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Immunogenicity of χ^{4127} *phoP*− *Salmonella enterica* serovar *Typhimurium* in dogs

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Abstract

Salmonellae are commonly isolated from dogs. The number of dogs infected with *Salmonella* spp. is surprisingly high and greater than the incidence of clinical disease would suggest. Salmonellosis is common in greyhound kennels. Morbidity can approach 100% in puppies and the mortality ranges to nearly 40%. To date, there has been little effort to evaluate the feasibility of a vaccine for control of this disease in dogs. In the studies described here, an attenuated strain of *Salmonella enterica* serovar *Typhimurium* (Se *Typhimurium*), χ^{4127}, was capable of establishing a limited infection in dogs. The χ^{4127}-attenuated salmonellae efficiently stimulated protective immune responses in serotype homologous, direct, oral challenge experiments. Morbidity in the wild-type-challenged dogs was 8.3% in immunized dogs but 100% in the non-vaccinated controls. In (9/12) control dogs, the disease involved both gastrointestinal and respiratory tracts with high fever (>40.2 °C) that persisted through 5 days after challenge. Serum IgG response against *S. typhimurium* lipopolysaccharide (LPS) significantly increased ($P < 0.01$) in vaccinated dogs and in non-vaccinated dogs after challenge. The non-vaccinated dogs had 3 to 4 logs higher numbers of Se *Typhimurium* in splenic and hepatic tissue than did the vaccinated dogs. This particular attenuated strain has potential for use as a vaccine for canine salmonellosis. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Salmonella; Mucosal immunization; Canine; Vaccine

1. Introduction

Salmonellae are frequently isolated pathogens from many species of animals. Despite improved sanitation and continued research, infections are still prevalent in humans and animals. Most infections originate from food substances and persistently infected carriers [1,2]. *Salmonella* spp. are common isolates from dogs [3]. The number of dogs infected with *Salmonella* is surprisingly high and greater than the incidence of clinical disease would suggest [4]. Large numbers of bacteria must be ingested by dogs to establish intestinal colonization [5], and stress has been documented to increase rates of infection in dogs [6]. Treatment of dogs with antibiotics also has been found to increase the risk of salmonellosis [6]. Eating infected raw meat, direct contact with feces of infected dogs and scavenging infected carcasses are likely sources of infection for dogs [7–9].

Salmonellosis (with concurrent isolation of *Salmonella enterica* serovar *Typhimurium* (Se *Typhimurium*)) is common among racing greyhounds [7,10,11] and racing sled dogs [12]. Morbidity can approach 100% in puppies, and the mortality may be 40% (Gabbert, personal communication, Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA). The direct economic impact of these losses to the industry is substantial. Less obvious losses are incurred from poor growth and performance. The surviving dogs often become carriers and may be public health threats to individuals in contact with them [5]. Salmonellae infections also may be immunosuppressive and partially responsible for severe, clinical, viral enteritis and vaccine failure [4].

There has been little effort to develop canine vaccines for salmonellosis. The risk of disease is low for the majority of dogs. However, dogs in some racing environments are

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at higher risk [7,10–12]. The attenuated strain χ^{4127} of Se *Typhimurium* was evaluated in beagle dogs as a potential vaccine for salmonellosis. This attenuated strain efficiently stimulated protective immune responses in serotype homologous, direct, oral challenge experiments.

2. Materials and methods

2.1. Bacteria

The strains of Se *Typhimurium* used in these studies for canine inoculation were maintained as frozen stocks in Luria broth (LB, Difco Laboratories, Detroit, MI) with 10% glycerol [13]. The KSU7 strain was isolated originally from a greyhound dog with enteritis (Dr. M.M. Chengappa, Kansas State Veterinary Diagnostic Laboratory). Infection of dogs with the χ^{4127} strain of Se *Typhimurium* has been described previously [14].

