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A TOXICOKINETIC/ACCELERATED FAILURE TIME ANALYSIS OF THE MARE REPRODUCTIVE LOSS SYNDROME (MRLS)

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ABSTRACT

This paper reports a toxicokinetic/statistical analysis of experimental and field MRLS data. Dose/time of abortion curves for late pregnant mares intubated with 100 and 50 g/day of Eastern tent caterpillars (ETC) and 100 g/day of irradiated ETC were fit by simple regressions with dose specific 'lag-time' delays in the abortion process.

Accelerated failure time (AFT) survival model analysis showed that the 'lag-time' depends on ETC dosage. The model takes the form: $T_i = \exp\{ß_0 + ß_1 x_{i1} + \ldots + ß_k x_{ik} + \sigma\varepsilon_i\}$ and includes an intercept term ($ß_0$) and an error term ($\sigma\varepsilon_i$). T_i is the time that abortion occurs for an individual *i*, and $ß_1$ through $ß_k$ are coefficients for covariates that might affect abortion time. Using the MRLS data, AFT Regression yields the following model:

$T_i = \exp\{5.477 - 0.017\ \text{dose}_i + 2.182\ \text{irradiatedi} + 0.526\ \varepsilon_i\}$

SE:	0.480	0.006	0.353	0.101
p:	<0.0001	0.0052	<0.0001	

where dose_i is the dose of caterpillars for individual *i*, and irradiated_i equals 1 if individual *i* was irradiated and 0 if not.

The analysis shows that time to abortion decreases as dose increases. Dose/time response curves from the AFT model fit the experimental dose/abortion data at a high level of significance. Estimated lag-times from the AFT model are 10, 20 and 80 h for the 100, 50, and 100 irradiated g/d doses of caterpillars, respectively. A family of dose/time abortion curves for varying doses of ETC was calculated, allowing interpretation of any ETC related sequence of abortions as 'intubated ETC equivalents'. The model suggests that all of the abortion events are quantitatively related and shows that the relationship between ETC dose and the abortion response is exponential. Exposure to high concentrations of ETC produces intense, focused outbreaks of ETC-related abortions, closely linked in time and place to migrating ETC, as occurred in Kentucky in 2001. Lower doses produce delayed and more diffuse responses, less closely related to ETC exposure. The model suggests that the initial exposure to ETC commenced on or shortly before 27th April, 2001 and was initially equivalent to about 5 g/day of intubated ETC, increasing to the equivalent of about 30 g/day of intubated ETC at the peak of the 2001 MRLS outbreak.

This statistical model throws light on the epidemiology, clinical presentations and manifestations of MRLS, allows quantitative interpretation of field and experimental MRLS and also throws light on the basic toxicological/pathological mechanisms underlying MRLS.

INTRODUCTION

In 2001, central Kentucky experienced acute transient epidemics of early and late fetal losses (EFL/LFL), pericarditis and unilateral endophthalmitis, together called the mare reproductive loss ssyndrome (MRLS). During late April and early March, EFT totalled ~2,500 cases and LFL more than 650 cases (Harrison 2001).

Some mares presented foals normally but showed evidence of amnionitis with yellowish discoloration and oedema of the placental membranes. Pathophysiologically, there was an intense placentitis associated with thickened

TABLE 1a: 2002 ETC intubation data: time of abortions and bacteria isolated from alantochorion and stomach contents following dosing with ETC (50 g/day x 10 days)

Parameters	Mare 220	Mare 78	Mare 398	Mare 390	Mare 395	Mare 393
Time of abortion	62 h	166 h	142 h	95 h	69 h	350 h
Bacteria isolated	*Enterobacter sakazaki*	*Serratia marcescens, Enterococcus* Spp.	*Enterobacter cloacae*	*Enterobacter cloacae*	*Enterobacter cloacae*	*Serratia marcescens Enterococcus species*

TABLE 1b: 2003 ETC intubation data: time of abortions and bacteria isolated from alantochorion and stomach contents following dosing with non-irradiated ETC (100 g/day x 10 days)

Parameters	Mare 647	Mare 668	Mare 638	Mare 305	Mare 630	Mare 10
Time of abortion	32 h	32 h	46 h	48 h	84 h	120 h
Bacteria isolated	*Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*

TABLE 1c: 2003 ETC intubation data: time of abortions and bacteria isolated from alantochorioan and stomach contents of the 3 aborted fetuses following dosing with irradiated ETC (100 g/day x 10 days)

Parameters	Mare #637	Mare 619	Mare 172	Mare 628	Mare 351	Mare BUC
Time of abortion	280 h	326 h	567 h			
Bacteria isolated	*Streptococcus spp.*	*Actinobacillus spp.*	*Streptococcus spp.*			

placental membranes. Funisitis associated with the amniotic section of the umbilical cords of these neonates was observed with ecchoymotic and petechial hemorrhage on the surface of the cords. Microbiologically, the consistent recovery of *alpha-hemolytic streptococci* and *Actinobacillus* sp. from umbilical tissue, lungs, and placentas of affected animals was almost a hallmark for defining the clinical entity of LFL/MRLS (Williams *et al.* 2002).

