PREVALENCE AND SPATIAL DISTRIBUTION OF ANTIBODIES TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM O ANTIGENS IN BULK MILK FROM TEXAS DAIRY HERDS

A Thesis

by

SHERRY LYNN GRAHAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2003

Major Subject: Veterinary Medicine and Surgery

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Approved as to style and content by:

James A. Thompson (Co-Chair of Committee)	Kerry S. Barling (Co-Chair of Committee)
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ABSTRACT

Prevalence and spatial distribution of antibodies to *Salmonella enterica* serovar Typhimurium O antigens in bulk milk from Texas dairy herds. (May 2003)

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The purpose of this study was to describe the herd antibody status to *Salmonella* Typhimurium as estimated from co-mingled milk samples and to describe the resulting geographical patterns found in Texas dairy herds. Bulk tank milk samples were collected from 852 Grade A dairies throughout Texas during the summer of 2001. An indirect enzyme-linked immunosorbent assay (ELISA) using *S.* Typhimurium lipopolysaccharide was performed with signal to noise ratios calculated for each sample. The ELISA ratio was used in fitting a theoretical variogram and kriging was used to develop a predicted surface for these ratios in Texas. A spatial process with areas of higher risk located in the panhandle and near Waller County was apparent. Lower risk areas included Atascosa, Cooke, Collin, Titus, Comanche and Cherokee Counties. Subsets representing large dairy sheds in northeast Texas, the Erath County area, and the Hopkins County area were also evaluated individually. Each result illustrated a spatial process with areas of low and high ELISA ratio predictions. Cluster analysis was performed for the entire state

with cases defined as herds having milk ELISA ratios greater than or equal to 1.8. Using this cutoff, the prevalence of herds with positive bulk tank milk ELISAs was 4.3%. Significant clustering of cases was demonstrated by the Cuzick and Edward's test. The spatial scan statistic then identified the two most likely clusters located in and near the Texas Panhandle. This study demonstrated that the distribution of S. Typhimurium antibodies in bulk tank milk in Texas is describable by a spatial process. Knowledge of this process will help elucidate geospatial influences on the presence of S. Typhimurium in dairy herds and enhance our understanding of the epidemiology of salmonellosis.

ACKNOWLEDGEMENTS

This thesis could not have been completed without the support and assistance of many wonderful people. I would first like to thank Dr. William Moyer for embarking on this joint U.S. Army – Texas A&M venture and allowing me into his graduate program. His vision and superb sense of humor were an inspiration throughout my time here.

Dr. James Thompson deserves the credit for initiating this endeavor and his brilliant understanding of spatial epidemiology was crucial to its completion. Thanks go to Dr. H. Morgan Scott, as well, for his expertise in epidemiology and his challenging insights that greatly enhanced this study.

This project certainly could not have been completed without the help of Dr. Suryakant Waghela and his laboratory staff. Dr. Waghela's expertise and innovation in developing a bulk tank milk ELISA for S. Typhimurium was critical to the success of this project.

Mr. James Fraley and the Texas Department of Health Milk and Dairy Products Division were also crucial to the success of this project. Dr. Michael Tomaszewski and his staff at the Texas Dairy Herd Improvement Association, Southwest office and Mr. Joe Pope, the Erath Country Dairy Extension Agent were of great assistance in verifying locations of dairy herds as well. Thanks also go to Drs. Robert Field and Steve Wikse for their support and encouragement throughout my time here.

And finally, but most importantly, my gratitude goes to Dr. Kerry Barling, who has been an extraordinary mentor and friend. His optimism has been inspiring and his advice invaluable to me throughout my time at Texas A&M.

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CHAPTER I

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) has a diverse host range which includes humans, cattle, pigs, sheep, horses, rodents and birds. (Kingsley and Baumler, 2000). Salmonellosis is a primarily foodborne enteric disease caused by non-typhoidal *Salmonella* organisms, including *S.* Typhimurium. In humans, *Salmonella* spp. are the second most commonly isolated pathogens during the diagnosis of diarrheal disease and *S.* Typhimurium accounts for most of these isolates (Hohmann, 2001; CDC, 2002).

S. Typhimurium infects adult cattle but rarely causes clinical disease. However, outbreaks in humans have been associated with milk and beef products and from cattle-induced environmental contamination (Sanchez, et al., 2002). In addition, the emergence of a multi-drug resistant type of *S.* Typhimurium, which accounted for 30% of the isolates from humans with diarrhea in 2000 (CDC, 2002), is believed to have occurred due to widespread antibiotic use in cattle (Hohman, 2001;Rabsch, et al., 2001;Threlfall, et al., 2000).

Recently, interest in the spatial distribution of *Salmonella* spp. in cattle herds has increased as new techniques have emerged to assist in assessing this distribution (Kabagambe, et al., 2000; Sato, et al., 2001). Such studies may result in an enhanced understanding of the epidemiology of salmonellosis in cattle, which could then be used

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This thesis follows the style and format of Preventive Veterinary Medicine.

to target surveillance programs, assess individual herd risk, and reduce the occurrence of this zoonosis.

Structure and Biology

Salmonella spp. are Gram-negative, intracellular, rod-shaped bacteria. *Salmonella enterica* is comprised of approximately 2500 serovars. Those serovars causing illness in humans or animals are generally of groups B and D, or occasionally, groups E and C (Kingsley and Baumler, 2000).

The serogroups are distinguished by their variable lipopolysaccharide (LPS) "O" antigens. *S.* Typhimurium is a member of serogroup B, which has O antigens 1, 4 and 12 (Smith, et al., 1995). Antigens O1 and O12 are shared with serogroup D1, however, this group has an O9 antigen instead of the O4 antigen. The LPS antigens are targeted during the host immune response, and the O antigens are the primary target (Kingsley and Baumler, 2000). Cross-reaction of immunity within and among serogroups has been studied. Within serogroups, cross-protection is often good (Kingsley and Baumler, 2000) and cross-reaction on serology often high (Smith, et al. 1995). Despite the common antigens, however, cross-protection between serogroups tends to be weak (Kingsley and Baumler, 2000) as does cross-reaction on serological tests (Barrow and Wallis, 2000).

S. Typhimurium invades and colonizes the gut associated lymphoid tissue (GALT) of the ileum and regional lymph nodes are infected via lymph drainage. In the immunocompetent host, the infection generally remains localized, but in normal individuals, it may become systemic, with the spleen and liver being the primary organs

affected. *S.* Typhimurium stimulates both a humoral and a cell-mediated immunity regardless of the extent of its invasion (Baumler, et al., 2000).

Salmonellosis of Humans

Salmonellosis of humans is predominantly a foodborne disease with recent outbreaks being linked to poultry, meat products, eggs, ice cream, alfalfa sprouts, milk, and cereal among others (Sanchez, et al., 2002). Person-to-person transmission via the fecal-oral route may also occur with infected persons shedding organisms in their feces for a month or more. Symptoms of disease vary with dose and host immune status, but generally include abdominal cramps, diarrhea and fever arising six to 72 hours after infection. Risk factors for clinical disease include decreased gastric acidity, altered intestinal flora, and reduced intestinal motility. Life threatening bacteremia may occur, especially in children and immunocompromised people. Infectious endarteritis involving heart valves and/or the aorta is a sequelae, frequently fatal, which requires both surgical treatment and long term, sometimes lifetime, antibiotic therapy (Hohman, 2001).

During 2000, there were 39,574 diagnosed cases of salmonellosis in the United States with the most commonly isolated serovar being *Salmonella enterica* serovar Typhimurium (CDC, 2002). Because of underreporting, however, the CDC estimates that 1.4 million cases of salmonellosis actually occur annually, with nearly 600 deaths, making this the most common cause of mortality associated with a foodborne disease (Rabsch, et al., 2001).

The emergence of multi-drug resistant types of *Salmonella* spp. has complicated treatment and increased the threat of serious complications in salmonellosis. In 1998, the multi-drug resistant phage type *S.* Typhimurium DT 104 was first isolated in the United States (Hohman, 2001). This organism is believed to have developed this resistance in cattle in the United Kingdom during the 1990s in response to antibiotic use in these animals (Hohman, 2001; Rabsch, et al., 2001; Threlfall, et al., 2000). Today, DT104 is considered a pandemic serovar (Sanchez, et al., 2002) and accounted for nearly 30% of *S.* Typhimurium isolated in the U.S. in 2000 (CDC, 2002). This is not the first resistant phage type to be associated with antibiotic use in cattle, and concerns about the emergence of additional multi-drug resistant phage types in livestock are growing.

Salmonellosis of Cattle

As a disease of adult cattle, salmonellosis is often subclinical or characterized by mild diarrhea, lethargy and decreased food consumption. Even so, it may cause significant economic and production losses through reduced weight gain, feed efficiency, and milk production (Huston, et al., 2002a; Kabagambe, et al., 2000). In a recent study of risk factors for clinical disease associated with *S.* Typhimurium on Dutch dairies, Veling, et al. (2002b) noted that symptoms were seen only in adult cows on 66% of the affected farms.

Dairy cattle are believed to be commonly exposed to *Salmonella* spp. through feed, water, wild birds, rodents, and persistently contaminated environments but the epidemiology of this disease is not yet understood (Kabagambe, et al., 2000; Warnick, et al., 2001; Wells, et al., 2001). Infected cattle may shed *S.* Typhimurium for up to 12

weeks after recovery, but rarely become chronic carriers. Farms, however, may be persistently infected for years by the continuous cycle of environmental contamination, cattle infection and fecal shedding (Anderson, et al., 2001; Huston, et al., 2002a). This chronic herd-level infection with subclinical individual animal illness and intermittent fecal shedding may pose a significant risk for humans through contamination of meat and milk products and of the environment (Anderson, et al., 2001). *Salmonella* may survive in freshwater systems for 56 days or more, depending on conditions (Murray, 2000) and there is concern that contamination of aquifers and surface waters through cattle waste may occur. Direct contamination of surface water, leakage or overflow from lagoons, milking shed wastewater disposal and runoff from pastures or barnyards can all contribute to local water contamination. The contaminated water may then be a reservoir for infection of humans, cattle or other species.

Diagnosis of infected herds is difficult. Fecal culture performed on clinically affected individuals is commonly used. *Salmonella* serovars commonly found in human disease have also been cultured from up to 8.9% of tested raw milk in the U.S. (Jayarao and Henning, 2001). Neither fecal nor milk culture is likely to diagnose chronic subclinical herd-level infection, however, as shedding of organisms is intermittent. Screening of herds for infection with *Salmonella* serovars Dublin and Typhimurium through serology of individuals or immunoassays of milk samples from both bulk tanks and individuals has been attempted with varying success (Hoorfar, et al., 1995; Hoorfar and Bitsch, 1995; Hoorfar and Wedderkopp, 1995; House, et al., 1993; Smith, et al., 1995; Veling, et al., 2000; Veling, et al., 2001; Veling, et al., 2002a). Sensitivities in

recent studies using LPS O-antigen ELISAs on milk for serovars Dublin and Typhimurium range from 54-100% and underlying factors affecting these differences have not yet been elucidated. Specificities, on the other hand, have been uniformly high, ranging from 98-100% (Hoorfar, et al., 1995; Hoorfar and Wedderkopp, 1995; Veling, et al., 2000; Veling, et al., 2001; Veling, et al., 2002a). Despite the potentially moderate sensitivity, the bulk milk ELISA has the advantage of identifying subclinical and previously infected herds rather than only clinically affected ones and is also less costly, labor intensive and invasive than individual animal serology, milk sampling or fecal cultures.

