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RAPID COMMUNICATION

Quantitative studies of the regular distribution pattern for *Salmonella enteritidis* in the internal organs of mice after oral challenge by a specific real-time polymerase chain reaction

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Abstract

AIM: To identify and understand the regular distribution pattern for *Salmonella enteritidis* (*S. enteritidis*) in the internal organs of mice after an oral challenge over a 3 wk period.

METHODS: Assays based on the serovar-specific DNA sequence of *S. enteritidis* from GenBank, and a serovar-specific real-time, fluorescence-based quantitative polymerase chain reaction (FQ-PCR) were developed for the detection of *S. enteritidis*. We used this assay to detect genomic DNA of *S. enteritidis* in the blood and the internal organs, including heart, liver, spleen, kidney, pancreas, and gallbladder, from mice after oral challenge at different time points respectively.

RESULTS: The results showed that the spleen was positive at 12 h post inoculation (PI), and the blood was at 14 h PI. The organism was detected in the liver and heart at 16 h PI, the pancreas was positive at 20 h PI, and the final organs to show positive results were the kidney and gallbladder at 22 h PI. The copy number of *S. enteritidis* DNA in each tissue reached a peak at 24-36 h PI, with the liver and spleen containing high concentrations of *S. enteritidis*, whereas the blood, heart, kidney, pancreas, and gallbladder had low concentrations. *S. enteritidis* populations began to decrease and were not detectable at 3 d PI, but were still present up to 12 d PI in the gallbladder, 2 wk for the liver, and 3 wk for the spleen without causing apparent symptoms.

CONCLUSION: The results provided significant data for understanding the life cycle of *S. enteritidis* in the internal organs, and showed that the liver and spleen may be the primary sites for setting itself up as a commensal over a long time after oral challenge. Interestingly, it may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis* over a 12 d period. This study will help to understand the mechanisms of action of *S. enteritidis* infection *in vivo*.

Key words: Fluorescence-based quantitative polymerase chain reaction; Internal organs; *Salmonella enteritidis*; Regular distribution pattern

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INTRODUCTION

A significant proportion of human salmonellosis is caused

by consumption of raw or partially cooked eggs[1-3]. *Salmonella enteritidis* (*S. enteritidis*) is one of the main causes of gastrointestinal infection in China. Incidence of infection is highest in children, elderly, and immunosuppressed individuals[4]. *S. enteritidis* is a facultative intracellular pathogen capable of causing disease in a wide range of host species. After oral ingestion, *S. enteritidis* cells rapidly reach the bowel and penetrate the macrophages, spread to the mesenteric lymph nodes, and in severe cases, can reach the circulatory system[5]. The macrophages carry the organism to the liver, pancreas, and spleen, where the bacteria are thought to replicate in both phagocytic and nonphagocytic cells[5]. Also, it was reported that the *S.* typhimurium can be shed into the gallbladder from the liver, where either an active infection (cholecystitis) or a chronic infection can develop[6,7].

Previous studies showed that *S. enteritidis* was usually cleared from internal organs more than 8 wk after inoculation of poultry. How *S. enteritidis* survives within macrophages is unclear. It seems likely that the type Ⅲ secretion system encoded by *Salmonella* pathogenicity island 2 may play a major role. Moreover, extensive studies of mouse have shown the role for T cells, natural killer cells, macrophages, neutrophils and numerous cytokines in *Salmonella* resistance[8,9]. Numerous studies indicate that *S. enteritidis* cells have evolved strategies to resist and overcome innate immune defenses[10,11]. Up to date, the mechanisms by which *S. enteritidis* and other serotypes persist within the host and the reasons for the absence of immune clearance are not known. As a result of the increased prevalence of *S. enteritidis* and its complex life cycle, identifying the regular distribution pattern of *S. enteritidis* in the internal organs over a 3 wk period, which is not described hitherto, will help to understand its mechanism of action.

