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A rabbit model of non-typhoidal *Salmonella* bacteremia

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Abstract

Bacteremia is an important cause of morbidity and mortality in humans. In this study, we focused on the development of an animal model of bacteremia induced by non-typhoidal *Salmonella*. New Zealand White rabbits were inoculated with a human isolate of non-typhoidal *Salmonella* strain CVD J73 via the intra-peritoneal route. Blood samples were collected at specific time points and at euthanasia from infected rabbits. Additionally, tissue samples from the heart, lungs, spleen, gastrointestinal tract, liver and kidneys were obtained at euthanasia. All experimentally infected rabbits displayed clinical signs of disease (fever, dehydration, weight loss and lethargy). Tissues collected at necropsy from the animals exhibited histopathological changes indicative of bacteremia. Non-typhoidal *Salmonella* bacteria were detected in the blood and tissue samples of infected rabbits by microbiological culture and real-time PCR assays. The development of this animal model of bacteremia could prove to be a useful tool for studying how non-typhoidal *Salmonella* infections disseminate and spread in humans.

Keywords

Bacteremia; non-typhoidal *Salmonella* infections; rabbit model

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1. Introduction

Bacteremia and sepsis constitute important sources of morbidity and mortality in humans. Several bacteria including non-typhoidal *Salmonellae* (NTS) are important causes of infections and have shown to result in disseminated infections, even in modestly compromised hosts. Such infections cause significant mortality in humans. Non-typhoidal strains of *Salmonella enterica* (*S. enterica*) serotypes Enteritidis or Typhimurium have been reported to be major causes of such illnesses [1–7]. In a study from Spain of 172 cases of *Salmonella* bacteremia (70% Enteritidis and 17% Typhimurium), 16% of patients developed septic metastases, and 16% died [8]. In the 1990s, in Massachusetts General Hospital, 18% mortality was observed in 45 patients affected with bacteremia induced by NTS [9].

S. enterica serotype Enteritidis is a facultative intracellular pathogen that is a frequently isolated serotype in the United States and accounts for nearly 15% of human cases of salmonellosis. An infection with *S. enterica* generally causes a localized intestinal infection. However, *S. enterica* serotypes can spread systemically in the elderly, in young children and in immunocompromised individuals causing serious conditions such as septicemia and septic shock [10,11]. *S. enterica* serotypes Typhimurium and Enteritidis have been reported to account for 79–95% of all bacteremic NTS infections in sub-Saharan Africa [12–14]. Reports from the United Kingdom suggest that infection with drug resistant serotype Typhimurium may result in greater morbidity and mortality than infection with other serotypes, further complicating the burden of *Salmonella*- associated bacteremia, and illness [15]. Since the pathogenesis of *Salmonella*-induced sepsis cannot be studied in humans due to ethical reasons, development of an animal model, which mimics such disease, is critical. An animal model could provide important insights into how NTS infections disseminate and spread in mammalian hosts.

A central goal of all sepsis models is to reproduce clinically relevant pathogenesis that is similar to disease seen in humans. We focused on the development of an animal model of *Salmonella* induced bacteremia. A murine enteric fever model has previously been developed to evaluate *Salmonella* pathogenicity. Collins and Carter [16] have shown that irradiated germ-free mice developed diarrhea when they were orally challenged with non-typhoidal *S. enterica* and died within 5–8 days of challenge. However, these animals may not be representative of conventional animals as these mice were germ free and were irradiated during the course of the study. Furthermore, previous *Salmonellae* infection studies in mice have reported varying results regarding spread of the organisms, as well as of induction of bacteremia in infected animals [17–22]. These studies postulated different mechanisms related to the absorption and spread of bacteria in infected mice (elaborated in the discussion section). The mouse model has other limitations; including an insufficient volume of blood available to detect bacteria, important for studies of pathogenesis as well as diagnostic assay development. Since detection of low-level bacteremia in humans requires large blood volume samples for evaluation, we decided to explore a rabbit model to study *Salmonella* associated bacteremia. The rabbit animal model enabled evaluation of a larger volume of blood, which is required to translate information gained from animal studies to human studies.

Previous studies have shown that rabbits developed illness comparable to human salmonellosis [23]. We performed initial pilot studies in 2 New Zealand White (NZW) rabbits in which animals were inoculated with human clinical isolates of 10^{11} colony forming units per milliliter (CFU/ml) of non-typhoidal *S. enterica* bacteria via the orogastric route following previously reported oral inoculation techniques [23]. Although rabbits in this initial pilot study shed *Salmonella* in their feces, we could not detect bacteria by blood culture (data not shown). Additionally, none of the animals showed any clinical signs of disease (fever, diarrhea, dysentery, dehydration, lethargy and weight loss). Similar studies by others produced very low frequency [23] or no bacteremia [21, 22] during the first few days of infection with oral inoculations.

We conducted another pilot study in 2 NZW rabbits where animals were inoculated with non-typhoidal *S. enterica* bacteria (10^{11} CFU/ml) via a different route (intra-peritoneal-i.p.). Although rabbits in this study developed fever and weight loss, they did not display bacteremia (data not shown). We accordingly conducted another i.p. infection study with a higher dose of bacteria (10^{13} CFU/ml) in 2 NZW rabbits. Both rabbits developed fever, weight loss, diarrhea as well as bacteremia (data not shown).

