ORIGINAL ARTICLE

Salmonella Oranienburg Isolated from Horses, Wild Turkeys and An Edible Home Garden Fertilized with Raw Horse Manure

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Impacts

- Routine faecal screening for *Salmonella* as part of the veterinary teaching hospital's infection control protocol facilitated identification of equine salmonellosis infections on a ranch in coastal Northern California.
- The *S.* Oranienburg clinical strain was found in multiple farm samples including faeces from symptomatic and asymptomatic stable mates, a healthy pet dog, wild turkeys, stored manure, water troughs and soil from the family's edible home garden.
- Viable *S.* Oranienburg persisted an estimated 210 days in garden soil fertilized with raw horse manure.

Keywords:
Animals, wild; disease outbreaks; electrophoresis, gel, pulsed-field; gardening; horses; organic; raw manure; *Salmonella*; turkey; Zoonoses

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Summary

In July 2010, a horse from a rural farm (Farm A) in coastal Northern California was diagnosed with S*almonella* Oranienburg infection following referral to a veterinary hospital for colic surgery. Environmental sampling to identify potential sources and persistence of *Salmonella* on the farm was conducted from August 2010 to March 2011. *Salmonella* was cultured using standard enrichment and selective plating. Pure colonies were confirmed by biochemical analysis, serotyped and compared by pulsed-field gel electrophoresis (PFGE) analysis. A total of 204 clinical and environmental samples at Farm A were analysed, and *Salmonella* spp. was isolated from six of eight (75%) horses, an asymptomatic pet dog, two of seven (28.6%) water samples from horse troughs, nine of 20 (45%) manure storage pile composites, 16 of 71 (22.5%) wild turkey faeces and four of 39 (10.3%) soil samples from the family's edible home garden. Well water and garden vegetable samples and horse faecal samples from a neighbouring ranch were negative. *S.* Oranienburg with a PFGE pattern indistinguishable from the horse clinical strain was found in all positive sample types on Farm A. The investigation illustrates the potential for widespread dissemination of *Salmonella* in a farm environment following equine infections. We speculate that a recent surge in the wild turkey population on the property could have introduced *S.* Oranienburg into the herd, although we cannot rule out the possibility wild turkeys were exposed on the farm or to other potential sources of *Salmonella*. Findings from the investigation indicated that raw horse manure applied as fertilizer was the most likely source of garden soil contamination. Viable *S.* Oranienburg persisted in garden soil for an estimated 210 days, which exceeds the 120-day standard between application and harvest currently required by the National Organic Program. The study underscores the need to educate the public about potential food safety hazards associated with using raw animal manure to fertilize edible home gardens.

Introduction

Zoonotic *Salmonella* spp. colonize the gastrointestinal tract of domestic and wild animals. Animal infections are usually asymptomatic, but *Salmonella* may cause septicaemia in newborn calves, lambs, foals and piglets. Salmonellosis in adult horses ranges from asymptomatic carriers to severe acute gastroenteritis characterized by fever and diarrhoea; infection may lead to life-threatening colic (Aiello and Moses, 2012). *Salmonella* is transmitted between animals through faecal–oral contact or through contaminated food and water (Hoelzer et al., 2011). Wildlife can serve as a source for domestic animal infections (Pedersen and Clark, 2007; Skov et al., 2008; Taylor and Philbey, 2010; Carlson et al., 2011). Human infection with zoonotic *Salmonella* spp. is most commonly attributed to consumption of contaminated foods of animal origin, but fresh produce is emerging as an important cause of outbreaks and recalls in the United States (Fatica and Schneider, 2011).

The resurgence in the popularity of home and community gardening in the United States is increasing access to fresh fruits and vegetables (Butterfield, 2009). A number of lay publications and websites promote the use of animal-based soil amendments as a sustainable practice for hobby farmers. But, amateur gardeners may inadvertently expose themselves to zoonotic enteric pathogens through improper handling and use of untreated raw animal manure as fertilizer (Cieslak et al., 1993; Mukherjee et al., 2006).