One of the main advantages of FQ-PCR is the ability to quantitate unknown samples. With this assay, it is possible to carry out a rapid quantitative analysis of DNA over a wide linear range, with an unknown template. The specific DNA fragment (Sdf I) of *S. enteritidis* was reported by Agron *et al*[12], which was screened for using the Supression Subtractive Hybridization method, and appears to only be found in serovar *enteritidis* strains[12,13]. Here, based on the study of Agron *et al*, we developed a standard curve (this methodology is the first time established which based upon the Sdf I DNA fragment), and then applied this to the study of internal organ distribution of *S. enteritidis* in mice.

MATERIALS AND METHODS

Bacterial strains

A total of 19 *Salmonella* strains were included in this study. Most strains were purchased from the National Center for Medical Culture Collection, including *S. enteritidis* (Human, No. 50041), *S. enteritidis* (Human, No. 50040), *S. enteritidis* (Mouse, No. 50338), *S. enteritidis* (Human No. 50100), *S. enteritidis* (Human, No.50128), *S. enteritidis* (Human, No. 50335), *S. enteritidis* (Mouse, No. 50336), *S. enteritidis* (Human, No. 50760), *S. choleraesuis* (No. 50191-1), *S. typhi* (No. 50013), *S. typhimurium* (No. 50115-13), *S. paratyphi* (No. 50001-24), *S. pullorum* (No. 50047-2), *S. anatum* (No. 50083-4), *S. gallinarum* (No. 50770), *S. dublin* (No. 50761). Three strains were isolated and maintained by the Avian Diseases Research Center, Collage of Veterinary Medicine of Sichuan Agricultural University, including *S. enteritidis* (Duck, No. MY_1), *S. enteritidis* (Duck, No. SC1), *S. enteritidis* (Chicken, No. CD1).

Preparation of bacterial samples and generation of standard templates

Briefly, 5 mL of bacterial culture was grown overnight in Luria-Bertani broth and 500 μL of the culture was harvested by centrifugation. The pellet was resuspended in 500 μL TE buffer (pH 8.0) and 2 μL of lysozyme solution (30 g/L) was added, followed by lysis using 10% SDS (80 μL) at 60℃ for 1 h. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Then, a 1/10 volume of 3 mol/L sodium acetate and 2 volumes absolute ethanol were added, and the nucleic acid was then pelleted by centrifugation, washed with 70% ethanol, and dried under vacuum. The DNA genomic pellet was resuspended in 30 μL TE buffer (pH 8.0), and stored at -20℃ until use.

A conventional PCR was carried out using a template from *S. enteritidis* (Chicken, No. CD1), with primers F_1 and R_1 (designed with Sdf I, Genbank Accession No. AF370707.1, generated by TakaRa Biotech, DaLian, China). The primer sequences from 5' to 3', were as follows: F_1, TGTGTTTTATCTGATGCAAGAGG; and R_1, CGTTCTTCTGGTACTTACGATGAC. Amplification was carried out in a total volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA Polymerase (TaKaRa Taq, TakaRa Biotech), 5 μL 10 × PCR buffer (with Mg^{2+}, 25 mmol/L), and 6 μL templates, then made up to a volume of 50 μL with deionized water. An initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 52.5℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. The product size was 293 bp.

Finally, the product was gel-excised and quantified with appropriate standards. Its concentration was determined spectrophotometrically using the Bio-Rad-Smartspec-3000 instrument, according to the manufacturer's instructions. The standards were diluted, divided into aliquots, and frozen before used.

Development of FQ-PCR and its products

The FQ-PCR assay, including volume, Mg^{2+} concentration, probe and primer concentrations, and annealing temperature were optimized initially. Subsequently, the sensitivity of the assay, the linear range and standard curve were determined by using known amounts of purified template DNA (generated as described above). The primers (F_2 and R_2) and TaqMan-probe (FP) of FQ-PCR were designed using an internal region of the 293 bp sequences (described above, generated by TakaRa Biotech), and were used as follows, from 5' to 3': F_2, TTGATGTGGTTGGTTCGTCACT; R_2, TCCCTGAA TCTGAGAAAGAAAAACTC; and TaqMan-probe (FP), FAM- TGCAGCGAGCATGTTCTGGAAAGC-TAMRA.

