Salmonella L-forms: formation in human bile in vitro and isolation culture from patients’ gallbladder samples by a non-high osmotic isolation technique

D. N. Wang1, W. J. Wu2,3, T. Wang2, Y. Z. Pan4, K. L. Tang5, X. L. She2, W. J. Ding2,3 and H. Wang2

1) Department of Medical Microbiology and Parasitology, Institutes of Medical Science, Shanghai Jiao Tong University School of Medicine, Shanghai, 2) Department of Medical Microbiology, Guiyang Medical University, 3) Guiyang City Maternal and Child Health Care Hospital, 4) Department of Surgery, Affiliated Hospital of Guiyang Medical University and 5) Department of Surgery, Guizhou Province People’s Hospital, Guiyang, China

Abstract

Bacterial L-forms have always been considered as osmotic-pressure-sensitive cell-wall-deficient bacteria and isolation culture of L-forms must use media with high osmotic pressure. However, isolation culture of stable L-forms formed in humans and animals is very difficult because they have adapted to the physiological osmotic pressure condition of the host. We use a non-high osmotic isolation technique to isolate stable L-forms of Salmonella Typhi and Salmonella Paratyphi A from bile-inducer cultures in vitro and from patients’ gallbladder specimens. Multiplex PCR assay for Salmonella-specific genes and nucleotide sequencing are used to identify the Salmonella L-forms in stable L-form isolates. Using this method, we confirmed that Salmonella Paratyphi A and Salmonella Typhi cannot be isolated from bile-inducer cultures cultured for 6 h or 48 h, but the L-forms can be isolated from 1 h to 45 days. In the 524 gallbladder samples, the positive rate for bacterial forms was 19.7% and the positive rate for Salmonella spp. was 0.6% by routine bacteriological methods. The positive rate for bacterial L-forms was 75.4% using non-high osmotic isolation culture. In the L-form isolates, the positive rate of invA gene was 3.1%. In these invA-positive L-form isolates, four were positive for the invA and flic-d genes of Salmonella Typhi, and ten were positive for the invA and flic-a genes of Salmonella Paratyphi A.

Keywords: Bacterial L-form, biliary diseases, gene assay, Salmonella Paratyphi A, Salmonella Typhi

Original Submission: 5 November 2014; Revised Submission: 15 December 2014; Accepted: 16 December 2014

Editor: D. Raoult
Article published online: 26 December 2014

Introduction

Bacteria can form L-forms in bacteriological media and in humans and animals either by induction or spontaneously. As a result, these variants exist widely both in natural environments and in humans and animals [1–5]. However, little is known about how the L-forms can be isolated from biological samples, and other samples, using non-high osmotic pressure. It has always been considered that the L-form can only grow in chemically undefined complex media with high osmotic pressure and high serum concentrations. As a result, isolation culture of a stable L-form in pure culture has been very difficult, even impossible, and the L-forms derived from high-osmotic-pressure-sensitive bacteria cannot grow in these media. Salmonella Typhi and Salmonella Paratyphi A are the most common bacterial species within the genus Salmonella and cause gastrointestinal infections worldwide. They can invade the human gallbladder and play an important role in cholecystitis, cholelithiasis and some serious diseases [6–11]. However, isolation culture of Salmonella spp. from gallbladder specimens...
has always shown a very low positive rate using routine bacterial isolation culture methods [6,7]. When Salmonella Typhi was inoculated into the gallbladder of an experimental animal, after 7 days the bacteria can spontaneously form the stable L-form [12]. In this study we present a new isolation culture technique used for isolation culture of stable L-forms. We use this method to isolate the stable L-forms from human bile-inducer cultures of Salmonella Typhi and Salmonella Paratyphi A in vitro and patients’ gallbladder specimens.