We conducted an epidemiologic and laboratory investigation of equine salmonellosis infections to identify potential environmental sources. Contamination and persistence of *Salmonella* Oranienburg was documented in soil from the family's fruit and vegetable garden fertilized with raw horse manure.

Materials and Methods

Our investigation had three components: (i) the initial diagnosis of equine salmonellosis following hospital admission for colic surgery, (ii) an epidemiologic investigation to identify potential environmental sources of *Salmonella* at the horse farm and (iii) a laboratory investigation to characterize and compare clinical and environmental *Salmonella* isolates.

Clinical investigation

On 4 July 2010, an 18-year-old Shire cross mare (Horse 1) developed a fever of 40°C, anorexia and clinical signs of mild colic at a rural, private horse ranch (Farm A) located in the northern coast of California. The mare had been placed on oxytetracycline because of a presumptive diagnosis of granulocytic anaplasmosis caused by tick-borne *Anaplasma phagocytophilum*, which is endemic in the area. The mare's condition worsened, and she was referred to the University of California, Davis (UCD) Veterinary Medical Teaching Hospital (VMTH), for colic evaluation on 11 July 2010. A diagnosis of colon displacement was made, and the mare was taken to surgery.

As part of the hospital's Infectious Disease Control protocol, a faecal sample was collected at admission during rectal palpation to screen for *Salmonella* by using a standard diagnostic protocol. Briefly, faeces were cultured by plating the sample directly onto xylose–lysine–tergitol 4 (XLT4) with incubation at 37°C for 48 h, as well as inoculating faeces into selenite enrichment broth overnight. The enrichment broth was then plated on XLT4 and incubated at 37°C for 48 h. Suspect colonies were replated for isolation and confirmed by polyvalent and group serology and biochemical testing.

According to the referring veterinarian, two stable mates at Farm A also had a history of fevers and mild diarrhoea that summer, but the diarrhoea was thought to be antibiotic-related following oxytetracycline treatment for fevers presumed to be caused by *A. phagocytophilum*. There were no other known cases of suspect salmonellosis in the surrounding equine community that summer. However, in October 2010, the referring veterinarian reported febrile illness in a horse at a neighbouring farm (Farm B) approximately five kilometres from Farm A. Faecal samples from four horses at Farm A and 10 horses at Farm B were submitted to the teaching hospital for *Salmonella* diagnostic testing in August 2010 and October 2010, respectively.

Epidemiologic investigation

The owners of Farm A invited UCD veterinarians to investigate potential sources of *Salmonella* at their property. The farm was visited on 5 August and 26 August 2010. Approximately 2–10 g of freshly deposited animal faeces (horse, dog, cat, wild turkey, wild rabbit) were collected from the ground with a sterile scoop and placed in a sterile, sealable plastic bag. Additionally, five ~2-gram grabs of dried turkey faeces or faeces from the horse manure storage pile were each pooled into ~10-gram composite samples. Water (well, horse trough, bucket with tadpoles) was collected in 100-millilitre sterile screwcap vials. Approximately 10 g of top soil and 25 g of vegetables (kale, potatoes and squash) were collected from the family's edible home garden and placed in sterile, sealable plastic bags.

To monitor persistence of *Salmonella*, the owners of Farm A volunteered to continue sampling fresh horse faeces, stored manure (pile), wildlife faecal samples and garden soil from November 2010 to March 2011. Samples were shipped monthly in August 2010, October 2010, November 2010, February 2011 and March 2011. We

trained the owners in aseptic sample collection, provided supplies (gloves, scoops and sealable sample collection bags) and gave instructions for overnight shipping to our laboratory. Samples were shipped on ice packs and stored at 4°C until processing.

