increased risk of carriage [8]. These epidemiological theories
are supported by laboratory-based investigations, which have shown that Salmonella can form biofilms and survive for prolonged period on gallstones [9,10].

There remains a significant burden of typhoid fever across Asia, yet the understanding of Salmonella carriage in these populations is limited. We have found previously that Salmonella play a pivotal role in the persistence of these pathogens in Kathmandu, Nepal [11]. We aimed to define the microbiology and epidemiology of invasive Salmonella carriage in Kathmandu. We demonstrate that S. Typhi and S. Paratyphi A are present in the gallbladder in a high concentration, are less common than other Gram-negative organisms, are not associated with lymphocytic infiltration in the gallbladder tissue, and do not exhibit resistance to multiple antimicrobials.

Results

Microbiological Examination of Bile from Cholecystectomy Patients

From June 2007 until October 2010, a total of 1,496 patients underwent cholecystectomy for acute or chronic cholecystitis at Patan hospital in Kathmandu. From the 1,496 patients, bile samples from 1,377 individuals were obtained and subjected to microbiological examination; 119 (8%) patients either denied consent or were unavailable for recruitment. A Gram-negative microbiological examination; 119 (8%) patients either denied consent or were unavailable for recruitment. A Gram-negative organism was isolated from 20% (274/1,377) of the bile samples. E. coli, Salmonella spp. and Escherichia coli were the most commonly isolated organisms, found in 78 (5.7%), 48 (3.5%) and 41 (3.0%) of the bile samples, respectively (Table 1). The remainder of the culture positive bile samples contained a range of organisms including Pseudomonas spp., Acinetobacter spp., Enterobacter spp., Citrobacter freundii, Vibrio spp. and Seratia marcescens (Table 1). Of the 48 Salmonella isolated, 24 (1.7%) were S. Typhi, 22 (1.6%) were S. Paratyphi A and two (0.1%) were S. enterica group C.

Forty-six Salmonella isolates were available for antimicrobial susceptibility testing by disc diffusion. Fifty-nine percent (27/46) of the Salmonella isolates were resistant to nalidixic acid, and a single S. Paratyphi A isolate was resistant to both nalidixic acid and ciprofloxacin. All S. Typhi and S. Paratyphi A strains were susceptible to ceftriaxone, chloramphenicol, gatifloxacin and ofloxacin and we identified no multi-drug resistant (MDR) (resistant to chloramphenicol, ampicillin and co-trimoxazole) isolates. One S. enterica group C isolate demonstrated resistance to nalidixic acid, ceftriaxone, gatifloxacin and chloramphenicol (Table 1).

Baseline data, stratified by microbiological culture result are shown in Table 2. Notably, fitting with the typical epidemiological characteristics of cholelithiasis, 77% (1,066/1,377) of the patients were female and the median age was 39 years (range: 16 to 76 years). The median age of those with positive bile cultures from any patients were Salmonella positive and, when questioned, only 15% (7/46) of the Salmonella bile-positive patients had a memorable history of typhoid, none of which had been confirmed by microbiological culture. From available records, 16% (7/43) of Salmonella bile-positive patients reported >5 days of fever on entry, 7% (3/46) were admitted with jaundice, 5% (2/41) had a palpable gallbladder and 4% (2/45) were admitted with pancreatitis.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Patients</th>
<th>Antimicrobial resistance n (%)</th>
<th>Aminoglycoside</th>
<th>Chloramphenicol</th>
<th>Ciprofloxacin</th>
<th>Gentamicin</th>
<th>Amikacin</th>
<th>Nalidixic Acid</th>
<th>Ofloxacin</th>
<th>Chloramphenicol</th>
<th>Cotrimoxazole</th>
<th>Gentamycin</th>
<th>Amikacin</th>
<th>Nalidixic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhi</td>
<td>24 (1.7)</td>
<td></td>
<td>0.23 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
<td>0.24 (0)</td>
</tr>
<tr>
<td>Paratyphi A</td>
<td>22 (1.6)</td>
<td></td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
<td>0.22 (0)</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>21 (1.5)</td>
<td></td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
<td>0.21 (0)</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>19 (1.4)</td>
<td></td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
<td>0.19 (0)</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>17 (1.2)</td>
<td></td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
<td>0.17 (0)</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>16 (1.2)</td>
<td></td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
<td>0.16 (0)</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>13 (0.9)</td>
<td></td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
<td>0.13 (0)</td>
</tr>
<tr>
<td>Other</td>
<td>34 (2.5)</td>
<td></td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
<td>0.34 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>226 (16.4)</td>
<td></td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
<td>226 (16.4)</td>
</tr>
<tr>
<td>Non-Salmonella</td>
<td>103 (7.5)</td>
<td></td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
<td>103 (7.5)</td>
</tr>
</tbody>
</table>
Bacterial Load of Salmonella in Bile

