Published in final edited form as:

Future Microbiol. 2015 ; 10(10): 1615–1627. doi:10.2217/fmb.15.93.

Contaminated water delivery as a simple and effective method of experimental *Salmonella* infection

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Abstract

Aims—In most infectious disease models, it is assumed that gavage needle infection is the most reliable means of pathogen delivery to the gastrointestinal tract. However, this methodology can cause esophageal tearing and induces stress in experimental animals, both of which have the potential to impact early infection and the subsequent immune response.

Materials and Methods—C57BL/6 mice were orally infected with virulent *Salmonella* Typhimurium SL1344 either by intragastric gavage preceded by sodium bicarbonate, or by contamination of drinking water.

Results—We demonstrate that water contamination delivery of *Salmonella* is equivalent to gavage inoculation in providing a consistent model of infection. Furthermore, exposure of mice to contaminated drinking water for as little as 4 hours allowed maximal mucosal and systemic infection, suggesting an abbreviated window exists for natural intestinal entry.

Conclusions—Together, these data question the need for gavage delivery for infection with oral pathogens.

Keywords

oral infection; water contamination; intragastric; gavage; natural route; alternative infection technique; experimental stress; *Salmonella*

1 Introduction

Many enteric pathogens survive in nutrient-poor environments and can persist in soil, sewage, lake water, food, or drinking water before transmission to a susceptible host [1]. Once accessing the mucosal tissues of the host, these pathogens express virulence mechanisms that allow for epithelial attachment and/or penetration [2–4]. Many pathogens have also evolved specific strategies that enable them to avoid or suppress innate and adaptive immune responses and this immune evasion is thought to facilitate in vivo

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replication [5–7]. Given the complexity of these early host-pathogen interactions it is preferable that the immunological response to infection is studied in an animal model that allows a natural route of infection. For enteric pathogens this typically involves oral infection of inbred mice, a process that is usually accomplished by delivering a microbial bolus directly to the stomach with an oral gavage needle [8]. This gavage needle delivery method was developed over 100 years ago to provide consistent and uniform dosing of chemical compounds in mice [9], but also provides a simple methodology to ensure accurate doses of an enteric pathogen are delivered to the gastrointestinal tract.

Salmonella contamination of food or fresh produce routinely causes outbreaks of self-limiting gastroenteritis in the US [10–13], and *Salmonella* is considered a Category B Select Agent by the US government due to the potential for intentional large-scale food or water contamination [14], however, the impact of *Salmonella* infection is substantially greater in developing nations [15–18]. *Salmonella enterica* Typhi and Paratyphi are human-restricted serovars that cause a systemic infection of the reticuloendothelial system called typhoid fever [19, 20]. Recent estimates suggest that typhoid fever affects 27.1 million people and causes 217,000 deaths annually, with most of these cases localized to south, and southeast, Asia [15, 21]. Further, serovars of *Salmonella* typically associated with gastroenteritis can cause a systemic non-typhoidal Salmonellosis in immune compromised individuals, which is particularly problematic in areas of Africa where the incidence of HIV infection is high. [22] *Salmonella* serovars are therefore responsible for a high level of morbidity and mortality, especially in geographical areas where sanitary facilities and clean water are limited [5, 23–25]. Greater understanding of the host response to natural *Salmonella* infection would assist the development of new vaccines to combat typhoidal and non-typhoidal Salmonellosis [26, 27].

Inbred strains of mice are often used to study the innate and adaptive immune response to *Salmonella* infection in the laboratory. C57BL/6 mice are highly susceptible to infection with *S. enterica* serovar Typhimurium and can be infected via systemic (intraperitoneal (IP) or intravenous (IV)), or mucosal (oral) routes [5, 25]. Direct systemic inoculation of *Salmonella* via IP and IV routes avoids an early host response in the intestinal mucosa and thus has the potential to introduce artificial variables. However, systemic administration also reduces variability between animals making it a useful approach to easily interrogate the systemic immune response to disseminated organisms. Oral infection is usually accomplished with a gavage needle and this can be preceded by fasting or pretreatment with bicarbonate to increase stomach pH. After gaining access to the intestinal lumen, *Salmonella* are able to penetrate specialized microfold (M) cells present over the epithelial surface of Peyer's Patches (PP) in the small intestine. Immediately beneath this PP epithelial layer, *Salmonella* infect dendritic cells and macrophages and can subsequently migrate via lymphatic vessels to establish a systemic infection of the bloodstream, spleen, liver, and bone marrow [4, 25, 28–30].

