Title:

Epidemiological investigation of bovine tuberculosis outbreaks in Uruguay (2011-2013)

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Abstract

Bovine tuberculosis (bTB) is a chronic disease of cattle caused by infection with the *Mycobacterium bovis*. While bTB prevalence in Uruguay has been low (<11 outbreaks/year) for the past 50 years as a consequence of a national control program, annual incidence increased in 2011 through 2013 to 15, 26 and 16 infected herds each year, raising concerns from livestock stakeholders and the government. The goal of this study was to assess the spatial dynamics of bTB in Uruguay from 2011-2013 and the association between bTB and potential demographic and movement risk factors at the herd level using data provided by the Uruguayan Ministry of Livestock, Agriculture, and Fisheries. Clustering of incident outbreaks was assessed using the Cuzick-Edwards' test and the Bernoulli model of the spatial scan statistic, and a conditional multivariable logistic regression model was used to assess risk factors associated with bTB in a subset of Uruguayan dairy farms. Significant ($P<0.05$) global clustering was detected in 2012, while high-risk local clusters were detected in southwestern (2011, 2012, 2013), northwestern (2012), and southeastern (2012) Uruguay. Increased risk of bTB in different regions of Uruguay suggests a potential role of animal movements in disease dissemination. Larger herds, higher numbers of animals purchased, and incoming steers to the farm were associated with increased odds of breaking with bTB, in agreement with previous studies but also suggesting other additional sources of risk. These results will contribute to enhanced effectiveness of bTB control programs in Uruguay with the ultimate objective of preventing or mitigating the impact of the disease in the human and animal populations of the country.

Keywords

Bovine tuberculosis; spatial cluster analysis; risk factors; Uruguay

INTRODUCTION

Bovine Tuberculosis (bTB) is primarily caused by *Mycobacterium bovis* and is a chronic disease that affects cattle and a wide range of livestock and wild species. The significance of bTB is due to its zoonotic nature, animal health risks, economic importance due to high cost of control and surveillance programs, and its impact on trade. bTB is prevalent throughout most of the world, including South America (de Kantor et al., 2008). Control programs based on test and cull, bTB-free farm certification, and slaughter-based surveillance have been implemented throughout the South American region (de Kantor and Ritacco, 2006; Max et al., 2011), though success has been variable. Cases of bTB in cattle herds is associated with a number of epidemiological factors that could explain this variability, including individual differences (age, gender, and breed) (Belchior et al., 2015; O'Hagan et al., 2015), herd-level factors (including herd size (Johnston et al., 2011; Winkler and Mathews, 2015), type of production and management (Griffin et al., 1993; Johnston et al., 2005; Álvarez et al., 2014; Bekara et al., 2016), and other factors such as presence of wildlife (Fitzgerald and Kaneene, 2013; McCluskey et al., 2014) or domestic reservoirs (Kaneene et al., 2010; Boadella et al., 2011; Zanardi et al., 2013; Guta et al., 2014; Pesciaroli et al., 2014). Previous studies have highlighted animal movements as an important factor in the maintenance and spread of bTB in a population (Gilbert et al., 2005; Clegg et al., 2013; Ribeiro-Lima et al., 2015; Vial et al., 2015), and some have hypothesized that certain types of movement may be higher risk (Gilbert et al., 2005). Given that bTB cases are often clustered spatially (Perez et al., 2002; Allepuz et al., 2011; de la Cruz et al., 2014), an assessment of the spatial heterogeneity in the distribution of bTB outbreaks is a prerequisite for understanding epidemiological factors related to disease occurrence.

Much of the landmass in Uruguay is utilized for agriculture, with 11.2% of the country's gross domestic product (GDP) attributable to agro-industrial-related production, almost half of which comes from livestock production (DIEA, 2015). The cattle industry includes more than 45,000 farms and ~12 million head of cattle. Farm size varies from <10 to 17,000 head, with a mean of 263 cattle per farm (VanderWaal et al., 2016a). Animals are typically managed outdoors in a variety of free-range systems, and production may be characterized broadly as beef and dairy, although some farms also have a combination of livestock and agricultural systems.