2.2. Dogs

The dogs used in these experiments were purpose-bred beagles from Ridglan Farms (Mount Horeb, WI). The dogs were from 12–16 weeks of age at the time of initial inoculations with χ^{4127} Se *Typhimurium*. The dogs were housed individually in cages in an indoor holding facility. Each treatment group of dogs was housed separately from other groups to prevent infection of controls. The dogs had continuous access to food and water. Each animal was exercised twice daily, and dogs within each group did intermingle during exercise periods. Dogs were vaccinated for canine distemper, hepatitis, parvo virus, and rabies according to facilities guidelines. All dogs were screened to be negative for *Salmonella* spp. by culture of rectal swabs and feces. Also, all dogs used had low, background titers to Se *Typhimurium* lipopolysaccharide (LPS) antigens by ELISA (see Section 2.5) before inoculation with χ^{4127} Se *Typhimurium*. Negative status was confirmed by sampling at least two times before inoculation. All dogs were allowed at least 1 week for acclimatization after shipment before studies began. Clinical observations were initiated 3 days before administration of the challenge inoculum (below). Animal experiments were conducted according to institutional animal care and use policies and supervision.

2.3. Immunogenicity studies

To investigate the immunogenicity of avirulent Se *Typhimurium* in homologous challenge studies, 12 dogs (as described above) were randomly divided into two groups of six animals. One group was immunized as described below and the second group was not vaccinated. This study protocol was repeated and data are presented as study A and B. The χ^{4127} strain was used as the hypothetical immunogen, and one group of the dogs was immunized with 5×10^8 CFU of bacteria per os in LB (as described above and below). All animals were observed daily for any change in demeanor or health. Blood samples for serum preparation were collected on days 0 (day of immunization), 7, 14 and 21. All animals were challenged on day 22 with 1×10^9 CFU of wild-type Se *Typhimurium* KSU7 (inoculum prepared and administered as described below). After challenge, the animals were examined closely at least three times per day, and vital data collected twice a day. Heart rate, respiration rate and rectal temperature were measured twice, both morning and evening. Blood samples and fecal swabs were collected on days 22 (before and after challenge administration), 23, 24, 25 and 27. Total and differential leukocyte counts as well as platelet counts were performed in the Clinical Pathology Laboratory at the Kansas State University Teaching Hospital. Blood for bacterial culture was collected twice per day. The animals were euthanized by bolus pentobarbital injection on day 27 and necropsies performed.

2.4. Oral animal inoculations

The KSU7 strain of Se *Typhimurium* used for inoculations to challenge the dogs was maintained as frozen stocks (−70 °C) after animal passage. Weanling BALB/c mice were inoculated intraperitoneally and sacrificied 2–3 days later. The liver and spleen were aseptically collected and used as sources of animal-passaged bacteria after homogenization in 9.0 ml of LB. For challenge preparation, an aliquot of the animal-passaged bacteria was thawed rapidly in a water bath at 37 °C and added to 10.0 ml of fresh LB. The LB culture was incubated overnight at 37 °C (16–20 h). This culture was diluted 1:75 into pre-warmed Luria–Bertani broth (LBB, Difco Laboratories, [15]) and incubated for an additional 6 h at 37 °C with limited aeration until the A_{600} was near 0.900. The bacteria were centrifuged at 10,000 × RCF for 10 min and re-suspended in phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl, PBS) with 1% gelatin (w/v) as described (buffered saline with gelatin [16]). Food was withheld for about 12 h before oral inoculation. Dogs were inoculated by gavage with 2.0 ml of the inoculum 10 min after administration of 10 ml of 10% sodium bicarbonate (w/v in H_2O, also by gavage). Each inoculum was tested to assure purity of culture and numbers of viable bacteria by plating on Hektoen Enteric Agar (HEA, Difco Laboratories). The χ^{4127} Se *Typhimurium* was maintained as above and stored frozen in LB (not passaged in mice).

2.5. Serology

An enzyme-linked immunosorbent assay (ELISA) used to quantitate serum antibody was similar to that published elsewhere [17,18]. The Immulon II (Becton Dickinson, Alexandria, VA) 96-well microtiter plates were coated with 100 ml of antigen (*S. typhimurium* LPS, Sigma) at 10 mg/ml