Spatial Distribution

Recently, *Salmonella* serovars causing clinical disease in dairy cows in California were found to be temporally and spatially clustered by analysis of fecal culture results. Most of these isolates were of serovar Typhimurium, the same serovar frequently causing disease in humans (Sato, et al., 2001). In other studies, fecal shedding of *Salmonella* spp. was also determined to be spatially clustered (Kabagambe, et al., 2000; Troutt, et al., 2001).

As *Salmonella* spp. may survive in the environment for a prolonged period of time, there may be a spatially distributed risk for salmonellosis. *Salmonella* has been found in bodies of water; manures and sewage sludge spread on pastures; and contaminated feeds (Murray, 2000). Environmental factors that enhance survivability of the organism, could result in a spatial pattern of disease risk. Identification of such a pattern would assist in elucidating the risk factors for salmonellosis in dairy herds and

could lead to the development of targeted surveillance systems as well as recommendations for minimizing the impact of these risks and reducing the incidence of salmonellosis.

CHAPTER II

THE SPATIAL DISTRIBUTION OF ANTIBODIES TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM O ANTIGENS IN BULK MILK SAMPLES FROM TEXAS DAIRIES

The objectives of this study were to describe herd antibody status to *Salmonella* Typhimurium as estimated from co-mingled milk samples and to describe the resulting geographical patterns found in Texas dairy herds. Knowledge of the spatial distribution of these antibodies will be valuable in determining geospatial influences on the presence of *S.* Typhimurium in Texas dairy herds that will enhance our understanding of the epidemiology of salmonellosis. Hypotheses generated will be further tested in future studies and may become important in surveillance, control and prevention programs.

Methods

Data and sample collection

Bulk tank milk samples were obtained from all Grade A permitted dairies in Texas during June, July and August 2001. Milk samples were collected by Texas Department of Health (TDH) milk inspectors during routine visits to dairy farms under their jurisdiction. Each sample was identified by TDH dairy number and maintained at -20º C until analyzed. TDH personnel also recorded the global positioning system coordinates of each dairy at the time of sample collection.

Laboratory analysis

An indirect enzyme-linked immunosorbent assay (ELISA) using *S.* Typhimurium lipopolysaccharide (LPS) (Sigma-Aldrich, Co.) was performed on each sample. Standard microwell plates (Dynatech Immulon® type 2, Fisher Scientific) were coated with the LPS antigen and maintained at -20°C until use. Plates were thawed and blocked with a high salt phosphate-buffered saline solution (PBS) plus 1%casein immediately prior to use. Positive and negative controls and the undiluted milk samples were added in duplicate wells on each plate and incubated at room temperature for one hour. The second antibody, horseradish peroxidase-labeled anti-IgG, diluted in PBS with 1% casein (confirmed negative for *Salmonella*), was added to each well and the plates incubated for an additional two hours. Finally, ABTS (Kirkegaard and Perry Laboratories, Inc.) was added as the chromogenic substrate and the optical densities were electronically read at 405 nm. Signal to noise ratios for each sample were calculated by dividing the average optical density of each sample by the average optical density of the negative control samples of the same plate. This ratio describes the intensity of antibody binding while accounting for background noise (Wright et al., 1993).

Mapping

Each dairy was identified by its latitude and longitude. These coordinates were used to plot the location of all dairies on a Texas map using a commercial GIS software program (ArcView® GIS 3.2, Environmental Systems Research Institute, Inc., Redlands, Ca.). The map with dairy locations was then projected into Universal

Transverse Mercator 1983 (UTM83), Zone 14 units. The UTM83 coordinates were exported and used for all statistical analyses.

Statistical analysis

Descriptive statistics were calculated using commercial statistical software (S-PLUS® 6 Professional Edition for Windows®, Release 2 (September 2001), MathSoft, Inc. Seattle, WA). Scatter plots with contours of observed ELISA ratios were produced to visually assess the first-order effect of trend. In cases where trend was suggested by the contour plot, loess plots of ELISA ratios versus location were created to further assess this possibility. Empirical variograms were calculated in the 0, 45, 90, and 135 degree directions and compared to broadly assess the presence of first- and second-order effects. Any data with apparent trend or zonal anisotropy was detrended using loess smoothing prior to modeling. Geometric anisotropy was corrected when present by adding an angle and ratio correction term to the variogram. Theoretical variograms were fitted to the observed data and the best fit model(s) selected based upon visual assessment and minimization of the objective function (residual sum of squares between the theoretical and empirical variograms) which was produced for each model. Ordinary kriging was performed, resulting in a predicted surface with standard error estimations for each fitted model. In cases where kriging on a rectangular grid would result in large areas with no observed locations, a convex hull was created around observed locations and kriging performed only within this area. The kriged predicted surface was imported into ArcView® and overlaid onto Texas maps obtained from Environmental Systems Research Institute, Inc., Redlands, California.

Because of the uneven distribution of dairy farms within Texas, data were additionally divided into three subsets encompassing known dairy sheds. The Hopkins area subset included all data points within or touching the boundaries of Delta, Hopkins, Franklin, Titus, Camp, Upshur, Wood, or Rains County. The Erath area subset included all data points within or touching the boundaries of Erath, Comanche, or Hamilton County. The northeast subset included all points east of X=486 (UTM83, zone 14) and north of Y=3434 (UTM83, zone 14). These areas were further evaluated in the same manner as for the entire state.

Results

In all, 1027 milk samples were received from TDH. Of these, 18 (1.8%) samples were lost in testing and two (0.2%) samples could not be identified. Duplicate and triplicate samples were received from a total of 52 locations and accounted for 60 (5.8%) total samples. The remaining 947 (92.2%) milk samples represented unique dairies. Of these samples, 852 (88.9%) could be matched with a geographic location provided by TDH personnel and were used in data analysis. The number of Grade A permitted dairies in Texas was dynamic and generally declining during the study period. The peak number of these dairies recorded by TDH was 954, resulting in an overall estimate of the proportion of dairies for which test results were generated of 89%.

State of Texas data

The 852 bulk milk samples were from dairies located in 109 Texas counties with the largest percentages found in Hopkins (16%) and Erath (14%) counties (Figure 1). The ELISA signal to noise ratio range was 0.547 to 3.868. The mean for these ratios

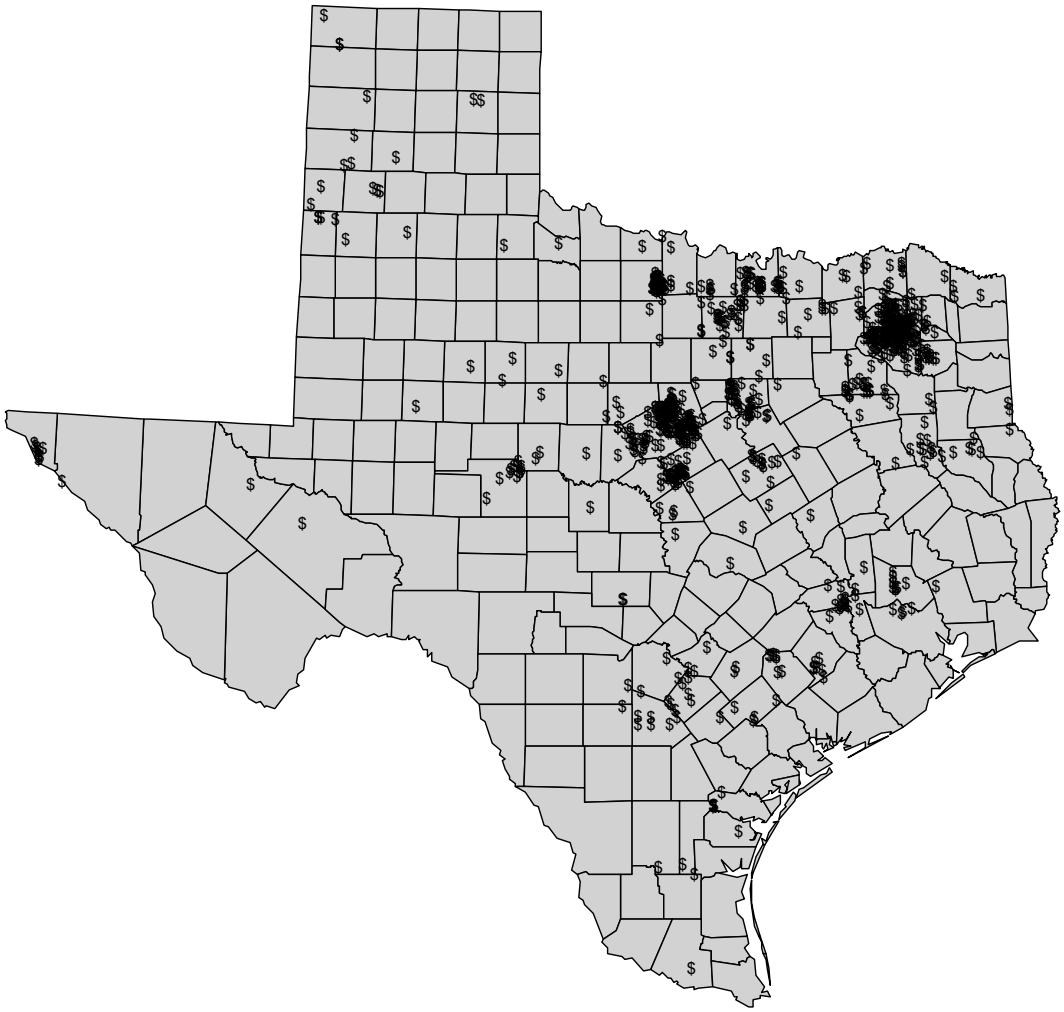


Figure 1
Locations of sampled Texas dairy herds.

was 1.166 (95% CI 1.142, 1.190) with a standard deviation of 0.359 and the median was 1.101.

A contour scatter plot of observed ratios did not suggest trend, however, directional variograms initially appeared to have generally increasing covariances without a sill in the 0 and 135 degree directions. This was further evaluated through plots of smoothed trend against the X- and Y-axes for both the original data and data rotated -45 degrees. Neither plot suggested the presence of trend; instead, it was concluded that the apparent increasing covariance was artifact due to the scarcity of data at that range.

Directional variograms were plotted again with the range restricted to 250 kilometers (km). Comparison revealed similar sills and ranges for the 90 and 135 degree directions and a nugget effect in the 45 degree direction. The 0 degree directional variogram initially appeared to have a range 1.5 times those of the 90 and 135 degree variograms, however, further exploration suggested that this was also due to the sparseness of points and not to true geometric anisotropy.

Spherical and exponential theoretical variograms were fitted to the omnidirectional empirical variogram (Figures 2 and 3). Best fit parameters for the spherical model included a range of 200 km, an absolute sill (actual sill minus nugget effect) of 0.032 and a nugget of 0.083. The objective function for this model was 0.0013. The best fit exponential theoretical variogram included a range of 125 km, absolute sill of 0.032 and nugget of 0.083. The resulting objective function for the exponential model was 0.0011.