The current bTB control program of Uruguay is based on intradermal caudal fold tuberculin (CFT) testing of all >12 month-old dairy animals, supplemented by passive surveillance of both beef and dairy cattle at slaughter plants. Additionally, cattle going to markets, expositions, fairs or quarantines for live-animal export are also tested using the CFT test. CFT testing is performed by private practice veterinarians, and positive or inconclusive reactors to the CFT test are re-tested within 10 days using the intradermal comparative cervical tuberculin (CCT) test. The CCT test is implemented by the official veterinary services to differentiate cross-reactivity resulting from exposure to environmental mycobacteria. Animals with a >4 mm increase in the skin fold thickness of the bovine Purified Protein Derivative (PPD) inoculation site compared to the avian PPD inoculation site are considered reactors and culled. Affected farms are consequently considered bTB outbreaks (i.e. positive-farms) and cannot send/receive animals until two negative CFT herd-tests at six-month intervals are obtained. Neighboring farms and farms directly connected with the positive-farm by animal movements undertaken within 2 years of the outbreak onset are also tested with CFT. Passive surveillance is intended to detect bTB-like macroscopic lesions (tubercles) during routine inspection conducted in slaughter plants. To confirm infection, lesions detected during inspection are sampled and

submitted to the National Veterinary Diagnostic Laboratory (DILAVE) in Montevideo for isolation of *M. bovis*. Farms in which bTB is confirmed through culture are also considered bTB outbreaks and subjected to the same procedure described above.

National control program efforts have resulted in a gradual decrease of bTB prevalence in the country over the last 50 years, with <11 outbreaks reported each year from 2003-2010. However, in 2011-2013 the mean number of annual outbreaks increased, with more than 15 bTB outbreaks reported every year, mostly in dairy farms, with no apparent cause for this change in the long-term trend.

The goal of this study was to assess the spatial dynamics of bTB in Uruguay in the 2011-2013 period and the association between bTB and demographic and movement factors in the dairy industry of Uruguay. Results will contribute to the design and implementation of disease management strategies intended to enhance the effectiveness of bTB control programs in Uruguay, with the ultimate objective of preventing or mitigating the impact of the disease in the human and animal populations of the country.

METHODS

Data source and case definition

Location, demographic characteristics and bTB status (positive/negative) of all cattle farms in Uruguay during the study period were provided by the Animal Health Department at the Ministry of Livestock Agriculture & Fisheries of Uruguay (MGAP). Due to confidentiality concerns, farms were de-identified in the database by eliminating their unique ID number.

Geolocations (UTM coordinates) were provided at the local unit (LU) level, the smallest administrative unit of the country. LUs aggregate into counties (n=261) and departments (n=19).

Although, spatial location of a farm will typically match that of one LU, some large farms may extend over more than one LU.

Farms included in the study were classified into six production types as described elsewhere (VanderWaal et al., 2016a). Briefly, there was one category for dairy farms and five sub-categories for beef production types, namely, farms with <5 animals (small farms) and farms with >5 animals were categorized based on the ratio of steers/breeding cows (Molina, 2012) into breeding (ratio <0.3), fattening (ratio >1.0), and complete cycle (ratio 0.3-1.0) farms. The remaining type of production comprising only heifers and/or calves was classified as growing production farms. Herd size (total number of cattle), age and gender demographics of animals in the herd [calves, heifers 1-2 years, heifers 2-3 years, steers 1-2 years, steers 2-3 years, bulls, and fattening and breeding cows (all above 3 years)], and presence of sheep in the farm (yes/no) were additional attributes included in the demographic dataset for each year.

bTB-positive farms were defined based on detection of at least one bTB-positive animal through either active (CFT confirmed using CCT) or passive surveillance (detection of lesions compatible with bTB in the slaughterhouse confirmed by isolation) activities, whereas farms were defined as bTB-negative otherwise. Onset date (defined as the date when the outbreak was confirmed) was also recorded for bTB-positive farms.