Of approximately 21,000 Thoroughbred broodmares in central Kentucky pregnant with 2001 and 2002 foals in 2001 (Dwyer *et al.* 2003), about 17% lost their foals. Coincident with the 2001 MRLS epidemics, small numbers of horses in central Kentucky exhibited panophthalmitis (n =~30) or pericarditis (n=~60). The endophthalmitis was always unilateral and progressed to blindness despite intensive therapy (Latimer 2002). Both of these syndromes are now considered part of the MRLS entity.

Concurrent with the 2001 EFL/LFL epidemic was a local population explosion of Eastern Tent Caterpillars (ETC), *Malacosoma americanum*, with large numbers dispersing in pastures and elsewhere when EFL/LFL peaked. Contemporary field evaluations pointing to strong time and location correlations between EFL and presence of ETC, and rigorous epidemiological surveys strongly suggested that ETC exposure played a critical role in the syndrome (Dwyer 2002; Cohen *et al.* 2003). These associations were confirmed by experimental approaches when ETC next appeared in central Kentucky during the spring of 2002 (Bernard *et al.* 2002; Webb *et al.* 2002; Sebastian *et al.* 2003).

This paper presents a retrospective toxicokinetic/statistical analysis of experimental data generated from field data during the 2001 MRLS outbreak and ETC experiments during the 2002 and 2003 ETC seasons. The analysis uses the accelerated failure time (AFT) survival model which is specifically designed to relate events to the time at which they occur. The ramification of combining experimental data from multiple years is that there could be differences in conditions between the years that are not accounted for. The prospective experimental data were used to create the AFT model, while the retrospective field data were examined post-hoc in light of the AFT model.

MATERIALS AND METHODS

ETC administration experiments included dosing of 50 g/day of ETC from northern Michigan for 10 days by stomach tube to 6 mares in late pregnancy in 2002 (Table 1a; Sebastian *et al.* 2003). In a 2003 experiment to evaluate the effect of irradiation on the abortigenic efficacy of ETC, 100 g/day of non-irradiated (Table 1b) and 100 g/day of irradiated (Table 1c) ETC from central Kentucky were administered for 10 days to 2 groups of 6 mares in late pregnancy via stomach tube.

Statistics: regression analysis and accelerated failure time model

Regression analysis

The data points from the 50 and 100 g/day experiments were first plotted as percent of total abortions against time, and best-fit logarithmic regressions were calculated. The x-intercepts (apparent 'lag-times') were calculated by setting the value of y equal to zero in the equation.

Accelerated failure time model

The data points were also fitted using the AFT survival model, because it is a regression model that allows for the prediction of the time at which events occur based on independent variables. In this case, the time at which abortions occur are predicted based on dose of ETC. The AFT model can also take into account censored data, ie when an event has not occurred by the time the experiment has ended. In these experiments, mares that have been dosed with ETC but have not aborted by the end of the data collection period are censored observations. A log-normal AFT model has been used to fit the ETC data. That is, if T is the time at which abortion occurs, log T is assumed to have a normal distribution, and thus T has a log-normal distribution. The model also assumes that the error term is normally distributed. The model used here assumes that the probability of an abortion event remains close to zero for a period of time after the first administration, which is dependent on the dose administered. This model takes the form:

$$T_i = \exp\{ß_0 + ß_1 x_{i1} + \ldots + ß_k x_{ik} + \sigma\varepsilon_i\}$$

The Xs are variables that might affect abortion time (dose of ETC). The ßs are coefficients that estimate the affect of the x variables. The ε is an error term that is assumed to be normally distributed, while σ is a scale parameter for the error term.

A likelihood ratio test of the fitted AFT model verses a null model (with no explanatory variables) yields a chi-square statistic of 27.9 with 2° of freedom. This statistic is significant at the $P<0.0001$ level, which indicates that the model with parameters ETC dose and irradiation is a significant improvement over the null model for predicting time until abortion. The individual chi-square tests for these effects are also highly significant, which tells us that both variables are important to the model.