The intention of experimental gavage delivery is to simulate an oral infection by placing a known quantity of bacteria directly to the stomach [25, 31]. However, a major drawback to this method is that the gavage needle has the potential to damage the esophageal lining, raising some concern that bacteria could use experimentally-induced abrasions to access the

host via an unnatural route [8, 32–34]. In addition, the restraint and manipulation required for effective oral gavage dosing induces significant stress in experimental animals [33, 35, 36], which could potentially impact the immune response [37–40] and/or susceptibility to infection [41–44]. Taken together, although oral gavage is an effective means of oral infection, the procedure itself has the potential to affect the route of bacterial entry and the subsequent host response.

The alternative approach of simply adding live vaccines or pharmacological agents to the water supply has been shown to be successful in livestock, poultry, and swine. [45] Furthermore, administration of medication to rodents in food or water has remained popular, particularly when repeated treatments are required [35, 46–48]. There has been recent interest in the administration of bacteria to mice in the food or water supply [49–51]. However, a systematic comparison of intra-gastric gavage and water contamination as a model of oral infection are currently lacking.

In this current study, we examined whether contaminated water delivery of *Salmonella* would be a useful experimental strategy for consistent laboratory infection of inbred mice. We show that C57BL/6 mice were infected efficiently and consistently by water contamination and that rates of infection and subsequent bacterial growth were comparable to gavage needle delivery. Using this natural infection approach, maximal host infection was completed within a relatively brief time window and continued exposure to contaminated water had little effect on infection. We conclude that contaminated water delivery is a highly effective approach to laboratory infection, does not require technical skill in animal handling, eliminates potential artifacts associated with gavage delivery, and is more likely to mimic the physiological conditions of natural oral infection with *Salmonella*.

2 Methods

2.1 Mice

C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8–16 weeks of age. Mice exposed to contaminated water were housed in groups, similar to gavage infected mice. All animal procedures were approved by the Institutional Animal Care and Use Committee (protocol 16932) at the University of California, Davis.

2.2 Bacterial strains and infection

The *Salmonella enterica* serovar Typhimurium strain used in this study was the virulent strain SL1344. *Salmonella* were grown overnight in Luria-Bertani broth (Becton Dickinson) culture at 37°C without shaking. Culture without shaking avoids bacterial overgrowth and has been used routinely in previous experiments by our laboratory in previous studies examining *Salmonella* immunity [52–54]. Bacterial concentration was estimated using a spectrophotometer, with bacteria being grown to an OD₆₀₀ of between 0.4–0.6. For both infection routes, an appropriate volume of overnight *Salmonella* culture was centrifuged for 30 minutes at 6000–8000 rcf at 4°C. Supernatant was discarded and bacterial pellets were resuspended in a total of 50ml of water. This concentrated bacterial suspension was then diluted to appropriate bacterial numbers in different water sources, as indicated. For

infection by water contamination, water was contaminated in a single volume, then used to refill individual water bottles. For oral infection by gavage needle, mice were pretreated with 5% NaHCO₃ solution ig before being infected with *Salmonella* diluted in PBS. In all experiments, the actual concentration of bacterial dose administered was confirmed by serial dilutions and plating onto MacConkey agar plates (Becton Dickinson).

2.3 Water sources

Various water sources were tested for their impact upon *Salmonella* survival after water contamination. These sources included 1) mouse cage bottles: unchlorinated, potable drinking water typically provided to mice by animal care staff, 2) laboratory faucets: unchlorinated, non-potable tap water from laboratory sink, 3) kitchen faucets: chlorinated, potable drinking water, as provided on campus.

