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Geostatistical analysis of biomarkers of genotoxicity in cattle, *Bos taurus* and *Bos taurus* × *Bos indicus*, sentinels near industrial facilities

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Abstract This study, performed at the behest of ranchers living and working down-prevailing wind from industrial facilities located in Calhoun County, Texas investigated locational risks to ecosystem health associated with proximity to specific industrial complexes. Concerns expressed were for potential genotoxicity in cattle resulting from the release of complex chemical mixtures. The Comet Assay and flow cytometric evaluation of variations in DNA content were utilized to evaluate DNA damage. Bayesian geo-statistical analysis revealed the presence of important spatial processes. The Comet assay's optical density provided a strong indication of increased damage down-prevailing wind from the industrial complexes. Results indicated that proximity to and location down-prevailing winds from industrial facilities increased the locational risk of genotoxicity in this sentinel species.

Keywords Chromosomal alterations · DNA–protein cross-linkage · Spatially oriented genotoxic response

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Introduction

Ranchers and landowners living in close proximity to and down-prevailing wind from two industrial companies expressed concern over the possibility of adverse health effects for themselves and their livestock. Their greatest concern was the perceived cluster of genotoxic responses including neoplasia in the human population and reductions in the reproductive and general health status of their livestock. The industrial operations located in Calhoun County, Texas included an aluminum smelting facility in operation since the 1950s and a plastics production facility (Maywald 2001). The plastics facility was built in the 1980s and has undergone numerous expansions since that time (Formosa Plastics 2007). According to the United States Environmental Protection Agency's (USEPA) toxic release inventory the two companies were responsible for the release of in excess of 635,000 kg of 43 toxic chemicals during 2002. The majority of the chemicals released were potential to known carcinogens with some released in high amounts. For example, there were in excess of 8,600 kg of 1,2-dichloroethylene and 7,700 kg of 1,3-butadiene released. Both of these are classified as probable human carcinogens and were listed as air emissions by the plastics facility in 2002 (USEPA 2007; USEPA 2004). In excess of 6,350 kg of the 1,2-dichloroethylene was classified as fugitive air releases with the remainder being point source emissions. Fugitive emissions are the result of leaks, evaporative losses from surface impoundments and spills, and releases from building ventilation systems. This type of emission is not released through a confined air stream and does not benefit from dispersion and dilution characteristics inherent in a point source release system and is expected to have the highest concentrations in close proximity to the source. Fugitive air emissions accounted

for 278,895 kg of the 581,121 kg of toxic chemicals released into the air by the plastics facility in the 2002 reporting year. In the same time period the aluminum company released 2.6 kg via fugitive air emissions and 770.21 kg through point source emissions systems (USEPA 2004).

Genotoxicity has been evaluated utilizing many different analytical modalities including flow cytometric methods and single cell gel electrophoresis. These methods are utilized to determine if chromosomal aberrations, single-strand DNA breaks, alkali-labile sites, DNA crosslinkages, or incomplete DNA repair are present. Chromosomal aberrations are structural or numerical changes to chromosomes which occur as a result of aging or exposure to genotoxic substances (Clark et al. 2000; Custer et al. 2000; Bickham et al. 1998). These changes are induced through DNA strand breaks, faulty replication associated with a damaged DNA template, and through inhibition of DNA synthesis (Albertini et al. 2000). Flow cytometric evaluation of variations in DNA content has been used to evaluate genotoxicity associated with exposure to an extensive list of chemicals and has been shown to correlate well with other chromosomal aberration assays (Neuparth et al. 2006; Matson et al. 2005a, b; Matson et al. 2004; Baciuchka-Palmaro et al. 2002; Custer et al. 2000; Bickham et al. 1998; Wickliffe and Bickham 1998; Lowcock et al. 1997; Shugart et al. 1989).

The Comet test has an extensive history in detection of single- and double-strand DNA breaks, alkali-labile sites, and incomplete DNA repair resulting from a wide variety of genotoxic chemical exposures (Sram et al. 1998; Fairbairn et al. 1995). With the comet test, cells are placed in molten agarose, exposed to detergents and high salt to provide accessibility to the DNA, and electrophoresis is performed. Neutral and alkaline electrophoresis solutions have been utilized with strong alkaline solutions shown to be preferred for detection of single- and double-strand DNA breaks and alkali-labile sites. With electrophoresis, damaged DNA strands migrate further than intact DNA yielding a "Comet" appearance. This method has been utilized with a wide variety of cell types from many different species and provides a sensitive indication of response to genotoxic exposure (Gabelova et al. 2004; Blasiak et al. 2004a, b; Marlin et al. 2004; Lemiere et al. 2004; Frenzilli et al. 2001; Albertini et al. 2000; Blasiak et al. 1999; Nacci et al. 1996).