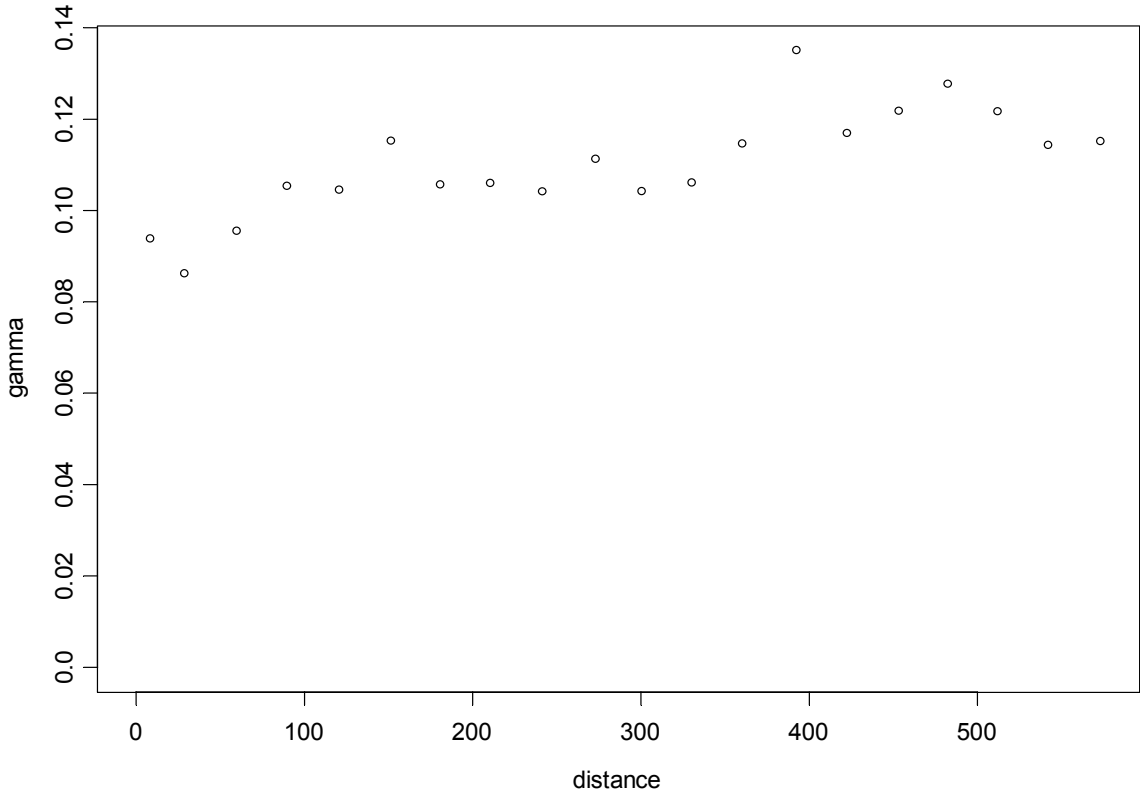


Figure 2
Omnidirectional empirical variogram for antibody to S. Typhimurium in bulk milk from Texas dairies (distance in km).

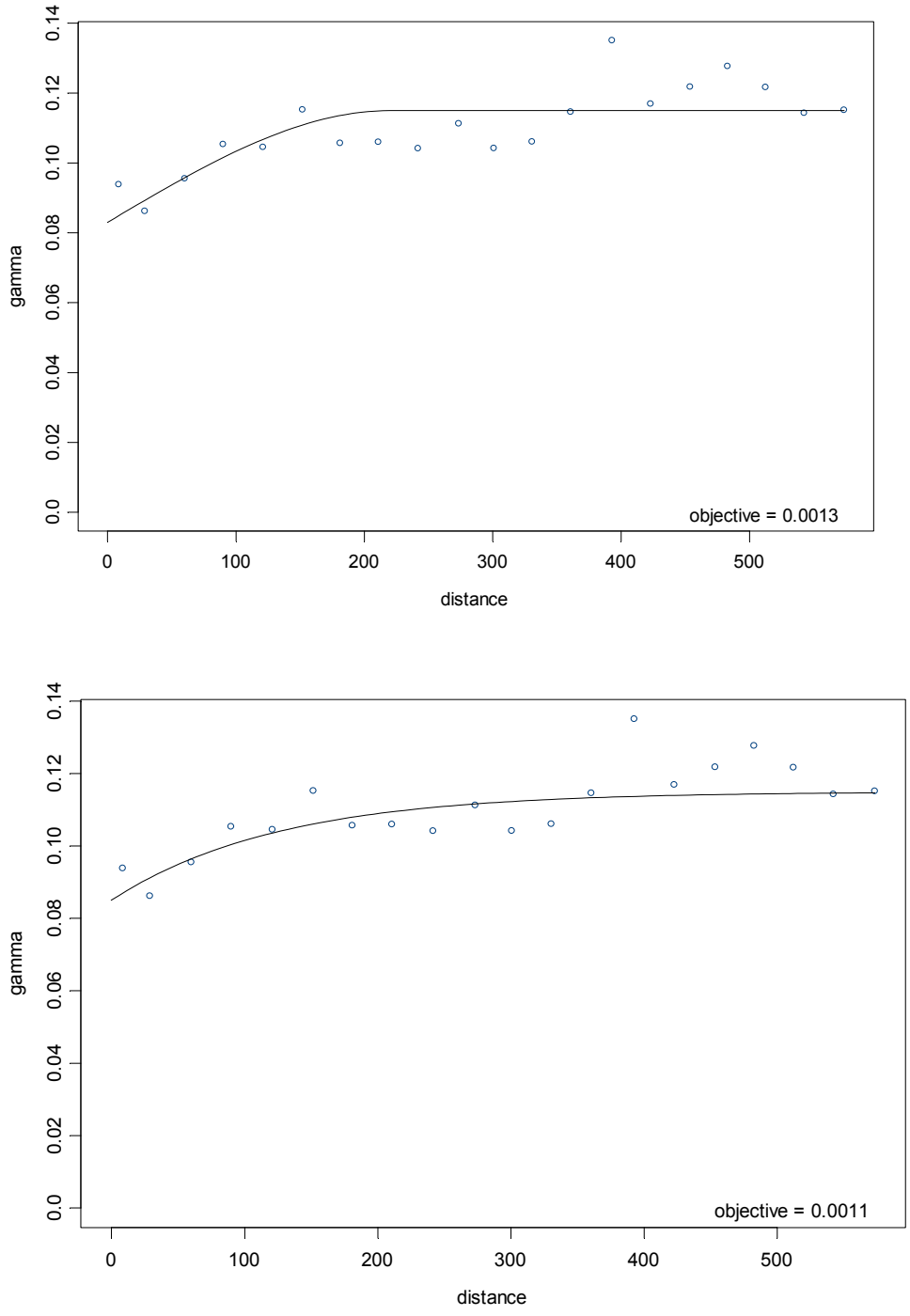


Figure 3
Fitted theoretical variograms for antibody to *S.* Typhimurium in bulk milk from Texas dairies (distance in km).

Ordinary kriging was performed for both the exponential and spherical theoretical variograms described above. Kriging predictions were then calculated for unsampled locations within a convex hull created to encompass all sampling locations (Figure 4). Kriging predictions and standard errors were generated for locations within this polygon, exported to ArcView®, and overlaid on the previously generated maps (Figure 5).

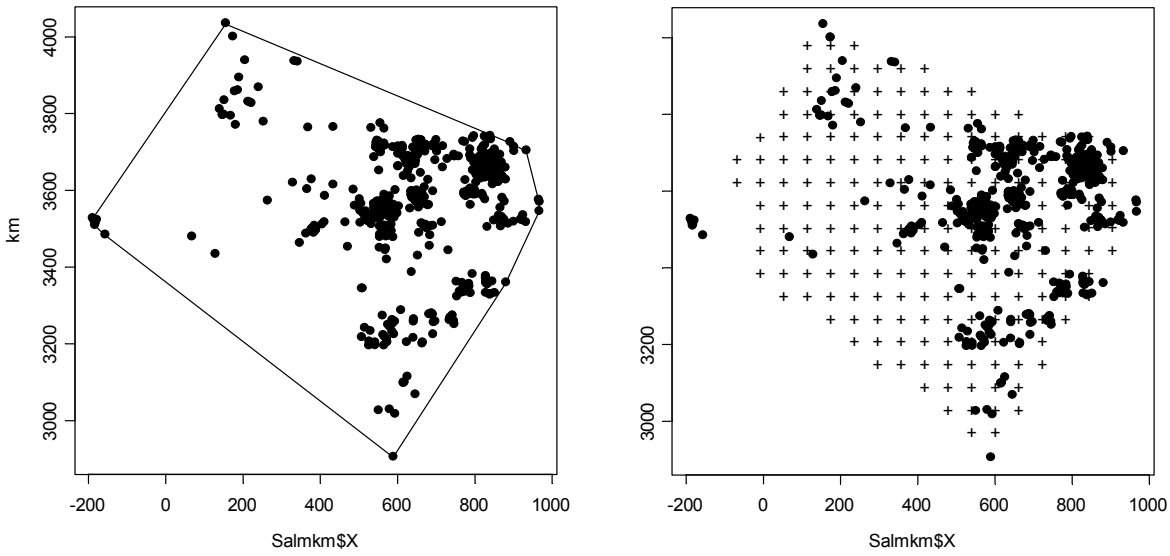


Figure 4
Convex hull based on locations of sampled dairies (left) and kriging prediction locations for the convex hull (right).

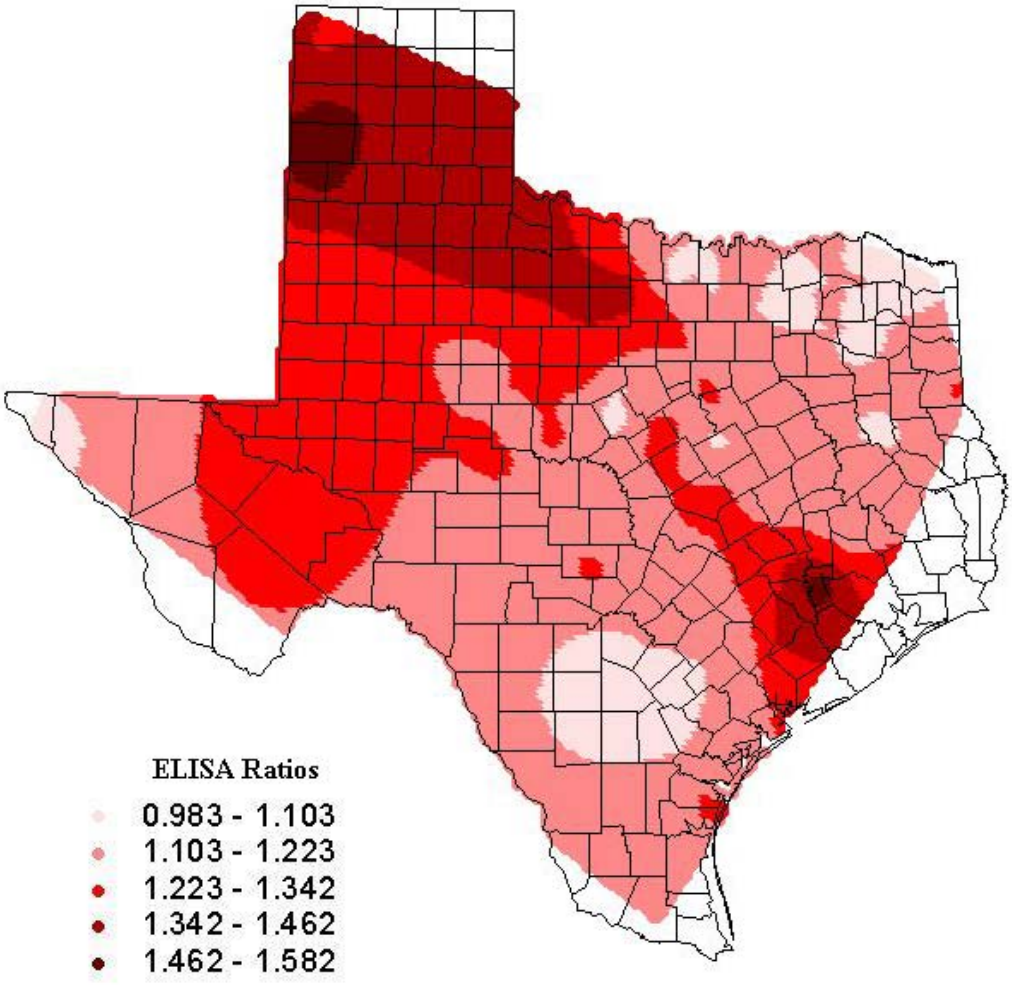


Figure 5
Contour plot of kriging predictions for antibody to S. Typhimurium in bulk milk from Texas dairies based on fitted spherical theoretical variogram.