2.4 Enumeration of bacteria in organs and water

For bacterial loads in organs, mice were euthanized, and the indicated organs from infected mice were harvested into 1X PBS (Gibco) on ice, homogenized and reconstituted in a known volume of PBS. For determination of bacterial survival in water, 100µl aliquots were removed from sample tubes after vortexing. Samples from organs or water were mixed thoroughly, and serial 1:10 dilutions were plated on MacConkey agar plates, incubated overnight at 37°C, and enumerated the following day for calculation of the number of cfu in the total organ or tube.

2.5 Statistical analyses

All statistical analyses were performed as described in the figure legends and results with GraphPad Prism versions 5 or 6. In brief, comparisons between 2 groups were made by 2-tailed student t-test, unless a significant difference in variance was indicated by F-test, in which case a t-test with Welch's correction was used (Fig 4). Survival was compared by the log-rank (Mantel-Cox) test (Fig 2). All error bars represent the mean ± SEM (standard error mean). ***p < 0.005, **p < 0.01, *p < 0.05, or p > 0.05 (ns)

3 Results

3.1 *Salmonella* survival in water is dependent upon water source and inoculation dose

Before determining the feasibility of water contamination as a route of *Salmonella* infection in mouse studies, we first examined bacterial survival in different water sources, since viability in drinking water is a necessary pre-requisite for infection. Initially, water taken from (i) animal housing cage bottles, (ii) laboratory faucets (non-potable), or (iii) kitchen faucets (potable, chlorinated) was inoculated with different doses of *Salmonella* (serovar Typhimurium, virulent strain SL1344) and examined for bacterial survival at one day (Fig. 1A) or one week (Fig. 1B). Contamination with 1×10⁹ bacteria/ml generated similar recovery of bacteria from all three water sources and displayed very little loss of bacteria at one day or one week time-points (Fig. 1). However, lowering the initial contaminating dose reduced bacterial survival in both laboratory and kitchen water, particularly when doses of 1×10³ and 1×10⁵ were used (Fig. 1). In contrast, water from animal housing cage bottles allowed efficient recovery of bacteria at all administered doses (Fig. 1).

We next examined the longevity of bacterial survival in this water source in more detail. At low doses (10^3 and 10^5) *Salmonella* were unculturable from contaminated water after 6–8 weeks, although this varied slightly in individual experiments (Fig. S1A and B). At the higher initial dose of 10^7 , bacteria persisted in water for up to 16 weeks after inoculation (Fig. S1C), and for more than 25 weeks when starting at 10^9 bacteria (Fig. S1D), although again this varied in individual experiments (Fig. S1). We conclude that *Salmonella* have the capacity to survive for long periods in water but display some variability depending on the water source and especially when seeded at a low initial doses. These data suggest a need to test individual water sources carefully prior to adopting a contaminated water delivery approach in the laboratory.

3.2 Water contamination is an effective means of experimental *Salmonella* infection

The data above demonstrate that bacterial inoculations of 1×10^7 – 10^9 bacteria/mL remain relatively stable in water for a substantial period of time and could therefore provide a consistent source of bacteria for oral infection. It has been established that an average C57BL/6 mouse (15g) consumes approximately 4mLs of drinking water per day [1, 55], allowing a rough estimate of an appropriate bacterial concentration for contaminated water delivery. We initially examined whether C57BL/6 mice could be consistently infected after exposure to contaminated water and whether the rate of death in these mice would differ from gavage delivery of a known bacterial dose. Mice that were administered 1×10^7 bacteria by oral gavage or exposed to drinking water containing 1×10^7 bacteria/mL succumbed to infection at a similar rate between day 7 and 10 post-infection (Fig. 2A). Administration of a ten-fold higher dose in drinking water resulted in similar survival, whereas administration of 1×10^8 bacteria by gavage modestly accelerated the time taken to develop a moribund state (Fig. 2B). These data demonstrate that C57BL/6 mice can be easily infected via drinking water and that the time to death approximates a comparable dose via gavage delivery.