**Methods**

**Bacterial strains and media**

The strains of Salmonella Typhi H901 and Salmonella Paratyphi A are stored in our laboratory. Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 were obtained as lyophilized cultures from the National Institutes for the Control of Pharmaceutical and Biological Products. L-form egg medium (LEM) plates, an L-form medium with high osmotic pressure, were prepared in our laboratory according to previously described methods [13]: containing 10.0 g polypeptone (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 5.0 g yeast extract (Tianhe Microbe Reagent Co., Ltd, Hangzhou, China), 5.0 g glucose, 50.0 g NaCl, 8.0 g agar, 50 mL egg white, 20 mL 50% egg yolk salt solution and 1.0 L distilled water. The peptone-glucose (PG) liquid medium (non-high osmotic medium) was prepared in our laboratory according to previously described methods [15]: containing 10.0 g polypeptone, 5.0 g yeast extract, 5.0 g glucose, 5.0 g NaCl, and 1.0 L distilled water, prepared by our laboratory. Blood agar plates were prepared with nutrient agar (Tianhe Microbe Reagent Co., Ltd) and 10% take off the fiber sheep blood in our laboratory. SS agar and the Sabouraud agar media were obtained from Hangzhou Tianhe Microbe Reagent Co., Ltd and were prepared as agar plates according to the manuals in our laboratory.

**Collection of bile samples**

Bile specimens from patients with biliary disease were collected by cholecystectomy at the Affiliated Hospital of Guiyang Medical College and the Guizhou Province People’s Hospital in China (permit number: TZJF-2011-26). Bile samples of 0.1 mL were inoculated into the blood agar plates and SS agar plates and incubated under normal air conditions at 37°C for isolation of aerobic bacteria and Salmonella spp.; into the LEM plates and incubated in 5–10% CO₂ air conditions at 37°C for isolation culture of bacterial L-forms adapted to high osmotic pressure conditions; into Sabouraud agar plates and incubated under normal air conditions at 37°C for isolation culture of Candida spp.; and into PG liquid medium and incubated under normal air conditions at 37°C for isolation culture of the L-forms adapted to non-high osmotic pressure conditions. Antimicrobial activities of bile samples against Staphylococcus aureus ATCC 25923 and E. coli ATCC 25922 were tested respectively by the agar-well diffusion method [14].

**Preparation of the bile inducer**

Five samples of bile with microbe-negative culture and without antimicrobial activity were mixed together, the mixture was centrifuged at 488 g for 5 min and then the supernatant liquid was run through a filter with a pore size of 0.22 μm. Forward (F) and reverse (R) primers were as follows, F: 5’-GTGAAAATTACGGCAACGTGGC-3’ and R: 5’-TCATCGACGTCGAAAGGAACC-3’ [10]; F: 5’-AATCAACACCAACTGCAGCG-3’, and R: 5’-GCATAGCCACCATATAACC-3’ [11]; F: 5’-AATCACAACACCTGCAGCG-3’ and R: 5’-TAGTGCTTATTG- TAGCCGCAAAGG-3’ [11]; and were synthesized by the Sangon Biotech Co., Ltd (Shanghai, China). They were used, respectively, to detect the invA, flICd, and flICA genes of Salmonella, Salmonella Typhi and Salmonella Paratyphi A in the bile mixture using multiplex PCR (multi-PCR). The bile mixture, in which no typical bacteria, Candida spp. and L-forms, no antimicrobial activity and no Salmonella, Salmonella Typhi or Salmonella Paratyphi A-specific genes had been detected, was the bile inducer used to induce the L-forms from Salmonella Typhi and Salmonella Paratyphi A in vitro.