Amplification of FQ-PCR was carried out in a total volume of 25 μL, containing 0.6 μL each primer (10 μmol/L), 0.75 μL dNTPs (10 mmol/L), 1.25 U EX Taq DNA Polymerase (TaKaRa EX Taq Hot Start Version, TakaRa Biotech), 5 μL 5 × PCR buffer (free Mg^{2+}), 0.8 μL TaqMan-probe (5 μmol/L), 0.5 μL Mg^{2+} (250 mmol/L) and 5 μL aliquot of the sample DNA templates (for the standards DNA templates, 1 μL was added), then made up to a volume of 25 μL with deionized water. Each run consisted of a 95℃ 5 min hot start, which activated the conjugated polymerase, followed by 45 cycles with a 94℃ denaturation for 30 s, 55℃ annealing for 30 s, and reading the fluorescent signal at this step.

The primers of FQ-PCR (F_2 and R_2) were used for conventional PCR with *S. enteritidis* (Chicken, No. CD1) DNA templates, in order to verify the specific amplification. Amplification was carried out in volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA polymerase (TaKaRa Taq, TakaRa Biotech), 5 μL 10 × PCR buffer (with Mg^{2+}, 25 mmol/L), and 6 μL templates, then made up to a volume of 50 μL with deionized water. An initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 49℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. A 10 μL aliquot of PCR product was electrophoresed on a 1.5% agarose gel for 40-50 min at 80 V, and visualized and photographed under UV illumination. Simultaneously, DNA sequences of the products were carried out by TakaRa Biotech.

FQ-PCR standard curve

Based on the previous studies to generate the standard curve as follows[14-16]: Standards DNA were used to establish a standard curve and the standards contained amplified target DNA in different quantities which were measured by fluorimetic analysis (iCyclerQ, Bio-Rad, USA). The Primers F_2 and R_2 were used for this amplification, the DNA was 10-fold serially diluted in nuclease-free water and standard curve was generated by using 1.0×10^5 to 1.0×10^{10} gene copies of standards DNA. Concentrations of the standards were measured by fluorimetic analysis, then an analysis of key cycler measurements were performed after each run to verify identical amplification efficiencies and conditions between runs. Finally, based on the data generated, a standard curve for the icycler was obtained and based on the standard curve to obtain the copies number of *S. enteritidis* for the samples.

Specificity,sensitivity and reproducibility of the FQ-PCR

All 19 bacterial strains were used to assess the specificity of the FQ-PCR. The phenol/chloroform/isoamyl alcohol method (described above) was used to prepare the DNA template, 6 μL of this aliquot was used in FQ-PCR.

To determine the detection limit of this FQ-PCR assay, different quantities of standard DNA of *S. enteritidis* was added. We used phenol/chloroform/isoamyl alcohol method to extract DNA of tissue from several control group samples (described below), and added 1.0×10^5-1.0×10^{-1} copies of the standard DNA for each. Finally the results were measured by fluorimetric analysis.

To evaluate the variability between experiments, three different known concentrations of DNA were amplified by performing the assay described above in triplicate. For each experiment, the crossing point, average crossing point, standard deviation, and coefficient of variation for each assay were calculated.