Herein we report the successful development of the NZW rabbit as an animal model of *S. enterica*, in which bacteremia was induced, producing pathology. This model adds to the current body of knowledge regarding NTS animal models, and could prove to be a useful tool for studying how NTS infections disseminate and spread in humans. The rabbit model may further help in developing improved and novel rapid diagnostic assays used for detecting low levels of bacteria in the blood of infected individuals.

2. Materials and Methods

2.1. Animals

Specific pathogen free NZW rabbits (*Oryctolagus cuniculus*) were purchased from an approved vendor (Covance Research Products, Denver, PA). Rabbits used in this study were free of bacteria including pathogenic *Escherichia coli*, *Clostridium difficile*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, *Treponema cuniculi*, *Clostridium piliforme*, cilia-associated respiratory bacillus, *Salmonella* spp., ectoparasites, and fungi. The study was conducted at the AAALAC-accredited animal facility overseen by the Program of Comparative Medicine at the University of Maryland School of Medicine. Animals underwent a 48-hour acclimatization period at the animal facility before being included in this study. Ten female adult NZW rabbits (3.5–5 kg) were utilized in this study. Fecal samples from all rabbits were pre-screened and tested negative for presence of *Salmonella* spp. prior to study initiation. Additionally, these rabbits were observed for signs of diarrheal illness (diarrhea/dysentery) and were found to be free of such clinical signs. Animals were single-housed in cages maintained in an animal biosafety level 2 containment facility (ABSL-2), and appropriate measures were taken to ensure safe handling practices while working with the animals and bacteria. All animal procedures were carried out under ABSL-2 practices. All procedures were reviewed and approved by the Institutional Care and Use Committee and the Institutional Biosafety Committee of the University of Maryland School of Medicine. These protocols followed strict guidelines of the *Guide for the Care*

and Use of Laboratory Animals [24] and the Biosafety in Microbiological and Biomedical Laboratories [25].

2.2. Bacteria

A human clinical isolate NTS strain, *S. enterica* serotype Enteritidis (CVD J73), was utilized in this study. This strain was provided by Dr. Myron Levine (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD). The bacterial suspension was prepared by plating the organism for single colony isolation on multiple trypticase soy agar plates with 5% sheep blood (TSA II 5% sheep blood, Becton Dickinson, Sparks, MD) and incubating overnight at 37°C. Single colonies were added to 10 ml of autoclaved, filtered (Microcon DNA Fast Flow Centrifugal Filter Unit with Ultracel membrane, EMD Millipore, Billerica, MA) and nuclease free water (Ambion, Life Technologies, Grand Island, NY). Bacterial suspensions were compared to McFarland standards (Remel, Lexena, KS) until the appropriate titer (10^{12} CFU/ml or 10^{13} CFU/ml) was achieved, and 1 ml was removed for plating serial dilutions of the bacterial suspension to ensure the suspension was at the correct titer.

2.3. Experimental Design

All rabbits were inoculated with the CVD J73 strain via the i.p. route on Day 0 of the study. Animals were divided into 5 groups (n=2 per group). Rabbits in Groups 1–4 (n=2 in each group; identified by rabbits numbers, R1–R8) received 1×10^{13} CFU/ml and rabbits in Group 5 (n=2; identified by rabbit numbers R9 and R10) received 1×10^{12} CFU/ml of the bacteria. Blood samples were collected from each animal on a daily basis of the study starting on Day 0 prior to infection. Animals were monitored twice daily for clinical signs of disease (fever, lethargy, weight loss, dehydration, diarrhea and/or dysentery). Rabbits were scheduled for euthanasia and tissue collection at specific time points during the study. Two rabbits were scheduled to be euthanized each day, Day 1 through Day 4; i.e., Group 1 was euthanized on Day 1, Group 2 on Day 2, Group 3 on Day 3 and Groups 4 and 5 on Day 4 of the study. However, if the animals met alternative end points (discussed under section 2.6), they were euthanized at earlier time points to prevent undue pain, suffering, and distress. Blood, tissues, and tissue swabs were collected at euthanasia. Blood samples were cultured using standard blood culture methodology (BACTEC™) by the Johns Hopkins Medical Institutions Clinical Microbiology Laboratory (Baltimore, MD). Tissue swabs were plated on TSA II with 5% sheep blood (Becton Dickinson, Sparks, MD), to determine if any recovered bacteria was consistent with recovery of Gram negative rods, indicative of *Salmonella*. Nucleic acid DNA extracted from these colonies were subjected to standard real-time PCR assays to confirm presence of *Salmonella enterica* in these samples. Tissue samples (liver, kidneys, sections of the gastrointestinal tract, heart, lung, and spleen) collected at necropsy were subjected to histopathological analysis.