Laboratory investigation

Environmental samples were processed at the UCD Western Institute for Food Safety and Security laboratory by selective enrichment and plating. Briefly, 10 g of faecal material were pre-enriched in 90 mL of buffered peptone water (BPW) for 20 h at 37°C with shaking at 100 rpm; 10 μL of BPW bacterial suspension was added to 1 mL Rappaport–Vassiliadis (RV) (BD Becton, Sparks, MD, USA) and incubated for 48 h at 42°C, followed by streaking of 10 μL of RV onto xylose–lysine deoxycholate (XLD) and incubation for 24 h at 37°C. Six suspect colonies per positive plate were streaked for isolation onto a secondary XLD plate and incubated for 24 h at 37°C. Isolated black colonies were biochemically confirmed using lysine, Simmons' citrate, triple sugar iron and urea. Purified colonies were stored on cryobeads at −80°C.

Pulsed-field gel electrophoresis

Up to six *Salmonella* isolates (n = 270) from each positive environmental sample were regrown and analysed by pulsed-field gel electrophoresis (PFGE) to compare the genetic relatedness of *Salmonella* strains isolated from Horse 1 to strains isolated from stable mates and environmental samples. The PFGE analysis was conducted according to the Center for Disease Control and Prevention's (CDC) PulseNet standard procedure (Ribot et al., 2006). Briefly, bacterial isolates were retrieved from storage and suspended in cold buffer containing 1 M NaCl, 10 mM Tris pH 8 and 10 mM EDTA for DNA isolation. DNA was digested in enzyme buffer with restriction enzyme *Xba*I. Images were analysed, and the similarity among different strains was characterized using GelComparII software (Applied Maths). Pattern comparisons were made based on criteria originally established by Tenover et al. (1995) and modified for food-borne pathogens by Barrett et al. (2006). Pulsotypes were assigned arbitrary consecutive numbers.

Serotyping

Salmonella isolates confirmed by polyvalent and group serology were submitted to the US Department of Agriculture National Veterinary Laboratory Services (NVSL) for serotyping. We assumed that strains belonging to the same group by serology and with indistinguishable PFGE patterns would likely belong to the same serotype; thus, to conserve resources, a subset of representative *Salmonella* strains (n = 50) from each unique serogroup and pulsotype were submitted to NVSL for serotyping.

Antimicrobial susceptibility testing

Salmonella isolates from Horse 1, two stable mates and a composite corral sample were assayed at the UCD VMTH clinical laboratory for susceptibility to 10 antimicrobials using the Sensititre® assay (Thermo Scientific, TREK Diagnostic Systems, Cleveland, OH, USA) following the recommendations of the Clinical Laboratories Standards Institute (CLSI, 2008).

Results

Clinical investigation

Salmonella was isolated from the admission faecal sample (Horse 1) collected on 11 July 2010. The isolate was identified as S*almonella enterica* subtype Oranienburg. *Salmonella* Oranienburg was cultured subsequently from Horse 1 daily faecal samples collected from admission through discharge (Table 1). *S.* Oranienburg was also confirmed in faecal samples from four horses at Farm A collected in early August 2010. *Salmonella* was not cultured from 10 horse faecal samples collected at Farm B in October 2010. The isolates from Farm A were found to be resistant to doxycycline with a minimum inhibitory concentration ranging from 2 μg/mL to greater than 8 μg/mL.

Epidemiologic investigation

Interviews with the owners of Farm A revealed that the index case (Horse 1) came from a closed herd with seven other draft horses aged 6–23 years. The horses were housed in a barn and adjacent dry lot corral pens (Fig. 1). The horses were periodically turned out for exercise onto 40 acres of fenced pasture in forested land. The stalls and paddocks next to the barn were cleaned daily, and manure was stacked in a storage pile adjacent to the barn.

According to the owners, the feed, water and husbandry practices had been identical for the past 15 years on their farm. Water for the house and barn was supplied by a sealed well. Hay was purchased from feed stores. No custom supplements or herbal products were being administered to the horses. No new horses had been admitted to the farm in the past 3 years, and there were no other domestic livestock or avian species housed on the farm. The family had a pet dog and cat that were healthy at the time of the investigation.