**Induction and isolation of L-forms derived from Salmonella Typhi and Salmonella Paratyphi A in vitro**

The 24-h cultures of Salmonella Typhi H901 and Salmonella Paratyphi A on nutrient agar plates were inoculated respectively into sterile normal saline and then diluted into bacteria-containing fluids with a McFarland standard value of 5 (about 15 × 10⁹ bacterial cells/mL). Five milliliters of bile inducer was added into the glass cell culture flask of each group, then 0.3 mL of the bacterial fluid for each bacterial species was inoculated into the flask with the bile inducer for its own group; bile-inducers containing microbes were named “bile-inducer cultures” of the bacteria species. Bile-inducer cultures were incubated under normal air conditions at 37°C for induction of the L-form. During incubation, 0.1-mL sub-samples were taken from the bile-inducer cultures at 1, 3, 6, 12 h and at 1, 2, 3, 5, 7, 15, 30, 45 and 60 days, and inoculated into blood agar plates, SS agar plates and Sabouraud agar plates, then incubated under normal air conditions at 37°C. The samples of bile-inducer cultures were filtered using a 0.22-μm pore size and then 0.1 mL of the filtered sample was inoculated into the LEM plate and incubated in 5–10% CO₂ air conditions at 37°C, or into a cell culture flask with 5 mL of PG liquid medium and incubated in an ordinary bacteriological incubator at 37°C. The growth phenomenon of the bacterial forms was observed with the
naked eye and the isolates were identified by routine bacteriological methods, including Gram stain, growth pattern on SS agar medium and double sugar iron medium and slide agglutination reaction with diagnostic sera for Salmonella. The growth pattern of the L-forms was observed respectively using an inverted microscope at low-power (100 × or 200 ×) and high-power (400 ×) magnification, the L-forms in the PG liquid media were filtered using a 0.22-μm pore size filter and then the filtrates were subcultured with the PG liquid medium more than three times to isolate the pure cultures of stable L-form [15].

Identification of L-forms derived from Salmonella spp.
Pure cultures of the stable L-form isolated from the bile-inducer cultures were identified according to characteristics of morphology, filterability and growth pattern in the PG liquid medium and on the blood agar, SS agar, Sabouraud agar and LEM soft agar media [15]. The Salmonella Typhi-specific and Salmonella Paratyphi A-specific O and H antigens of the bile-induced L-forms were detected by the microscopic agglutination test with diagnostic sera for Salmonella spp. (Lanzhou Institute of Biological Products Co., Ltd, Lanzhou, China). The parentage of the stable L-form subcultures was further identified by the multi-PCR assay for the invA, flIC-d and flIC-a genes of Salmonella spp., and the nucleotide sequence of the positive PCR products was determined by the Sangon Biotech Co., Ltd (Shanghai, China).

Isolation of microbes and stable L-forms in human gallbladder

We collected 524 gallbladder specimens, including gallbladder tissues (436 cases), bile (23 cases) and gallstones (65 cases), taken by cholecystectomy from 524 patients with biliary diseases at the Affiliated Hospital of Guiyang Medical College, the Guizhou Province People’s Hospital, the Zunyi City People’s Hospital and the Guizhou Southwest State People’s Hospital in Guizhou province in China (permit number: TZJF-2011-26). Most of the samples were collected from patients during the operation and then immediately inoculated into the media for isolation of microbes. A few samples were stored at 4°C within 7 days in the hospital and then collected and inoculated immediately into the media for isolation of the microbes in our laboratory. The gallbladder tissues and gallstones were pretreated by disinfection of the surface with iodophor, washed with PG liquid medium, and then broken by extrusion [15]. The pretreated specimens of gallbladder tissues and gallstones and the 0.1-mL bile samples were respectively inoculated into various media for isolation culture of Salmonella spp., aerobic bacteria, Candida spp. and bacterial L-forms according to the above methods. Once the growth phenomenon of L-form cells in the non-high osmotic culture was observed by microscopy, the L-form isolates were filtered through a 0.22-μm pore-size filter and subcultured with the PG liquid medium. After more than three subcultures without reverting to bacteria growth, the L-form isolates were defined as stable L-form pure cultures isolated from the human gallbladder specimens.

