ORIGINAL ARTICLE

Spatial Epidemiology of *Escherichia coli* O157:H7 in Dairy Cattle in Relation to Night Roosts Of *Sturnus vulgaris* (European Starling) in Ohio, USA (2007–2009)

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Impacts

- Results from recent studies have suggested European starlings (*Sturnus vulgaris*) act as vectors for pathogen transmission among farms.
- Our results suggest that starling night roosts may act as point sources for dissemination of *Escherichia coli* O157:H7 among dairy cattle farms in northeastern Ohio, USA.
- This study highlights the need to investigate the role of starling control as part of pre-harvest food safety programmes.

Keywords:
Escherichia coli O157:H7; dairy cattle; *Sturnus vulgaris*; starlings; spatial scan statistics

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Summary

The goal of our study was to use spatial scan statics to determine whether the night roosts of European starlings (*Sturnus vulgaris*) act as point sources for the dissemination of *Escherichia coli* O157:H7 among dairy farms. From 2007 to 2009, we collected bovine faecal samples (n = 9000) and starling gastrointestinal contents (n = 430) from 150 dairy farms in northeastern Ohio, USA. Isolates of *E. coli* O157:H7 recovered from these samples were subtyped using multilocus variable-number tandem repeat analysis (MLVA). Generated MLVA types were used to construct a dendrogram based on a categorical multistate coefficient and unweighted pair-group method with arithmetic mean (UPGMA). Using a focused spatial scan statistic, we identified statistically significant spatial clusters among dairy farms surrounding starling night roosts, with an increased prevalence of *E. coli* O157:H7-positive bovine faecal pats, increased diversity of distinguishable MLVA types and a greater number of isolates with MLVA types from bovine-starling clades versus bovine-only clades. Thus, our findings are compatible with the hypothesis that starlings have a role in the dissemination of *E. coli* O157:H7 among dairy farms, and further research into starling management is warranted.

Introduction

Escherichia coli O157:H7 is a foodborne bacterial pathogen that causes major public health problems throughout North America (Scallan et al., 2011; Thomas et al., 2013). Infections can lead to gastroenteritis, haemorrhagic colitis and/or haemolytic uraemic syndrome, which can be fatal (Ochoa and Cleary, 2003). Human infections have been associated with contact with shedding animals and humans, consumption of contaminated produce, water and various foods of bovine origin (Bell et al., 1994; CDC, 2001; Olsen et al., 2002; Galanis et al., 2003; Honish et al., 2005).

Cattle are the major reservoir of *E. coli* O157:H7 in North America (Wells et al., 1991). Nevertheless, this

pathogen has also been isolated from other species such as deer, rodents and wild birds, including European starlings (*Sturnus vulgaris*) (Sargeant et al., 1999; Nielsen et al., 2004; Wetzel and LeJeune, 2006; LeJeune et al., 2008). Currently, the exact role of this and other wildlife species in the dissemination of *E. coli* O157:H7 among livestock operations remains unclear.

Based on data from molecular studies, researchers have suggested that starlings have a role in the dissemination of this pathogen among livestock operations (LeJeune et al., 2008; Gaukler et al., 2009; Williams et al., 2011). For example, Williams et al. (2011) isolated indistinguishable *E. coli* O157:H7 subtypes using multilocus variable-number tandem repeat analysis (MLVA) from both bovine and starling samples on several farms. Moreover, Cernicchiaro et al. (2012) discovered a significant association between the number of starlings per milking cow and the prevalence of *E. coli* O157:H7 shed by cattle on dairy farms. Collectively, these findings support the hypothesis that starlings have an epidemiological role in the dissemination of *E. coli* O157:H7 among livestock operations.

During the late summer and early autumn, starlings congregate in night roosts numbering in the tens to hundreds of thousands of birds (LeJeune et al., 2008). Each day, flocks emanating from these roosts disperse to multiple farms to feed, water and gather in shelter areas (Linz et al., 2007; LeJeune et al., 2008). Radio-tracking studies have shown that the night roosts are usually 10–40 km from the areas used for daily activities, to which strong daily fidelity is shown (Homan et al., 2010, 2013; Gaukler et al., 2012). When going back to the roost at the end of the day, starlings often stage temporarily at dairy farms lying on the routes leading back to their roosts (LeJeune et al., 2008). Consequently, these dairy farms have potential to be visited by a large variety of individuals over a broad area. If starlings were contributing to the epidemiology of *E. coli* O157:H7 in cattle on dairy farms, it is expected that, compared with dairies farthest from a roost, those closest would have both a higher prevalence and a greater diversity of molecular subtypes of *E. coli* O157:H7 being shed by their dairy cattle.

