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SHORT REPORT
Molecular detection of the index case of a subclinical *Salmonella* Kentucky epidemic on a dairy farm

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SUMMARY

Salmonella enterica commonly colonizes the intestinal tract of cattle and is a leading cause of foodborne illness. A previously described investigation into the prevalence of *S. enterica* on a dairy farm revealed an 8-year-long asymptomatic *S. enterica* epidemic caused by serotypes Cerro and Kentucky in the lactating herd. To investigate the source of the *S.* Kentucky strains, the genomes of two *S.* Kentucky isolates were sequenced; one collected prior to the epidemic (2004) and one collected during the epidemic (2010). Comparative genomic analysis demonstrated significant polymorphisms between the two strains. PCR primers targeting unique and strain-specific regions were developed, and screening of the archived isolates identified the index case of the asymptomatic *S.* Kentucky epidemic as a heifer that was raised off-site and transported onto the study farm in 2005. Analysis of isolates collected from all heifers brought onto the farm demonstrated frequent re-introduction of clones of the epidemic strain suggesting transmission of pathogens between farms might occur repeatedly.

Key words: Food safety, genomics, *Salmonella*.

Salmonella spp. are a major cause of morbidity and mortality in humans and domesticated animals worldwide. The aetiological agents of most salmonellosis cases in humans and other mammals are members of the *Salmonella enterica* species, and the intensity or level of illness is often serotype-specific [1, 2]. All *S. enterica* serotypes are considered potentially pathogenic to humans, but not all serotypes cause symptomatic infections in other mammals. Non-human hosts may subclinically harbour these organisms and therefore pose an under-appreciated public health risk to humans.

Salmonella enterica subsp. *enterica* serovar Kentucky is an occasional pathogen of humans and was the 44th most isolated serotype from diagnosed salmonellosis cases in humans from 1999 to 2009 in the United States [3]. Currently, human infections with highly drug-resistant *S.* Kentucky ST198 strains have spread globally suggesting it is a serotype with clear global health significance [4, 5]. *S.* Kentucky is

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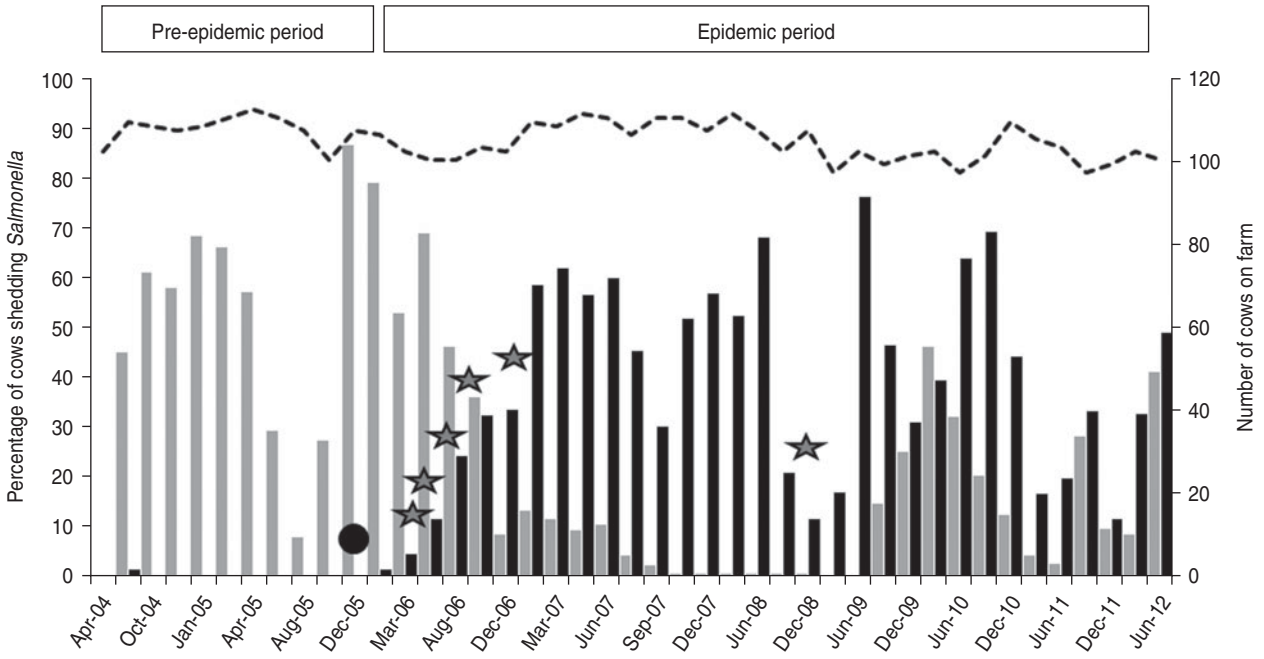