3.3 Water contamination delivery preserves a natural route of oral infection

Next, we examined the timing of bacterial dissemination to different tissues after gavage delivery or the exposure of mice to *Salmonella* in drinking water. As expected, administration of 1×10^9 *Salmonella* by gavage needle caused bacterial colonization of the mesenteric lymph nodes (MLN) within 1 day of infection and elevated numbers of bacteria were detected in this tissue at every subsequent time point (Fig. 3B). In this same group of mice, the spleen and liver were seeded with *Salmonella* between 2 and 3 days post-infection, after which bacterial replication occurred in both tissues (Fig. 3B). Mice that were exposed to contaminated drinking water containing 1×10^9 bacteria/mL displayed an almost identical pattern of infection in the MLN, spleen, and liver (Fig. 3B), suggesting that bacterial entry and dissemination occur very similarly using both experimental approaches.

A similar situation occurred in mice administered a lower dose of bacteria (1×10^7) by gavage, or exposed to contaminated water with 1×10^7 bacteria/mL. Although both groups displayed lower bacterial burdens and had increased mouse-to-mouse variability, the overall tempo of infection was comparable in the spleen and liver between gavage and contaminated drinking water (Fig. 3A). We next focused on the day 4 time point after infection to complete a more rigorous comparative analysis between contaminated water delivery and

gavage administration at 1×10^7 and 1×10^9 infection doses. At both doses, there was no statistically significant difference in the bacterial burden in the MLN, spleen, or liver whether bacteria were administered by gavage needle or water contamination (Fig. 4). An F-test for variance showed significantly higher variance in the gavage-infected mice at the 10^9 dose for all organs, thus significance was analyzed by T-test with Welch's correction.

Interestingly, in the above experiments, the only mouse with detectable bacteria in the spleen and liver at day 1 after gavage delivery also displayed oral bleeding during the gavage procedure, perhaps suggesting that esophageal abrasions account for early systemic infection. We tried to examine this issue directly in a subsequent experiment by simply sacrificing mice on day 1 after infection and stratifying the mice that had displayed any evidence of complication (bleeding or liquid aspiration) during the gavage procedure, compared with those that had uncomplicated infection. In this particular experiment, we had a relatively inexperienced staff member perform the gavage inoculations under supervision in order to increase the number of complications. Mice that displayed complications during gavage delivery clearly had detectable bacteria in the MLN, spleen, and liver, 1 day following infection (Fig. S2). In contrast, mice that displayed no complications during gavage delivery, or had been administered bacteria in water, had no systemic bacteria at this early time point (Fig. S2). Together, these data suggest one area where contaminated water delivery may be advantageous over a gavage infection approach. While gavage and water contamination approaches are both able to efficiently cause systemic infection, use of a gavage needle appears to be associated with early entry of bacteria to blood and systemic tissues, most likely as a result of esophageal abrasions (Fig. 5).

3.4 Short exposure to contaminated water is sufficient for maximal infection

The data above demonstrate that exposure to contaminated water is an effective and consistent experimental approach to infect C57BL/6 mice with *Salmonella*. One major difference between water contamination and gavage delivery is the extended exposure time that is afforded by our water exposure methodology. To determine the length of exposure that is required for maximal infection, mice were exposed to contaminated water for different time periods, before clean water was supplied for the remainder of the experiment. Since mice are diurnal and drink more during the dark cycle, the first exposure to contaminated water was initiated after dark [56]. Mice that were exposed to contaminated water for only 30 minutes had relatively high bacterial loads in the MLN, spleen, and liver (Fig. 6), demonstrating that a short exposure can be sufficient to initiate a disseminated infection. However, not all of these mice were successfully infected in this 30 min cohort (Fig. 6). Indeed, it took 4 hours of exposure to contaminated water before all mice were consistently infected in the MLN, spleen, and liver (Fig. 6). In this 4 hour group, bacterial loads in the MLN, spleen, and liver were identical to mice that had continued access to contaminated water for up to 4 days. These data suggest that the *Salmonella* infection window is relatively short even during continuous exposure to contaminated water and that super-infection (increased infection due to continual entry of organisms over a long period of time) is unlikely to be a contributing factor to bacterial loads in tissues.