Several methods have been developed using space, time, or space and time to study the epidemiology of diseases. The spatial scan statistic, which describes the existence of disease clusters in space, has been used to study both infectious and non-infectious diseases (Pearl et al., 2006; Christian et al., 2011). Spatial scans are employed for general tests, which are concerned with the overall pattern of disease over a study region, where the location of clusters is unknown (Tango, 1995). When a more specific hypothesis is being tested under the assumption that the focal point of contamination is known (e.g. a starling night roost), a focused clustering test may be more powerful (Tango, 1995; Kulldorff, 2010).

Our study objective was to examine the role of European starlings in the dissemination of *E. coli* O157:H7 among dairy farms in northeastern Ohio by applying a focused spatial scan statistic to determine whether starling night roosts in the area were foci for disseminating this bacterium among dairy cattle. Under the assumption that starlings were vectors and that roosts were foci, we hypothesized that dairies closer to night roosts would display the following patterns in *E. coli* O157:H7 prevalence: (i) higher prevalence of bovine faecal pats positive for *E. coli* O157:H7, (ii) higher prevalence of *E. coli* O157:H7 isolates from starling-bovine clades versus bovine-only clades and (iii) higher molecular diversity (as measured by species richness) of *E. coli* O157:H7, demonstrated by higher numbers of distinguishable genetic subtypes based on multilocus variable-number tandem repeat (VNTR) analysis (MLVA).

Materials and Methods

Sample collection, isolation and MLVA typing

This study took advantage of data from previous studies, which looked at the association between the presence of starlings and the on-farm prevalence of *E. coli* O157:H7 in dairy herds and the role of these birds as biological vectors among these herds in Ohio. All MLVA typing and epidemiological data were obtained from these previous studies (Williams et al., 2011; Cernicchiaro et al., 2012). A detailed description of sample size calculation, farm recruitment and sampling is available in Cernicchiaro et al. (2012).

Briefly, 150 dairy farms in northeastern Ohio were sampled twice a year, first, during summer (June–August) and subsequently during autumn months (September–November), with the exception of one farm that was visited three times. The visits took place from 2007 to 2009. At each visit, fresh bovine faecal pat samples were taken from the first 30 milking cows seen defecating, and up to 30 starlings were captured and euthanized per farm.

Escherichia coli O157:H7-positive samples were isolated from enrichment samples of bovine faeces and starling intestinal contents by immunomagnetic separation, standard microbiological methods and latex agglutination tests. Additionally, suspect colonies were confirmed using polymerase chain reaction (PCR) to detect the presence of the *rfb*E gene. Positive samples were MLVA-typed using eight VNTR loci (3, 17, 34, 19, 9, 36, 25 and 37) (Hyytia-Trees et al., 2006). MLVA types were generated to construct a dendrogram based on a categorical multistate coefficient and unweighted pair-group method with arithmetic mean (UPGMA) (Hyytia-Trees et al., 2006). We used all data collected from farms over the 3-year period in spatial analyses; relationships among isolates were based on the constructed dendrogram by Williams et al. (2011); Table 1).

Table 1. Clades containing *E. coli* O157:H7 MLVA types isolated from both starling and bovine samples

Clade	MLVA type	Species	No. farms
1*	1	S/B	7
	2	B	1
	3	B	1
2	4	B	1
	5	B	1
	6	B	1
	7	B	1
	8	B	2
	9	B	1
	10	B	1
	11	B	1
3*	12	B	1
	13	B	1
	14	S/B	3
	15	B	1
	16	B	1
	17	B	1
	18	B	1
	19	B	1
4	20	B	1
	21	B	1
	22	B	1
	23	B	1
	24	B	1
	25	B	1
	26	B	1
	27	B	1
	28	B	1
5	29	B	1
	30	B	1
	31	B	1
	32	B	1
	33	B	1

Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) types of *E. coli* O157:H7 isolates from starling (S) and bovine (B) origin, and the number of farms where each MLVA type was isolated between 2007 and 2009, in Ohio. Table modified from dendrogram constructed by Williams et al. (2011).