Northeast Texas subset

The northeast subset was comprised of 708 dairies representing 83% of the total sample (Figure 6). The approximate area encompassed by this subset was 500 km east to west and 350 km north to south. The minimum signal to noise ELISA ratio was 0.547 and the maximum was 3.115. The median value was 1.095 and the mean was 1.150 (95% CI 1.124, 1.175) with a standard deviation of 0.341.

Neither a contour scatter plot of observed ratios nor a loess plot of ratios versus location suggested trend. Comparison of directional variograms revealed similar sills and ranges for the 90 and 135 degree directions and a nugget effect in the 45 degree direction. The 0 degree directional variogram, however, appeared to have a range of approximately 1.5 times that of the 90 and 135 degree variograms suggesting geometric anisotropy in this direction. Further analysis suggested that this could be remedied by adding an angle correction of 0, 15, 30 or 45 degrees with a ratio correction of 1.05, 1.15. or 1.25. As variograms resulting from each of these corrections were similar, the correction of 15 degrees with a ratio of 1.25 was used for fitting of the theoretical variogram.

Best fit parameters for the spherical theoretical variogram included a range of 130 km, an absolute sill of 0.031 and a nugget of 0.092. The objective function for this model was 0.0016. The best fit exponential theoretical variogram included a range of 70 km, absolute sill of 0.035 and nugget of 0.092. The resulting objective function for the exponential model was 0.0019.

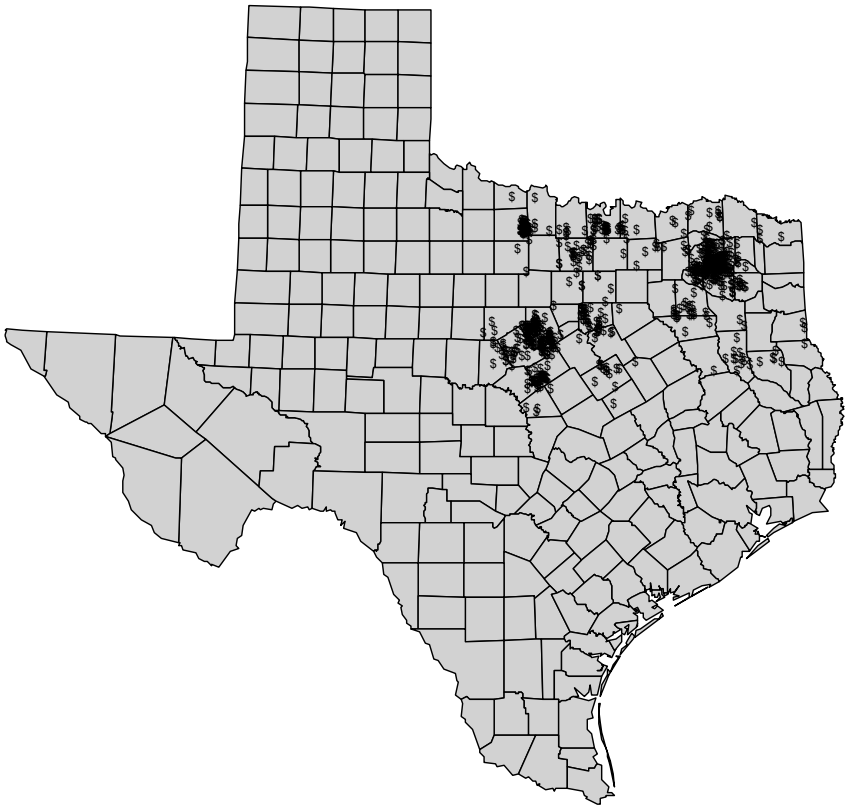


Figure 6
Locations of Texas dairies representing northeast Texas subset.

Ordinary kriging was attempted for both the exponential and spherical theoretical variograms described above but was unable to be able to be completed. Error messages citing a covariance matrix problem continued to occur despite increasing the nc argument to its maximum. Theoretical variograms without the anisotropy correction (Figure 7) were able to be kriged, however. The spherical theoretical variogram parameters fitted to the uncorrected model and used for kriging were a range of 90km, sill of 0.019 and nugget of 0.083, resulting in an objective function of 0.0012. The exponential variogram fitted and used had a range of 75km, sill of 0.02, and a nugget of 0.084, yielding an objective function of 0.0011.

Ordinary kriging was performed using each theoretical variogram and kriging predictions were calculated for unsampled locations within a rectangular grid encompassing all sampling locations. Kriging predictions and standard errors were generated for this grid, exported to ArcView®, and overlaid on the previously generated maps (Figure 8).

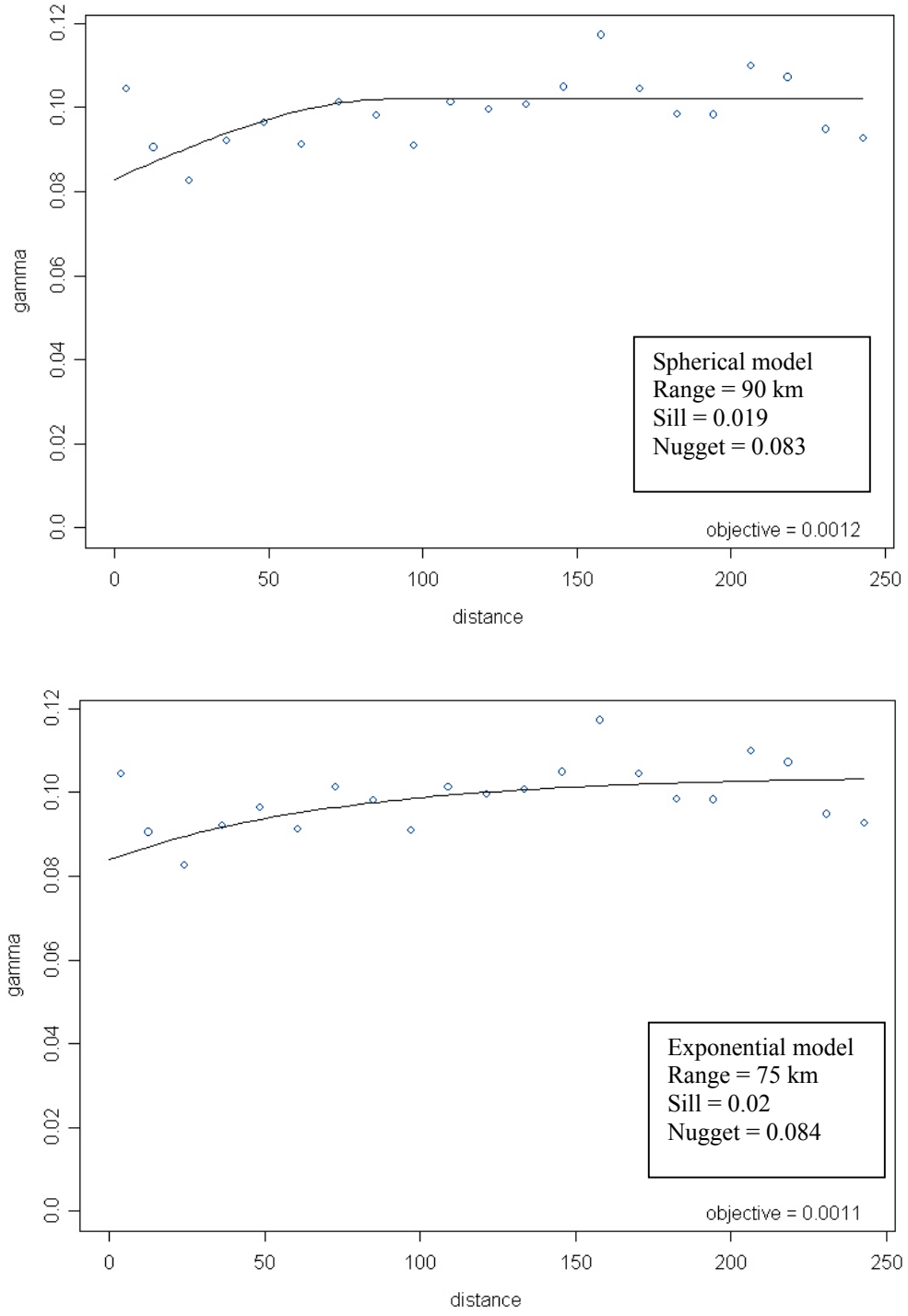


Figure 7
Fitted theoretical variograms antibody to *S.* Typhimurium in bulk milk from northeast Texas dairies (distance in km).

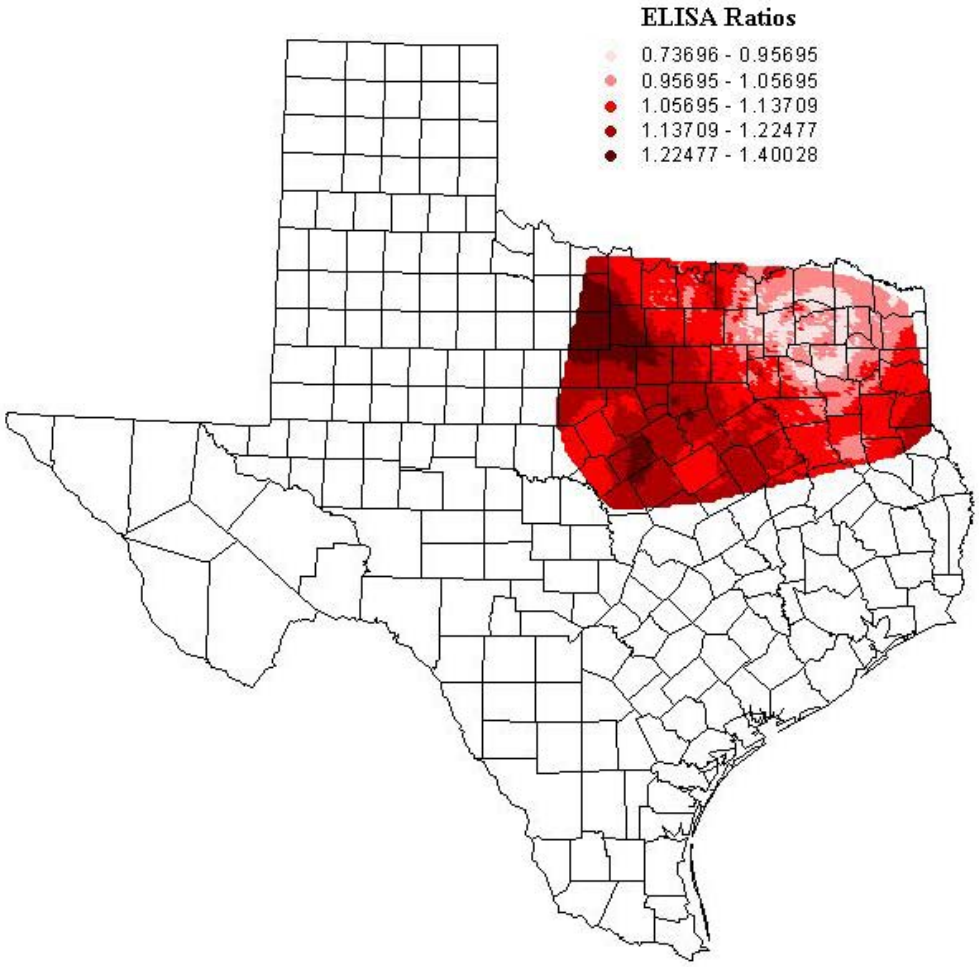


Figure 8
Contour plot of kriging predictions for antibody to S. Typhimurium in bulk milk from northeast Texas dairies based on the spherical theoretical variogram.