Experimental infection of mice

Our infection model was based on the previous studies, which showed that orally introduced *S. enteritidis* had a rapid transit time through the intestine and established itself within the walls of the gut in more than 3 d[17,18]. 80 mice (age 9 wk, specific-pathogen-free) were purchased from the Animal Center of Sichuan University, China. In brief, a group of 60 mice was oral infected with a virulent *S. enteritidis* strain (Chicken, No. CD1, LD_{50} is 4.0×10^8 cells after oral challenge), at 4.0×10^6 cells per mouse (In our preliminary experiments, the results showed that this dose of challenge can induce clinical signs and pathology in mice, but can not cause death). Another group of 20 mice was treated with an equal volume of water as a control. Blood, heart, liver, spleen, kidney, pancreas, and gallbladder were analyzed by FQ-PCR at different post-inoculation time points, at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h, 24 h and 36 h and 2 d, 3 d, 6 d,9 d,12 d and 2 wk,3 wk.

Three mice from infected group and one from control group were sacrificed at each time point (There was a potential complication of *S. enteritidis* DNA in the blood for the tissue results, particularly in the spleen and liver where large amounts of blood could be accumulated, so the mice were perfused before the tissue collection) and its organs were aseptically harvested and immediately placed in 1.5 mL labeled snap-cap tubes, and frozen. The blood sample, 200 μL, was obtained from the vena caudalis with syringe aseptically before sacrificed. For the bile sample, 200 μL was obtained from gallbladder with syringe aseptically after sacrificed, and frozen.

DNA extraction from tissue samples was described below: Briefly, 0.5 g of the tissue sample was ground up using a tissue grinder in the 1.5 mL Eppendorf tube. The pellet was resuspended in 500 μL TE buffer (pH 8.0) with 10 μL Proteinase K (30 g/L) and incubated at 37℃ for 2 h. Finally, with a conventional phenol/chloroform/isoamyl alcohol method (described above), to extract the genomic DNA of *S. enteritidis* from tissue, used 5 μL aliquot of DNA template for FQ-PCR detection.

For the bile and blood samples, taking 200 μL to be harvested by centrifugation, then the pellet was resuspended in 500 μL TE buffer (pH 8.0) with 3 μL Proteinase K (30 g/L), and incubated at 37℃ for 1 h. Finally, used the phenol/chloroform/isoamyl alcohol method (described above) to extract DNA template and used 5 μL to be detected.

Comparative the differences between FQ-PCR and the traditional bacterial culture method

All the samples of mice were detected by the traditional bacterial culture method in order to identify the accuracy of this FQ-PCR method. Firstly, taking 0.1 g or 20 μL tissue sample, then used the selective enrichment media-

Selenite Cystine Broth, incubated at 37℃ for 20 h. And then, transferred to *Salmonella* Shigella Agar (SS agar). two differential media, Macconkey agar and Triple sugar iron agar were streaked and incubated at 37℃ for 24 h. Suspected colonies were picked up for biochemical and serological tests. The following tests were used glucose, maltose, arabopyranose, mannitol, glycitol, Hydrogen sulfide, MR, lysine decarboxylase, argininedecarboxylase, orinithinedecarboxylase, *et al.*

Serotypes were identified by multivalence serum of O_{A-F} and $O_{1,9,12}$ factor serum. The results were referred to[19,20] incomplete sentence-authors must complete.

Statistical analysis

All samples were analyzed three times with a mean and standard error. Data were analyzed on an IBM compatible personal computer using SPSS version 11.0. Effects were considered to be significant if $P < 0.05$. Finally, based on the standard curve (described aboved) to obtain the copies number of *S. enteritidis*. The *S. enteritidis* DNA copy concentrations were expressed as the mean $\log_{10}$ copies genome numbers per 0.5 g or 0.2 mL of tested tissue or blood and bile, respectively.

RESULTS

Specific verification of FQ-PCR products

The primers of FQ-PCR were used for conventional PCR with *S. enteritidis* (Chicken, No. CD1) DNA templates, in order to verify the specific amplification. Results showed that the PCR produced an intense band with the expected 130 bp for *S. enteritidis*, which indicated 100% specificity. Also, Sequence analysis was carried out using BLASTn and BLASTn Programs of National Center for Biotechnology Information, and resulted in 100% homology with DNA of *S. enteritidis*, Genbank Accession No.AF370707.1.