2.4. Bacterial Inoculations, Animal Monitoring and Sampling

Prior to bacterial inoculation, rabbits were maintained fasting overnight. On Day 0, water was removed two hours prior to the inoculation and blood (volume equivalent to 0.2% of the animal's body weight) was collected by venous access from each animal (pre-inoculation

baseline sample). Animals were anesthetized with 35–50 mg/kg Ketamine (KetaVed Ketamine HCl Injection, USP Vedco, St Joseph, MO) combined with 3–5 mg/kg Xylazine (AnaSed Injection, Ben Venue Laboratories, Bedford, OH) given subcutaneously (SQ). For the i.p. injection, the hair near the peritoneal area was clipped with a # 40 blade, and scrubbed with povidone-iodine and rinsed with 70 % alcohol. Bacteria (10^{12} and 10^{13} CFU/ml) suspended in 1 ml of autoclaved and filtered nuclease-free water was injected i.p. using a 20 gauge needle to rabbits (identified by R1–R10) belonging to Groups 1–5. All animals were administered 0.02–0.05 mg/kg of buprenorphine (Buprenorphine Hydrochloride Injection, PharmaForce, Columbus, OH) via the SQ route on Day 0 post-infection to reduce pain and distress. Post-inoculation, rabbits were placed on Gaymar thermostatically controlled warm water heating pads for thermal support, until they were able to remain sternal upon recovery from sedation. Food and water was made available to the animals upon return to housing and when recovered from sedation. Animals were monitored on a daily basis (twice daily) to check for clinical signs of disease (fever; body temperature of $>39.5^{\circ}\text{C}$, lethargy, weight loss, dehydration, diarrhea and/or dysentery). Animals displaying lethargy, dehydration, weight loss of 5% compared to pre-infection body weight, and/or diarrhea were treated with fluids-25ml/kg of Ringer's lactate solution (Lactated Ringer's Solution-Lactated Ringer's Injection USP, B Braun Medical, Scarborough, Ontario, Canada) administered SQ daily and analgesics (0.02–0.05 mg/kg buprenorphine given every 6–12 hours SQ) until signs resolved. Body weights and body temperatures of each animal were measured on a daily basis. Blood samples (volume equal to 0.2% of the animal's body weight) were collected from each animal on a daily basis post-infection until the end of the study. Larger blood volumes (25–30 ml) along with tissue samples and tissue swabs were collected from the rabbits at necropsy.

2.5. Euthanasia

All rabbits were scheduled to be euthanized at specific time points in this study (i.e., Group 1 rabbits were to be euthanized on Day 1, Group 2 on Day 2, Group 3 on Day 3 and Groups 4 and 5 on Day 4 of the study). We based these time points on preliminary studies in rabbits, where we noticed the presence of bacteria on Days 1–4 post-inoculation and clearance of bacteria thereafter (data not shown). This serial pattern of euthanasia enabled us to achieve collection of blood samples from infected rabbits throughout the course of the infection and provided us with large blood volumes at euthanasia important for detection of lower levels of bacteria present in blood. Prior to euthanasia, animals were anesthetized with 35–50 mg/kg Ketamine combined with 3–5 mg/kg Xylazine given subcutaneously using a 25G needle. Rabbits were euthanized by an intra-cardiac injection of sodium pentobarbital (100mg/kg) using a 25-G needle.

2.6. Alternative end points

The alternative euthanasia end points included weight loss >15 % of pre inoculation body weight, hemorrhagic colitis that was more than intermittent (formed stool present with blood spotting clots, not just hemorrhage or blood clots), inability to eat or drink for more than 12 hours or lack of movement around cage when manually stimulated (toe or ear touch/pinch).

2.7. Histopathology and tissue analysis

Tissues (heart, liver, lung, spleen, kidneys and sections of the gastro-intestinal tract) collected from rabbits at necropsy were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. Histopathologic findings were evaluated by a board-certified veterinary pathologist. Sterile culture swabs were collected from the tissues and subjected to microbiological culture via plating and confirmation of recovery of bacteria consistent with Gram negative rods/*Salmonella* and real-time PCR assay.

2.8. Blood Culture Analysis

Blood specimens collected daily (pediatric blood culture bottles – BD BACTEC™ Peds Plus™/F Culture Vials, Becton Dickinson, Sparks, MD) and at euthanasia (adult blood culture bottles – BD BACTEC™ Standard 10 Aerobic/F, Becton Dickinson, Sparks, MD) were cultured using standard blood culture methodology to identify the presence of bacteria in whole blood samples by the Johns Hopkins Medical Institutions Clinical Microbiology Laboratory (Baltimore, MD).

2.9. Real-Time polymerase chain reaction (PCR) for detection of *Salmonella* species

Tissues removed from rabbits at the time of necropsy including the heart, lungs, liver, spleen and kidneys were cut into 1 mm³ sections utilizing a sterile scalpel for each tissue section, and each tissue section was incubated in 200 µl of 20 mg/ml Proteinase K (Roche Diagnostics, Indianapolis, IN) for 24 hours at 65°C followed by Proteinase K inactivation at 95°C for 20 minutes. Whole blood and tissue swabs were plated on TSA II agar with 5% sheep blood and plates were incubated at 37°C for 24 hours. Any bacterial colonies that grew were picked from the plates and were resuspended in 1 ml of autoclaved, filtered and nuclease free water and stored at –80°C. Nucleic acid extraction, utilizing the Roche MagNA Pure LC Total Nucleic Acid Extraction Kit per manufacturer instructions, was initially performed on all tissue samples from each organ from each rabbit (N=60) for testing with real-time PCR to confirm *Salmonella enterica*. Positive controls for each extraction run consisted of *Salmonella enterica* bacteria, and negative controls for each run consisted of autoclaved, filtered and nuclease free water as *Salmonella enterica* negative tissue samples were not available.