in 0.06 M sodium carbonate buffer (pH = 9.6). After incubation at 37 °C for 18 h, plates were washed with 200 ml of PBS with 0.05% Tween 20 (Sigma) three times. Next, plates were blocked with PBS with 0.05% Tween 20 and 1% bovine serum albumin (BSA). Sera to be tested were diluted 1:200 in PBS and assayed in duplicate. Diluted serum was added to each well in 100 ml volumes. The plates were incubated for 2.0 h at 37 °C. The plates were emptied, washed three times with 200 ml of PBS and reblocked with 200 ml of the BSA blocking solution. The plates were emptied and 100 ml of goat-anti-canine IgG–alkaline phosphatase conjugate (0.5 mg/ml in PBS, Kirkegaard and Perry, Gaithersburg, MD) with 0.05% polyoxyethylenesorbitan monolaurate (PESM)) was added. The plates were washed twice with PBS with 0.05% PESM. After incubating at 60 min at 37 °C, a volume of 100 ml of ABTS peroxidase substrate (Kirkegaard and Perry) was added to each well. The plates were incubated for 30 min at 22 °C (protected from light). The ABTS peroxidase stop solution (Kirkegaard and Perry) was added and absorbance at 405 nm was recorded. Control negative sera were collected from newborn puppies. The control positive serum was obtained from a mature dog injected with heat-killed (65 °C, 60 min) suspension of logarithmic phase culture of a Se *Typhimurium* (canine clinical isolate) in LB that was mixed with adjuvant (monophosphoryl lipid A, trehalose dicorynomycolate, tubercule cell wall skeleton emulsion, Sigma). The negative A_{405} values (mean + 2 S.D.) from control sera were subtracted from the values from test sera. The means of duplicate assays were recorded for further analysis. Each ELISA plate had both serum negative and positive controls as well as reagent controls.

2.6. Necropsy examinations

Necropsies were performed on all dogs at the end of the in-life experimental phase. Dogs were placed on their backs with legs extended laterally. The abdomen and thorax were opened in an aseptic manner. Tissues were examined for gross pathological lesions. Portions of the tissue samples were placed in 10% buffered formalin, and others were placed in sterile containers on ice for transport to the laboratory. All samples were collected by aseptic technique.

2.7. Histological analysis

Fixed visceral organs in 10% buffered formalin solution were processed for paraffin embedding, and 4 mm-thick sections were cut and attached to glass microscope slides. After re-hydration, the slides were stained with hematoxylin and eosin. These stained sections were examined for pathologic changes. Tissues analyzed were liver, spleen, intestine, colon, mesenteric lymph nodes, Peyer's patches, lung, bronchial lymph nodes, adrenal glands, kidneys and heart. All tissues were collected during the respective necropsies (Section 2.6).

2.8. Culture techniques for host animal study specimens

Rectal swabs, 0.5 g of feces and heart blood swabs were used to inoculate 5.0 ml selenite brilliant green sulfa broth (SBGSB, Difco) for selective enrichment for *Salmonella* isolation. These broth cultures were incubated at 37 °C for 48 h and plated on HEA plates. Observations of *Salmonella* on the HEA plates were recorded as positive results. Standard methods for biochemical and serological typing of isolated bacteria were used [19]. In addition, the isolates were tested for the presence of acid phosphatase activity [20]. The χ^{4127} *phoP−* Se *Typhimurium* strain has no detectable acid phosphatase activity.

Colonic fecal material and spleen and liver tissue samples were collected aseptically. From these fresh tissues, 1 g was placed in sterile, 20 ml tubes. These samples were homogenized as 1:10 dilutions in brain heart infusion broth (BHIB, Difco). Decimal dilutions were performed in BHIB. Dilutions were plated in drops on HEA. Detectable salmonellae (positive growth on HEA) indicated the presence of 10^2 CFU. The initial 1:10 dilution in BHIB was inoculated in SBGSB for enrichment as described above. This step allowed detection of less than 10^2 CFU (recorded as 10 CFU). The dilutions were plated in triplicate and geometric means calculated [21]. Biochemical, serological and acid phosphatase tests were used to confirm species and wild-type status as described previously [19–22].

2.9. Statistics

Instat@ computational software (GraphPad, San Diego, CA) was used to calculate descriptive statistics (mean, S.D., S.E. of the mean) and analysis of variance (ANOVA) with the Student–Newman–Keuls (SNK) post-test statistic for evaluating differences of treatment means [23]. If no significant differences occurred between experimental replicates, some treatment data were pooled for presentation and analysis purposes.