FQ-PCR standard curve

By using standards template containing from 1.0×10^5 to 1.0×10^{10} copies, accurate results for a series of samples were obtained, based on the data used to generate the standard curve with the iCycler IQ Detection System (Bio-Rad; USA). The correlation coefficient for the associated standard curve was 1.000 and PCR efficiency was 98.2%, which indicated that the crossing threshold values for the standards fell within accurate range. Through the formula as follows, we could quantitate the DNA copies of *S. enteritidis* for unknown samples, Y= -3.366X + 44.914 (where Y is the threshold cycle, and X is the log of the starting quantity) (Figure 1).

Specificity, sensitivity and reproducibility of the FQ-PCR

All 19 bacterial strains were used to assess the specificity of the FQ-PCR, indicated that only *S. enteritidis* strains genomic showed the positive results, while there was no positive results with none *S. enteritidis* strains.

A range from 1.0×10^5 to 1.0×10^{-1} copies of *S. enteritidis* standards template was used, the limit of detection was 10 copies/μL.

Three different, known concentrations of DNA, 1.0×10^9 to 1.0×10^7 copies/μL, were amplified by performing the assay described above in triplicate. Analysis of these values proved that the assay was reproducible, as the coefficient of variation was statistically low, at < 1.8%, the standard deviation was 1.1%, and the threshold cycle for each concentration was difference between 0.2 cycle and 0.4 cycle, highly reproducible.

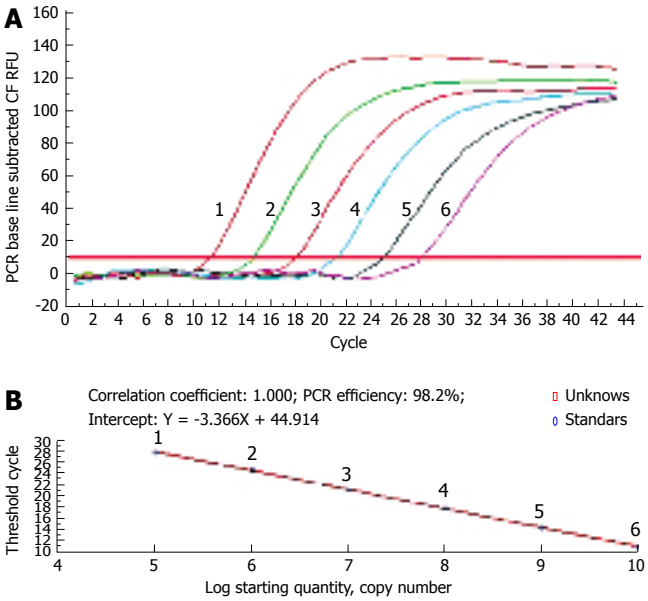

Figure 1 Used the standards DNA template with 10-fold serial dilutions to develop standard curve. **A**: Amplification curve; **B**: Standard curve; 1, 1.0×10^{10} copies/μL; 2, 1.0×10^9 copies/μL; 3, 1.0×10^8 copies/μL; 4, 1.0×10^7 copies/μL; 5, 1.0×10^6 copies/μL; 6, 1.0×10^5 copies/μL.

Clinical signs and gross necropsy

S. enteritidis-inoculated mice appeared to be clinically normal, and there were no sings of depression or diarrhea, and feeding and drinking behavior remained normal at 30 min to 8 h, and 6 d to 3 wk PI. However, mice developed significant clinical signs of *S. enteritidis* infection at 12 h to 3 d PI. At necropsy, gross lesions were observed in all of the mice at this period, such as hyperemia of intestine, swelling of gallbladder[19,20].