The only significant environmental change reported by the owners was increased sightings of wild turkeys (*Meleagris gallopavo*) visiting the property during summer 2010.

Table1. *Salmonella* recovery from clinical and environmental samples collected during investigation of equine infections at a rural Northern California farm, July 2010 to March 2011

Source	No. samples tested	No. positive (%)	Serotype	Pulsotype
Farm animals				
Horse 1 – hosp*	10	10 (100)	Oranienburg	1
Horse 1†	6	6 (100)	Oranienburg	1, 2, 3, 4, 5, 6, 7
Horse 2	3	1 (33.3)	Oranienburg	1
Horse 3	4	1 (25)	Oranienburg	7
Horse 4†	1	1 (100)	Oranienburg	1, 3
Horse 5	2	1 (50)	Oranienburg	3
Horse 6	2	1 (50)	Oranienburg	1
Horse 7	3	0		
Horse 8	6	0		
Horse composite‡	3	1 (33.3)	Oranienburg	3
Horse –farm B§	10	0		
Cat	1	0		
Dog†	2	2 (100)	Oranienburg	1, 3, 7
Barn area				
Manure pile†	20	9 (45)	Oranienburg	1, 3, 6, 7
Water troughs	7	2 (28.6)	Oranienburg	1, 3, 7
Rabbit feces	1	0		
Turkey feces†,¶	71	16 (22.5)	Oranienburg 11:d-	1, 3, 6, 7 9
Turkey feathers	2	0		
Tadpoles	4	0		
Well	7	0		
Garden				
Soil†	39	4 (10.3)	Oranienburg Enteritidis	1, 3 8
Vegetables	10	0		
Total	214	55 (25.7)		

*Horse 1 faecal samples were collected daily for 10 days from admission to the teaching hospital through discharge; one isolate from admission (11 July, 2010) was available for pulsotyping.

†More than one pulsotype found in multiple picks from the primary culture.

‡Composite sample of horse faeces from corral pen floor.

§Neighboring horse ranch.

¶Two positive samples were composited from dried turkey faeces.

They described seeing groups of up to 30 wild turkeys in the pastures, in the barns, in the feeders and drinking from the horse's water troughs. In previous years, they recalled seeing groups of five or fewer wild turkeys at a time visiting the property. They also reported seeing other types of wild birds at an on-site garbage pit used for disposal of kitchen scraps and other organic material at a neighbouring ranch (Farm B) located about 5 km from Farm A. According to owners of Farm B, the pit had been present for approximately 30 years.

A fenced fruit and vegetable garden (~12 × 8 m) was located in front of the house, approximately 200 m from the horse barn (Fig. 1). The owners did not observe wild turkeys or their faeces in the garden or around the house. But, the garden was fertilized routinely with raw (untreated) horse manure from the manure storage pile next to the barn. Following diagnosis of equine salmonellosis on their property, the owners immediately stopped applying raw manure to the garden. Additionally, a commercial, treated chicken manure–green waste compost blend was applied to the garden that summer, but was not available for testing. In November 2010, the owners tilled the garden soil and left it fallow through March 2011. In November, they also moved, randomly turned and tarped the manure storage pile relocated approximately 1000 m from the garden area (Fig. 1).

Laboratory investigation

A total of 204 clinical and environmental samples at Farm A were analysed, and *Salmonella* spp. was isolated from six of eight (75%) horses, an asymptomatic pet dog, two of seven (28.6%) water samples from horse troughs, nine of 20 (45%) manure storage pile composites, 16 of 71 (22.5%) wild turkey faeces and four of 39 (10.3%) soil samples from the family's edible home garden. (Table 1). Well water,