Following real-time PCR analysis of tissue samples, recovered bacterial isolates from tissue swab samples were extracted and tested for any tissue sample that tested negative by real-time PCR for *Salmonella enterica* (N=20). *Salmonella enterica* bacteria were utilized as positive controls, and autoclaved, filtered and nuclease free water as *Salmonella enterica* negative controls. Extractions were performed as above, with *Salmonella enterica* bacteria utilized as positive controls, and baseline whole blood samples from Day 0 utilized as negative controls.

Nucleic acid extracts from tissues (N=60) and bacterial colonies recovered from tissue swabs (N=20) were analyzed by a *Salmonella* spp. specific PCR to confirm recovery of *Salmonella*. Additionally bacterial isolates recovered from whole blood (N=6) were also tested with real-time PCR for *Salmonella enterica*. A previously reported [26] real-time PCR assay was conducted with modifications. Briefly, each real-time PCR reaction

contained: 25 µl Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 2 µl of 10 µM for each primer and probe (400 nM final concentration), 0.5 µl of Amplitaq Gold (Applied Biosystems), 8.5 µl of nuclease free water (Ambion, Austin, TX) and 10 µl of sample. Primers and probes utilized to detect *Salmonella* were: LC Sal 1 (5'AACTTCATCGCACCGTCA3'), LC Sal 2 (5'TATTGTCACCGTGGTCCA3') LC Sal Probe (5'6FAM-TCGGCATCAATACTCATCTGTTTACCG-BHQ13'). Cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds, 60°C for 1 minute. Positive real-time PCR reactions were defined as reactions having a C_T value and an exponential increase in fluorescence above baseline. Positive amplification was confirmed through analysis of multicomponent data. A positive PCR result indicated the presence of *Salmonellaenterica*.

2.10. Statistical Analysis

Descriptive statistics analysis was performed. Linear regression analysis using generalized estimating equation [27] to account for intra-subject correlations was applied to determine if the temporal change in body weights and body temperatures in rabbits was significant with time. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary North Carolina).

3. Results

3.1. Clinical signs

Experimentally infected rabbits in this study displayed clinical signs of disease (fever; body temperature of more than 39.5°C, weight loss, dehydration and lethargy) as early as day one post-infection. Although a group size of 2 per group was utilized in the experiment, an overall of 10 rabbits were infected with *Salmonella* bacteria. As shown in Tables 1a and 1b, data points of the 10 rabbits were recorded making it a total of 32 data points. Linear regression analysis using generalized estimating equation to account for intra-subject correlations was applied on all available data points of body weights and temperatures of 10 rabbits ($n=32$) to determine if the temporal change in body weights and temperatures were was significant with time. All rabbits (10/10) displayed significant weight loss throughout the study period. Body weights of infected rabbits decreased 0.08 kg daily on average ($p<0.001$) with weight loss ranging from 5–20% among different groups when compared to their baseline weights on day 0 [Table 1a]. Significant weight loss was observed from day 0 to day 1 ($p<0.001$), day 1 to day 2 ($p<0.001$), and day 2 to day 3 ($p=0.020$). Infected rabbits showed elevated body temperatures during days 1–4 when compared to their baseline temperatures on day 0 of the study. Significant body temperature increase was observed from day 0 to day 1 ($p<0.001$) and day 2 to day 3 ($p<0.001$). Ninety percent (9/10) of rabbits exhibited fever (body temperature as high as 41.5°C; normal body temperature of rabbits ranges from 38–39.5°C) during the study [Table 1b]. Data were analyzed using generalized estimating equations approach for regression analysis. Five rabbits (one belonging to Group 2, two belonging to Group 3, and two belonging to Group 5) were euthanized due to meeting alternative end point criteria. These rabbits displayed significant weight loss, lethargy, lack of appetite and were unresponsive to stimuli. Clinical signs of disease in infected rabbits

suggested a high (90–100%) attack rate of infection attributable to the *Salmonella*-associated bacteremia.

3.2. Recovery of bacteria from biological samples

Day 0 (pre-inoculation baseline) blood samples for all rabbits tested negative as expected by blood culture. For blood samples collected at euthanasia, 100% (10/10) tested positive by blood culture. Blood samples that were collected daily post Day 0, and pre-euthanasia were 50% positive (5/10) [Table 2]. Times to culture positivity by the BACTEC™ varied from 5 hours and 56 minutes to 23 hours and 28 minutes, with a mean time to positive blood culture result of 15 hours and 28 minutes. A total of 60 tissue swab samples (10 rabbits; with six samples per rabbit) were analyzed for presence of bacteria via culture and PCR assays. Seventy percent (42/60) of tissue samples collected at necropsy grew bacteria consistent with *Salmonella* spp. (Gram negative rods and creamy white colonies in TSA II agar with 5% sheep blood) [denoted by Growth or No Growth in Table 3]. One hundred percent (10/10) of the spleen, 80% (8/10) of liver, 70% (7/10) of lung and kidney and 30% (3/10) of heart samples tested positive (growth of bacterial colonies in TSA II agar).