In addition, individual information on movements to/from all farms during the 2008 to 2013 period, stratified by the six production types described previously, was provided by the MGAP (Directory of Animal Identification System, SIRA) to assess farm-level network characteristics (listed in table S1) regarding bTB risk.

111 *Data analyses*

112 *Spatial cluster detection.*

113 Farms were assumed to be located at the centroid of the LU; an average centroid was
114 computed for farms whose surface extended over more than one LU.

115 Two tests for detection of spatial clustering were used, namely the Cuzick-and-Edwards'
116 test for inhomogeneous populations (Cuzick and Edwards, 1990) and the spatial scan statistic test
117 (Kulldorff and Nagarwalla, 1995), which were implemented using ClusterSeer software (version
118 2.5) and SaTScan software v9.3 (<http://www.satscan.org/>), respectively. Details on these
119 techniques are available elsewhere (Ward and Carpenter, 2000). Briefly, the Cuzick-and-
120 Edwards' test is a test for detection of global clustering that makes no assumption on the
121 underlying distribution of the susceptible population. The test compares the sum of the observed
122 cases that are nearest-neighbors to another case at different levels of neighborhood to
123 expectations under the null hypothesis of random distribution of cases and controls. The
124 Bernoulli model of the spatial scan statistics is a test for detection of local clusters that delimits a
125 circular window of variable sizes on a map around each possible location of cases, and compares
126 the observed number of cases and controls within each window with the expected assuming a
127 random distribution of cases and controls. Expected distributions for both the Cuzick-and-
128 Edward's test and the spatial scan statistics were generated via 999 Monte Carlo simulations in
129 which case and control labels were assigned randomly to each location, and log-likelihood tests
130 were used to assess the significance of results.

131 To avoid possible incidence/prevalence bias resulting from differences in time-to-recover
132 bTB free farm status as a consequence of diverse attitudes toward risk from farmers (e.g.
133 depopulation), data from each of the three years were analyzed independently; therefore, only

new positive farms reported each year were considered bTB-positive farms. Surveillance strategies applied during the three years of study were constant in terms of bTB diagnostic tests implemented, responsible staff, and national regulations. Therefore, disease misclassification bias due to limited sensitivity of tests used for bTB detection (CFT, CCT, slaughter detection) (Monaghan et al., 1994; Farnham et al., 2012; Álvarez et al., 2012a; Garcia-Saenz et al., 2015; Von Gehlen, 2015) was assumed to be non-differential across years.

ArcGIS software 10.2.2 (Redlands, CA, US) was used for visualization of the farm distribution and the results of the spatial analyses.

Identification of herd-level risk factors

All bTB-positive dairy farms (cases) and three controls per case (farms that were bTB-negative throughout the study period) were included to identify factors associated with an increased risk of bTB outbreak. Control farms were matched to case farms by production type (i.e., dairy farms), location (closest dairies to the case farm), and year that the positive farm was detected.

After selection, a formal assessment of the spatial independence of bTB in the selected population was verified by running a third-order nearest-neighbor Cuzick-and-Edward's test and the Bernoulli model spatial scan statistics.

Demographic variables for the year in which the outbreak was detected, and cattle movement variables (table S1) summarized across the three years prior to the onset date were initially explored to evaluate the distribution and proportions of bTB-positive and negative farms for each variable in the study. Continuous variables were categorized in thirds or dichotomized (presence vs absence) based on their distribution. Because farm-betweenness (a network

centrality measure) was right-skewed, the variable was ranked giving the largest value to the highest betweenness index.

Strength of univariable associations between bTB-status and epidemiological factors was estimated using a univariable conditional logistic regression. Covariates with P-values ≤ 0.2 were selected for potential inclusion in multivariable models, and a Spearman's correlation coefficient was used to assess collinearity between variables. If two variables were found to be correlated (Spearman's test > 0.7), the one with the lowest Akaike information criterion (AIC) value in the univariable model was selected and included as a main effect in the multivariable conditional regressions. The final model was selected using AIC and a backward stepwise elimination procedure (P > 0.1 to be excluded from the model). Two-way interactions between variables retained in the final model were also assessed. Statistics analyses were conducted using R (v3.1.0, R Foundation for Statistical Computing).