To quantify the bacterial load in the bile, real-time PCR was performed on total nucleic acid extracted from the bile of six *S. Paratyphi A* positive individuals and 12 *S. Typhi* positive individuals. All qualitative serovar specific PCR data corresponded precisely with the culture data. The median target copy numbers/bacterial loads were $9.3 \times 10^4$ (IQR $5 \times 10^4$–$2.3 \times 10^5$) CFU/ml for *S. Paratyphi A* and $5.2 \times 10^4$ (IQR $2 \times 10^4$–$7.28 \times 10^5$) CFU/ml for *S. Typhi*. The difference in bacterial load between the two organisms was non-significant ($p=0.93$, Mann-Whitney U test), yet, were approximately two and three orders of magnitude greater than those previously reported in bone marrow and blood, respectively [12,13].

Hematological and Biochemical Characteristics

The 1,377 individuals undergoing cholecystectomy were divided into three groups on the basis of their bile culture results: *Salmonella* positive, culture negative, and culture positive for non-*Salmonella*. Individuals that were *Salmonella* positive were more likely to have experienced continuous right upper-quadrant pain (10%, 5/48) compared to those that were culture negative (3%, 37/1,151) ($p=0.008$, chi squared test) and those that were culture positive for non-*Salmonella* (2%, 5/214) ($p=0.008$, chi squared test). Hematology and biochemistry data from the patients were compared using the Mann-Whitney U test (Table 3). There was no significant difference in liver enzyme or bilirubin levels between the *Salmonella* positive group and the other two groups. Yet, the *Salmonella* positive group had a higher median neutrophil count and a lower median lymphocyte count than the culture negative group and the culture positive non-*Salmonella* group (Table 3).

Surgical and Histopathological Characteristics

The major surgical and post-surgical characteristics of the gallbladders from the three groups were compared using Fisher’s exact test (Table 4). The majority of *Salmonella* positive individuals had gallstones (96%, 46/48); yet, there was no significant difference in the proportion of individuals with gallstones between the three groups. We did, however, identify several gallbladder characteristics that were associated with the presence of *Salmonella*. Namely, gallbladder distension and inflammation was more frequently observed in the *Salmonella* positive group than the culture negative group and the non-*Salmonella* culture positive group (Table 3). Furthermore, the presence of an empyema (pus within the gallbladder cavity) was also more common in the *Salmonella* positive group than the other two groups. Inflammation was more likely to be due to polymorphonuclear infiltration than lymphocytic infiltration in the *Salmonella* infected gallbladder tissue, with 13% (6/48) of the *Salmonella* positive gallbladder specimens having massive neutrophil infiltrate near the lumen, compared to 4% (51/1,151) and 5% (10/214) of the culture negatives and the non-*Salmonella* culture positives, respectively. Furthermore, an additional 15% (7/48) of the *Salmonella* positive gallbladder specimens had acute-on-chronic cholecystitis (neutrophil infiltrate near the lumen with lymphocyte infiltrate and dysplasia in the mucosa) compared to 5% (10/214) and 7% (14/214) of the culture negatives and the non-*Salmonella* culture positives, respectively (Table 3). Correspondingly, chronic inflammation without large neutrophil infiltrate was not observed in gallbladder tissue from the *Salmonella* positive group.