Identification of L-forms derived from Salmonella spp. in the bacterial L-form isolates
The parentage of bacteria and Candida was identified by routine bacteriological/fungal methods; identification of the bacterial L-forms isolated from the patients’ gallbladder specimens depended on their morphological characteristics, filterability and growth phenomenon [15]. Molecular detection was used to further identify the L-forms derived from Salmonella Typhi and Salmonella Paratyphi A in the pure cultures of bacterial L-forms isolated from gallbladder specimens, including multi-PCR assay for the invA gene of Salmonella, the invA, flIC-d, flIC-a genes of the Salmonella Typhi and Salmonella Paratyphi A.

Results

The L-forms induced by human bile in vitro

By the routine bacteriological methods, the Salmonella Paratyphi A could be isolated until 6 h of culture and the Salmonella Typhi could be isolated until 48 h of culture from the bile-inducer cultures. No L-form could be isolated from these bile-inducer cultures by the high osmotic isolation technique. Conversely, the L-forms could be isolated from the bile-inducer cultures of Salmonella Paratyphi A that were cultured from 1 h to 45 days and of Salmonella Typhi that were cultured from 1 h to 30 days. The stable L-forms could not grow on the blood agar, SS agar, Sabouraud agar and LEM soft agar media. Most of the stable L-form cells in the culture of PG liquid medium showed single cells, some of them could be arranged in pairs or in chains (Fig. 1a, b). The L-form cells grew at the bottom of the PG liquid media, they showed no motility and no adhesion to the wall of the glass cell culture flask, and always showed clarified and sterile cultures when observed with the naked eye. Scanning electron microscopy revealed that the L-form cells had a rough surface (Fig. 1c, d). The L-forms isolated from the bile-inducer cultures of Salmonella Typhi and Salmonella Paratyphi A had different biological characteristics and surface antigens from their parental bacteria, but the variants showed positive reactions in the multi-PCR assay for genes invA (molecular weight (284 bp), flIC-d (750 bp), flIC-a (329 bp) of Salmonella Typhi or Salmonella Paratyphi A (Table 1 and Fig. 2). Nucleotide sequencing showed that coincidence ratio of the nucleotide sequences of invA (GenBank: CP007598.1), flIC-
d (GenBank: L21912.1), filC-a (GenBank: FM200053.1) of Salmonella Typhi or Salmonella Paratyphi A were respectively 99%. The coincidence ratio of the nucleotide sequences between the bile-induced L-forms and their parental bacteria was 99%.

The microbes and the L-forms in the gallbladder specimens

By the routine bacteriological/fungal methods and the high osmotic isolation technique, no Candida spp. and bacterial L-forms could be isolated from these patients’ gallbladder samples. There are three strains of Salmonella spp. and 100 strains of other bacterial species were isolated from 100 specimens in the 524 gallbladder samples by routine bacteriological methods, the positive rate of microbes was 19.1% (100/524) and in which the positive rate of Salmonella spp. was 0.6% (3/524). These microbial species were respectively 24 strains of E. coli, nine strains of Klebsiella spp., eight strains of Enterobacter aerogenes, seven strains of Citrobacter spp., one strain of Salmonella Typhi and two strains of Salmonella Paratyphi A, three strains of Proteus spp., three strains of Neisseria sicca, three strains of Moraxella spp., 17 strains of Enterococcus spp., seven strains of Staphylococcus aureus and 19 strains of Staphylococcus epidermidis. By the non-high osmotic isolation technique, 395 specimens of patient gallbladder samples gave positive isolation cultures of bacterial L-forms, the positive rate was 75.4% (395/524). Twenty-nine specimens of 524 patient gallbladder samples were negative isolation cultures of bacterial form and L-form, they accounted for 5.53% (29/524). No L-form was isolated

TABLE 1. Identification results of the bile-induced L-forms of Salmonella Typhi and Salmonella Paratyphi A by the agglutination test and gene assay