For PCR detection, tissue samples (N=60) were initially screened, and 40% (24/60) of these tissue samples were positive for *S. enterica*. These were tissues from which nucleic acid was extracted and directly subjected to real-time PCR. Tissue swabs were tested (from tissues which tested negative) for presence of bacteria via PCR.

Hence 36 such tissue swabs were tested (since 24/60 tested positive as mentioned above). For tissue swab testing, the swabs were plated in TSA II agar with 5% sheep blood and observed for growth of bacterial colonies. The bacterial colonies were then subjected to nucleic acid extraction and screened for *S. enterica* via real-time PCR. Of the 36 tissue swabs, 20 grew bacterial colonies, whereas the remaining 16 did not grow colonies. The bacterial colonies (from the 20 tissue swabs) were subjected to PCR and 90% (18/20) of these samples were positive for *S. enterica*.

In terms of detection of bacteria in various organs of infected rabbits with the real-time PCR assays (denoted by + or – in Table 3), 100% (10/10, 3 tissue samples and 7 bacteria recovered from tissue swabs) of spleen, 80% (8/10, 5 kidney pair tissue samples and 3 bacteria recovered from kidney pair swabs) of kidney, 70% (7/10, 6 liver tissue samples and 1 bacteria recovered from liver swab) of liver and lung (3 tissue samples and 4 bacteria recovered from lung swabs) and 20% (2/10, 2 tissue samples) of heart samples tested positive for *Salmonella enterica* by real-time PCR, which detected nucleic acid directly extracted from tissues (denoted by +* in Table 3) or from bacterial colonies growing on TSA II agar (supplied with 5% sheep blood) when tissue swabs were plated on it (denoted by +[#] in Table 3). Of the bacteria recovered from whole blood samples, 67% (4/6) tested positive for *Salmonella enterica* by the real-time PCR assay. There are different possibilities to account for the samples that had bacterial growth, but were negative by PCR. PCR inhibitors could have been introduced during the nucleic acid extraction process, there could have been poor nucleic acid extraction efficiency for those samples resulting in the amount of nucleic acid extracted being below the level of detection for the and lastly, because the assay utilized was slightly adapted for this study, there could variations in PCR reaction

efficiency that could have generated the false negative results. A summary of the microbiological culture and real-time PCR assay results from all infected rabbits are displayed in Table 3. The real-time PCR, blood culture results, as well as microbiological findings from the swabs collected from various tissue samples confirmed the presence of *Salmonella enterica* bacteria in blood and multiple organs in the infected rabbits, indicating the development of bacteremia produced by *S. enterica* serotype Enteritidis.

3.3. Gross and histopathology

Gross and histopathology was observed in several organs of infected rabbits. At necropsy, rabbits exhibited gross pathological findings including congested heart and lungs, mottled appearance of kidneys and friable/discoloration of the liver. Histopathological findings included areas of infiltration of neutrophilic cells in the ileum (Figure 1a) and lymphoid necrosis (Figure 1b) indicative of acute/subacute enteritis. Grossly, the liver exhibited areas of congestion, petechial hemorrhages and necrosis (Figure 2a). Histopathological lesions of this liver showed areas of neutrophilic infiltration (Figure 2b) and necrosis (Figure 2c). The spleen of infected rabbits showed areas of lymphoid depletion and congestion. Overall histopathological and gross findings displayed pathology of multiple organs (liver, kidney, lungs, heart and areas of the gastrointestinal tract) indicative of acute to subacute enteritis and hepatitis due to septicemia/bacteremia associated with the *S. enterica* experimental infection.

4. Discussion

Bacterial infections can cause septicemia/bacteremia and have serious consequences especially in the young, elderly or immunocompromised human populations. Further understanding of the pathogenesis and dissemination of bacterial infections is required. The goal of our study was to develop an animal model of *Salmonella* bacteremia. Such a model has value both for studying the pathophysiology of NTS infection, but also could help evaluate new diagnostic tools for NTS and other bacteria in blood.

In our current study in rabbits we found that we could reliably induce bacteremia using nontyphoidal *Salmonella* spp. with an i.p. injection of a large number of viable organisms, overwhelming the natural defense of the peritoneal cavity. We were able to reduce the time course to development of bacteremia (as might occur with a peritoneal abscess), achieving acute bacteremia over a period of 1–4 days after inoculation. This model resulted in 100% of the inoculated animals developing bacteremia. Rabbits in our study developed clinical signs of disease characterized by fever, weight loss, dehydration and lethargy. Histopathological and gross findings showed extensive pathology indicative of enteritis and acute hepatitis. Several animal models of bacteremia/septicemia have been previously advanced [17–23, 28–32]. The murine enteric fever model has been utilized extensively to evaluate *Salmonella* pathogenicity. With that model, however, the absence of clinical signs which mimic disease seen in humans, has been limiting. Collins and Carter conducted a study in germ-free mice, in which mice when given *Salmonella* bacteria via the intragastric route, showed localized growth of bacteria in the intestinal lumen with no systemic infection [16]. However, when host resistance in these mice was altered via whole-body irradiation when infected with the *Salmonella* bacteria, they developed severe diarrhea and died within 5–8 days as a result of