Hopkins area subset

There were 232 dairies located in the eight counties represented by this subset (Figure 9). The area encompassed was approximately 110 km east to west by 105 km north to south. The minimum signal to noise ELISA ratio was 0.608 and the maximum was 2.474. The median value was 1.020 and the mean was 1.089 (95% CI 1.052, 1.126) with a standard deviation of 0.284.

A contour scatterplot of observed ratios did not suggest trend. Comparison of directional variograms revealed similar sills and ranges for the 0 and 30 degree directions and a nugget effect in the 45 and 90 degree directions. There was no evidence of geometric anisotropy.

Spherical and exponential theoretical variograms were fitted to the omnidirectional empirical variogram (Figure 10). Best fit parameters for the spherical model included a range of 18 km, an absolute sill of 0.018 and a nugget of 0.057. The objective function for this model was 0.0005. The best fit exponential theoretical variogram included a range of 10 km, absolute sill of 0.020 and nugget of 0.055. The resulting objective function for the exponential model was 0.0005.

Ordinary kriging was performed for both the exponential and spherical theoretical variograms described above. Kriging predictions were then calculated for unsampled locations within a rectangular grid encompassing all sampling locations. The sampled and prediction locations were plotted on a scatterplot. Few locations were predicted in areas without sampled locations, thus kriging predictions and standard

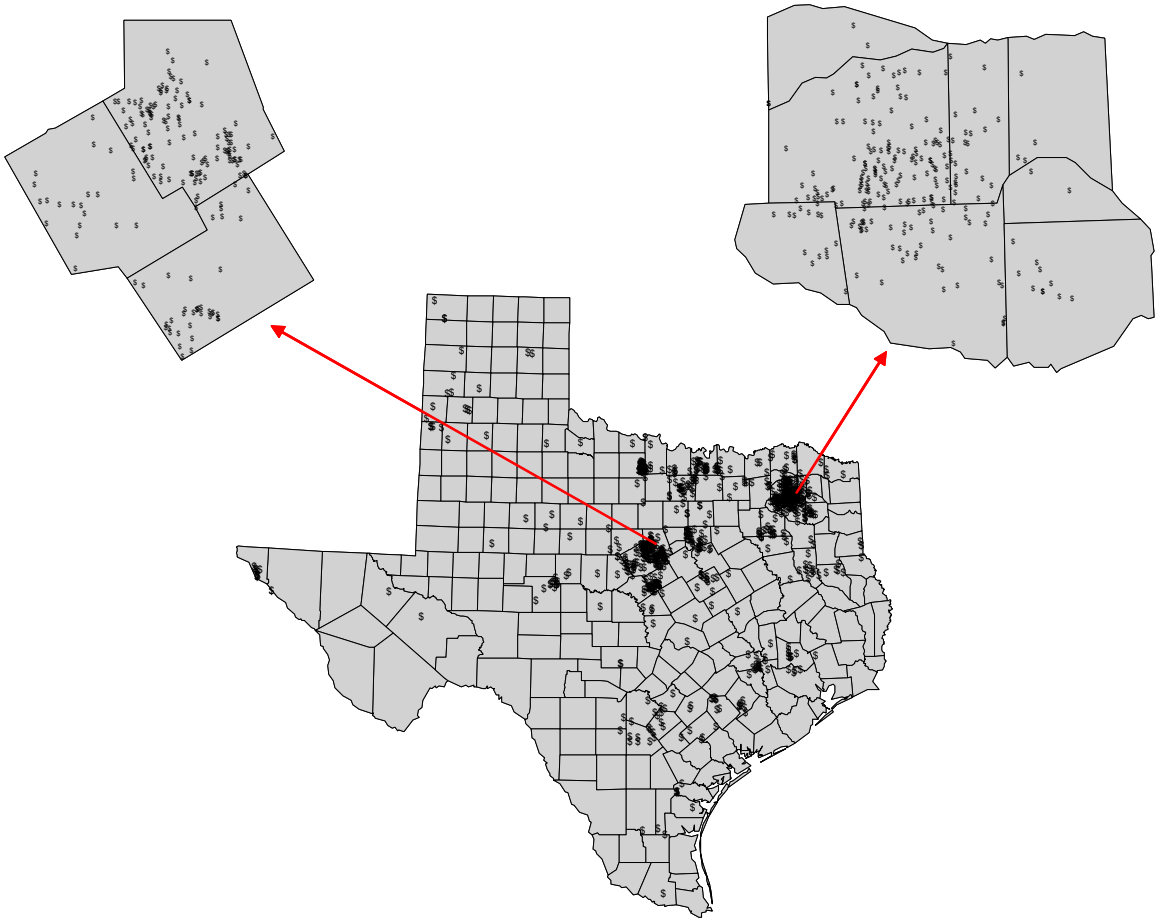
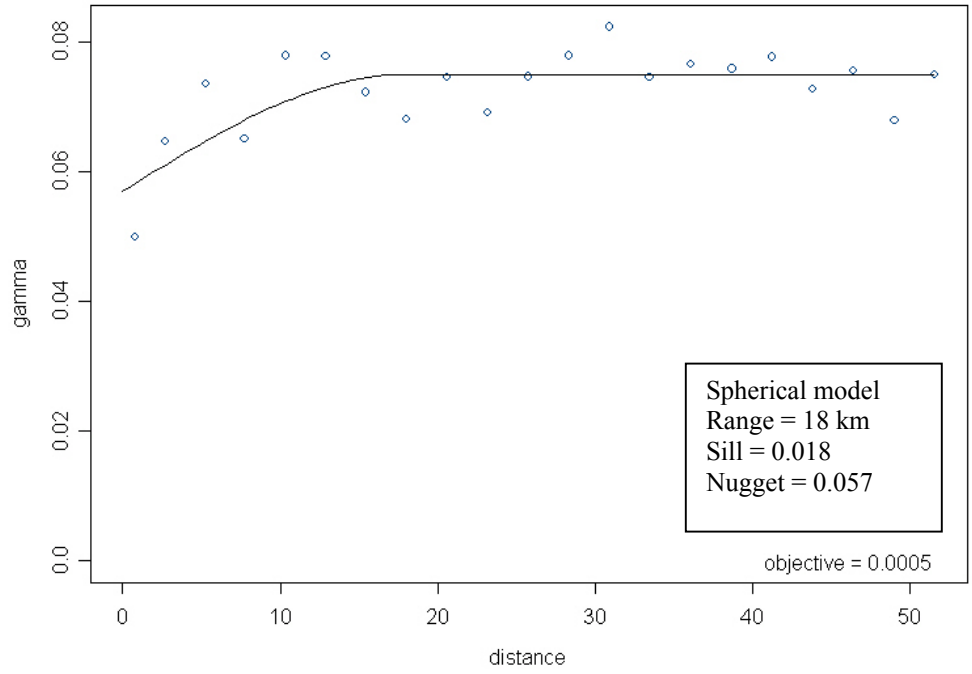


Figure 9
Additional subsets of data chosen for individual kriging showing locations of sampled dairies for Erath County subset (left) and Hopkins County subset (right).



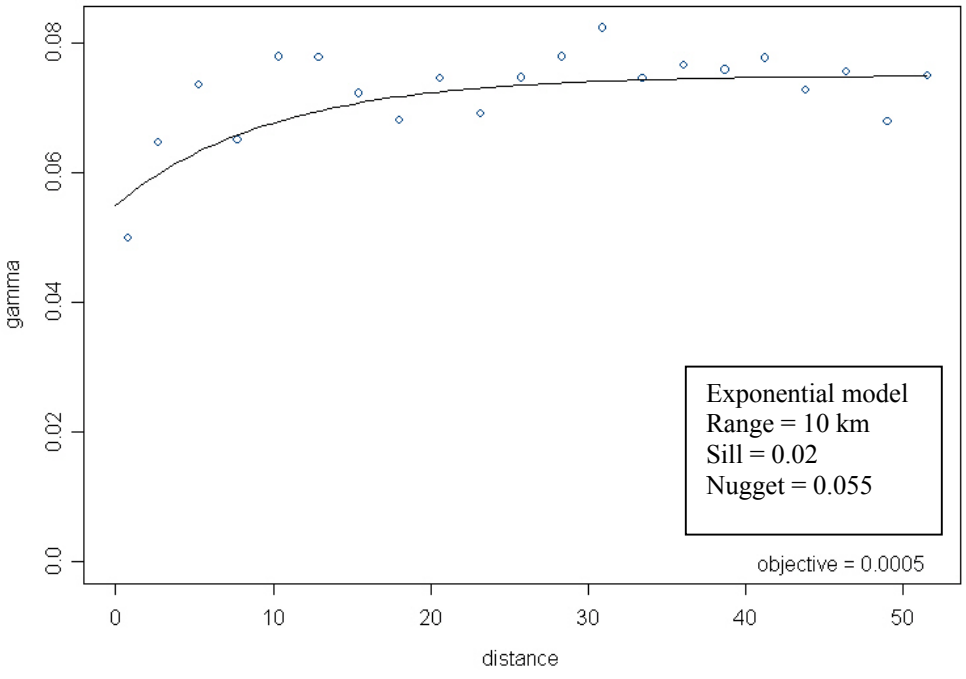


Figure 10
Fitted theoretical variograms antibody to *S.* Typhimurium in bulk milk from Hopkins County, Texas area dairies (distance in km).

errors were generated for locations within this grid, exported to ArcView®, and overlaid on the previously generated maps (Figure 11).

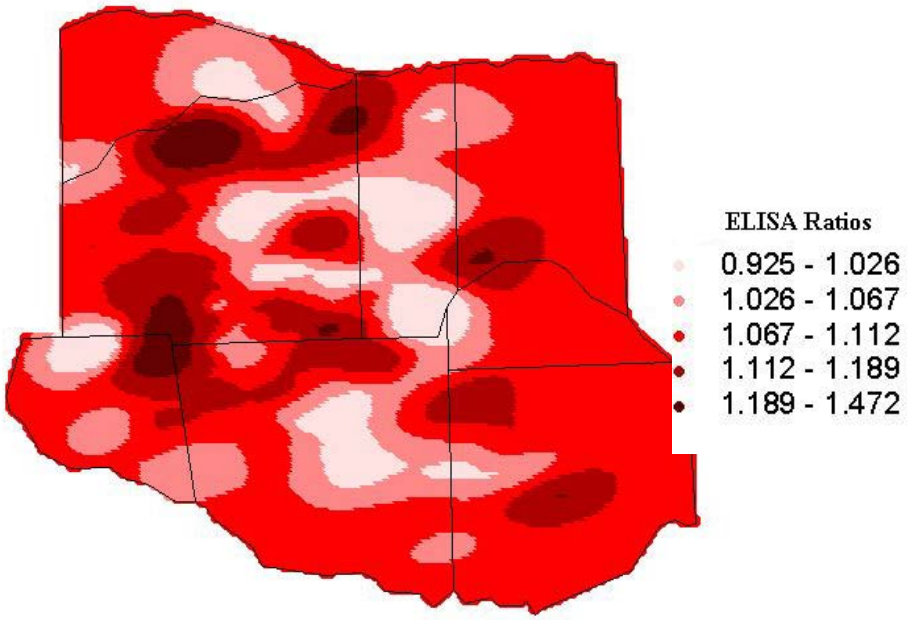


Figure 11
Contour plot of kriging predictions for antibody to S. Typhimurium in bulk milk from Hopkins County area dairies based on the spherical theoretical variogram.