*Clades containing MLVA types of *E. coli* O157:H7 isolates of both bovine and starling origin.

LeJeune et al. (2008) previously identified three wetland roosts (Morton, Lime Lakes and South Rittman) and one upland roost (Apple Creek) in the study area (Fig. 1). The Morton roost was the smallest of the four night roosts, containing approximately 1000 starlings. This roost was an infrequently used satellite roost of the much larger South Rittman roost that housed between 10 000 and 20 000 starlings. These two roosts were in close proximity (≈1.8 km apart). Apple Creek was a seasonal roost comprised of mostly young-of-the-year starlings numbering from 300 000 to 500 000. The largest roost was Lime Lakes with greater than one million birds. Unlike the other three night roosts, Lime Lakes was a mixed-species roost including red-winged black birds (*Agelaius phoeniceus*), common grackles (*Quisculus quiscula*) and European starlings. We used the locations of these four night roosts for all focused spatial analyses to examine whether the roosts were foci for the dissemination of *E. coli* O157:H7 among dairy cattle on the 150 farms (Fig. 1). The latitude and longitude of all farm and roost locations were recorded using a GPS unit (eTrex Vista HCx, Garmin Olathe, KS, USA). All collected samples were georeferenced to their farm locations for subsequent analyses. All work was conducted with required government permits and approval from the Ohio State University Institutional Animal Care and Use Committee.

Statistical methods

We used focused spatial cluster analysis to identify statistically significant ($\alpha \leq 0.05$) spatial clusters focused around the four night roosts based on a grid file containing roost-site coordinates. We used SATSCAN™ software (Kulldorff, 2010) for the analyses and selected the following models:

1. For binary data, a Bernoulli model was used to identify statistically significant clusters of bovine faecal pats positive for *E. coli* O157:H7 and isolates from starling-bovine clades versus bovine-only clades.
2. For count data, a Poisson model was used to identify statistically significant clusters of high numbers of distinguishable MLVA types from bovine faecal pats. A population file was included in this analysis, which provided an offset for the model and was set as the number of bovine faecal pats collected per farm ($n = 60$ or $n = 90$).

The spatial scan of isolates from starling-bovine clades was conducted using only *E. coli* O157:H7-positive farms, whereas the other spatial scans were conducted using all study farms.

We conducted all analyses for each individual study year as well as the entire study period (2007–2009) using the spatial scan statistic. The maximum spatial scanning window was set at 50% of the population, the maximum allowable level (Kulldorff and Nagarwalla, 1995). For each analysis, 9999 replications were generated to ensure appropriate power in estimating the significance levels of the identified clusters (Kulldorff, 2010). The data were fit to the Gumbel distribution using the method of moment's estimation (Abrams et al., 2010). The *P*-value was then calculated as the probability that this best-fitting distribution generated a value greater than the maximum likelihood ratio observed for the most probable cluster from the observed data set (Kulldorff, 2010). We calculated the likelihood ratio and the relative risk based on the specified model (i.e. Bernoulli or Poisson) to determine whether the

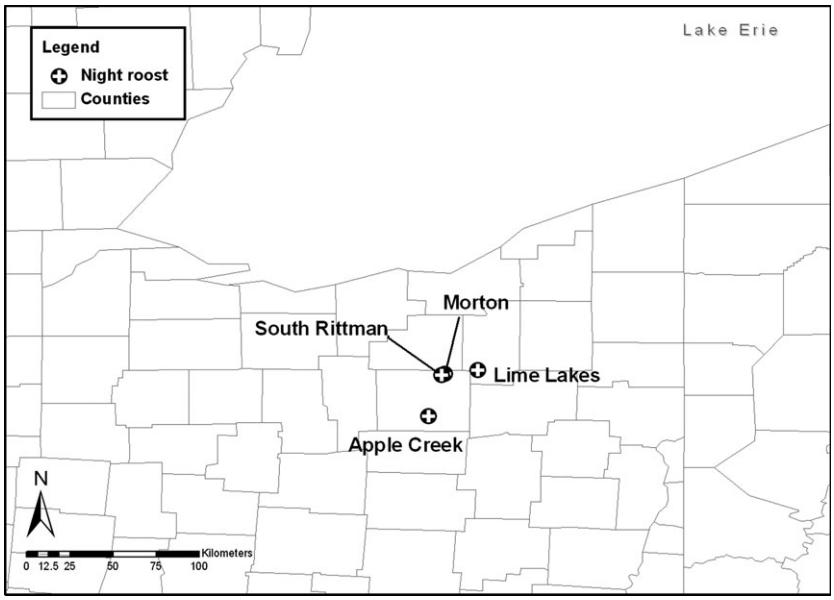

Fig. 1. The location of starling night roosts identified within the study region in northeastern Ohio for the study period 2007–2009.