Discussion

The mechanism of gallbladder infection/colonization remains contentious, and it is unknown if *Salmonella* promote gallbladder...
damage during chronic infection or if the organisms exploit existing gallbladder damage to stimulate colonization [14]. Bile is typically sterile, and consists of organic and inorganic compounds, bile acids, cholesterol, phospholipids and the pigment biliverdin. Sterility is partially maintained by the secretion of IgA and mucus, preventing bacterial survival and adhesion to the surface of the lumen and the major bile duct, respectively [15]. Here, we found a wide array of organisms in the bile of individuals undergoing cholecystectomy, some of which have been previously isolated from the gallbladder [16–18]. Again, whether these organisms invade the gallbladder lining or are simply transient inhabitants, their presence may influence the outcome of the disease process.

Table 3. The haematological and biochemical characteristics of the Salmonella positive, culture negative and the culture positive for non-Salmonella bile culture groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Culture negative</th>
<th>Culture positive non-Salmonella</th>
<th>Salmonella positive</th>
<th>p1*</th>
<th>p2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell (x10⁶/μL)</td>
<td>n = 953</td>
<td>median 7.9, IQR 6.6–9.5</td>
<td>188</td>
<td>7.85, IQR 6.45–10.15</td>
<td>42</td>
</tr>
<tr>
<td>Neutrophil (x10⁶/μL)</td>
<td>917</td>
<td>66, 58–74</td>
<td>177</td>
<td>65, 58–75</td>
<td>41</td>
</tr>
<tr>
<td>Lymphocyte (x10⁶/μL)</td>
<td>914</td>
<td>31, 24–38</td>
<td>175</td>
<td>31, 23–38</td>
<td>40</td>
</tr>
<tr>
<td>Monocyte (x10⁶/μL)</td>
<td>270</td>
<td>1, 1–2</td>
<td>70</td>
<td>1.5, 1–2</td>
<td>9</td>
</tr>
<tr>
<td>Eosinophil (x10⁶/μL)</td>
<td>641</td>
<td>2, 1–4</td>
<td>131</td>
<td>2, 1–4</td>
<td>22</td>
</tr>
<tr>
<td>Basophil (x10⁶/μL)</td>
<td>54</td>
<td>0, 0–1</td>
<td>14</td>
<td>0, 0–0</td>
<td>1</td>
</tr>
<tr>
<td>Total bilirubin (mg/mL)</td>
<td>965</td>
<td>0.8, 0.68–1</td>
<td>190</td>
<td>0.8, 0.7–1</td>
<td>42</td>
</tr>
<tr>
<td>Conjugated bilirubin (mg/mL)</td>
<td>950</td>
<td>0.2, 0.19–0.26</td>
<td>186</td>
<td>0.2, 0.18–0.24</td>
<td>41</td>
</tr>
<tr>
<td>AST (u/L)</td>
<td>961</td>
<td>30, 23–41</td>
<td>190</td>
<td>29.5, 23–40</td>
<td>42</td>
</tr>
<tr>
<td>ALT (u/L)</td>
<td>960</td>
<td>30, 21.9–43</td>
<td>188</td>
<td>29.5, 21–43.5</td>
<td>42</td>
</tr>
<tr>
<td>ALP (u/L)</td>
<td>941</td>
<td>122, 82–209</td>
<td>188</td>
<td>150.5, 94.5–232.5</td>
<td>42</td>
</tr>
<tr>
<td>Amylase (u/L)</td>
<td>136</td>
<td>61.5, 41.5–252.5</td>
<td>20</td>
<td>57.5, 38–86.5</td>
<td>9</td>
</tr>
</tbody>
</table>

*pMann-Whitney U test, boldface indicates p<0.05.