RESULTS

Descriptive results:

The total number of farms with complete information in all datasets (demographics, bTB-status, and movements) was 42,759 (2011), 43,213 (2012), and 42,186 (2013), whereas 2,884 (6.3 %), 2,392 (5.2 %), and 1,971 (4.5%) farms were removed from the analysis due to missing information in each year, respectively. The number of bTB outbreaks detected each year was 16 (2011), 26 (2012), and 16 (2013), for an annual cumulative incidence of 4, 6 and 4 herds per 10,000 each year. bTB-positive farms were located in eleven of the nineteen departments of the country (Figure 1). Most of the positive farms were dairies (57/58) and all of them were detected by active surveillance (CFT confirmed by CCT testing).

Cluster detection results:

Significant ($P < 0.02$) clustering of positive herds up to the second level of neighborhood was detected with the Cuzick-and-Edward's test in 2012, whereas no global spatial pattern was observed on any of the other two years. The Bernoulli model of the spatial scan statistic test identified one, three and one significant ($P < 0.05$) clusters of variable size in 2011, 2012 and 2013 respectively (Table 1) (Figure 1).

Factors associated with bTB-positive herd status

A total of 57 bTB-positive dairy farms and three-matched controls per farm were included in the multivariable conditional logistic regression. As expected, no association between location and bTB status in the selected herds was detected using the CE and spatial scan statistics tests (Figure 2) after the matched selection. Ten demographic and 51 movement-related factors were initially assessed as factors potentially associated with the occurrence of bTB outbreaks in dairy farms.

Seventeen variables were selected in the univariable analysis due to their association ($P < 0.2$) with bTB status, of which five were excluded due to correlation with other predictors with a lower AIC in the univariable model [incoming animals from fattening herds, incoming dairy calves, contacts introduced (total/dairy/non-dairy)], leaving 12 covariates for the multivariable stage (Table 2).

The best-fit multivariable conditional logistic regression model included three epidemiological factors: herd size, total incoming animals to the farm, and incoming steers (Table 3). Two-way interactions were not significant.

DISCUSSION

Although the bTB control program of Uruguay has been in place since the late 1800's (Casas Olascoaga, 2013) and has nearly eradicated the disease, bTB incidence has increased in the country since 2011. Results from spatial and risk factor analyses presented here suggest that the observed incidence increase was likely related to animal movements and changes in management practices favoring disease spread, rather than bTB persistence and reemergence in confined regions of the country. Results will help to design strategies aimed at improving bTB control and surveillance based on risk-targeted strategies, which will help to inform the efficient allocation of resources in Uruguay.

Clustering of outbreaks in distinct areas in consecutive years is inconsistent with localized disease persistence and spread. If local disease spread had occurred, the locations of clusters would be expected to be similar in consecutive years. For example, the spatial relationship of annual clusters of vesicular stomatitis identified in the U.S. was interpreted as consistent with local disease persistence, which has also been confirmed using virus sequencing (Perez et al., 2010). However, clusters identified in this study in consecutive years were located far apart from each other, suggesting long distance disease spread of bTB into naïve areas (suggested by global clustering observed in 2012). Consequently, long-distance transmission factors likely through animal movements, may have contributed to the unexpected increase of bTB outbreaks recently observed in Uruguay. The distance between clusters was noticeably greater than the median distance described for Uruguay cattle shipments (VanderWaal et al. 2016a). That finding suggests that outbreaks may have been associated with some specific farms with high network connectivity (engaged in long distance movements) that may have served as “super-spreader” farms (VanderWaal et al. 2016a; VanderWaal et al., 2016b).