Distribution of S. enteritidis in the internal organs

The distribution of *S. enteritidis* within the internal organs after oral challenge was determined by means of FQ-PCR, over a 3 wk period at intervals. Results showed that the spleen was positive at 12 h PI, with about 2.95×10^2 copies/0.5 g. Then, blood had positive results at 14 h PI with about 2.75×10^2 copies/0.2 mL, and the organism was detected in the liver and heart at 16 h PI, pancreas was positive at 20 h PI, and the last organ to show a positive results were the kidney and gallbladder at 22 h PI. The copy numbers of *S. enteritidis* in each tissue reached a peak at 24-36 h PI, with the liver, spleen containing high concentrations of *S. enteritidis*, about 2.00×10^4 to 3.31×10^6 copies/0.5 g, whereas the blood, heart, kidney, pancreas and gallbladder had low concentrations, ranked from 5.13×10^2 to 1.70×10^5 copies/0.5 g (0.2 mL). Numbers of bacteria decreased at 3 d-9 d, the level of *S.*

Table 1 The distribution and quantity of *S. enteritidis* within different internal organs and blood after oral challenge at different times

Time	Blood	Heart	Liver	Spleen	Kidney	Pancreas	Gallbladder
30 min	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	2.47	0.00	0.00	0.00
14 h	2.44	0.00	0.00	2.80	0.00	0.00	0.00
16 h	2.68	2.72	2.83	3.22	0.00	0.00	0.00
18 h	2.91	3.21	3.31	3.61	0.00	0.00	0.00
20 h	3.41	3.46	3.76	3.85	0.00	2.43	0.00
22 h	3.75	3.62	3.93	4.20	2.50	2.88	2.44
24 h	3.94	3.97	4.30	4.36	2.80	3.12	2.82
36 h	4.11	5.28	6.46	6.52	4.46	4.16	2.71
2 d	3.78	3.01	4.11	5.59	2.75	2.82	2.60
3 d	2.50	2.81	3.80	5.22	0.00	2.27	2.52
6 d	0.00	0.00	3.02	4.02	0.00	0.00	2.36
9 d	0.00	0.00	2.69	3.78	0.00	0.00	2.22
12 d	0.00	0.00	2.32	3.52	0.00	0.00	2.10
2 wk	0.00	0.00	2.10	2.82	0.00	0.00	0.00
3 wk	0.00	0.00	0.00	2.48	0.00	0.00	0.00

$\log_{10}$ copies per 0.2 mL for Blood and bile; $\log_{10}$ copies per 0.5 g for others.

enteritidis clearly decreased, with the blood, heart, kidney, and pancreas not having a positive result. The mice were capable of carrying *S. enteritidis* cells were present up to 12 d PI in the gallbladder, 2 wk for the liver, and 3 wk for the spleen without causing any apparent symptoms. Importantly, the level of *S. enteritidis* cells number compared to the other organs at 2-12 d PI, the gallbladder can contain about 1.25×10^2 to 7.94×10^2 copies/0.2 mL. It may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis*, and *S. enteritidis* can persist over a 12 d period within it. Also, the control group did not have any positive results at any time in any location. The details are shown in Table 1.

The results of the traditional bacterial culture method

All the samples from different time points were detected by traditional bacterial culture method, and the results showed that the traditional bacterial culture method had positive results when the *S. enteritidis* target DNA concentrations were > $10^{4.02}$ copies per 0.5 g or 0.2 mL. Apparently, the FQ-PCR assay provides a more sensitive and accurate method for the this study.

DISCUSSION

Once attached to the host cells, *S. enteritidis* can manifest athletic phenomenon of invasion, such as phagocytized by cell invagination. After fused by macrophage, *S. enteritidis* cells will appear in cytoplasm, survive and multiply there[21]. Humoral immunity contributes to serve as the first line of defense against *S. enteritidis* infection. After the *S. enteritidis* cells are swallowed by macrophage and heterophil granulocyte, both of them happen to outbreak of breathing and produce oxygen free radical, for instance H_2O_2, OH-, O_2^-[22], which can kill and wound the bacterium. The cell factors, such as IL-1, IL-6 *et al*, also have the bactericidal effect for the *S. enteritidis* invasion[23]. In contrast, cellular immune responses have been rarely investigated, although such responses are crucial for protective immunity against *S. enteritidis*. The mechanisms by which *Salmonella*-specific $CD4^+$ T cells contribute to protective immunity are incompletely understood, but T-cell proliferation, the sine qua non of $CD4^+$ T-cell activation, and the production of gamma interferon can be regarded as in vitro indicators of these essential elements of protective immunity[24]. Our data (Table 1) showed that the *S. enteritidis* populations decreased obviously or were not detectable in most detected-samples at 6 d PI, without causing apparent symptoms. Perhaps, the results of this study indicate that the mice have an efficient protective immunity after oral challenge.