Table 4. The gallbladder characteristics within the Salmonella positive, culture negative and the culture positive for non-Salmonella bile culture groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Culture negative</th>
<th>Culture positive non-Salmonella</th>
<th>Salmonella positive</th>
<th>p1*</th>
<th>p2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallbladder tissue thickness</td>
<td>n = 1,103</td>
<td>n = 214</td>
<td>n = 48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thick (&gt;4 mm)</td>
<td>173 (15.7)</td>
<td>36 (16.8)</td>
<td>10 (20.8)</td>
<td></td>
<td>0.350</td>
</tr>
<tr>
<td>Normal (4 mm)</td>
<td>493 (44.7)</td>
<td>86 (40.0)</td>
<td>21 (43.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thin (&lt;4 mm)</td>
<td>74 (6.7)</td>
<td>12 (5.6)</td>
<td>1 (2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallbladder size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contracted</td>
<td>108 (9.8)</td>
<td>25 (11.7)</td>
<td>1 (2.1)</td>
<td></td>
<td>0.026</td>
</tr>
<tr>
<td>Distended</td>
<td>221 (20.0)</td>
<td>52 (24.3)</td>
<td>15 (31.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall stones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>19 (1.7)</td>
<td>6 (2.8)</td>
<td>3 (6.3)</td>
<td></td>
<td>0.101</td>
</tr>
<tr>
<td>Single</td>
<td>344 (31.2)</td>
<td>62 (29.0)</td>
<td>14 (29.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>684 (62.0)</td>
<td>133 (62.1)</td>
<td>28 (58.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>93 (8.4)</td>
<td>17 (7.9)</td>
<td>8 (16.7)</td>
<td></td>
<td>0.046</td>
</tr>
<tr>
<td>Empyema</td>
<td>90 (8.2)</td>
<td>21 (8.8)</td>
<td>10 (20.8)</td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>Sludge</td>
<td>57 (5.2)</td>
<td>8 (3.7)</td>
<td>1 (2.1)</td>
<td></td>
<td>0.338</td>
</tr>
<tr>
<td>Mucocele</td>
<td>50 (4.5)</td>
<td>7 (3.3)</td>
<td>1 (2.1)</td>
<td></td>
<td>0.427</td>
</tr>
</tbody>
</table>

*Fisher’s exact test, boldface indicates p<0.05.

p1: Comparing culture-negative to Salmonella-positive patients.
p2: Comparing culture-positive, Salmonella-negative to Salmonella-positive patients.
doi:10.1371/journal.pone.0047342.t003

doi:10.1371/journal.pone.0047342.t004

Invasive Salmonella in the Human Gallbladder
functionally stimulate cholecystitis or cholelithiasis, or merely have
the ability to colonize damaged gallbladders, remains unclear. Our
data confirm that non-Salmonellae organisms, with a spectrum of
pathogenic potential, are as equally adept at colonizing the
gallbladder and surviving within the bile as typhoidal Salmonella.
Yet, non-Salmonellae appear not to stimulate the same pathology as
Salmonella; Salmonella infected tissue was more commonly associated
with systemic and local acute inflammatory responses. Mouse
experiments, utilizing Salmonella Typhimurium, have shown that
Salmonella can replicate within the epithelial cells of the gallbladder
[19], and that colonized gallbladders displayed evidence of the
epithelial destruction and local neutrophil infiltrate. Here, we also
find extensive neutrophil infiltrate, yet are unable to confirm if the
bacteria are damaging the tissue or colonizing previously damaged
tissue. However, as shown by an increased prevalence of
gallbladder distention, right upper quadrant pain, empyema and
a raised systemic neutrophil count, there is an evident association
of invasive Salmonella in the gallbladder with an acute inflamma-

tory response.

We have previously noted the presence of individuals in
Kathmandu with Salmonella in their gallbladder, highlighting the
Paratyphi A has received much less attention than that of S. Typhi
and it is unknown as to what extent chronic gallbladder carriage is
contributing to the increasing burden of S. Paratyphi A across
many parts of Asia [20]. Enteric fever caused by S. Paratyphi A
increased from 17.5% (155/883) in 1993 to 34% (926/2,718) in
2003 in the location of this study [21].

Furthermore, we found an almost equal ratio of S. Typhi and
S. Paratyphi A (1:0.9) isolated from bile, yet the isolates from blood
can be attributed from patients over the same period is lower (1:0.4)
[22]. This disparity may result from a multitude of factors, but
may predict that S. Paratyphi A is more adept at inducing carriage
in this population, or, once in the gallbladder, may be more likely
to induce an acute inflammatory response, requiring a surgical
intervention, than S. Typhi.