3. Results

3.1. Clinical and pathological observations following challenge

The χ^{4127} strain of Se *Typhimurium* stimulated an adaptive immune response that was protective in challenge experiments. Morbidity (defined herein as vomiting, diarrhea, anorexia or persistent coughing) in the wild-type-challenged dogs was 8.3% (1/12) in immunized dogs but 100% in the non-vaccinated control dogs. In (9/12) control dogs, the disease involved both gastrointestinal (vomiting and diarrhea) and respiratory (coughing) tracts with high fever (>40.2 °C) that persisted until day 27 (Fig. 1). The non-vaccinates responded to the infection with a PMN-predominant leukocytosis at 60 h (Table 1). The leukocyte counts of vaccinated

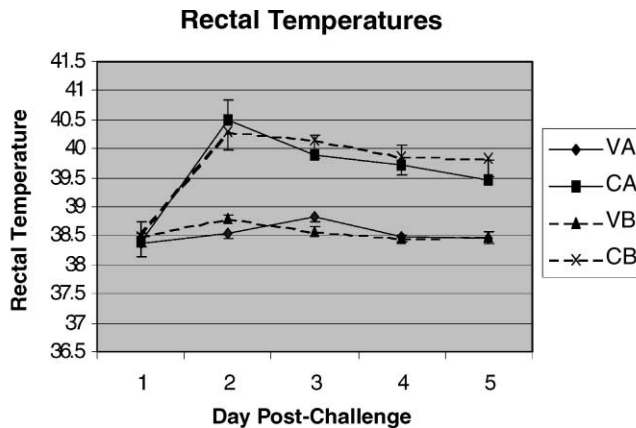

Fig. 1. Rectal temperatures (°C) of dogs infected orally with the KSU7 strain of *S. typhimurium* (1×10^9 CFU) after immunization with χ^{4127}. Each data point represents the mean value from six dogs from measurements taken twice both morning and evening. VA: vaccinates, study A; CA: non-vaccinates/controls, study A; VB: vaccinates, study B; CB: non-vaccinates/controls, study B. On days 2 through 5, the means of rectal temperatures of vaccinates were significantly less than rectal temperatures of non-vaccinated controls ($P < 0.001$). For non-vaccinates, the change in rectal temperatures from day 1 to 2 was significant ($P < 0.001$).

Table 2
Platelet counts (1000×) per ml from *S. typhimurium*-infected dogs at the time of challenge and on day 25 (60 h post-challenge)

Treatment	Day 22 (time 0)	Day 25 (60 h)	*P*
Vaccinates-A	480.2 ± 75.2	319.3 ± 35.1	>0.05
Non-vaccinates-A	433.2 ± 49.9	98.8 ± 19.4	<0.001
Vaccinates-B	479.8 ± 66.9	412.7 ± 41.3	>0.08
Non-vaccinates-B	419.7 ± 34.8	138.3 ± 40.0	<0.01

The *P*-value is the probability that the differences from day 22 to 25 are significantly different.

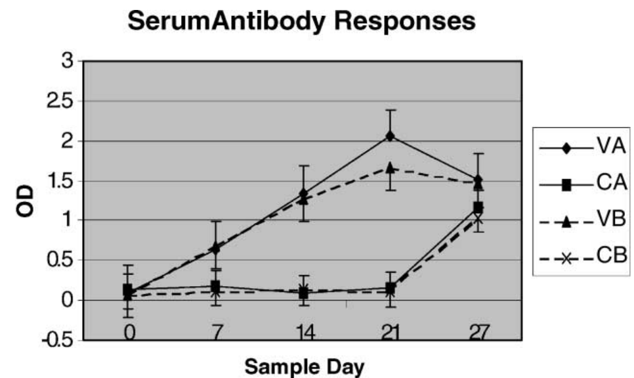

Fig. 2. Serum antibody as measured by an indirect ELISA to *S. typhimurium* LPS. Serum was obtained from blood collected at the times indicated. The absorbance at 405 nm was recorded and corrected by subtracting negative control values. Negative control A_{405} values were always below 0.085, and the positive control values were greater than 1.520. Vaccinates were inoculated orally on day 0; controls were not vaccinated. VA: vaccinates, study A; CA: non-vaccinates/controls, study A; VB: vaccinates, study B; CB: non-vaccinates/controls, study B. Vaccinates' serum antibody activity (means of OD) was significantly higher than the antibody activity of non-vaccinates' sera on days 14 and 21 ($P < 0.05$).

dogs also increased, but the increase was not significant ($P > 0.05$) and only barely above laboratory normal values. Blood platelet counts were lower 60 h after challenge in the non-vaccinated dogs (Table 2). The decreased platelet numbers were below laboratory normal ranges (>200,000 ml), but no purpuric-like symptoms were observed. Serum IgG response against Se *Typhimurium* LPS significantly increased ($P < 0.05$) in vaccinated dogs and in non-vaccinated dogs after challenge (Fig. 2).