Fig. 1. Percentage of cows shedding *Salmonella enterica* on the study farm. Black bars indicate percentage of cows shedding *S.* Kentucky and grey bars indicate percentage of cows shedding *S.* Cerro (primary y-axis). Dotted black line shows the number of cows on the farm (secondary y-axis). The black circle indicates initial introduction of the epidemic strain onto the farm. Grey stars indicate introductions of heifers shedding the epidemic strain.

often subclinically shed from dairy cattle and previous studies demonstrated the incidence of infection in cows on *S.* Kentucky-positive farms ranging between 1·8% and 97% [6–9]. Thus, its frequent isolation from dairy farms and ability to cause illness in humans suggests that monitoring this serotype, among others, may limit the public health risk of zoonotic salmonellae for humans who consume dairy and beef products and those who work in the dairy industry or have contact with animals at agricultural fairs.

As part of an on-going longitudinal investigation (2004–2012) into the presence of human foodborne pathogens on dairy farms in the northeast United States, a commercial dairy farm in Pennsylvania was investigated for the presence of *S. enterica* [9]. A long-term *S. enterica* epidemic in the herd was observed and the two primary serotypes were identified as Cerro and Kentucky [9]. Further work demonstrated that *S.* Kentucky was prevalent on other dairy farms surrounding the study farm demonstrating a regional presence of this serotype [10]. From April 2004 to December 2005, the majority of recovered *S. enterica* isolates from the study farm were serotyped as Cerro. Two cows were shedding *S.* Kentucky and four were shedding *S.* Typhimurium for only one sample collection date each, as reported previously [9]. During this period, *S.* Kentucky was isolated 16 times (twice from mature cows, 11 times from composite manure samples, once from flies, once from a tractor tyre swab, and once from standing water near the tractor tyre) (Fig. 1). *S.* Typhimurium became extinct on the farm in June 2004 and, from January 2006 onwards, *S.* Kentucky was isolated with increasing frequency [9]. A shift from Cerro to Kentucky dominance was gradually observed, followed by co-existence of the two serotypes on the farm (Fig. 1) [9]. Cows shedding either serotype did not display symptoms of an infection. Sporadic isolations of *S.* Oranienburg (two cows), *S.* Enteriditis (one cow), *S.* Muenster (one cow) and *S.* Montevideo (one cow) were observed during the study period [9].

Identification of the source, or index case of *S. enterica* epidemics and contamination is integral to preventing larger outbreaks as well as identifying agricultural management practices that promote transmission or contamination. The aim of this study was to identify the source of the *S.* Kentucky on this dairy farm using comparative genomics followed by PCR identification of epidemic-associated genotypes.

Monitoring of the herd and environmental samples (feed, source and trough water, composite manure, environmental waters and streambed sediments in the

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surrounding area, and flies) was conducted 4–6 times per year [11, 12]. The herd size ranged between 98 and 112 lactating cows. Calves were monitored post-weaning prior to leaving the home herd for a heifer-rearing facility, and prior to their re-introduction as pre-fresh heifers to their home herd. Processing of samples and identification of *S. enterica* were as described previously [11, 12]. For each sample at least six randomly chosen presumptive *Salmonella* colonies were selected for confirmation. Serotyping was determined on select isolates [13]. Farm management records were collected during the course of the study.

To identify potential unique genomic markers of the outbreak strain we compared the genome sequence of a 'pre-epidemic strain' to that of an 'epidemic strain'. The pre-epidemic strain (strain 0253) was isolated from a randomly selected cow in 2004 and the epidemic strain (strain 5349) was isolated from a different randomly selected cow in 2010. Genomic DNA from each strain was isolated from overnight cultures using a DNeasy blood and tissue kit (Qiagen, USA). The genomes were sequenced using the Genome Sequencer FLX+ 454 Life Sciences (Roche, USA) and the Sequencing Reagents XL+ kit according to the manufacturer's instructions. *De novo* assemblies were performed using Roche Newbler software v. 2.6 (Roche). The assembled genomes were annotated using rapid annotations based on subsystem technology (RAST) [14]. To identify significant regions of non-homogeneity between the two *S.* Kentucky isolates, the genome of *S.* Kentucky 5349 was aligned with the *S.* Kentucky 0253 genome using BLASTP.