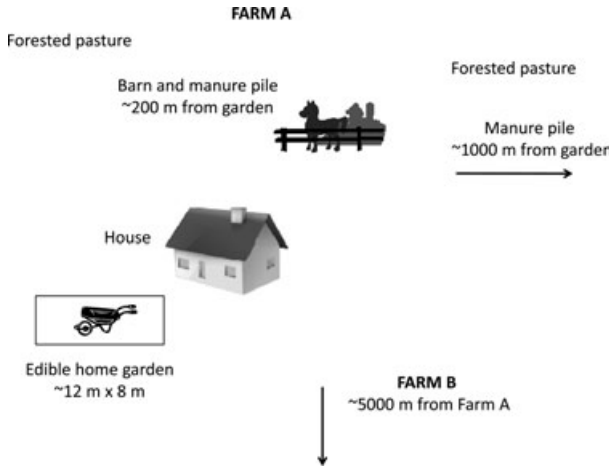

Fig. 1. Schematic of Farm A. Clip art images from Microsoft Office Gallery, 2010.

garden vegetable samples and horse faecal samples from a neighbouring ranch (Farm B), as described above, were negative. All strains were serovar Oranienburg except one turkey faeces sample collected on 23 August 2010, which contained serovar 11:d-, and one garden sample collected on 26 November 2010, which contained serovar Enteritidis (Table 1, Fig. 2).

The PFGE patterns of environmental isolates were compared with the *S.* Oranienburg strain cultured from Horse 1 at admission to the hospital (pulsotype 3, Fig. 2). Altogether, seven *S.* Oranienburg pulsotypes were identified, and PFGE patterns indistinguishable from the horse clinical strain were found in stable mate (Horse 4, Horse 5), dog, turkey, water trough, manure pile and garden soil samples (Fig. 2). Multiple pulsotypes (range 2–3) were found in 16 (36%) of the individual samples (Table 1), and 67% of the strains belonged to pulsotypes 1 and 3 (Fig. 2). Interestingly, closely related pulsotypes (1, 2, 4, 5) differing by 1–3 bands from pulsotype 3 were cultured from Horse 1 after discharge from the hospital (Fig. 2). We do not know whether Horse 1 was shedding other pulsotypes during hospitalization because by the time we initiated the study, all of the clinical isolates were discarded except the original isolate from the sample taken at admission on 11 July 2010.

Recommendations

Our recommendations to the owners to mitigate the human and animal health risk on Farm A included the following: (i) reduce or discourage the turkey population by hunting and scare tactics, (ii) clean and disinfect horse water troughs and feed bins exposed to horse manure and wild turkey faeces with a 1 : 10 commercial bleach solution, (iii) thoroughly wash hands with soap and use good personal hygiene when handling the horses and manure, (iv) do not use raw horse manure or any other untreated animal manure as fertilizer in the fruit and vegetable garden and (v) rinse fresh produce from the garden in cool, running, potable water before consumption. Additionally, the owners discontinued off-farm activities with Horse 1 such as parades due to her status as a chronic *Salmonella* carrier.

Discussion

Potential sources and persistence of viable *Salmonella* on a rural horse farm in coastal Northern California were investigated following diagnosis of *S.* Oranienburg infection in a horse hospitalized for colic surgery. The index case did not present with classical signs of *Salmonella*-related acute gastroenteritis, although a history of fever and colic have been associated with *Salmonella* shedding in horses (Dargatz and Traub-Dargatz, 2004; Ernst et al., 2004; Dallap Schaer et al., 2012; Hartnack et al., 2012). Diagnosis was credited