risk/rate within the scanning window was different from outside the window (Kulldorff and Nagarwalla, 1995). For all analyses, the most likely and secondary statistically significant ($P < 0.05$) clusters were presented. However, during the analysis, geographical overlap was allowed using the criterion of no cluster centres in another cluster to identify all significant clusters involving a starling night roost even if they overlapped in space.

Computer software

Multilocus variable-number tandem repeat analysis patterns were compared using the commercial VNTR script within the BIONUMERICS® software (Ver. 6.6.; Applied Maths, Austin, TX, USA). All data management requiring the merging of files was performed in STATA® version 11.0 for Windows (Stata Corporation, College Station, TX, USA). All scan statistical analyses were performed using SATSCAN™, version 9.0 (Kulldorff, 2010). Spatial scan statistical analyses were visualized using the geographical information system (GIS) software ARCMAP 9.2 (ESRI, Redlands, CA, USA). All maps used in the figures were accessed through the Data Resource Center at the University of Guelph (http://www.lib.uoguelph.ca/resources/data_resource_centre/).

Results

Additional data relevant to the study farms (e.g. demographics, management and environmental characteristics) have been described in earlier papers (Williams et al., 2011; Cernicchiaro et al., 2012).

Spatial clustering of *E. coli* O157:H7-positive bovine faecal pats

Over the 3-year study period, 9000 bovine faecal samples were collected. Of these samples, 88 (1.0%) samples, from 33 of the 150 sample farms, were positive for *E. coli* O157:H7. Five starlings (1.2%) from 430 captured birds also tested positive for *E. coli* O157:H7.

Statistically significant spatial clusters of bovine faecal pats positive for *E. coli* O157:H7 with starling night roosts as foci were found in 2009 and across the entire study period (Table 2, Fig. 2). The significant clusters identified for 2009, and the entire study period had radii of 60.2 and 41.0 km, respectively, and both clusters encompassed all four starling night roosts. The Apple Creek night roost was identified as the focus of the significant cluster detected for 2009. In contrast, the South Rittman night roost was identified as the focus of the significant cluster across the study period.

Spatial clustering of *E. coli* O157:H7 isolates from bovine faecal pats that have MLVA types shared by starlings and cattle

Three statistically significant spatial clusters of similar size were identified focused on the Apple Creek night roost in 2007, 2009 and for the entire study period (Table 2; Fig. 3). Two secondary statistically significant spatial clusters were identified focused on the Lime Lakes night roost in 2009, and for the entire study period. The significant cluster centred on the Lime Lakes night roost in 2009 had the largest radius of all identified clusters and encompassed all other night roosts.

Table 2. Spatially significant spatial clusters focused on starling night roosts found in northeastern Ohio, 2007–2009

Analysis	Model	Year(s)	Night roost*	Radius (km)	No. of farms	Observed/expected	*P*-value
Positive bovine faecal pats	Bernoulli	2009	AC	60.2	32	2.68	<0.001
	Bernoulli	2007–2009	SR	58.6	114	2.79	<0.001
Isolates from faecal pats with MLVA types from starling-bovine clades versus bovine clades	Bernoulli	2007	AC	17.3	9	1.99	0.009
	Bernoulli	2009	AC	17.1	3	3.78	0.016
	Bernoulli	2009	LL	68.9	5	4.06	0.028
	Bernoulli	2007–2009	AC	17.2	12	2.07	0.010
	Bernoulli	2007–2009	LL	29.4	17	2.04	0.046
Greater numbers of distinguishable MLVA types	Poisson	2007–2009	SR	42.8	33	1.52	0.014

The radius (km), number of farms included, relative risk and significance of the statistically significant spatial clusters focused on starling night roosts in northeastern Ohio for each analysis and model for the study period 2007–2009.
*Starling night roost: AC, Apple Creek; SR, South Rittman; LL, Lime Lakes.