We found that 3.5% of the individuals undergoing gallbladder
surgery had invasive Salmonella in their bile in this area with a high
incidence of enteric fever [23]. A report from a similar patient
demographic in India suggest an equivalent rate of <5%, and in
Chile, 7.3% of bile cultures were found to be positive for Salmonella
[7]. The long-term carriage of invasive Salmonella in the gallbladder
is thought to be central to the maintenance and transmission of
these human-restricted pathogens [14]. However, data from our
previous work in Kathmandu suggests that direct transmission
drives a negligible role in acute infections, and we have
hypothesized that carriers merely act as a reservoir for maintaining
local strain diversity in areas of high endemicity [22]. Here, we
found antimicrobial resistance to only nalidixic acid in the
Salmonella from the gallbladder. Although nalidixic acid resistance
often precedes resistance to other fluoroquinolones, these isolates
were susceptible to gatifloxacin and ofloxacin. Firstly, these data
show that infection with an antimicrobial resistant organism is not
likely to be associated with Salmonella carriage. Secondly, if one
considers nalidixic acid resistance as a proxy marker of contem-
porary strains, the organisms in the gallbladder have probably
been there for some time (i.e. from a period when nalidixic acid
resistance was less prevalent) [21,24]. Nalidixic acid resistance is
a growing problem in Kathmandu. From an ongoing clinical trial
enrolling enteric fever patients over the last two years at Patan
Hospital, 80% (171/214) of invasive Salmonella isolates demon-
strated resistance to nalidixic acid, which is greater than the
proportion (59%) found from bile isolates in the current study
(unpublished data). This evidence supports our current hypothesis
of gallbladder carriage playing a limited role in the acute
transmission of typhoid in Kathmandu.

Whilst we argue that in locations such as Kathmandu, the role
of carriers in typhoid fever transmission may be negligible, it is
reasonable to suggest that those shedding invasive Salmonella play
a vital important role in low transmission setting. In the USA, up
to 30% of typhoid fever infections are anticipated to result from
contact with a chronic carrier [25]. Therefore, these individuals
will become increasingly important as indirect transmission this
area begins to subside after the introduction of an effective
intervention strategy. However, currently there is no appropriate
diagnostic test for the detection of long-term carriers [26]. Bile
cultures from string devices are considered effective [27], but are
impractical for screening large cohorts [28]. The presence of
gallbladder disease is, perhaps, currently the best clinical predictor
carriage of invasive Salmonella [7]. However, it remains unclear
to why some patients progress to become chronic shedders and
others do not. The development of a rapid diagnostic for the
detection of invasive Salmonella carriage should accelerate regional
elimination of typhoid and add insight into the epidemiological
role of these individuals.

One of the major caveats of our study that limits the
generalizability of our findings is the fact that our passively
acquired patient population may not accurately reflect the general
population of Kathmandu. Additionally, all patients in the study
had some form of gallbladder abnormality, although it is unclear
whether such abnormalities had been induced by the infecting
organisms. Nevertheless, in the absence of an alternative
methodology, our study represents a reasonable estimation of
the burden and mechanism of invasive Salmonella carriage.

In conclusion, we have calculated a prevalence of 3.5% of
invasive Salmonella in bile from patients undergoing cholecystec-
tomy in Kathmandu, Nepal. We demonstrate that S. Paratyphi A
is almost as prevalent as S. Typhi in the gallbladder in this
population and that carriage is not driven by antimicrobial
resistance. The overall role of invasive Salmonella carriage in
settings such as Kathmandu is not understood, and we suggest that
organisms in the gallbladder may not play a dominant role in
acute typhoid fever in this location. We predict, however, that
carriers will become more important if current transmission
mechanisms are disturbed; prospectively identifying these indivi-
duals is paramount for rapid local and regional elimination.

Methods

Ethics Statement

This study was conducted according to the principles expressed
in the Declaration of Helsinki and was approved by the
institutional ethical review boards of Patan Hospital, The Nepal
Health Research Council and The Oxford University Tropical
Research Ethics Committee (OXTREC, Reference number:
2108). All enrollees were required to provide written informed
consent for the collection and storage of all samples and
subsequent data analysis. In the case of those under 18 years of
age, a parent or guardian was asked to provide written informed
consent.