Concern over the potential for genotoxic damage associated with exposure to industrial emissions varied by location within the study area with close proximity and location down-prevailing wind increasing the concern. Evaluation of locational risks of environmentally induced biologic response has been made possible by the adaptation of geo-statistical techniques originally developed for the field of mineral exploration. Geo-statistical modeling utilizes geographical information systems (GIS) technology to produce continuous surface prediction maps from limited numbers of sampling points. The ability to produce accurate maps from limited data has led to it's utilization for disease mapping and also makes the methodology ideal for environmental investigations utilizing biomarkers in sentinel species. The production of response prediction maps identifies geographical areas with increased risks, and also potentially identifies sources of genotoxic substances (Biggeri et al. 2006; Diggle and Ribeiro 2007). Geo-statistical methods have been improved through the application of Bayesian statistical methods. Bayesian statistical methods are gaining increased acceptance in the scientific community due to perceived advantages over traditional "frequentist" methods (Fernandez and Green. 2002). These advantages are of significant value when applied to environmental investigations such as this study. One of the advantages is the ability to deal with correlation between sampling points. Frequentist statistics often assume that each sampling location is independent of other sampling locations. This assumption of independence makes frequentist methods less than ideal for use in environmental investigations. When pollutants are released in the environment they travel from the point of release depending on the dispersion characteristics of the chemical, the matrix the chemical is released in, and environmental conditions such as wind and water patterns present at the time of release (Scott et al. 2003; Chen et al. 2003; Janssen et al. 2001). This dispersion leads to spatial correlation on an unknown scale. Bayesian geo-statistical methods are designed to assess and quantify this spatial correlation (Thompson et al. 2005; Boyd et al. 2005). Bayesian spatial modeling using generalized linear kriging expanded to include a nugget or random effect for each location allows for the possibility of varying random and spatial effects (Best et al. 2005; Spiegelhalter et al. 2002; Diggle and Ribeiro 2007). This technique has been especially useful in modeling farm animal data that considers all animals in a herd to be at the same location (Thompson and Scott 2007). Comparison of a base model, which includes temporal and random effects, with an extended model which also contains spatial effects allows inferences to be made on whether the data are affected by an important spatial process (Spiegelhalter et al. 2002). Bayesian prediction can then be performed allowing the development of risk maps across the entire ecosystem under consideration (Spiegelhalter et al. 2003). Utilization of these methods for response variables allows integrated locationally-based conclusions to be drawn from the data.

The primary objective of this study was to investigate a specific veterinary concern of spatially oriented genotoxic responses. The objective was to be achieved with a geo-

statistical analysis of biomarkers of genotoxicity namely genetic damage detected by single cell gel electrophoresis and chromosomal aberrations detected by flow cytometry. The secondary objective was to provide information on environmental quality that could be gleaned using cattle as sentinel species.

Materials and methods

Sample collection location and animal selection

The study area was defined geographically as the area surrounding two industrial facilities with a radius of approximately 18 km. The study area was confined by the predominance of cropland to the north and east and the marine environment to the south and west. Herds were selected for inclusion based on location within the study area and owner willingness and ability to gather their cattle for sampling purposes at 30-day intervals between July and September 2002. Twenty-one herds were included in the study. Coordinates of the livestock processing facilities at each herd location were obtained using hand-held global positioning system (GPS) locators and used for statistical analysis. Livestock processing facilities were used due to the near-central location of these facilities. The locations of the processing facilities are provided in Fig. 1a. Five adult female *Bos taurus* or *Bos taurus* × *Bos indicus* inter-species cattle from each herd were randomly selected for inclusion in the study. All animals included in the study were between the three to seven years of age. Five animals from one herd were *Bos taurus*. All others were *Bos taurus* × *Bos indicus* inter-species cattle. Each animal was uniquely identified with a numbered ear tag. Sampling was started in July 2002 and was repeated at 30-day intervals through September 2002.

Whole blood samples were obtained via caudal venipuncture with EDTA vacutainer tubes. Fifty microliters were then placed in 2 ml (mls) of Hanks balanced salt solution and flash frozen in liquid nitrogen as per the protocol of Tice and Vacquez (1999). The remainder of each blood sample was transferred to cryo-vials and placed on dry-ice for transport to laboratory facilities. All samples were labeled with a unique identifier generated by a random number generator to provide blinding of laboratory personnel. Samples were then stored at −80°C pending analysis.