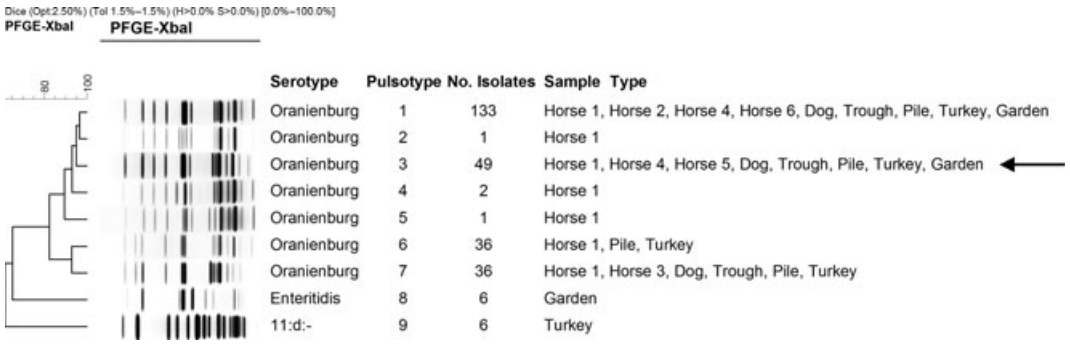

Fig. 2. Diversity in *Salmonella* pulsotypes (*Xba1* restriction) isolated from horse clinical and farm environmental samples, 11 July 2010 to 1 March 2011. Individual horses are numbered Horse 1 to Horse 6; environmental samples include manure storage pile and composite faeces from horse pen floor ('pile'), turkey faeces ('turkey'), water from horse trough ('trough'), dog faeces ('dog') and garden soil ('garden'). Arrow indicates the clinical strain isolated from Horse 1 on the day of admission to the hospital (11 July 2010).

to the teaching hospital's infection control protocol that dictates routine faecal screening for *Salmonella* and isolation of positive horses at the time of admission. The history of fevers among some stable mates, and culture confirmation of *S.* Oranienburg shedding in symptomatic and asymptomatic horses at Farm A, suggest that the infection spread through the herd during summer 2010. The report also highlights to potential difficulty differentiating fevers caused by equine salmonellosis from granulocytic anaplasmosis in regions where the tick-borne infection is endemic.

There are over 2500 serotypes of *Salmonella* spp. described (CDC, 2011a). Many of these are zoonotic and belong to *S. enterica* subspecies *enterica* serogroup 1, including *S.* Oranienburg. According to CDC's National *Salmonella* Surveillance database, *S.* Oranienburg represents 2.2% of all serotypes from human sources reported in 2009, the most recent available data (CDC, 2011b). Human outbreaks from *S.* Oranienburg appear to be rare, but have been linked epidemiologically to Tyrolean cheese, fruit salad and possibly green onions (Deeks et al., 1998; Allerberger et al., 2000; CDC, 2007). There were no reported human illnesses linked to the equine salmonellosis outbreak described in this study.

Animal illnesses due to *S.* Oranienburg have been reported in the veterinary literature including outbreaks among dairy calves and a hybrid mouse colony (Lentsch et al., 1983; Kaneene et al., 2010). Traub-Dargatz et al. (1990) conducted a study of *Salmonella* shedding among hospitalized horses and found that Oranienburg and Newport were the most common serovars in that population. A survey of *Salmonella* recovery from domestic animal faeces in the Culiacan Valley of Mexico also found that *S.* Oranienburg was the dominant serotype among food animals in the region (Jimenez et al., 2011).

Infections with multidrug-resistant *Salmonella* strains have been reported among horses and dairy calves (Dargatz and Traub Dargatz, 2004; Hartmann et al., 1996; Kaneene et al., 2010). The *S.* Oranienburg clinical isolate in our study, and several other related strains cultured from stable mates, were resistant to doxycycline. This is not surprising as the horses had a history of intermittent fevers and treatment for suspected granulocytic anaplasmosis. Because the environmental strains from Farm A were closely related based on PFGE findings, we did not test any additional *S.* Oranienburg isolates for antibiotic resistance.

The clinical strain (pulsotype 3) and multiple related strains were found distributed widely on Farm A (Table 1, Fig. 2). Given current knowledge of *Salmonella* molecular epidemiology, the genetic variations among *S.* Oranienburg isolates are consistent with the 7-month time frame of the study (Barrett et al., 2006). The findings demonstrate genetic changes that can occur in *Salmonella* PFGE *Xba*1 macrorestriction patterns during the course of a clinical infection and across time in the farm environment, most likely from creation or loss of a restriction site (Tenover et al., 1995).