4 Discussion

It is increasingly clear that the initial route of pathogen entry can affect pathology and disease progression, as well as the type, strength, duration, and location of the immune response [6, 57–62]. Non-natural routes of infection are often utilized in the laboratory simply because they can reduce experimental variables or increase reproducibility, factors that are often important in experimental design. However, if these laboratory infections are to appropriately model natural infection then care should be taken that the natural context is preserved as much as possible.

Several studies have infected mice with oral pathogens using experimental approaches that closely mimic natural infection, such as contamination of food or water [63–66]. In 1908, a gavage delivery approach for mice was described that allowed oral administration of chemicals in defined quantities [9], and today this methodology is widely used in experimental models of oral infection. The assumed advantage of gavage delivery is that it will improve the accuracy and consistency in the delivery of an infectious dose via the oral route [64, 65, 67]. However, given the stress of the gavage procedure and the possibility for esophageal tearing, it is important that this assumption is actually tested [33, 35]. Stress has a number of physiological effects in mice that could potentially impact microbiological and immunological studies [38, 41, 44]. Further, the possibility that a pathogen is introduced to the bloodstream or lung during the gavage procedure could also impact the kinetics of infection and subsequent immune response [57, 60].

Our data demonstrate that simple contamination of drinking water is an effective means of infecting mice with *Salmonella*, and that this experimental approach can be as accurate and consistent as gavage delivery. This experimental approach works well because of the ability of *Salmonella* to survive in such a nutritionally poor environment as water. However, since C57BL/6 mice were maximally infected within 4 hours of exposure to water, *Salmonella* do not actually need to survive for very long in drinking water for this methodology to be useful. Our studies do show significant variability in bacterial survival that is dependent on the water source, thus despite the short exposure time required, bacterial survival should be tested empirically before utilizing this approach. In particular, care should be taken when administering low doses of bacteria since our data show greater variability occurs when bacterial numbers are decreased below 10^7 cfu/mL. It will obviously be important to perform similar studies of short-term viability in water before examining whether water contamination is an appropriate experimental alternative for gavage delivery of other microbial pathogens. By infecting mice with a range of doses for each route we were able to demonstrate an approximate relationship between the infectious dose used in the standard gavage and the concentration of bacteria to use when contaminating water. This information will likely be critical both for investigators wishing to transition from gavage to water inoculation from established protocols, and should allow direct comparison of data between these two infection approaches. Our data suggest that the dose administered by gavage per mL can be approximated by a similar amount of bacteria/mL in water, i.e. 1×10^n cfu ig can be approximated by 1×10^n cfu/mL in contaminated water.

Our data directly compared gavage bolus delivery of *Salmonella* with water contamination using challenge doses previously used to infect mice in our laboratory [68]. Importantly, all mice succumbed to infection regardless of the challenge methodology and the time to the development of a moribund state was similar between methods. A small, but statistically significant, difference in survival was noted at the lower 10^7 dose suggesting a small survival advantage may exist for mice infected by water contamination. However, given the similar bacterial loads between gavage and water contamination at this dose, this small impact on survival does not appear to correlate with a higher burden of bacteria. It is conceivable that gavage delivery causes greater systemic inflammation due to the introduction of bacteria directly into the bloodstream via abrasions or that these mice succumb more rapidly due to the combination of stress from infection and extensive animal handling [37, 44]. Overall, water contamination appears to be at least as consistent as gavage delivery and the subsequent disease progression is similar using either approach. Furthermore, the kinetics of infection followed a similar pattern beginning with bacteria detectable solely in the MLN and then spreading quickly to the spleen and liver, where numbers subsequently increased at similar rates. This kinetic was strikingly similar at higher doses and likely reflects the bottleneck in intestinal entry with high numbers of bacteria [32]. However, the overall kinetics of infection, and the percentage of mice that were infected over time, show greater variability with lower doses using both experimental approaches. To directly compare the different methodologies, we focused more carefully on bacterial loads at the day 4 time-point and found that these were comparable between methods, but again widely variable within groups at lower doses. At the higher doses variance within groups was reduced, however an F-test for equal variances demonstrated that the variance for gavage-infected mice was significantly greater than water contaminated mice.