Flow cytometry

Flow cytometric measurement of cellular DNA content was performed as per published protocols (Darzynkiewicz and Juan 1997). Samples were thawed in a warm water bath and cells were lysed, digested with trypsin, exposed to RNAs and stained with propidium iodide. Cells were incubated in propidium iodide for a minimum of twenty minutes prior to analysis with a Becton-Dickson FACSCalibur Flow Cytometer. The flow cytometer was set for excitation with blue light and detection of propidium iodide at red wavelengths and fluorescent microspheres analyzed prior to sample evaluation to insure proper flow cytometer set-up and function. Cells were gated on side scatter, forward scatter, and the ratio of peak to integrated fluorescence to allow evaluation of lymphocytes. Ten-thousand cells (lymphocytes) meeting all gating parameters were measured per sample and the variation in DNA content reported as the half-peak coefficient of variation.

Comet assay

The alkaline single cell gel assay more commonly known as the Comet Assay, was performed as per the protocol developed by Tice and Vacquez (1999). Samples were thawed in a warm water bath and slides prepared as per the referenced protocol. Slides were allowed to cool and then placed in cold and freshly made lysing solution for a minimum of one hour. Slides were then exposed to an alkaline buffer solution with a pH of greater than 13 to allow un-winding of DNA. Electrophoresis was then performed. Following electrophoresis, slides were placed in a neutralization buffer and allowed to drain. This was repeated three times. Slides were then stained with ethidium bromide and 100 cells scored with Kinetic Imaging's Komet analysis.

Statistical analysis

Each herd-location was identified by the latitude and longitude of the cattle processing facilities. These coordinates were used to plot the location using a commercial GIS software program.[1] The map was then projected into Universal Transverse Mercator 1983 (UTM83), Zone 14 units. The UTM83 coordinates were exported and used for all statistical analyses. To evaluate the risk of location within the study area a base model was compared to an extended model using the Deviance Information Criteria (DIC) statistic (Spiegelhalter et al. 2002). The base model contained temporal terms for the measurements taken at three time periods and a spatially random or nugget term to account for the five observations taken at each location. The extended model included a spatially dependent factor for herd as described by Diggle and Ribeiro (2007). The models used a Bayesian method of inference, with vague

[1] ArcGIS®Version 9.1, Environmental Systems Research Institute, Redlands, Ca.