<table>
<thead>
<tr>
<th>Salmonella</th>
<th>Diagnostic sera for Salmonella</th>
<th>Multi-PCR assay for</th>
<th>A-F group O polyvalent serum</th>
<th>O</th>
<th>H</th>
<th>invA</th>
<th>filC-d</th>
<th>filC-a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhi L-form</td>
<td></td>
<td></td>
<td>A-F group O polyvalent serum</td>
<td>2</td>
<td>9</td>
<td>a</td>
<td>d</td>
<td>Vi</td>
</tr>
<tr>
<td>Paratyphi A L-form</td>
<td>Bacterial form</td>
<td></td>
<td>A-F group O polyvalent serum</td>
<td>2</td>
<td>9</td>
<td>a</td>
<td>d</td>
<td>Vi</td>
</tr>
</tbody>
</table>
| Note: +, positive reaction; −, negative reaction; N/A, not applicable.
from the 100 samples with bacteria-positive cultures by the non-high osmotic isolation technique.

**The Salmonella L-forms in the stable L-form isolates of gallbladder samples**

The stable L-form subcultures isolated from patient gallbladder samples showed the same morphology, growth characteristics and cell structures as the bile-induced L-forms of *Salmonella Typhi* and *Salmonella Paratyphi A* (Fig. 3a–c). The small L-form cells (elementary bodies) can be observed by scanning electron microscopy (Fig. 3d). Gene detection of *invA*, *flIC-d* and *filC-a* for the bacterial L-form subcultures by multi-PCR showed that the positive rate of *Salmonella invA* gene was 3.1% (16/524) or 4.1% (16/395). In these *invA*-positive bacterial L-form isolates, 25% (4/16) was identified as *Salmonella Typhi* (*invA* and *flIC-d* positive) and 62.5% (10/16) was identified as *Salmonella Paratyphi A* (*invA* and *filC-a* positive).

**Discussion**

The cell-wall-deficient bacteria, as for the spore [16] and viable but non-culturable state of bacteria [17], are special life states of bacteria under extreme conditions. The L-forms, like the ‘fastidious bacteria’ [18] are very difficult to isolate and culture

---

**FIG. 2.** The results of agarose gel electrophoresis of the multi-PCR assay for the genes of the stable L-forms isolated from the bile-inducer cultures of *Salmonella Typhi* and *Salmonella Paratyphi A*. The multi-PCR assay for the genes of *Salmonella* L-forms. Lane M: DNA ladder maker. Lane 1: *invA* and *flIC-d* genes of bacterial form of *Salmonella Typhi*. Lane 2: *invA* and *flIC-d* genes of the stable L-forms isolated from the bile-inducer cultures of the *Salmonella Typhi*. Lane 3: *invA* and *flIC-a* genes of bacterial form of *Salmonella Paratyphi A*. Lane 4: *invA* and *flIC-a* genes of the stable L-forms isolated from the bile cultures of *Salmonella Paratyphi A*. Lane 5: Negative control.