Evidence of deep tissue invasiveness of KSU7 strain was observed during the necropsies of the control dogs. Splenomegally, hepatomegally, and mesenteric lymphadenopathy were observed. Focal hemorrhages in the liver, Peyer's patches and mesenteric lymphoid tissues were also observed. The lungs were edematous, and numerous 0.5–2.0 cm foci of hemorrhagic consolidation were observed. The spleen and liver had numerous foci of perivascular mononuclear inflammatory reactions. The lung had similar mononoclear-predominant lesions in the interstitial spaces. The vaccinated dogs were free of gross lesions in deep tissues. However, the mesenteric lymph nodes and spleen were moderately enlarged in the vaccinated dogs as would be expected 5 days after oral challenge.

The necropsy observations were consistent with findings from quantitative cultures of colonic feces, spleen, liver and

Table 1
Blood leukocyte counts (1000×) per ml from *S. typhimurium*-infected dogs at the time of challenge and on day 25 (60 h post-challenge)

Treatment	Day 22 (time 0)			Day 25 (60 h)		
	Total (1000×)	PMN[a]	Bands[b] (%)	Total	PMN	Bands (%)
Vaccinates-A	11.17 ± 4.04	7.50 ± 1.10	<1	14.45 ± 3.74	11.32 ± 3.77	<1
Control-A	10.45 ± 4.20	7.47 ± 3.68	<1	24.05 ± 6.475	21.28 ± 5.98	9.2
Vaccinates-B	7.86 ± 1.88	6.27 ± 1.18	NO[c]	12.63 ± 2.654	10.23 ± 2.25	<1
Control-B	9.97 ± 2.93	7.85 ± 2.41	NO	26.43 ± 7.413	22.88 ± 6.32	6.9

Total leukocyte counts and PMN counts were statistically higher ($P < 0.05$) in non-vaccinates than vaccinates at 60 h.
[a] Polymorphonuclear neutrophils (PMN).
[b] Percentage of PMN counts.
[c] None observed: NO.

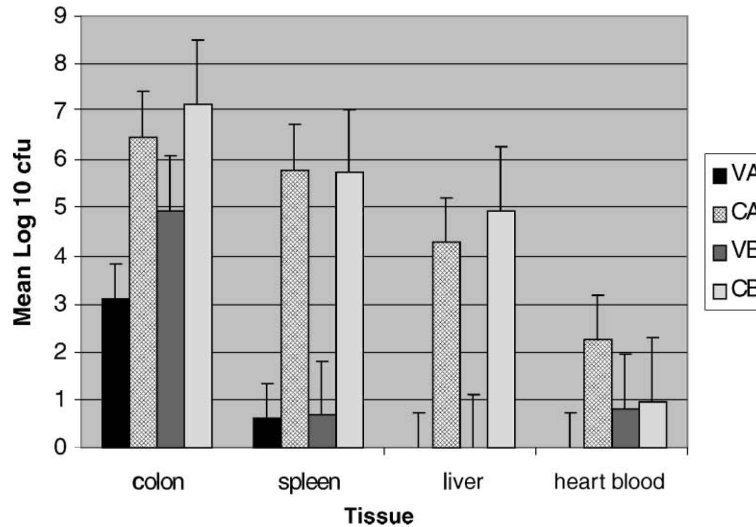

Fig. 3. Log 10 CFU of viable *S. typhimurium* from dogs infected with *S. typhimurium.* The results are log 10 CFU/g of colonic feces, spleen or liver (taken aseptically at necropsy). Vaccinates were inoculated orally with χ^{4127} *S. typhimurium* and subsequently challenged with KSU7 *S. typhimurium*. Tissues were collected at necropsy on day 27, 6 days after challenge. Differences in mean CFUs from feces and heart blood were not significantly different between vaccinates and non-vaccinates ($P > 0.05$). Differences in mean CFUs from spleens and livers from vaccinates and non-vaccinates were significantly different ($P < 0.005$).