Clusters were mostly identified in the southwestern region of the country, which corresponds to the densest dairy production region of Uruguay (DIEA, 2015), and close to the border with Argentina (Figure 1). In Uruguay, the dairy cattle industry is more intensively managed and is characterized by higher farm densities than the beef industry. Thus, the observed spatial aggregation of cases could be explained by use of specific management practices, such as production systems, culling rates, animal movements, diagnostic frequency as described elsewhere (Menzies and Neill, 2000; Amanfu, 2006; Munyeme et al., 2008; Humblet et al., 2009), and also a differential detection sensitivity driven by testing strategies used in each industry. Despite proximity to the Argentine border, transboundary transmission is unlikely to have occurred. Legal live cattle imports into Uruguay are negligible (<5 animals per year) (DIEA, 2011-2013), and follow the World Organization for Animal Health (OIE) recommendations for animal trade (www.oie.int) reducing the risk of bTB introduction. Furthermore, the Uruguay River, which separates Uruguay from Argentina, is sufficiently wide in the areas where clusters were located, so illegal transboundary animal movements were unlikely to occur.

The positive association between bTB and farm size (OR: 4-51, $P < 0.05$) is consistent with previous findings in a number of countries (Griffin et al., 1996; Brooks-Pollock and Keeling, 2009; Bessell et al., 2012). Large herds may result in high density at the paddock and long periods of time waiting in the pre-milking area, increasing the likelihood of cattle-to-cattle transmission (Menzies and Neill, 2000). Alternatively, because specificity of intradermal tests is imperfect, the larger the number of animals tested, the higher the probability of false positive results. The number of animals moved onto the farm was also associated with higher risk of bTB-positive results (Table 3) in agreement with previous reports in the UK (Gilbert et al.,

2005), northern Italy (Marangon et al., 1998), and Ireland (Clegg et al., 2013). Limited sensitivity of the diagnostic test could lead to the introduction of infected animals into the herd, which may be more likely to occur in large compared to small farms because number of shipments was positively correlated with farm size. Additionally, the association between bTB and purchasing steers (OR = 2.88, $P < 0.05$) may suggest that certain management practices, such as the presence of beef production on dairy farms, may increase risk. This management practice, although infrequent (13% of farms under study) may increase bTB risk because beef cattle are not routinely tested for bTB in Uruguay.

Our results present an opportunity for targeting bTB surveillance activities to high risk farms that are characterized by factors associated with bTB (movement of steers, large size, large frequency and number of movements). Targeting high risk farms may increase the probability of detecting disease and mitigate its impact. In that context, results here can also help to inform modeling efforts intended to assess the cost-effectiveness of alternative surveillance strategies prior to their implementation.

Because of the chronic nature of the disease, time of infection with *M. bovis* is unclear under field conditions. Because this study was focused on incident farms, and did not address the duration of the outbreak after detection, we assumed that exposure with *M. bovis* occurred within the same calendar year from the time of initial detection on the farm given that intradermal testing was performed annually. In addition, farm sizes are highly correlated across years (Pearson's $\rho = 0.94$, VanderWaal et al., 2016b). Thus, even though detected farms may have been infected in the previous calendar year for cases detected in January, we do not expect that the demographics of the farm to be substantially different between the year of infection versus detection. We decided to be consistent with the selection of controls to avoid bias. This situation

only occurred in 3/57 outbreaks, and thus it is unlikely to have had a major impact on the results. Another limitation of the study is that active surveillance was only performed in dairy farms. Dairy farms represented ~10% of the total farms in the country, leaving a high proportion of the cattle population unassessed through CFT testing, hence these cattle's role in bTB dynamics is unknown. Further studies are needed to address risk factors in the entire cattle population in Uruguay.

In conclusion, our results suggest that long-distance spread, likely due to cattle movements, was a more important feature of the spread of bTB in Uruguayan dairy farms than local spread. Uruguay, like many other countries globally, faces disparate challenges regarding control of bTB, a chronic disease with serious diagnosis limitations. Study results suggest that implementation of a surveillance strategy targeted to risk factors found here, along with maintenance of a comprehensive traceability system, industry awareness, and government support, could contribute to the prevention and mitigation of the incidence of the disease in the country, ultimately leading to eradicate bTB in Uruguay.

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FIGURE CAPTIONS

Figure 1. Location of BTB-positive farms reported by Uruguay's Animal Health Bureau, (MGAP) in 2011 (+), 2012 (●) and 2013 (▲). BTB clusters detected in Uruguay per year of study are also represented; 2011(dashed), 2012(solid) and 2013(cross-hatched).