**FIG. 3.** Stable L-form cells isolated from human gallbladder. (a) The stable L-form cells with obvious growth phenomena in PG liquid medium (light microscopy, 200 ×). (b) The L-form cells have rough surfaces and are approximately 2.2–5.0 μm in diameter. They are arranged in short chains or irregularly interconnected by their surface structures (scanning electron microscopy (SEM), 8000 ×). (c) The two L-form cells are approximately 3.2–4.1 μm in diameter and some bud-like structures can also be seen on their surfaces (SEM, 20 000 ×). (d) The large L-form is approx 5.2 μm in diameter and many small L-form cells (elementary bodies) are spherical, ovoid or short rods (indicated by the arrow) and are approx 0.12–0.38 μm in diameter, most of them are arranged in irregular clusters (SEM, 10 000 ×).
using routine bacteriological methods because of their special biological properties. It is considered an important mechanism by which bacteria can protect themselves from lethal conditions, and also results in variants that can express biological properties that are different from those of their parental bacteria [1–5,15,19–23]. According to past research, Gram-positive bacteria and Gram-negative bacteria, including Staphylococcus aureus, Bacillus subtilis and E. coli, could show similar penicillin sensitivity and rules of L-form formation on agar media with different osmotic pressure conditions [24]. Some L-form cells might be stable in fried-egg colonies induced by penicillin on L-form medium, and cultures of antibacterial tests in vitro were resistant to the lower osmotic pressure conditions [25,26]. It has been confirmed that the stable L-forms formed in human and animal bodies as well as in cultures with non-high osmotic pressure conditions—such as the L-forms derived from obligate aerobes (Bacillus subtilis [27], Mycobacterium tuberculosis [19,28]), facultative anaerobes (Staphylococcus aureus [29], Corynebacterium diphtheriae [20], Neisseria gonorrhoeae [30], Streptococcus pneumoniae [31], Salmonella typhimurium [32]), and obligate anaerobes (Clostridium tetani [23], Clostridium perfringens [22], Helicobacter pylori [22])—have adapted to the non-high osmotic pressure conditions or physiological osmotic pressure conditions of the host. This has resulted in these stable L-forms expressing some new biological characteristics and as a result they cannot be isolated and cultured using routine bacteriological methods and high osmotic isolation techniques. However, they can be isolated and cultured using the non-high osmotic technique under normal air conditions [19–22]. These stable L-forms derived from various bacterial species have atypical biochemical reactions but usually lose the biochemical reaction characteristics and cell-wall-related surface antigens of their parent bacteria. This results in stable L-forms that cannot be identified by routine bacteriological methods [15,21–23,27–29]. Stable L-forms, by means of their changed surface charge and rough surface structure, can adsorb and invade host cells and have cytotoxic effects in them [12,19,20], and as a nidus they were also responsible for the formation of stones in the gallbladder and urinary tract [12,15,33]. In this research, it was further confirmed that the Salmonella Typhi and Salmonella Paratyphi A could be induced rapidly into stable L-forms by human bile in vitro. Human bile was a good inducer of Salmonella spp. L-forms, resulting in Salmonella spp. becoming the latent bacteria in human bile and negative isolation culture of Salmonella Typhi and Salmonella Paratyphi A by routine bacteriological methods. Using the non-high osmotic isolation technique, the stable L-forms can be isolated from the Salmonella-free bile-inducer cultures. The parentage of the stable L-forms derived from Salmonella Typhi and Salmonella Paratyphi A can be identified by multi-PCR and by nucleotide sequencing for Salmonella-specific genes.