Erath area subset

The Erath area subset was comprised of 180 dairies located in three counties (Figure 9). The approximate area this subset encompassed was 115 km east to west and 125 km north to south. The minimum signal to noise ELISA ratio was 0.547 and the maximum was 3.115. The median value was 1.118 and the mean was 1.155 (95% CI 1.1036, 1.207) with a standard deviation of 0.351.

A contour scatter plot of observed ratios did not suggest trend. Comparison of directional variograms revealed similar sills for all directions. Ranges were difficult to detect; indeed, all directional variograms were generally flat and appeared to be constituted by pure nugget effect alone.

Spherical and exponential theoretical variograms were fitted to the omnidirectional empirical variogram (Figure 12). Best fit parameters for the spherical model included a range of 12 km, an absolute sill of 0.015 and a nugget of 0.085. The objective function for this model was 0.0031. The best fit exponential theoretical variogram included a range of 12 km, absolute sill of 0.02 and nugget of 0.085. The resulting objective function for the exponential model was 0.0026.

Ordinary kriging was performed for both the exponential and spherical theoretical variograms described above. Kriging predictions were then calculated for unsampled locations within a rectangular grid encompassing all sampling locations. The sampled and prediction locations were plotted on a scatterplot. Few areas of predicted

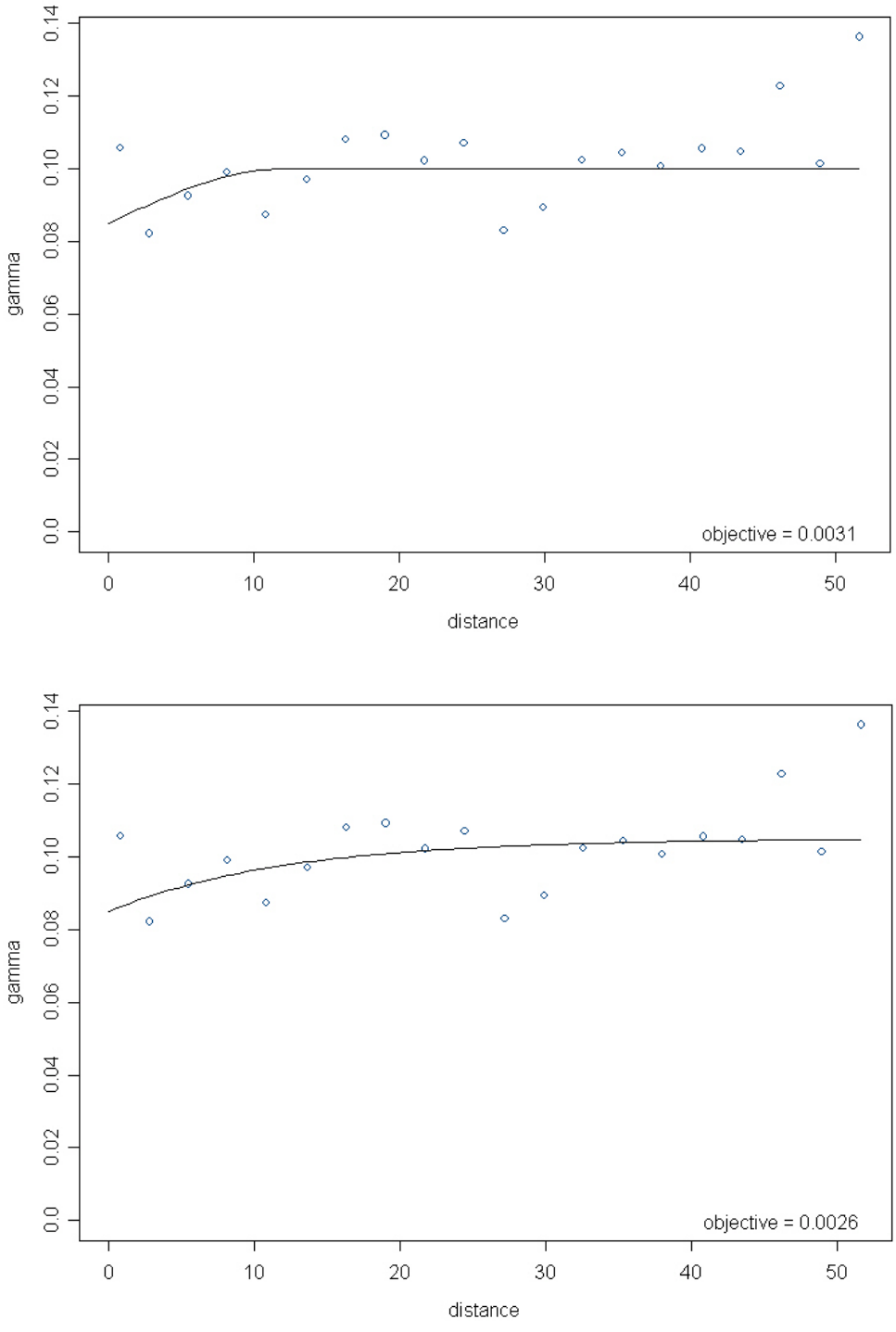


Figure 12
Fitted theoretical variograms antibody to *S.* Typhimurium in bulk milk from Erath County, Texas area dairies (distance in km).

locations were present that did not also have sampled locations, so kriging predictions and standard errors were generated for locations within this grid, exported to ArcView®, and overlaid on the previously generated maps (Figure 13).

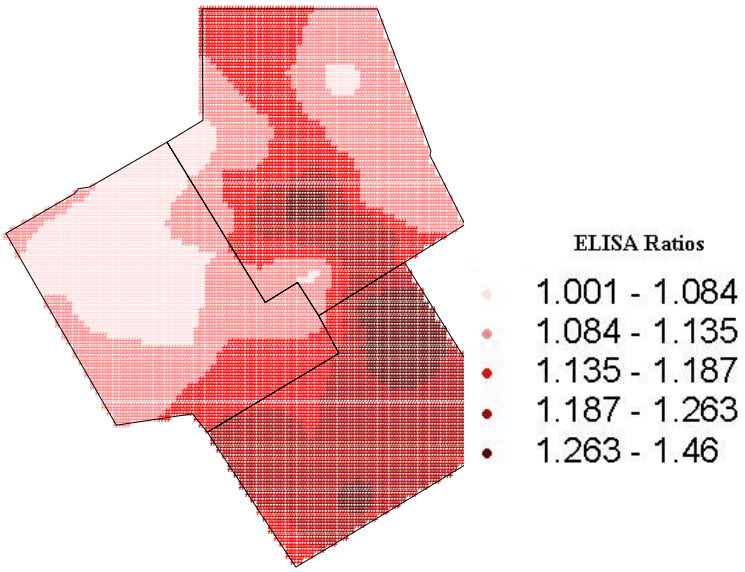


Figure 13
Contour plot of kriging predictions for antibody to S. Typhimurium in bulk milk from Erath County, Texas area dairies based on the spherical theoretical variogram.

Discussion

Bulk tank milk samples were obtained for approximately 89% of Grade A dairies operating in Texas during the time of sample collection. Milk samples lost in laboratory analysis did not appear to be from any one area and were not expected to bias results.

Kriging predictions for both the entire state and the subsets revealed a spatial pattern for the ELISA ratios suggesting that ecologic risk factors for salmonellosis in

dairies may exist. Previous studies have consistently shown that herd size is correlated with the prevalence of *Salmonella* spp. on dairies, however (Huston, et al., 2002b; Kabagambe, et al., 2000; Warnick, et al., 2003). It would have been beneficial to know the approximate size of the herds from which the samples were collected, as this may be a significant confounder to this analysis.

Seasonality of shedding of *salmonella* has also been demonstrated (Kirk, et al., 2002; Sato, et al., 2001; Troutt, et al., 2001). In the south central region of the U.S., shedding in cull cows at slaughter has been shown to be low in the winter and high in the summer (Troutt, et al., 2001). Cull cows are not necessarily representative of those remaining in the herd, and transportation, commingling and stress prior to slaughter may increase shedding of *Salmonella*. However, if the seasonal difference in shedding described in cull cows is representative of the presence of salmonellosis in the herd, collection of samples for this study during the summer should have capitalized on this seasonality. On the other hand, Purdy (2002) isolated *Salmonella* spp. more frequently from feedlot playas in Texas during the winter than during the summer. While feedlots are also not representative of dairy herds, and the time of entry of cattle into the feedlot in relation to the sampling time may be critical, this study suggests that summer might not have been the optimal time for bulk tank milk collection. In any case, the seasonality of *salmonella* shedding may not coincide with antibody presence, however, nor with lactation status of the cattle. Effects of season on the presence of antibody in milk samples need to be further considered before conclusions may be drawn.

Attempts to elucidate additional risk factors for clinical or subclinical salmonellosis in dairy herds have produced mixed results (Anderson, et al., 2001; Bender, et al., 1997; Huston, et al., 2002a; Kabagambe, et al., 2000; Kirk, et al., 2002; Sato, et al., 2001; Warnick, et al., 2001). Although additional risk factors may interact with the spatial distribution of salmonellosis and could be potential confounders or covariates, it seemed unwise to attempt to include them in this exploratory study. Their potential presence, however, should be remembered as more focused studies are developed.

The sensitivity of the bulk tank milk ELISA may have been a limiting factor for this study. Previous studies using similar tests have reported sensitivities ranging from 54-100% in detection of *S.* Typhimurium or *S.* Dublin in milk (Hoorfar and Wedderkopp, 1995; Hoorfar, et al., 1995; Veling, et al., 2000; Veling, et al., 2001; Veling, et al., 2002a). Although high sensitivity is generally desired or even required for a screening test such as this, the bulk milk ELISA does have certain advantages over the few other available testing methods. First, it theoretically had the potential to identify subclinical herds and herds with recent exposures, something that has been a serious limitation in studies based on cultures. Second, it has a high specificity for *S.* Typhimurium (Hoorfar and Wedderkopp, 1995). Recent studies culturing *Salmonella* species from cull cows and dairy farms have demonstrated that the majority of *Salmonella* present in cattle are host adapted species that are not zoonotic (Galland, et al., 2001). In order to be reasonably certain that cross-reaction with antibody to these species was not falsely elevating test results, a highly specific test was needed. Previous

studies have credited bulk milk ELISAs with specificities of 98-100% (Hoorfar and Wedderkopp, 1995; Hoorfar, et al., 1995; Smith, et al., 1995; Veling, et al., 2000; Veling, et al., 2001). Third, obtaining bulk milk samples was non-invasive and was less labor intensive and less expensive than alternative tests, which allowed larger numbers of herds to be sampled. This is of great importance in a spatial study, in that the number of sampled locations and the total area represented by the samples is a limiting factor in the accuracy of many spatial statistics.