systemic disease. Moreover, animals in this study were not representative of healthy hosts, as their host resistance was reduced by means of sub-lethal, whole-body gamma irradiation before challenge. Prior comparative studies which have utilized oral, i.p. and subcutaneous routes of inoculation with *Salmonella* in mice have generated variable results with spread and dissemination of bacteria. [17–22]. As an example, when mice in these studies were orally inoculated with 10^9 CFU of *S. enterica* bacteria, they developed bacteremia, but no clinical signs. However, entirely different findings were obtained from an i.p. inoculation of the same dose of *S. enterica* in similar mice, with blood and spleen samples from all (100%) of the infected animals demonstrating presence of bacteria [18]. This study by Gerichter (18) reported the dissemination of *Salmonella* bacteria through the organs of mice infected with the bacteria via the orogastric, subcutaneous, intramuscular and i.p. routes. Their results showed that the bacteria pass from the alimentary tract into the blood stream and reach various organs but do not multiply in these organs and hence do not produce secondary bacteremia. These results differed from those obtained by Orskov *et al.* (21). Their data showed that oral administration of *Salmonella* bacteria lead to localization/restriction of the bacteria in mesenteric lymph nodes. Gerichter's study (18) also reported the presence of bacteria for at least 16 days in the jejunum of infected mice. This was in contrast to Orskov *et al.*'s study (21), which showed that the bacteria disappeared rapidly from the intestines. These studies postulated different mechanisms related to the absorption and spread of bacteria from the gastro-intestinal tract to the blood stream. Other limitations of the mouse model have been reported. A review by Zhang *et al.*, (33) showed that NTS infections in mice and in humans cause dramatically different intestinal pathologies, elicit different host responses, and result in different disease syndromes. These differences make it difficult to improve the understanding of the pathogenesis of NTS infection in humans by extrapolating from data obtained using the mouse model.

Most clinical isolates of NTS from immunocompetent hosts can infect a broad range of potential vertebrate hosts (34). Because it offers genetic homogeneity, the mouse model is the typically used animal model in the determination of genes for virulence and host resistance. But other models including human volunteers, calf, cow ileal loops, rabbits, the nematode, and chickens are used to study human typhoid fever and gastroenteritis (35, 36). Several mouse strains have been used and were classified according to their susceptibility to *Salmonella* infection (37). Genetically modified murine strains offer great opportunities in understanding *Salmonella* disease pathogenesis. As an example, toll-like receptor deficient mice have been utilized to understand the role of specific immune responses to *Salmonella* infections (38). Inbred mice susceptible to *Salmonella* infection, may not be an accurate model because of the heterogeneity of human populations. However, studies in outbred animals pose significant variability within subjects. We conducted our study to explore the pathogenesis of *Salmonella* induced bacteremia in a larger animal (rabbit) model.

Animal models are comparative in nature and meant to provide data relevant to human cases. The wider the range of genetic backgrounds represented by different mouse strains or, as in this case, a different species, the rabbit, all using the same infectious agent leads to an increase in the relevance of the results as valuable animal models.

In our first pilot study we utilized NZW rabbits inoculated orally with 10^{11} CFU/ml of *S. enterica*. Although the rabbits shed bacteria in their feces they did not develop clinical signs of disease (fever, weight loss, lethargy, diarrhea and/or bacteremia). Hanes *et. al* [23] have reported diarrheal disease and bacteremia in NZW rabbits inoculated orally with non-typhoidal *S. enterica*. Bacteria were isolated from intestines, livers, spleen and blood of these animals although the percentage or number of animals that developed bacteremia, was not presented or discussed. Our initial oral infection studies in NZW rabbits did not produce bacteremia. Since the i.p. route of infection has consistently produced bacteremia in mice [18], we chose to further explore the i.p. route of infection in NZW rabbits. We inoculated rabbits via the i.p. route, at dose ranges 10^{11} – 10^{13} CFU of non typhoidal *S. enterica* and followed them for up to four days post infection. The short time frame for this study was based on our observation with previous studies in rabbits, wherein animals cleared bacteria in blood within 4–5 days after being infected, as well as prior observations that bacteria in blood of infected mice frequently clears during a similar time period, post-infection [18].