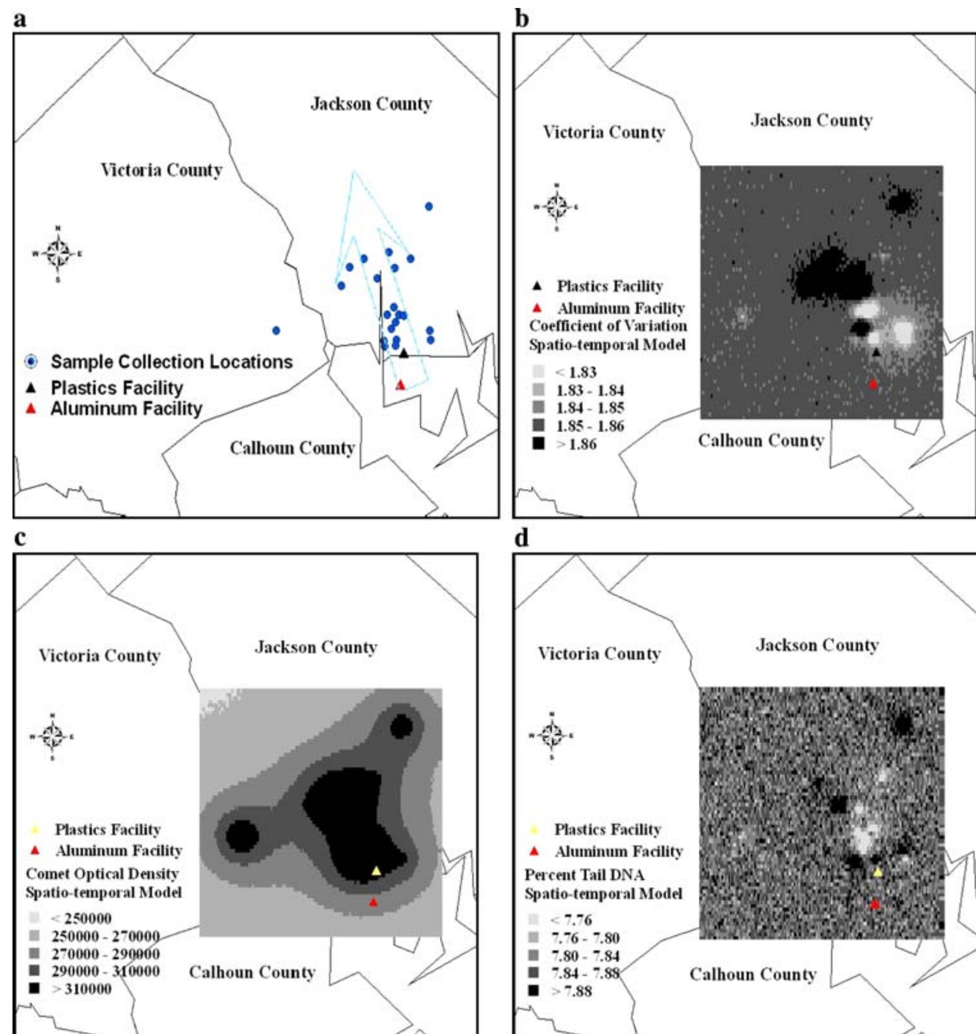

Fig. 1 Maps of (**a**) herd locations and prevailing winds, (**b**) predicted spatial distribution of coefficients of variations, (**c**) predicted spatial distribution of comet optical density, and (**d**) predicted spatial distribution of predicted percentages of DNA in the comet tail

prior beliefs and Markov Chain Monte Carlo (MCMC) implementation. The MCMC implementation was performed by use of a readily available software package (Spiegelhalter et al. 2003). The prior distributions used included a non-informative normal distribution for the intercept and temporal effects with means = 0 and precision = 0.0001, and vague gamma priors (Gamma[0.01, 0.01]) for variance components, including the range and nugget (spatially random location effect) and spatial effects (spatially dependent location effect). For all models, the distance-based variance function was exponential with the covariance between location$_i$ and location$_j$ modeled as a function of the distance between the two locations d_{ij} and the rate of decline of covariance (ϕ) as follows:

$$f\left(d_{ij}, \phi\right) = \exp\left(-\left[\phi d_{ij}\right]\right)$$

Convergence was evaluated by visual examination of the history plots of the two chains and visual examination of the Brooks, Gelman and Rubin statistics. For parameter estimation, the initial 500 iterations were discarded to allow for convergence then every 10th iteration was retained until 1,000 iterations had been saved. For each biomarker, the base and extended model were compared by use of the DIC. An improvement of greater than 3.0 in the DIC for the extended model was considered to indicate an important spatial process.

For those parameters fit best with the extended model, Bayesian spatial prediction was performed for a grid of points with each point representing the centroid of a 0.50-km by 0.50-km area encompassing the study area. One chain was utilized for predictions. A one thousand-iteration burn-in was performed. An additional one thousand iterations were performed and retained for the posterior distribution. Results of prediction modeling were imported into Arcview imagery of the study area.[2] The font size at each prediction location was adjusted to provide a continuous prediction surface of square pixels. Prediction maps

[2] ArcGIS®Version 9.1, Environmental Systems research Institute, Redlands, Ca.

were generated for the value of the parameter of interest. Parameters modeled included the comet optical density, tail length, percentage of DNA in the tail, olive tail moment, and the half-peak coefficient of variation.

Results

Flow cytometry results were fit best with the extended model as were the alkaline single cell gel electrophoresis parameters comet optical density and the percentage of DNA in the tail. Model comparison results are provided in Table 1. Maps of herd locations and the predicted values for parameters fit best with the extended model are provided in Fig. 1.

Evaluation of the map of predicted coefficients of variation indicated an area of decreased variation in DNA content in close proximity to and to the north and east of the industrial facilities. There was also a cluster of reduced variation to the north–northwest of the facilities. There were three clusters of increased DNA damage as measured by flow cytometry. The largest of these clusters was centered approximately 7 km down-prevailing wind from the industrial facilities. Of the two smaller clusters of increased damage, one was located in a similar direction and in closer proximity to the facilities with the other being located approximately fifteen kilometers to the north–northeast (Fig. 1b).

Evaluation of the map of predicted comet optical densities revealed three clusters of increased values and a clear spatial gradient across the study area. The largest cluster of increased DNA damage was oriented in close proximity to the plastics facility and extended approximately 10 km in a down-prevailing wind direction. The two smaller clusters were located to the north–northeast and west of the industrial facilities (Fig. 1c).