There were also some significant disadvantages to using the bulk milk ELISA. First was in selecting the parameter to use to describe the results. There are several methods for interpreting the optical densities resulting from an indirect ELISA test (Wright, et al., 1993). The percent positivity (optical density of sample divided by optical density of a high positive reference standard, expressed as a percentage) has been recommended (Wright, et al., 1993), however, this relies on the availability of a strong positive reference standard. The signal to noise ratio, on the other hand, is not dependent on a reference standard. Instead, it compares the sample result to background noise that might occur for a variety of reasons in the test or in the sampled population. It is more difficult to intuitively understand the meaning of a signal to noise ratio than the percent positivity, however. Also, it is quite difficult to compare results using signal to noise ratios with those of studies using the more common percent positivity value.

Second, the bulk milk ELISA only reflected the status of lactating cows. Dry cows, heifers and calves were not represented. While the intermingling of these populations and the sharing of a common environment would seem to lead to consistent

antibody status among these groups, this should not be assumed. A recent study by Velig, et al. (2002) found that the herd sensitivity for bulk milk LPS ELISAs for antibody to *Salmonella enterica* serovar Dublin was 79% when clinical disease was seen only in lactating cows, but dropped to 54% when antibody was present in non-lactating cows as well.

Finally, bulk tank milk ELISAs may be compromised by the effect of dilution which would be related to herd size, numbers of seropositive lactating cows, and potentially even individual milk production, although the latter would likely have an insignificant effect. At present, studies considering the effect of these factors on bulk milk ELISA results have not been reported.

Despite these problems with the ELISA, it was the most logical choice given the alternatives and the resulting continuous variable was quite appropriate for geostatistical analysis. There were, of course, some limitations to the analysis itself, primarily in that the dairy population is not dispersed evenly throughout the state. This resulted in large areas of Texas with few or no observed values where kriging predictions tended to default to the mean. However, one could argue that the risk surface is irrelevant in these areas, at least from a risk management standpoint as there are no dairies in them to be "at risk." On the other hand, a more complete picture of the risk surfaces would enhance the ability to correlate risk with ecologic features that might be important. Also, the predicted surface can be visually misleading, as they are represented by the mean value as opposed to no value. It is important to keep in mind the underlying spatial distribution of the population itself.

As an example of this problem, the question of potential northwest-southeast trend in *S.* Typhimurium risk in Texas would be more easily answered if there were more observed locations in central Texas. There is, in a manner of speaking, a "missing link" there and, although kriging will produce predictions in this area, they reflect the mean and are not useful in considering the potential trend. On the other hand, having observed locations too near to each other can also be disadvantageous. The kriging function relies on a covariance matrix that may not be able to be resolved when locations are very close to each other. This can sometimes be corrected by increasing the size of the covariance matrix, but there is an upper limit which cannot be exceeded. This problem tends to occur less with kriging based on a spherical theoretical variogram than on an exponential one, but can occur with either. It was primarily an issue in kriging the northeast area subset during this study, seemingly due to the attempted correction for geometric anisotropy. However, because the apparent anisotropy was small, a variogram could be acceptably fitted without using the correction factor, and ordinary kriging could be performed.

With respect to analysis of the subsets, it is important to note the issue of the Modifiable Area Unit Problem (MAUP). This refers to the possibility that apparently significant spatial effects occurring within a given area may disappear if the area is re-drawn to include additional data. Additionally, the selection of locations to include in these subsets was subjective. Subsets were selected prior to any investigation into test results in order to minimize bias, however. Edge effects may be prominent and in

retrospect could have been addressed by creating a guard around the selected subsets, as values would have been known for those shields.

In general, however, kriging predictions for the subsets seemed to compare well to those for the entire state. The Hopkins area seemed to have a comparatively large range of predicted values, but the significance of this is unknown. The Erath area, conversely, seemed to have a relatively flat predicted surface. This subset encompassed a smaller area than the others, however, which may have led to the lack of contour. This apparent lack of contour is not uninteresting, however. There is a large number of dairies in this area and further evaluation of their structure, environment and management could provide valuable insight into risk factors for salmonellosis in that similarities may reflect protective measures and differences may suggest factors unimportant in salmonellosis.

Although assessment of the spatial distribution of *S.* Typhimurium antibody in bulk tank milk was hindered by the lack of a reliable, accurate and efficacious screening test and by the overdispersion of dairy farms themselves, it was able to be performed successfully. There were definite areas of elevated and decreased risk for antibody to *S.* Typhimurium. These areas should be further evaluated for potential ecological associations including water sources and vegetation.

In addition, smaller scale, focused studies performed on one or two areas should be performed. These would have the potential bias of the MAUP, but their more manageable size would allow for the comparative use of tests and the consideration of additional variables. At a minimum, herd size and operation type (confinement vs.

grazing) should be considered as potential covariates in further analysis. While management factors such as these should be accounted for in the nugget of the theoretical variogram, their tendency to be spatially correlated themselves may confound analysis and must be considered.

If feasible, comparative testing of at least a portion of the observed herds should also be performed. Although there is no true "gold standard" test for salmonellosis, particularly subclinical disease, individual animal milk antibody tests and serology are promising (Hoorfar and Bitsch, 1995; House, et al., 1993; Smith, et al., 1989) and may be of value here. Combinations of methods that produced excellent results (herd sensitivities of 91% to 99%) were recently discovered by Veling, et al. (2002a).

Finally, potential ecologic risk factors related to the spatial distribution of *S.* Typhimurium antibodies modeled in this study, initially focusing on waterways and aquifers, should be evaluated. *Salmonella* may survive in water sources for a month or more and these may contribute to persistent or recurrent infections. Water may also provide a source of infection for neighboring herds, as well. On the other hand, if no correlation is found between water sources and *Salmonella* antibodies, the theory that cattle are a source of human salmonellosis via contaminated water supplies is uncorroborated and justification for legal measures protecting against such contamination is diminished.

CHAPTER III

PREVALENCE AND CLUSTERING OF TEXAS DAIRY HERDS WITH POSITIVE BULK MILK ELISA TESTS FOR ANTIBODY TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM O ANTIGENS

Spatial clustering occurs when more cases are present at a particular location than would be expected by chance. It may occur for a variety of reasons, including communicability of the disease, point source exposures, and common demographics. Clustering may or may not also be associated with an underlying spatial process.

This study was performed to determine if clusters of dairy herds with positive bulk milk ELISA tests for *S.* Typhimurium were present in Texas. Areas of likely clusters, if present, may be good candidates for additional studies. Lack of clustering could also provide insight into the epidemiology of salmonellosis in these herds.

Methods

Data and sample collection

The 852 bulk milk samples described in the previous chapter were used for this study. Each represented one Texas dairy with a unique geographic location and was identified only by TDH number. The data represented approximately 89% of the Grade A permitted dairies in Texas during the summer of 2001.

Laboratory analysis

An indirect enzyme-linked immunosorbent assay (ELISA) with *S.* Typhimurium lipopolysaccharide (LPS) as the antigen was performed on each sample as previously

described. Herds were then classified as bulk milk antibody positive or negative based upon the signal to noise ratio, which was derived by dividing the optical density reading of the sample by the optical density of the negative reference standard (Wright et al., 1993). Positive samples were defined as those with signal to noise ratios greater than 1.8.

Statistical analysis

Descriptive statistics were calculated using commercial statistical software (S-PLUS® 6 Professional Edition for Windows®, Release 2 (September 2001), MathSoft, Inc. Seattle, WA). Overall prevalence of bulk milk antibody to *S.* Typhimurium LPS was calculated by dividing the number of dairy herds with signal to noise ratios greater than or equal to 1.8 by the total number of dairy herds in the sample.

Clustering among herds with positive bulk milk antibody results was examined by Cuzick and Edwards test for heterogenous populations (Cuzick and Edwards, 1990). This was performed using a commercial software package (Clusterseer™ v. 2.03, Terraseer Inc., Ann Arbor, MI, 2001). Cases were defined as herds with signal-to-noise ratios of greater than or equal to 1.8 and were coded as 1; controls were those with ratios less than 1.8 and were coded as 0. The number of nearest neighbors (k) for k =1-10 was calculated and the resulting test statistic T_k was compared with the expected statistic $E(T_k)$ under the null hypothesis of no clustering of cases. Significance was tested using the z statistic and defined as an upper-tail $p < 0.05$. To correct for multiple comparisons, a Bonferroni adjusted p-value was calculated with statistical significance defined as $p < 0.05$.

Locations of likely clusters were investigated through a spatial scan statistic (Kulldorf and Nagarwalla, 1995) using a freeware program (SaTScan™ v. 3.0: Software for the spatial and space-time scan statistics, Kulldorff M. and Information Management Services, Inc., Bethesda, MD, National Cancer Institute, 2002). The spatial scan statistic is based on a likelihood ratio test for statistical significance. In brief, it superimposes a circular window centered on each data point in the study area in turn and varies its radius between zero and an upper limit defined by the investigator. The number of cases within each window is compared to the Monte Carlo estimation of expected cases that would occur if the cases were randomly distributed given the total population (Kulldorf and Nagarwalla, 1995). Despite its obvious advantages, the use of the spatial scan statistic in veterinary epidemiology is a recent development, with its debut occurring in 2000 (Carpenter, 2001).

The Bernoulli model was used for the likelihood function with the null hypothesis that rates within each window were less than or equal to those outside of the circle. Cases and controls were coded as for Cuzick and Edward's test. The maximum spatial cluster size examined was set at the recommended default of 50% of the total population. Secondary clusters were prohibited from having their centers within another cluster. Statistical significance was set at $p < 0.05$ with the p-value calculated through Monte Carlo hypothesis testing with 999 simulations.

Results

The overall prevalence of positive bulk milk ELISAs was 4.3% (37/852). ELISA signal to noise ratios ranged from 0.5472 to 3.8679. The mean signal to noise ratio was 1.1660 (95% CI 1.1419 -1.1902) and the median was 1.1005.

Cuzick and Edward's test (Table 1) resulted in a significant z-statistic ($p < 0.01$) for each T[k] above $k = 3$. The Bonferonni adjusted p value was also significant ($p < 0.001$).

K	T[k]	E[T]	Var[T]	z	p-value
1	2	1.56522	2.28162	0.28784	0.386735
2	6	3.13043	4.77963	1.31256	0.094666
3	13	4.69565	7.31366	3.07070	0.001068
4	17	6.26087	9.87870	3.41680	0.000317
5	19	7.82609	12.4124	3.17159	0.000758
6	23	9.39130	14.9768	3.51647	0.000219
7	26	10.9565	17.6000	3.58585	0.000168
8	26	12.5217	20.2051	2.99850	0.001357
9	30	14.0870	22.8496	3.32900	0.000436
10	37	15.6522	25.4250	4.23373	0.000011

Table 1
Cuzick and Edwards' test for clustering of dairy farms with positive bulk milk antibody test results.

The spatial scan statistic identified two clusters with significant log-likelihood ratios. The most likely cluster ($p = 0.001$) had centroid coordinates 252.993 and 37772 (UTM 83, Zone 14) and a radius of 306 kilometers. It encompassed 88 (10.32%) of the total population with 15 of these being cases and had an overall relative risk of 3.9 versus the area outside of the cluster. The significant secondary cluster ($p = 0.016$) had centroid coordinates of 552.187 and 36503 (UTM 83, Zone 14) and a radius of 63.5km.

This cluster encompassed 36 (4.22%) of the total population with 9 cases among them and an overall relative risk of 5.75 versus the area outside of the cluster (Figure 14).