Figure 2. Location of the 57 BTB-positive farms (stars) and 171 controls (i.e. BTB-negative farms) (●) selected to assess risk factors with a conditional multivariate logistic regression model assessment.

Table 1. Cluster results from the Bernoulli model of the spatial scan statistic for BTB-positive farms (2011, 2012 and 2013).

<i>Year</i>	<i>Cluster</i>	<i>BTB-farms in cluster/total*</i>	<i>Cluster location (UTM)</i>	<i>Radius (km)</i>	<i>Obs-to-Exp ratio</i>	<i>RR</i>	<i>P-value</i>
2011	1	16 /16	6245740, 476808	102.9	4.7	∞	<0.001
2012	1	7/26	6250320, 776662	8.3	443.5	52	<0.001
	2	4/26	6371400, 413092	47.3	15.1	3.9	0.016
	3	2/26	6220520, 444885	1.5	1108.7	4	0.031
2013	1	15/16	6247350, 372388	234.9	2.6	27.1	0.036

*Number of BTB-positive farms included in the cluster out of the total BTB-positive farms reported in each specific year.

Table 2. Epidemiological factors selected in the bivariate analysis ($P < 0.2$) after exclusion of correlated factors. Percentages (%) are based on the total number of BTB-farms (57), and 171 matched controls.

<i>Epidemiological Factor</i>	<i>Type of factor</i>	<i>Category</i>	<i>N</i>	<i>Cases (%)</i>	<i>Controls (%)</i>	<i>P-value</i>
<i>Incoming animals from Compete cycle farms</i>	Movement	Yes	30	13 (23)	17 (10)	0.016
<i>Incoming steers</i>	Movement	Yes	30	18 (31.6)	12 (21)	<0.001
<i>Incoming calves</i>	Movement	Yes	78	28 (49.1)	50 (29.2)	0.007
<i>Incoming calves from breeding farms</i>	Movement	Yes	37	17 (28.9)	20 (11.7)	0.002
<i>Incoming calves from complete cycle farms</i>	Movement	Yes	14	6 (10.5)	8 (4.7)	0.120
<i>Fattening cows in herd</i>	Demographic	Yes	39	17 (29.8)	22 (12.9)	0.004
<i>Steers in herd</i>	Demographic	Yes	93	30 (52.6)	63 (36.8)	0.037
<i>Total incoming animals</i>	Movement	≤ 1	78	34 (59.6)	44 (25.7)	<0.001
		$>1 \leq 44$	71	10 (17.5)	61 (35.7)	
		>44	79	13 (22.9)	66 (38.6)	
<i>Herd size (cattle)</i>	Demographic	<116	76	3 (5.3)	73 (42.7)	<0.001
		116-360	73	16 (28)	57 (33.3)	
		>360	79	38 (66.7)	41 (24)	
<i>Total number of bulls in herd</i>	Demographic	None	75	14 (24.6)	61 (35.7)	0.005
		≤ 2	102	21 (36.8)	81 (47.3)	
		>2	51	22 (38.6)	29 (17)	
<i>Betweenness (ranked)</i>	Net. centrality	Ranked	N/A			0.005
<i>In-closeness</i>	Net. centrality	Continuous	N/A			0.055

Table 3. Odds Ratio (OR) with their 95% confidence interval (95% CI) and P-value of the variables selected by the multivariate conditional logistic regression model.

<i>Variable</i>	<i>Type of factor</i>	<i>Category(N)</i>	<i>OR</i>	<i>95% CI</i>	<i>P-value</i>
<i>Herd size (cattle)</i>	Demographic	<116 (76)	1.00		
		116-360 (73)	5.79	1.58-21.21	0.008
		>360(79)	14.38	4.06-50.90	<0.001
<i>Incoming steers</i>	Movement	No (198)	1.00		
		Yes (30)	2.88	1.12-7.37	0.027
<i>Total incoming animals</i>	Movement	≤1 (79)	1.00		
		>1-≤44 (71)	0.77	0.29-2.06	0.608
		>44 (78)	1.90	0.81-4.44	0.138