Setting and Study Population

The study was conducted at Patan Hospital, a 318-bed
government hospital located in the Lalitpur Sub-Metropolitan
City in the Kathmandu valley, Nepal. Patan Hospital provides
both emergency and elective inpatient services. Typhoid fever is
a common complaint at Patan Hospital and S. Typhi and
S. Paratyphi A are the most common bacteria cultured from blood
of febrile patients in this location. Antimicrobials are available without prescription in the community in a variety of public and private outlets and there are numerous private physician clinics where patients may seek advice and clinical diagnosis for febrile disease. There has been no widespread implementation of a typhoid vaccine in this area, yet a generic typhoid Vi vaccine is available for purchase in some health care settings. However, at the time of this investigation there was limited community uptake of the vaccine.

The surgical department performs approximately 400 cholecystectomies annually. For the purposes of this study, consecutive patients admitted to the surgical ward from June 2007 to October 2010 for either open cholecystectomy or laparotomy surgery for symptomatic cholelithiasis between 0 am and 4 pm were approached for participation. All patients who gave written informed consent were eligible for the study; there were no exclusion criteria. All enrollees were also required to provide written informed consent for the collection, use and storage of the tissue removed during surgery samples. A questionnaire related to the patient’s health and demographics was administered prior to surgery along with a stool sample for microbiological culture. Surgeons collected bile samples and gallbladder tissue during the procedure.

Gallbladder Morphology and Histopathology

Patients were routinely examined by ultrasonography before surgery to assess the presence of gallstones and to detect inflammation. The surgeon performing the procedure curated a report, assessing the thickness of the gallbladder wall (stratified into three categories, thick: >4 mm, normal: 4 mm and thin: <4 mm), the presence and the number of gallstones, the presence and characteristics of fluid (pus: empyema, mucoid/clear/watery: mucocoele and sludge) and overall morphology (contracted or distended). Hematocrit, total leukocytes with differential count, total bilirubin, conjugated bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and amylase were measured prior to surgical intervention. All extracted tissue was subjected to a histopathological examination to assess/confirm the inflammation. The surgeon performing the procedure curated criteria. All enrollees were also required to provide written informed consent for the collection, use and storage of the tissue removed during surgery samples. A questionnaire related to the patient’s health and demographics was administered prior to surgery along with a stool sample for microbiological culture. Surgeons collected bile samples and gallbladder tissue during the procedure.

Microbiological Culture, Antimicrobial Susceptibility Testing and Real-time PCR

Bile and stool were collected for culture from all patients undergoing cholecystectomy. Bile was inoculated into equal volumes of Selenite F broth and Peptone broth and incubated at 37°C overnight. Broths were sub-cultured onto MacConkey agar and Xylene Lysine Deoxycholate (XLD) agar. After overnight incubation at 37°C the plates were examined for the growth of Gram-negative bacteria and colonies were identified by standard microbiological methods and identified by API20E manufactured by bioMérieux, Inc. S. Typhi and S. Paratyphi A isolates were confirmed by slide agglutination by specific antisera (Murex Biotech, Biotech, England). The antimicrobial sensitivity profile was performed by Kirby Bauer disc diffusion method using standard BSAC and CLSI guidelines [29]. The antimicrobials tested were amoxicillin, chloramphenicol, co-trimoxazole, nalidixic acid, ciprofloxacin, oxolinic acid and azithromycin by E-test (AB Biodisk, Sweden). Susceptibility to ciprofloxacin and oxolinic acid were evaluated using newly suggested susceptibility breakpoints for these antimicrobials; ≥0.125 μg/ml and ≥0.25 μg/ml for ciprofloxacin and oxolinic acid respectively [30]. Real-time PCR was performed using a standard curve for quantitation as previously described [12], using DNA extracted from 200μl bile samples as template.

Data Analysis

Data were entered into a database using Excel 2007 (Microsoft) and analyzed using Stata/IC version 9.2 (StatCorp, TX, USA). Chi-square and Fisher’s exact tests were used to compare proportions between groups and Mann-Whitney U tests were used for continuous non-parametric data. P-values ≤0.05 were considered to be statistically significant.

Acknowledgments

We wish to acknowledge the ongoing efforts of the microbiology laboratory and the surgical support staff as well as the histopathology department, who were essential for the completion of this study.

Author Contributions

Conceived and designed the experiments: SD SB. Performed the experiments: SC TVTN PTD PM SP VJKY SV. Analyzed the data: SD CT. Contributed reagents/materials/analysis tools: AK AA SK NSK GD JF CD BB. Wrote the paper: SB SD CT.

References