Discussion

Many studies have attempted to assess the prevalence of salmonellosis among dairy herds in recent years, but results have been extremely variable. This study found that the overall prevalence of antibody to *S.* Typhimurium in Texas Grade A Dairy bulk milk tanks was 4.3%. Wells, et al. (2000) found that 5.4% of lactating cows and 21 % of dairies were positive for *salmonella* on fecal culture but *S.* Typhimurium only represented 2.8% of the isolates. Huston, et al. (2002b) found that up to 99% of clinically healthy dairy cows from five Ohio herds were shedding *Salmonella* in their feces, but this included all *Salmonella* species. Galland, et al. (2001) and Troutt, et al, (2001) isolated *Salmonella* spp. from 26.8% of cull dairy cows and *S.* Typhimurium was typed in 3.3% of these. Gay et al. (1994) estimated the prevalence of fecal shedding of *Salmonella* spp. in cull dairy cattle as between 4.6 and 9.2%. Finally, Jayarao and Henning (2001) cultured bulk milk tanks and isolated *Salmonella* spp.from 6.1% of them.

A priori, it was expected that the bulk milk ELISA would underestimate the true prevalence of *S.* Typhimurium in dairy herds, because its sensitivity is allegedly low. It appears, however, that the prevalence estimate from this study is similar to or slightly higher than others reported in the literature. This may have occurred because all tests for *S.* Typhimurium in cattle tend to have low sensitivities. It could also be that the true

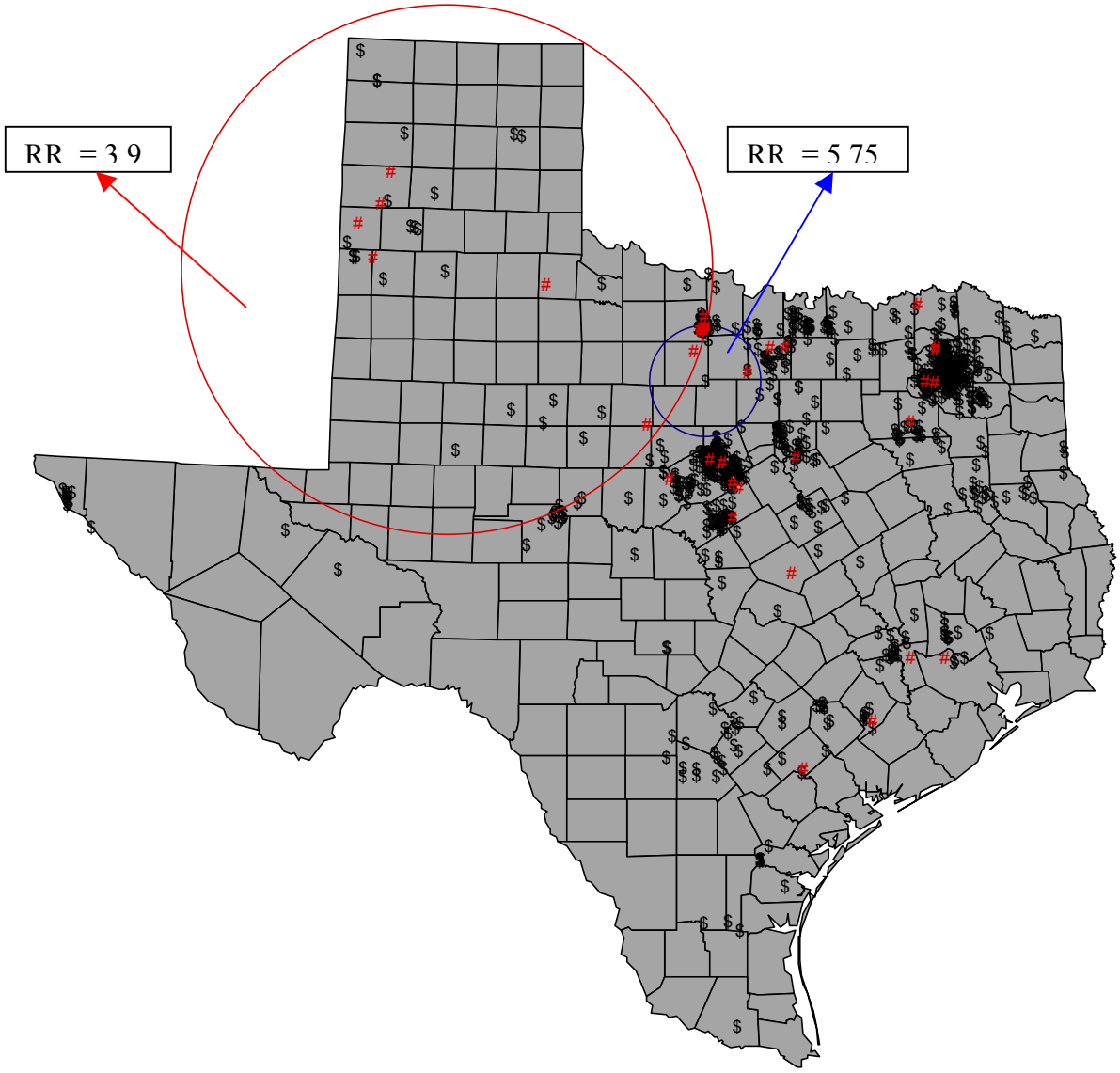


Figure 14
Locations of dairy herds with greater than or equal to 1.8 (●) or less than 1.8 (▲) signal to noise ratios on ELISA and locations of significant clusters of "case" herds.

prevalence is actually lower than believed and ours is an accurate estimate. On the other hand, this could have been just due to serendipity in the selection of a cut-off value used to define a positive test. Until an effective means of diagnosing both clinical and subclinical salmonellosis in dairy cattle is developed, this question will remain a subject of debate.

Both Cuzick and Edward's test and the spatial scan statistic agreed that significant clustering of cases occurred within the study region. These are complementary tests, both of which have the capability to account for a heterogenous population at risk. Cuzick and Edward's test looks for global clustering but does not locate clusters. Results of this test suggested that cases did cluster at the order of three nearest-neighbors or higher.

The spatial scan statistic considers local clustering and reports locations of the most likely cluster and any potential secondary clusters, in order of decreasing likelihood. The most likely cluster located encompassed a rather large area (~600km radius) and had widely dispersed farms within it. Because the spatial scan statistic only considers circular areas when searching for clusters, this result is somewhat misleading. The cluster located actually refers to the observed farms, not the entire area, within the circle. When overlaid on the kriged predicted surface from the previous chapter, however, concordance between the located cluster and an area of elevated risk was evident. The only other likely cluster identified by the spatial scan statistic was a much smaller area (63 km radius) with a high density of dairies. This cluster was also in agreement with the predicted surface.

Both likely clusters occurred in the northern area of Texas. There still may be a directional trend present as suggested by visual examination of the kriged surface. One explanation for the failure of such a trend to be identified statistically might be the lack of observed locations within the central region as described in the previous chapter. Other factors that may certainly play a role in the existence of these clusters include herd size and type of operation; population structure of the herd (herd "demographics"); common management measures such as suggested by local authorities or by local tradition; or common ownership or management of farms. On the other hand, these clusters might also be associated with an ecologic feature such as water source, or other spatially dependent variable, such as a common source of feed or trucking companies.

Because the test result/ herd status was not known when subsets were established for additional consideration, neither cluster was singled out for additional investigation. It seems logical that a spatial analysis be conducted for these regions in specific. It would be of value to define a guard around the clusters as well, to help minimize edge effects, for the analysis. These may also be potential targets for more detailed epidemiologic investigations and additional/ confirmatory testing.

CHAPTER IV

CONCLUSION

The presence of *S*. Typhimurium in dairy herds has long been a subject of consternation for health care professionals, veterinarians, producers and the public. *Salmonella* spp. are the second most common pathogen isolated from diagnostic samples from people with enteric disease in the U.S. (Hohmann, 2001; CDC, 2002) and may cause serious systemic or even fatal disease in some individuals. The potential association of human salmonellosis with cattle has long been a subject of debate. Outbreaks have been traced to the consumption of dairy or beef products, but a causal association with environmental contamination by cattle herds remains questionable. Environmental and animal rights groups as well as politicians and the public are quick to blame cattle operations when outbreaks occur, however, and legislation intended to minimize the impact of cattle on the environment continues to become law. The economic effect complying with these constantly changing regulations this has on the producer may be significant.

In addition, salmonellosis does cause clinical disease in both calves and adult cows. Cattle with salmonellosis may present with enteritis or may be depressed, with lethargy, anorexia or decreased feed consumption and an associated drop in milk production. Once *salmonella* infects a herd, it may persist for months or years through repeatedly infections. Diagnosis is difficult, especially in subclinical or persistently

infected herds, and prevention appears to be even harder. Even though the role of environmental contamination in human disease should be challenged, its role in the epidemiology of cattle disease is well established.

Many epidemiologic studies have been undertaken in an attempt to elucidate risk factors for salmonellosis in these herds but few definitive links have been found. Herd size is one factor that has been has been significantly correlated with the presence or shedding of various *Salmonella* spp by dairy cattle in repeated investigations (Huston, et al., 2002b; Kabagambe, et al., 200; Warnick, et al., 2003). Several other suggested risk factors have been considered, including the presence of wild and domestic animals or birds; contamination of feed or water; treatment with antimicrobials; movement of animals in and out of the herd; and fertilization of pasture or hay fields with manure or treatment sludge (Anderson, et al. 2001; Bender, et al., 1997; Huston, et al., 2002b; Kabagambe, et al., 2000; Kirk, et al., 2002; Sato, et al., 2001; Warnick, et al., 2001). Conflicting results from these studies have frustrated researchers, however, and the search for risk factors continues.

Recently, differences in rates of fecal shedding of *salmonella* have been noted to have a seasonal and geographic relationship (Kirk, et al., 2002; Sato, et al., 2001; Troutt, et al., 2001). Techniques to assess spatial associations and clustering of disease in populations have become more applicable to veterinary medicine, especially with the development of techniques that can account for heterogenous populations, commonly found in livestock production.

The results of this study are encouraging in that they reveal both clustering and a spatial distribution of *S*. Typhimurium in dairy herds in Texas. The models and predicted surfaces generated may form a basis for additional studies considering the ecologic factors that may be influencing this spatial process. This is an area that should be further considered. One of the main drawbacks in this study, however, was the heterogenous distribution of dairies in Texas. The consideration of data subsets addresses this problem by focusing specifically on dairy sheds. An alternative approach would be to sample other cattle populations that are present in areas without dairies for antibody to *S.* Typhimurium.

Another area that should be further investigated is the presence of the clusters identified in Chapter II. These appear to coincide with high risk areas from the kriged predictions. Performing a more "traditional" epidemiologic investigation in one or both of these areas might help to elucidate the reasons for these clusters. Again, a spatial analysis of *S.* Typhimurium in an alternate population might be of value. Clusters or kriged risk surfaces similar to those found in the dairies could elevate the suspicion of an environmental risk factor or factors, and common activities among the two populations could suggest potential factors to consider.

Finally, new techniques for modeling the spatial distribution of disease risk are frequently created. Methods of spatial analysis used in diverse fields are being adapted to veterinary and medical epidemiology. Bayesian methods which incorporate prior knowledge of spatial or disease processes into models and the ability to create risk surface predictions bases on binomial variables may be useful in the continued

investigation into the spatial distribution of *S.* Typhimurium in dairy herds in Texas. As models are generated, additional information is learned about the epidemiology of the disease in question – as much through the modeling process itself as through the outcomes. Eventually, risk factors for salmonellosis in dairy cattle will be determined and appropriate measures to reduce or eliminate these risks may be taken.

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