Salmonella cells have to attach to or form a close association with the intestinal epithelium in order to establish and persist in the gut and subsequently invade the underlying tissues[25,26]. *S. enteritidis* usually do not cause a disseminated systemic disease in humans, but clinically manifest as gastroenteritis and diarrhea[27]. The probability of establishment of persistent infection may involve a subtle interplay between host susceptibility and the challenge strain and does of *S. enteritidis*[10,28]. It has been reported that *S. enteritidis* can rapid distribution throughout the gastrointestinal tract, translocation to the mesenteric lymph nodes and spread to the liver and spleen of the mice. In the liver, the bacteria are ingested by the Kupffer cells, and then they invade the hepatocytes[29, 30]. In our studies, the spleens were positive at 12 h PI, but not the blood. It may be an indication that *S. enteritidis* cells were drained by the lymphatic system circulation (especially the Peyer's patchs in gut) priority. Also, recent studies have shown that some systemic spread of *salmonella* can occur without drainage through the lymphatic system and blood circulation[31]. Pathogen sampled subepithelial or even luminally by dendritic cells or CD18-expressing phagocytes can be transferred directly to the liver and spleen[32,33].

Previous studies showed that there is a association between *Salmonella* and intracellular survival in macrophages, which can be regarded as safe sites for bacterial multiplication[34,35]. However, how *S. enteritidis* survives within macrophages is unclear. It seems likely that the type Ⅲ secretion system encoded by *Salmonella* pathogenicity island 2 may play a major role[36,37]. One of the functions of *Salmonella* pathogenicity island 2 is to inhibit NADPH oxidase-dependent killing of *Salmonella*[38] . Also, it has been reported that the *S.* typhimurium genome encodes many mechanisms that allow resistance to the stressful environment encountered within the macrophage. Such stress include the production by NADPH oxidase of superoxide anion (O_2^-) and other reactive oxygen species (ROS), and by inducible nitric oxide (NO) synthase (iNOS) of NO and other reactive nitrogen species (RNS)[39,40]. A number of important bacterial pathogens infect, replicate, and persist within nucleated cells of the host, T-cell-mediated immunity has proven to be a critical factor in the effective clearance of many such intracellular bacterial pathogens. However, *Salmonella* can induce suppression of cellular responses[41]. Simultaneously, it has been

reported that chicken macrophages display differences in their responses to *S. enteritidis* and *S.* typhimurium, and contribute to the differential pathogenesis of these *Salmonella* serovars[42]. Also, it has been reported that *S. enteritidis* infection induce less inflammation resulting in a more commensal, while *S.* typhimurium infection can be cleared more rapidly by induction of inflammatory[43,44]. *S. enteritidis* resulted in increased splenic CD3 and reduced B populations, it was difficult to associate this increase with *S. enteritidis* clearance due to lack of any significant changes in $CD4^+$ of $CD8^+$ cells[45].The functions of the spleen and liver in filtration, immune responsiveness and activation of complement have been well documented. Spleen is a container for the lymphocyte-rich white pulp and macrophage-rich red pulp; it is comprised of distinctive B cells and macrophages. So, what we described above may be the reason for why significant number of *S. enteritidis* cells can persist over a long time in liver and spleen without causing apparent symptoms *in vivo* in this study. Up to day, the colonization mechanism of *S. enteritidis* in the gut and the internal organs are not clear and further studies should be carried out.