We chose to expand this study and conduct the present study in a larger group of NZW rabbits (N=10) using higher doses of *S. enterica* (10^{12} and 10^{13} CFU/ml) given via the i.p. route. Results from this study showed the development of clinical signs of disease (fever, weight loss, lethargy and dehydration) in all experimentally infected rabbits. Furthermore, evaluation of blood samples and various organs (heart, spleen, kidneys, lungs and liver) via blood culture, microbiological and real-time PCR assays yielded positive detection of bacteria. Finally, blood culture analysis of all infected rabbits taken at euthanasia tested positive (10/10) for the presence of bacteria [Table 2]. Blood culture samples taken at various points prior to study end point or euthanasia (5/10) also tested positive for presence of *S. enterica* indicating the development of bacteremia at varying time points (from days 1–4) post-infection in the rabbits [Table 2]. This may also indicate that larger blood volumes are required for detecting low level bacteremia in infected animals (since the 25–30 ml of blood was collected at necropsy whereas 5 ml of blood was collected prior to euthanasia). Microbiological culture and real-time PCR analysis of tissue samples collected at euthanasia from infected rabbits revealed the presence of bacteria in the heart, lung, liver, spleen and kidney samples of infected rabbits. All rabbits (100%) tested positive for the presence of bacteria in their spleens and 70–80% of the rabbits tested positive for the presence of bacteria in their livers. Varying rates of positivity in these organs may be related to the critical role these organs play in the clearance of bacterial infections [39–43]. It has also been reported that different bacteria distribute differently within the reticuloendothelial system [44]. Consistent with that observation, we noticed varying distribution of the *S. enterica* bacteria within the hearts, lungs, kidneys, spleens and livers of the infected rabbits [Table 3].

Our findings indicated dissemination of *Salmonella* in blood and various organs of infected rabbits. Gross and histopathological lesions in infected rabbits indicated that multiple organs were affected by the bacteremia induced by the *S. enterica* serotype Enteritidis bacteria. Limitations of our study include the large numbers of organisms that were required to produce bacteremia in the experimental animals. Alternate routes of infection may be evaluated. Earlier studies have reported the use of such routes in a continuous intravenous

infusion of bacteria [29, 30] or induction of septicemia with an intra-peritoneal blood clot, wherein implantation of a bacteria-laden fibrin clot into the peritoneum resulted in septicemia in a porcine model [28].

In our experience lower numbers of bacteria (10^8 and 10^{11} CFU) given orally, were unable to produce bacteremia in adult NZW rabbits (data not shown). The use of younger rabbits for oral infection studies resulting in bacteremia may be warranted. However, utilization of younger or smaller-sized rabbit models was hindered by our requirement to obtain larger volumes of blood samples for purposes of detection of low-level bacteremia. To a degree, the i.p. infection we describe here for *Salmonella* is artificial in comparison to natural infection. Although this is true, it is one way in which the pathogenesis of bacteremia can be evaluated separately from the intestinal infection, albeit in this case following an i.p. infection. Since the bacteremia in our study occurs over a number of days (1–4 days), it is not simply a result of the number of bacteria inoculated, rather it is an active infection set up in the peritoneal cavity, which could overwhelm the immunological barriers and result in the development of bacteremia. Our current study was able to establish a NZW rabbit model of *Salmonella*-induced bacteremia. This model may serve as a valuable tool to better understand the underlying pathological mechanisms involved in the dissemination and spread of bacteria via the hematogenous route.

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Highlights

Rabbit model of bacteremia

Non typhoidal Salmonella strain CVD J73

Detection of Salmonella with PCR

Figure 1a:

Figure 1b: **Figure 1.**

Figure 1a: Hematoxylin and eosin stained section of the ileum of a New Zealand White rabbit infected with 10^{13} CFU of *Salmonella enterica* serotype Enteritidis via the intraperitoneal route. This ileum section shows areas of acute inflammation with neutrophilic infiltration (arrow). Magnification 20X.

Figure 1b: Hematoxylin and eosin stained section of the ileum of a New Zealand White rabbit infected with 10^{13} CFU of *Salmonella enterica* serotype Enteritidis via the intraperitoneal route. This ileum section shows areas of lymphoid necrosis (arrow) with infiltration of neutrophils indicative of acute/subacute enteritis. Magnification 40X.

Figure 2a:

Figure 2b:

Figure 2c:

Figure 2.

Figure 2a: Gross pathology of the liver observed in a New Zealand White rabbit infected with 10^{13} CFU of *Salmonella enterica* serotype Enteritidis via the intraperitoneal route. The liver is friable and has extensive congestion and dark discoloration of the parenchyma combined with areas of necrosis. Figures 2b and 2c show histological sections of this liver.

Figure 2b: Hematoxylin and eosin stained section of liver of a New Zealand White rabbit infected with 10^{13} CFU of *Salmonella enterica* serotype Enteritidis via the intraperitoneal route. This liver section shows inflammatory areas with infiltration of neutrophils and macrophages (arrow). Magnification 40X.

Figure 2c: Hematoxylin and eosin stained section of liver of a New Zealand White rabbit infected with 10^{13} CFU of *Salmonella enterica* serotype Enteritidis via the intraperitoneal route. This liver section shows areas of necrosis (arrow). Magnification 40X.

Table 1a

Body weights of infected rabbits (R1–R10 denote rabbit numbers belonging to Groups 1–5) inoculated with *S. enterica* serotype Enteritidis bacteria via the intra-peritoneal route. Rabbits R1–R8 belonging to Groups 1–4 respectively, were inoculated with 10¹³CFU and rabbits R9 and R10 (Group 5) with 10¹² CFU of the bacteria. Five rabbits in the cohort (R3, R5, R6, R9 and R10) met their alternative end points and were euthanized prior to their scheduled euthanasia time points. Linear regression analysis using generalized estimating equation [27] to account for intra-subject correlations was applied to determine if the temporal change in body weights was significant with time. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary North Carolina).