Evaluation of the map of the predicted percentages of DNA in the tail of the comet revealed one cluster of decreased predicted values to the north of the facilities. There were three small clusters of increased predicted values in close proximity to the industrial facilities. There were also two additional clusters of increased predicted values locate at a greater distance from the facilities. One was located to the north–northwest and approximately 6 km from the industrial facilities. The second was located at a greater distance from and to the north of the facilities (Fig 1d).

Table 1 Comparison of model fit provided by base and extended models

Outcome	DIC base model	DIC extended model
Flow cytometry		
Coefficient of variation	547.125	538.602*
Comet assay		
Comet optical density	8094.73	7902.93*
Tail length	2077.22	2076.7**
% of DNA in tail	1611.6	1605.8*
Olive tail moment	898.637	901.527**

* Considered to provide a significant improvement in model fit

** Not considered to provide a significant improvement in model fit

Discussion

While concern over the effects of industrial pollutant exposure is heightened in close proximity to industrial facilities, few studies address the risk of adverse response associated with location. Wind patterns in this study area are dominated by a consistent on-shore flow with prevailing winds being from the southeast. Location north to northwest of and close proximity to the industrial facilities increased the degree of concern expressed by participating ranchers.

The distribution of predicted coefficients of variation indicated that factors affecting this testing modality extended across multiple herds. In the case of the cluster of decreased predicted DNA damage, proximity to the industrial facilities appeared to be protective with the lowest predicted values occurring nearest to industrial activities. The large cluster of increased predicted DNA damage was located down-prevailing wind but at a greater distance from industry. This increase in coefficients of variation across multiple herds is consistent with increases in chromosomal aberrations in this area which is suggestive of an environmental cause. Possible explanations for the spatial distribution of coefficients of variation identified during this study include confounders such as non-industrial environmental conditions and management practices or a non-linear distribution of pollution induced DNA damage. When pollutants are released into either air or water many factors affect how they are distributed in the environment. These include the physical properties of the chemicals released, the type of release system utilized, and weather conditions present at the time of release (Lawson et al. 2003).

Of the various comets test parameters evaluated the spatial distribution of comet optical density results provided the greatest cause for environmental concern in this study. Spatio-temporal modeling of comet optical density results provided strong evidence for the presence of a spatial orientation of DNA damage downwind of the industrial facilities. These results were indicative of an increase in locational risks for genotoxicity in this area. Comet optical densities have been shown to increase in the presence of protein–DNA cross-linking which has been

associated with exposures to acetaldehyde (Merk and Speit 1999; Speit et al. 2000). There was in excess of 907 kg of acetaldehyde fugitive air emissions by the plastics facility in 2002 (USEPA 2004). One possible explanation for the observed spatial distribution was DNA damage resulting from the uncontrolled release of acetaldehyde or other industrial pollutants.

The comet test parameter, percentage of DNA in the tail, is a measure of the amount of damaged DNA in a cell. As single strand breaks or alkali-labile additions occur, they result in smaller fragments of DNA which travel a greater distance than intact strands of DNA during electrophoresis. The spatial distribution of the predicted percentage of DNA in the tail of the comet was dominated by local correlation and did not demonstrate a generalized spatial orientation as the two parameters discussed above with the majority of the prediction area having a randomly distributed appearance. One possible explanation for this finding may be an inappropriate classification of which model provided the best model fit. An improvement in DIC of three or more with the extended model was considered as providing an indication of important spatially dependent location effects. This value was arbitrarily chosen and may have been insufficient in identifying substantial improvement associated with inclusion of the spatial term. The improvement in DIC for the percentage of DNA in the tail was 5.8 as compared to improvements of 8.5 and 81.8 for coefficients of variation and comet optical density, respectively.

While this study was not designed to answer questions concerning elevations in DNA damage in response to exposure to particular chemicals, it did address the potential for experiencing genotoxicity with increased DNA damage being present in close proximity and down-prevailing wind from industrial facilities. There were increased locational risks for genotoxicity in cattle as measured by the biomarkers utilized with coefficients of variation and comet optical density being elevated in common areas. When considering the results together, our conclusion is that in this study area, location down-prevailing wind resulted in increased risk of genetic damage in cattle. For the comet optical density, proximity also appeared to result in increased risks. Genotoxic responses in this sentinel species provide evidence that the environmental quality may be compromised in similar areas with industrial emissions being one explanation for the genetic damage found. This study provides support for the need to perform additional research on the clinical significance of the increase in evaluated parameters, the body-burdens of pollutants in cattle in this study area, and the association between body burden of pollutants, Comet assay parameters, variations in DNA content, and the genomic changes associated with exposures.

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