Interestingly, the gallbladder is a site of carriage in this study, it is also the storage site for bile. Our studies may be the first to report that *S. enteritidis* can persist for as long as 12 d PI in gallbladder in mice. Bile is produced in the liver and consists of many components, including bile salts, cholesterol, and bilirubin. Bile salts are detergents that aid in degradation and dispersion of lipids, and such make bile a good antimicrobial agent[7]. It has been reported that *Salmonella* is resistant to high concentrations of bile and individual bile salts[46]. From the liver, the *S.* typhimurium can be shed into the gallbladder, and this infection is frequently associated with gallbladder abnormalities, such as gallstones, also, this infection is often asymptomatic and can last for many years[5,6]. Biofilms have recently been implicated as the cause of many chronic infection in human[47]. *S. enteritidis* are capable of shielding themselves from environmental stress, host immune response and phagocytosis by the secretion of an apparently amorphous matrix of secreted polysaccharides[47-50]. This matrix provides a very stable environment and results in high levels of resistance to antimicrobial agents[46,50]. Often, it is difficult to clear the infection unless the substrate to which the bacteria are attached is removed[7]. In our studies, *S. enteritidis* cells can persist for 12 d PI with low concentrations, about 1.25×10^2 to 6.61×10^2 copies/0.2 mL. The gallbladder appeared to be gross lesion (such as swelling) at 20 h to 2 d PI. Interestingly, there were not any significant gross lesions over the 3-12 d PI period, although there was nearly the same number of *S. enteritidis* cells over the 12 d period.

FQ-PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy[51,52]. However, variation results may be due to either the PCR inhibitors, or a large amount of DNA from background organism DNA. In preliminary experiments, we used phenol/chloroform/isoamyl alcohol method to extract DNA of tissue from several control group samples (described above), and added 1.0×10^5 copies of the standard DNA for each. Finally, fluorimetic cycler measurements were performed as described above. The results showed that all the tests can obtain the expected data and the variability was statistically low, at < 4.3%. So, this methodology is very accuracy for studying on the distribution of *S. enteritidis* in the internal organs.

In conclusion, our results provide significant data for helping to clarify the pathogenic mechanism of *S. enteritidis* in the internal organs, and show that the liver and the spleen are the primary sites of invasion in normal mice after oral challenge, interestingly, the gallbladder is a site of carriage over a 12 d period in this study, to our knowledge, this is the first time reported, and future studies should be carried out.

COMMENTS

Background

There are over 2500 serovars in the genus *Salmonella*. It has been a public health concern for over 100 years, and the incidence of *Salmonella* infections has risen dramatically, especially those caused by *S. enteritidis*. Therefore, knowledge about *Salmonella* infection could be an additional means for decreasing the incidence of infection. Infection with *Salmonella* is usually started by oral ingestion of the pathogen, and is followed by bacterial colonization of the gut and invasion of internal tissues. As a matter of course, it is necessary to understand its mechanisms of action in the internal organs.

Research frontiers

To date, the regular distribution pattern for *S. enteritidis* in the internal organs of mice after oral challenge has not been established. FQ-PCR, as a rapid, sensitive technique for precise quantitation of nucleic acid, will be an ideal method to study the distribution of *S. enteritidis* in the internal organs.

Innovations and breakthroughs

These studies have identified the regular distribution pattern of *S. enteritidis* invasion in the internal organs, which is not described hitherto. Moreover, it may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis* over a 12-d period.

Applications

This study will provide significant data for clarifying the pathogenic mechanisms of *S. enteritidis* in the internal organs, and may ultimately lead to new insights in prevention and therapy.

Peer review

The manuscript by Deng and colleagues represents a clear and straight-forward study into the distribution and persistence on *S. enteritidis* in the internal organs of infected mice. The hypothesis and methods are clearly presented, and the results will help future studies.

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