heart blood. The non-vaccinated dogs had 3 to 4 logs higher numbers of Se *Typhimurium* in these tissues than did the vaccinates (Fig. 3). At the time of challenge, none of the dogs had positive rectal or fecal cultures after SBGSB enrichment culture. The non-vaccinated dogs had much higher numbers of bacteria in the spleen, liver and heart blood following challenge. Two vaccinated dogs had detectable Se *Typhimurium* in the spleen. However, (12/12) non-vaccinated dogs had bacteria in the spleen, and (9/12) had bacteria in the liver. These findings were supported by blood culture results. Only one vaccinated dog had a positive wild-type blood culture (at 3 days post-challenge), but (10/12) non-vaccinates had at least one positive blood culture. On day 27, (3/12) vaccinated dogs had a positive fecal swab culture for wild-type Se *Typhimurium* while (11/12) non-vaccinates had positive rectal swabs at day 27. All of the Se *Typhimurium* isolated after challenge were acid phosphatase test positive, indicative of the wild-type phenotype. In this study, no χ^{4127} strain Se *Typhimurium* organisms were isolated from feces or tissues after challenge.

4. Discussion

Salmonellae can cause significant clinical infections in dogs [3,7,8,11,12]. Canine salmonellosis is not common, but can be a very serious problem if dogs are exposed to high numbers of bacteria. The disease is usually an acute, self-limiting gastroenteritis [3,5]. There is evidence that dogs may shed salmonellae in significant numbers in feces [3,9,11]. It is not clear under what circumstances salmonellae can be more invasive and cause an enteric fever-like disease with septicemia or a persistent, sub-clinical infection. The data presented herein indicates that some strains of wild-type Se *Typhimurium* can be invasive, particularly in young dogs. The KSU7 strain caused significant morbidity when given via oral inoculation. The *phoP*− χ^{4127} strain colonized dogs, and was not invasive to deep tissues. As previously described [14], χ^{4127}-infected dogs intermittently shed these strains in feces for several weeks after high dose inoculation, although the numbers of bacteria isolated and the frequency of isolation decreased with time. Dogs infected with χ^{4127} were asymptomatic.

When the χ^{4127} strain was administered to beagle puppies, it was protective against subsequent challenge by KSU7 Se *Typhimurium*. This protection was observed as reductions in acute inflammatory responses, morbidity and deep tissue invasiveness. The χ^{4127} contains a *phoP* deletion mutation. Other studies support the attenuation and protective capacity of *phoP* deletion strains [21,23–26]. The strain χ^{4127} has potential to be a safe vaccine for canine salmonellosis as well as a potential vector for other antigens where mucosal immune responses are desirable.

For the challenge infections conducted in these experiments, we used relatively large numbers of bacteria. This approach is consistent with earlier reports that higher numbers of bacteria are required to establish clinical infections in dogs [5]. However, the high dose inoculations did result in invasive, patent infections. A lower dose challenge model should be developed to evaluate the pathogenesis of enteritis and intestinal infection. Other studies in other species have demonstrated the ability of attenuated Se *Typhimurium* strains to reduce post-challenge fecal shedding after low-dose challenges [21,24–26].

Se *Typhimurium* is invasive and virulent in dogs when delivered orally. These infections may be clinically significant (especially in young dogs) and can be repeated under laboratory conditions. A more complete characterization of tissue changes associated with pathogenesis of canine salmonellosis is needed. The observed reductions in blood platelet counts are interesting but the data do not provide a mechanistic explanation. The reduction may have been a result of immune-mediated clearance of antigenemia associated with septicemia. Also, the virulence mechanisms that account for gastrointestinal lesions, septicemia, tissue localization and persistent infection in dogs have yet to be characterized. It is likely that these mechanisms are similar among salmonellae that differentially infect various host species (reviewed in [27,28]). The avirulent, *phoP*− strain χ^{4127} of *S. typhimurium* was used successfully as a live oral vaccine to protect against invasive salmonellosis in dogs. This vaccine may be useful to reduce the incidence and severity of disease in dogs with significant exposure to Se *Typhimurium* in food. However, the χ^{4127} strain was not able to significantly reduce fecal shedding after the high dose, wild-type challenge with strain KSU7 Se *Typhimurium*. Therefore, vaccination would not replace the need for good personal hygiene, sanitary management and elimination of the use of contaminated meat for food [29].

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