Day	Rabbits											
	Post-infection		<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>	<u>R5</u>	<u>R6</u>	<u>R7</u>	<u>R8</u>	<u>R9</u>	<u>R10</u>
		<u>Group 1</u>		<u>Group 2</u>	<u>Group 3</u>		<u>Group 4</u>		<u>Group 5</u>			
		<u>Body weight (kilograms)</u>										
0		3.82	4.41	3.95	3.43	4.19	4.07	3.90	3.47	3.96	4.27	
1		3.07	3.95	3.82	3.29	4.04	3.90	3.89	3.28	3.83	4.16	
2					3.29	3.93	3.64	3.79	3.23	3.73	4.00	
3								3.70	3.10		4.00	
4								3.66	3.01			

Table 1b

Body temperatures of infected rabbits (R1–R10 denote rabbit numbers belonging to Groups 1–5) inoculated with *S. enterica* serotype Enteritidis bacteria via the intraperitoneal route. Rabbits R1–R8 belonging to Groups 1–4 respectively, were inoculated with 10¹²CFU and rabbits R9 and R10 (Group 5) with 10¹² CFU of the bacteria. Fever was defined by a body temperature of > 39.5°C. Five rabbits in the cohort (R3, R5, R6, R9 and R10) met their alternative end points and were euthanized prior to their scheduled euthanasia time points. Linear regression analysis using generalized estimating equation [27] to account for intra-subject correlations was applied to determine if the temporal change body temperatures was significant with time. P < 0.05 was considered statistically significant. Statistical analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary North Carolina).

Day	Rabbits											
	Post-infection		<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>	<u>R5</u>	<u>R6</u>	<u>R7</u>	<u>R8</u>	<u>R9</u>	<u>R10</u>
		<u>Group 1</u>	<u>Group 2</u>	<u>Group 3</u>	<u>Group 4</u>	<u>Group 5</u>						
		<u>Body temperature (°C)</u>										
0		38.3	38.7	38.9	39.7	39.2	38.9	39.3	39.1	39.4	39.6	
1		40.0	40.3	40.4	39.3	39.5	40.9	39.9	38.8	40.0	41.3	
2					37.8	39.8	41.0	41.2	39.9	40.1	40.6	
3								41.4	40.7		41.1	
4								40.4	41.5			

Table 2

Detection of *Salmonella enterica* from whole blood samples of infected rabbits (R1–R10) with BD BACTEC™ blood culture system.

	Group 1		Group 2		Group 3		Group 4		Group 5	
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
RABBIT										
Day 0	–	–	–	–	–	–	–	–	–	–
Day 1	+	+	+	+	–	+	–	–	+	+
Day 2	n/a	n/a	n/a	n/a	+	+	–	+	+	+
Day 3	n/a	n/a	n/a	n/a	n/a	n/a	+	–	n/a	n/a
Day 4	n/a	n/a	n/a	n/a	n/a	n/a	+	+	n/a	n/a

+: Bacterial growth

–: No bacterial growth

n/a= not applicable as the specific rabbits were euthanized on prior days

Table 3

Detection of *Salmonella enterica* from tissues and bacterial isolates obtained from tissue swabs from infected rabbits using microbiological culture and real-time PCR assays. For the microbiological assays, sterile swabs collected from tissues at necropsy were plated on trypticase soy agar (TSA) II with 5% sheep blood and plates were incubated at 37°C for 24 hours. Plates were observed for growth of colonies after 24 hours of incubation. Nucleic acid extracts from tissues and bacterial colonies, were then analyzed by a previously described [26] *Salmonella* spp. specific real-time PCR assay to confirm recovery of *Salmonella enterica* bacteria.

	Group 1		Group 2		Group 3		Group 4		Group 5	
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
RABBIT										
Heart	Growth/+*	Growth/+*	No Growth/-	Growth/-	No Growth/-	No Growth/-	No Growth/-	No Growth/-	No Growth/-	No Growth/-
Lung	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	No Growth/-	No Growth/-	No Growth/-	Growth/+*	Growth/+*
Liver	Growth/+*	Growth/+*	Growth/-	Growth/+*	No Growth/-	Growth/+*	Growth/+*	No Growth/-	Growth/+*	Growth/+*
Spleen	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*
R. Kidney	Growth/+*	Growth/+*	Growth/+*	Growth/+*	Growth/+*	No Growth/+*	No Growth/-	No Growth/-	Growth/+*	Growth/+*
L. Kidney	Growth/+*	Growth/+*	Growth/+*	Growth/+*	No Growth/+*	Growth/+*	No Growth/-	No Growth/-	Growth/+*	Growth/+*

Growth = Organism growth observed in TSA II with 5% sheep blood

No Growth = No organism growth observed TSA II with 5% sheep blood

+ = positive for *S. enterica* with real-time PCR assay; +* = positive for nucleic acid extracted from tissue and +[#] = positive for nucleic acid extracted from bacterial colony growing in TSA II agar with 5% sheep blood from tissue swab

- = negative for *S. enterica* with real-time PCR assay; both for nucleic acid extracted from tissue or tissue swab.