We speculate that wild turkey faeces were the source of the horse infections at Farm A because an increase in wild turkey numbers was the only change noted by the owners of Farm A. Additionally, the clinical strain (pulsotype 3) and closely related strains were detected in fresh and dry wild turkey faeces (Table 1, Fig. 2). Moreover, wild birds are common reservoirs of *Salmonella* and have been associated with transfer on horse and other livestock farms (Quevedo et al., 1973; Cizek et al., 1994; Kirk et al., 2002; Pedersen and Clark, 2007; Taylor and Philbey, 2010; Carlson et al., 2011). Beyond this study, there is limited information on the occurrence of *Salmonella* in wild turkeys (Howerth, 1985; Tizard, 2004). Additional prevalence studies of zoonotic *Salmonella* in wild turkeys and the importance of their interactions with domestic animals are needed to better understand the ecology and epidemiology of *Salmonella* in these populations. Notably, reports of damage and conflicts with wild turkeys have increased as their numbers expand in urban and agricultural areas (CDFG, 2004).

Although wild turkeys represent a plausible hypothesis for the source of the equine infections, we cannot rule out the possibility that the turkeys acquired their infection on Farm A or were exposed to an outside source and transiently carried it onto Farm A. For example, we tested the well water used in the horse water troughs, and the samples were negative, but we did not test feed because the owners had not changed their source. Feed has been suspected as a source in previous equine salmonellosis outbreaks (Walker et al., 1991). Notably, the local veterinarians were not aware of any other salmonellosis cases in the surrounding area, and *Salmonella* was not detected in faecal samples from horses at a neighbouring property, Farm B.

Salmonella Oranienburg persisted in the garden for an estimated 210 days following the last application of raw manure in summer 2010. Because the owners denied seeing wild turkeys or their faecal material in the garden, we believe *S.* Oranienburg recovery from the garden soil represents persistence, not re-introduction. *Salmonella* is known to persist in animal manure and slurries for long periods of time depending on temperature, pH, UV exposure from the sun and other conditions (Islam et al., 2004; Holley et al., 2006). The cool, coastal climate at Farm A may have contributed to survival of *S.* Oranienburg over a prolonged period of time.

It appears tilling and leaving the garden fallow for 4 months was insufficient to remediate the problem. Treatment with Ca(OH)2 (lime) to inactivate soil contaminated with *Salmonella* has been described, but may not be practical in an edible garden (Nyberg et al., 2001). While there

are no established standards related to soil amendments for home or community gardens, the National Organic Program (7 CFR § 205.203) specifies that untreated animal manures must be applied at least 90 days prior to harvest for crops whose edible portions do not come in contact with the soil and at least 120 days prior to harvest of crops whose edible portions do come in contact with the soil. Within two on-farm studies, following inadvertent commercial application of naturally contaminated soil amendments, *Salmonella* and enterohaemorrhagic *Escherichia coli* survival was found to persist beyond the 120-day pre-harvest interval; growth and incorporation of a cover crop was preliminarily associated with substantial reduction (or practical elimination) in the pathogen from the soil (T. V. Suslow, personal communication). Such on-going research and the results from our study clearly indicate that additional remediation research is needed to refine these standards, especially if manure is sourced from ill animals and applied to soil in cool, coastal climates.

In summary, the investigation illustrates the potential for widespread dissemination of *Salmonella* in a farm environment following symptomatic and asymptomatic equine infections. Moreover, the findings underscore the need to educate the public about food safety hazards associated with using raw manure to fertilize edible home gardens. The owners acknowledged that prior to the diagnosis of salmonellosis among their horses, they believed raw horse manure was safe, even though they admitted being aware of food-borne pathogen risk from raw ruminant manure (cow, goat, sheep). Educational materials addressing risk from the use of animal-based manure amendments in home and community gardens should be distributed widely to prevent illnesses from zoonotic food-borne pathogens.

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