PCR primers were developed to target unique regions of the epidemic strain. To amplify DNA from an indel region of *nupG*, primers *nupG*-F 5′-ctcactaccctgggctcgta-3′ and *nupG*-R 5′-tcaggaagaacggaatggtc-3′ were used. To amplify the DNA from an indel region of the putative transport protein primers PTP-F 5′-ccgattctgcagtggttttt-3′ and PTP-R 5′-acaataagatttgcggcaatg-3′ were used. Annealing temperature for *NupG* was 55 °C and the annealing time was 60 s. Annealing temperature for PTP was 53·3 °C and the annealing time was 60 s. PCR products were separated by size in a 1% agarose gel and visualized under UV light. Amplicon sizes were estimated by comparison to PCR ladders of known sizes. To determine the source of the epidemic strain all *S.* Kentucky isolates from the beginning of sampling in September 2004 onwards were screened until an isolate with both epidemic-specific indels was found.

Nucleoside permease subunit G (*nupG*), a housekeeping gene conserved among Gram-negative bacteria, encodes an inner membrane porin that is involved in transport of nucleosides. Mutants of this gene in *S.* Typhi have been demonstrated to be viable and can grow on minimal media [15]. The BLASTP analysis of strains 5349 and 0253 demonstrated this gene to encode a tandem 159 bp duplication of the sequence between nucleotides 158 and 318 resulting in an amino acid (aa) that is 53 aa longer in the epidemic strain (strain 5349) than that of the pre-epidemic strain (strain 0253). Another open reading frame (ORF) annotated as a putative transport protein exhibited a 93 bp deletion in the epidemic strain when aligned with the pre-epidemic strain.

From the beginning of the study period until the index case was detected, 1212 cow faecal, 152 composite manure, 97 water (trough and source), 90 feed (mixed and components), and 452 other (flies, waste lagoon, tyres, etc.) samples were collected. Of these, only 16 samples were positive for *S.* Kentucky. The first isolate with the markers of the epidemic strain was recovered from the faeces of a heifer that had been moved from an off-site independent heifer-rearing facility to the study farm in November 2005 (Fig. 1). The sample was collected prior to this animal entering the study farm indicating that the strain colonized the intestinal tract of the heifer prior to contact with other animals and the environment of the study farm. All 16 *S.* Kentucky isolates recovered prior to this event, like the sequenced pre-epidemic strain, did not encode the markers of the epidemic strain suggesting that, although the pre-epidemic strain was persistent on the farm, it did not become established in the bovine population. Farm records also indicate that, at this time, the heifer-rearing facility from which the epidemic strain originated was newly contracted thereby exposing heifers from the study farm to a novel population of heifers from multiple farms. Further, PCR targeting the epidemic-strain markers of *Salmonella* isolates collected from eight separate heifers brought onto the farm between January 2006 and November 2008 resulted in positive amplicons for six heifers, demonstrating this strain was carried onto the farm multiple times after the initial introduction (Fig. 1). It is important to note that more heifers were brought onto the farm during this time-frame, but only eight were sampled. During this period, no calves leaving the study farm were positive for *S.* Kentucky, indicating that epidemic-strain infections originated at the heifer-rearing

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facility and subsequently were transported onto the study farm.

Heifer raisers specialize in rearing female calves away from their birth farm in a facility that is often utilized by several farmers within a region. The heifers from the study farm were moved to the heifer-rearing facility after weaning (~6–7 months) and were generally returned to their home farm within 16–18 months, shortly before giving birth to their first calf. Previous studies demonstrated that off-site rearing of heifers is significantly associated with the introduction of *Salmonella* serotypes to a herd that were not previously identified in that herd [16–18]. This indicates a potential biosecurity issue as specialized heifer-rearing operations are becoming more common. Currently about 1 in 10 dairy farms in the United States utilizes heifer-rearing operations [19].

Monitoring transported cattle for carriage of human infectious agents, such as *S. enterica*, prior to their integration in a herd may help reduce the risk of pathogen transmission between herds. This may identify cows that carry agents harmful to other members of the herd or the human population that work with the herd or consume the meat and dairy products produced by the herd. Although, such monitoring remains economically infeasible for most dairy operations, decreasing costs of rapid molecular monitoring may allow for more rapid detection of pathogens in the future.

Results of this study demonstrate genomically that livestock movement, commingling with livestock from other farms, or utilizing facilities used by several other farms may result in transmission of pathogens to novel geographical locations and potentially increase the risk of pathogen exposure to larger human and animal populations. Results of this study also demonstrate a need to further evaluate the genomic variability within *Salmonella enterica* subsp. *enterica* serovars to better elucidate those elements and/or polymorphisms in the genome that result in emergence of specific strains within a susceptible host population.

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DECLARATION OF INTEREST

None.

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