Salmonella Typhi and Salmonella Paratyphi A, as bacterial species of the intestinal flora, are considered to be common infecting microbes of the gallbladder in humans [6–11]. In the epidemiology of enteric fever, carriers of Salmonella species (convalescent carriers and healthy carriers) are considered to be the most important source of Salmonella infection; the gallbladder and biliary tract are also common organs harbouring these organisms. However, using routine bacteriological methods, the positive rate of isolation culture of Salmonella spp. and other microbial species in gallbladder specimens taken from patients with chronic biliary diseases was only 5–20% [6,7]. These findings suggest that most gallbladders with chronic cholecystitis and cholelithiasis are undergoing an aseptic inflammatory pathological reaction, but it is difficult to explain why species of Salmonella and other microbes can colonize the gallbladder and persist in an asymptomatic carrier state that is frequently associated with the presence of gallstones and chronic biliary diseases and even other serious illnesses. Nor can these findings explain why the positive reaction of the molecular assay for Salmonella-specific genes can often be shown in gallbladder samples with Salmonella-negative isolation culture. In this research, we isolated microbes from gallbladder specimens of patients with chronic cholecystitis and cholelithiasis using routine bacteriological methods. Results showed that 80.9% (424/524) of these patients’ specimens were bacteria-negative in isolation culture, and the positive rate for Salmonella spp. was only 0.6% among the 524 gallbladder samples. Hence it is further confirmed that the gallbladders of most patients with chronic cholecystitis and cholelithiasis are sterile and very few people are Salmonella carriers in Guizhou China. Many bacterial species that are common in the biliary tract were isolated from the gallbladder specimens in this study, and Neisseria sicca, a member of the normal flora of the human respiratory tract, was isolated from the human gallbladder samples for the first time. It has been reported that some species of the non-gonorrhoeae Neisseria can be isolated from specimens of expressed prostatic secretions and from semen of patients with chronic prostatitis-like symptoms and these microbes were considered to be associated with male genital tract infection [34]. Hence many microbial species in the human intestinal tract can invade the gallbladder by ascending from the duodenum; also, via the haematogenous route from the hepatic portal vein, some microbial species of the human upper respiratory tract can invade the gallbladder through the blood circulation. Using the non-high osmotic isolation technique to isolate bacterial L-forms from gallbladder specimens of patients with chronic biliary diseases, a high positive rate of bacterial L-form infection was found in bacteria-free gallbladder specimens.
In the bacterial L-form-positive isolation cultures, the stable L-forms derived from *Salmonella Typhi*, *Salmonella Paratyphi* A can be identified by the multi-PCR assay for *Salmonella*-special genes. The positive rate of *Salmonella* L-forms in non-high osmotic isolation technique isolates was higher than that of classical *Salmonella* spp. using routine bacteriological methods, so it could be considered that the gallbladder, as a carrier of *Salmonella* L-forms, may be an important latent source of *Salmonella* infection. These L-variants are also an important factor in the positive reactions of molecular assays for specific genes of *Salmonella* Typhi and *Salmonella* Paratyphi A found in the gallbladder samples with *Salmonella*-negative isolation culture.

In clinical diagnostic microbiology, the molecular assay has been used widely for early and rapid diagnosis, but isolation culture of the causative agent is still considered the most important evidence of accurate diagnosis for bacterial infection. However, bacterial L-forms are the cell-wall-deficient variants of bacteria and non-normal bacteria, so they can express many special biological properties that are completely different from those of fastidious bacteria and other classical bacteria. According to research [1,15,20,28,30,31], the L-forms retain the internal antigenic substances and the nucleotide sequence of genes on the chromosome of their parental bacteria. Thus, molecular assay and immunological assay can be used to identify the parentage of stable L-forms. In this research, it was shown that the bile-induced L-forms of *Salmonella Typhi* and *Salmonella Paratyphi* A in *in vivo* have similar morphologies and biological properties. Their special characteristics are also similar to those of stable L-forms derived from other bacterial species isolated from gallbladder specimens. However, these stable L-forms derived from different species retained unique nucleotide sequences of specific genes on their chromosomes, which were the same as those of their parental bacteria. As a result, the parentage of L-forms derived from *Salmonella Typhi* and *Salmonella Paratyphi* A can be identified from stable L-form isolates by the PCR assay for specific genes of *Salmonella* bacterial forms. The procedures of isolation culture and identification for the stable L-forms derived from *Salmonella Typhi* and *Salmonella Paratyphi* A in gallbladder samples are summarized in Table 2.

### Author contributions

DNW, WJW, TW and HW performed the analyses of microbiology data and gene detection data. WJD, YZP and KLT collected the gallbladder specimens and extracted information from the patients’ clinical notes. WJW, TW and XLS were responsible for the isolation of the microorganisms, DNA extraction and PCR. HW supervised and managed the study. HW and DNW wrote the paper and approved the final version.

### Transparency declaration

The authors declare no competing financial interests.

### Acknowledgements

We thank the Guiyang Medical University Key Laboratory (XLL) for scanning electron microscopy. The study was supported by a grant from the CHN Guizhou High-level Personnel Province Scientific Research Fund Project (grant number TZJF-2011-26).

### References