When performing kinetic studies, it was noted that an individual animal that had blood in its mouth post-gavage was also an outlier with high bacterial loads in all organs and that additional mice had detectable bacteria in systemic sites before MLN infection. We hypothesized that these mice experienced some esophageal damage during gavage and that bacteria may have been introduced directly to systemic sites. Indeed, mice that had observable blood or aspirated liquid had higher bacterial loads in MLN and other sites of dissemination by day 1 post-infection. Although this potential problem will likely be highly dependent on the technical competence of the animal handler, it does highlight one area where water contamination may be superior to gavage delivery.

It was of some interest to determine the timing of the infection window with respect to water contamination, especially since there was the possibility that this approach could result in greater variability due to staggered timing of the initial infection or even super-infection due to continuous exposure. Surprisingly, as little as 30 minutes exposure was sufficient to infect half of the mice examined and within 4 hours all mice were consistently infected. Since mice consume most of their water during the dark cycle [56], this means that mice exposed to contaminated water are maximally infected during the first night of exposure and can simply be administered clean water the following day. This has important practical considerations but might also suggest that super-infections are prevented by rapid initial immune responses in the intestine [69]. It will be of interest to examine this window in mice lacking components of the innate or adaptive immune system.

Based on the data above, we suggest that water contamination is a suitable alternative to intra-gastric gavage as an oral route of infection for *Salmonella*. Further work needs to be done to examine whether this holds true for other microbes and may be dependent on the ability of the organism to survive in water for long enough to achieve uniform infection. Importantly, water contamination is simpler and easier to perform, requires less skill and training, is less stressful for the mice, and avoids complications that may arise during gavage. Furthermore, this method could be adapted for performing experiments that would not be possible with a gavage needle approach, such as oral infection of very young mice or for oral pathogens that use pre-gastric mucosal surfaces as portals of entry. This methodology may therefore be useful for examining natural exposure to oral pathogens in an experimental setting.

Conclusions and Future Perspectives

It is usually assumed that gavage needle infection is the most reliable means of pathogen delivery in models of intestinal infection. Our data demonstrate that simple water contamination provides a consistent model of *Salmonella* infection that is comparable to gavage delivery. Given the potential for esophageal tearing and the stress of animal handling to accommodate gavage inoculation, we suggest that future experiments should examine whether natural water contamination is a more appropriate technique. This methodology may also be useful for other infectious diseases where minimal restraint of animals would be advantageous during oral infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive Summary

1. *Salmonella* survival in water is dependent upon water source and inoculation dose
 - *Salmonella* survive long-term in water when starting at sufficient dose
 - *Salmonella* show differential ability to survive in different water supplies
2. Water contamination is an effective means of experimental *Salmonella* infection
 - Addition of *Salmonella* to water is as effective as gavage delivery
 - Time to death is comparable for both methodologies
3. Water contamination delivery preserves a natural route of oral infection
 - Water contamination preserves the tempo of infection observed with gavage
 - Gavage delivery results in more rapid systemic inoculation
4. Short exposure to contaminated water is sufficient for maximal infection
 - Four hours of exposure is sufficient for maximal infection of mice

Figure 1. *Salmonella* survival in water is dependent upon water source and initial inoculum dose Water from various sources was contaminated with *Salmonella* Typhimurium (SL1344) at a final density of 10^3 , 10^5 , 10^7 and 10^9 cfu/mL. Water sources shown here include: potable, chlorinated kitchen tap water (gray square), non-potable tap water from the laboratory sink (open triangle), and water supplied to the mice by animal care staff (cage water, filled black circle). Contaminated water was sampled for bacterial enumeration on MacConkey agar at days 1 (**A**) and 7 (**B**) after contamination. Limit of detection is 10 cfu/mL. Each point

represents an individual water tube from 3 combined experiments, with 3 tubes per group. Error bars represent the mean $\pm$ SEM of log-transformed data. SEM, standard error mean.