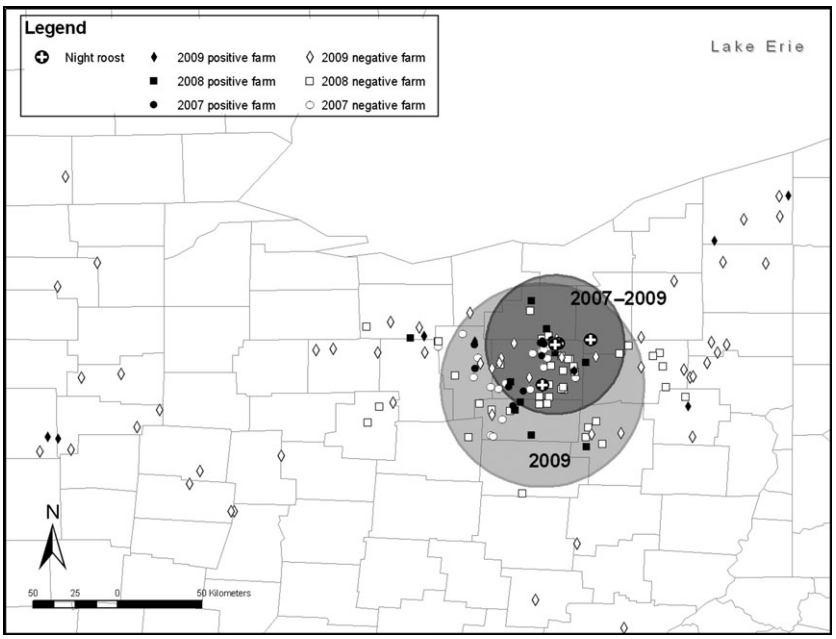

Fig. 2. The location of statistically significant spatial clusters, around starling night roosts in northeastern Ohio, of *E. coli* O157:H7 positive bovine fecal pats based on focused spatial scans of data from each year and the entire study period (2007–2009) using a Bernoulli model.

Spatial clustering of farms with higher numbers of distinguishable MLVA types

One statistically significant cluster of farms that tested positive for a greater number of distinguishable MLVA types was identified when the entire study period was examined (Table 2, Fig. 4). The spatial cluster was identified focused on the South Rittman night roost and contained all other night roosts.

Discussion

European starlings are the most common and numerous avian species found at dairy farms in Ohio (LeJeune et al., 2008). The frequent presence of birds in feed bunks and watering troughs is associated with the increased likelihood of foodborne pathogens in cattle (Sargeant et al., 2004; Carlson et al., 2011a). Correspondingly, Carlson et al. (2011b) has shown that starlings were effective mechanical vectors of *Salmonella* within cattle feeder operations. Based on our results, we hypothesize that starlings may similarly biologically disseminate *E. coli* O157:H7 among dairy farms in northeastern Ohio. We statistically demonstrated that starling roosts were spatially associated with increased prevalence of *E. coli* O157:H7 infection in dairy cattle, a greater diversity of distinguishable MLVA types and higher number of isolates with MLVA types from starling-bovine clades versus bovine-only clades. The radii of all identified