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Figure 2. Similar survival rates after oral infection with virulent *Salmonella* by either method Groups of C57BL/6 mice were orally infected with 10^7 (A) or 10^8 (B) cfu of SL1344 by gavage needle (solid line) or by water contamination (dotted line) with 10^7 (A) or 10^8 (B) cfu/mL, and euthanized when moribund. Graphs show combined data from at least two experiments, with at least eight mice per group in each experiment. Statistical significance was observed by the log-rank (Mantel-Cox) test. ** $p < 0.01$, and $p > 0.05$ (ns).

Figure 3. Kinetics of infection and dissemination are similar by either oral infection method
 C57BL/6 mice were orally infected with 10^7 (A) or 10^9 (B) cfu of SL1344 by gavage (solid line) or 10^7 (A) or 10^9 (B) cfu/mL by water contamination (dotted line). 4 mice were euthanized from each group each day for 5 days and bacterial loads were determined for MLN (left), spleen (center), and liver (right). Points on the graph represent individual mice, and lines follow the means for each group of log-transformed data. MLN, mesenteric lymph node.

Figure 4. Water contamination as a route of infection results in similar bacterial loads and improved consistency over intragastric gavage route

C57BL/6 mice were orally infected with 10⁷ (A) or 10⁹ (B) cfu of SL1344 by gavage (filled squares) or 10⁷ (A) or 10⁹ (B) cfu/mL by water contamination (open circles). Bacterial loads in MLN (left), spleen (center), and liver (right) were determined at day 4 after SL1344 infection. Data is combined from 2 (A) or 3 (B) individual experiments with at least 7 mice per group total. Statistical significance between groups was examined by two-tailed t-test (A) or t-test with Welch's correction due to unequal variances, as determined by an F-test (B), with no significant differences ($p > 0.05$) observed between any groups. Asterisks (*) represent samples below the limit of detection. Error bars represent the mean $\pm$ SEM of log-transformed data. MLN, mesenteric lymph node; SEM, standard error mean.

Figure 5. Intestinal infection by water bottle contamination or oral gavage route of infection

In the small intestine, *Salmonella* enter mainly through M (microfold) cells that overlay Peyer's Patches or solitary isolated lymphoid tissues (SILTs), or those scattered throughout the small intestine as intravillus M cells. After entering mucosal lymphoid tissues, *Salmonella* are taken by phagocytes, such as dendritic cells (DCs), and transported via afferent lymphatics to mesenteric lymph nodes (MLNs) (a). Although a *Salmonella*-specific immune response is activated quickly in mucosal lymphoid tissues, *Salmonella* are able to penetrate systemic sites, most notably the spleen, liver, and bone marrow (b). However, oral *Salmonella* infection by gavage needle can cause esophageal damage that could facilitate *Salmonella* penetration into blood vessels and lead to a more rapid systemic infection (c).

Figure 6. Infection by water contamination occurs rapidly

Mice were given water contaminated with 10^9 cfu/mL SL1344 for indicated numbers of hours during the dark cycle. Contaminated water was then removed and replaced with clean water. At 84 hours (3.5 days) after initial exposure all mice were euthanized and bacterial loads were determined for MLN (A), spleen (B), and liver (C). Dotted lines represent the limit of detection for each organ. The error bars indicate mean $\pm$ SEM for log-transformed

data. Data combined from 2 experiments with 4 mice per timepoint in each experiment.
MLN, mesenteric lymph node; SEM, standard error mean.

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