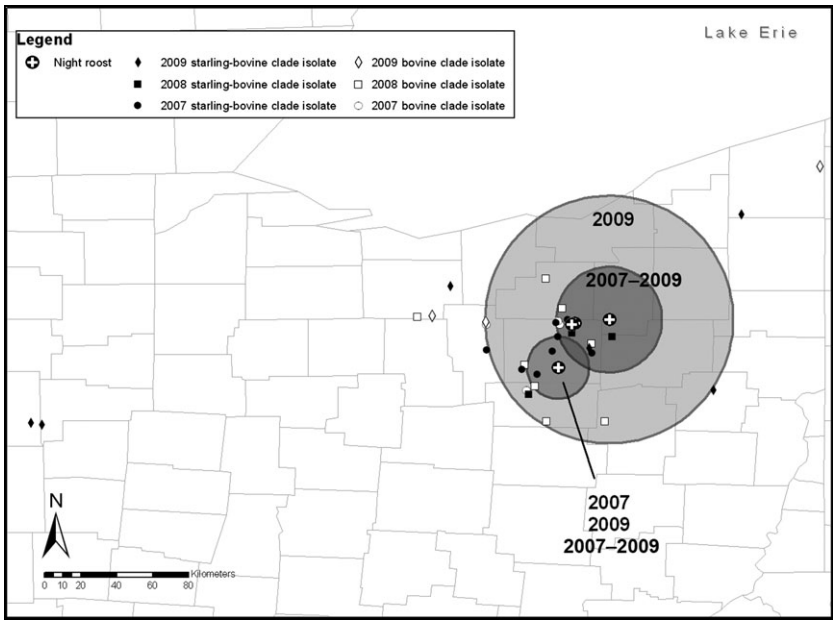

Fig. 3. The location of statistically significant spatial clusters, around starling night roosts in northeastern Ohio, of *E. coli* O157:H7 isolates from clades containing starling-bovine isolates versus bovine-only clades from each year and the entire study period (2007–2009), using a Bernoulli model with only *E. coli* O157:H7 positive farms.

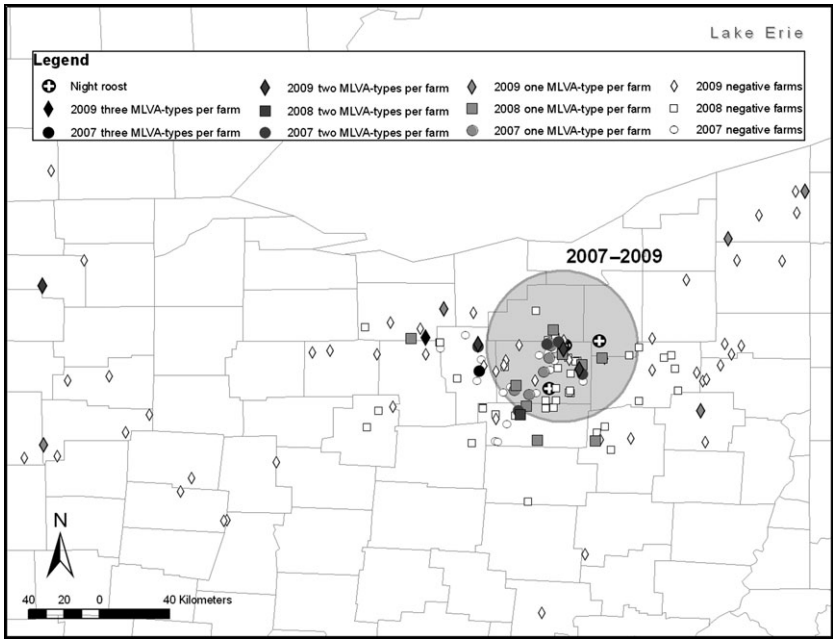

Fig. 4. The location of statistically significant spatial clusters, around starling night roosts in northeastern Ohio, of farms with greater numbers of distinguishable *E. coli* O157:H7 MLVA-types based on focused spatial scans of each year and the entire study period (2007–2009) using a Poisson model. In 2008 there were no farms identified with three or greater distinguishable *E. coli* O157:H7 MLVA-types.

significant spatial clusters were between 17 and 69 km, all of which are plausible daily flight distances for starlings (Homan et al., 2010). Thus, our spatial results are compatible with the hypothesis of starling night roosts acting as foci for the dissemination of this bacterium among dairy operations.

Previous studies conducted in the same area found that starlings tended to fly directly from their roosts to preferred dairies; whereas the return flights back to the roosts were more leisurely, with the birds often stopping at farms that were along the flight routes or nearby to staging areas (LeJeune et al., 2008; Homan et al., 2013). This dichotomy in behaviour should limit farm-to-farm dissemination of multiple distinguishable subtypes at remote sites while increasing the number of distinguishable genetic subtypes at sites closer to roosts. This is based on the increased probability that dairies nearer to roosts, which are aggregating the local populations, should have a greater number of visitors from a more spatially diverse array of farm sites. Consistent with this hypothesis, the prevalence of distinguishable genetic subtypes in our study was clustered among cattle from dairies closer to roosts. Although Cernicchiaro et al. (2012) did not find an association between the prevalence of *E. coli* O157:H7 in dairy cattle and distance to the nearest starling roost, we suspect the effect of distance in these models may actually depend on the specific roost and the flying routes used by these birds.

Consistent with previous studies using pulsed-field gel electrophoresis (PFGE) analysis, the majority of *E. coli* O157:H7 MLVA types identified in our study were farm specific, but there were farms sharing indistinguishable MLVA types (Wetzel and LeJeune, 2006). The large distance found between farms sharing indistinguishable MLVA types has often been cited as evidence to suggest the role of birds in the transmission of this bacterium (Wetzel and LeJeune, 2006; Williams et al., 2011). However, our findings of an increased diversity of distinguishable MLVA types and MLVA types from clades that share both bovine and starling types and which are closer to starling night roosts suggests starlings may have a role as vectors of this pathogen among dairy farms.

Three potential biases exist in our study related to farm recruitment, which must be considered when interpreting our results. First, farms were recruited to participate on a voluntary basis; thus, our study did not contain data from all farms in northeastern Ohio, nor even a random subset of farms. For selection bias to occur, the selection process has to be associated with both the *E. coli* O157:H7 status of the animals in the herd and the location of these herds relative to these night roosts. However, cattle are asymptomatic carriers of *E. coli* O157:H7, and presently, faecal analysis is the only practical means of confirmation (Wells et al., 1991). Therefore, it is unlikely that selection bias influenced our findings of statistically significant clusters, as dairy farmers would not be aware of the *E. coli* O157:H7 status of their herds, and thus, they could not volunteer based on this status. Second, due to the lack of random selection of farms, there was a higher density of farms sampled that were in close proximity to starling night roosts. However, this would likely make it more difficult to determine a difference among the farms, than if the farms were distributed equally across the study area. Therefore, we would expect that if this sampling distribution impacted our results, the bias would be towards the null. Thus, adding strength to our findings that the night roosts were the focus of statistically significant spatial clusters. Third, although we are confident all large night roosts were identified in the area, it is possible that a night roost may have been missed. However, if this was the case, it would have made it more difficult to identify foci of spatial clusters around known roosts because increased levels of positive faecal pats would have surrounded unidentified night roosts. Consequently, any biases associated with unidentified night roosts would have biased our results towards the null.

In a previous study using these data, Cernicchiaro et al. (2012) identified a number of herd factors related to cattle being positive for *E. coli* O157:H7, including number of birds per milking cow, type of manure storage, frequency of manure removal and number of ventilation systems. Therefore, it is possible that the location of clusters may have been confounded by identified herd-related factors. Using a multivariable regression analysis and replacing the faecal sample number with the expected number of positive faecal pats, it would have been possible to control for the herd-related factors that had been identified (Kleinman et al., 2005). However, this approach was not considered for this project as many of the variables identified in this previous risk analysis study may have been intervening variables and/or proxies for proximity to roosting site. For example, the number of starlings per milking cow is likely an intervening variable. Also, the number of ventilation systems used and types of manure storage practices were difficult to interpret and in retrospect may be proxies for proximity to these roost sites. Consequently, we chose to explore the spatial relation using the raw data, to avoid controlling for potential intervening or proxy variables.

It should also be noted that in this study, *E. coli* O157:H7 was only isolated from the gastrointestinal tract of birds and samples were not collected from their feet or feathers, which would be considered a mechanical means of transmission. Therefore, the low prevalence of *E. coli* O157:H7 identified in this study population of starlings may underestimate the role of these birds in spreading this bacterium or the diversity of the MLVA types mechanically carried by them (Williams et al., 2011; Cernicchiaro et al., 2012). We likely would have found more MLVA types shared by cattle and starlings if we had also considered mechanical transmission. It should be noted that even with a low prevalence, a large population of birds in the area suggests that the possibility for transmission was quite high.

Our data are consistent with the hypothesis that starlings have a role in the dissemination of *E. coli* O157:H7 among dairy farms. In particular, starling night roosts are statistically significant foci for the spread of this bacterium among our study farms. Accordingly, starling control should be examined at both the farm and the night roost locations and compared to assess both farm and regional level measure to control the on-farm prevalence of *E. coli* O157:H7 among cattle. These studies could be important for implementation of new policies to decrease the dissemination of *E. coli* O157:H7 among farms, thus potentially having a role in the prevention of foodborne illness.

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