Based on the AFT analysis, time courses of abortion responses were projected for doses of 5 to 500 g/day of ETC. The AFT model and this family of dose response curves allows dose and time response interpretation of any ETC-related sequence of abortions in terms of the dosage unit used in these experiments, which have been defined as 'intubated ETC equivalents'.

A constraint in this model is that none of the ETC dosings were continued beyond 10 days, while AFT analysis assumes that dosing/exposure to the caterpillars is continuous. Because of this, the mathematical fit of the AFT model to the data is presumably more accurate at the higher doses of ETC than at the irradiated ETC, which exhibited an apparently much lower abortigenic activity. A further constraint is that the duration of field exposure to dispersing ETC at any given location is rarely longer than 20 days.

TABLE 2: Estimated number of hours at which 0, 50, and 100% of abortions occur following various doses

Predicted % aborted	100 irradiated	Dose (g) 1	5	50	100	200	300
0	80	55	50	20	10	2	0.3
50	470	285	266	124	53	9.8	1.8
100	4900	3000	2800	1300	550	100	19

Results

Regression

In the first experimental reproduction of the LFL syndrome (Sebastian *et al.* 2003), 50 g/day of ETC were administered for 10 days, and all treated mares aborted rapidly. The first abortions occurred within 72 h, and all mares aborted within 14 days (Table 1a). In the 100 g/day ETC experiments, mares receiving non-irradiated ETC began aborting within 32 h, and all mares had aborted by Day 5 (Table 1b). In contrast, mares receiving irradiated ETC did not begin aborting until Day 12, two days *after* the last dose of ETC (Table 1c). Furthermore, the most recent abortion from this group occurred 24 days after initial exposure to ETC, which was 14 days after the last administration of ETC. No further abortions had been recorded in this group at the time of writing, about 70 days after the initial dosing (15th May 2003). Regressions for each dose/abortion time data set were then calculated.

The abortion curves for each of these experiments were similar in shape and apparently were related. The 100 g/day curve was shifted to the left compared to the 50-g/day curve, suggesting a dose response effect. Classical dose response curves show parallel slopes, but the slopes of these curves were markedly different, with the steeper slope of the 100 g/day curve presumably relating to the higher dose of ETC administered.

Survival analysis

Application of the data points to AFT analysis (LIFEREG procedure in SAS version 8, SAS Institute, Inc., North Carolina, USA) produced a very satisfactory data fit. The AFT analysis assumes that the data points are log-normally distributed and also reflects the fact that the probability of an abortion remains close to zero for a dose-dependent period of time after dosing begins (the apparent 'lag-time'). The AFT model was then used to estimate the number of hours after dosing for abortions to occur. Using the data presented above, AFT regression yielded the following model:

$$T_i = \exp\{5.477 - 0.017\ \text{dose}_i + 2.182\ \text{irradiated}_i + 0.526\ \varepsilon_i\}$$

SE:	0.480	0.006	0.353	0.101
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where dose_i is the dose of caterpillars for individual *i*, and irradiated_i equals 1 if individual *i* was fed irradiated and 0 if not. The coefficients indicate that the time until abortion decreases with increasing dose and increases if the ETC were irradiated. All coefficients were highly significant in the model, as indicated by the *p* values.

The model was used to explore the relationships between dose of ETC and daily abortion rate. The analysis shows that exposure to 100 g/day of ETC rapidly causes abortions that peak 3–4 days after exposure, and essentially all of the exposed mares will abort by Day 10. At a dose of 30 g/day, abortions peak at ~6 days after exposure and are complete within ~18 days. If the abortigenic dose of caterpillars is reduced to an amount equivalent to that present in the irradiated 100 g/day ETC, the first abortions do not begin to appear until about Day 8, and the abortions will not peak until Day 20.

The bacteria isolated from the aborted fetuses were *Serratia marcescens*, *Enterococcus* spp. and *Enterobacter* spp. in the 50 g/day study (Table 1a), *Serratia marcescens* in the 100 g/day study (Table 1b), and *Streptococcus* and *Actinobacillus* spp. in the irradiated study (Table 1c). An unusual aspect of MRLS is this very wide spectrum of bacterial species isolated from aborted foals (Donahue *et al.* 2002). The close fit of the experimental data to the AFT model may suggest that the role of different bacterial species in the abortion events is not a critical determinant of the time to abortion, or of the fundamental mechanism that drives this syndrome.

Discussion

Because the AFT survival analysis is appropriate for constructing models that relate an event to the time at which it occurs, it was selected to fit these data points, and all coefficients were statistically significant. It was then used to estimate dose/abortion time curves for a series of doses from 5 to 500 g/day. The very good fit of the ETC dose/abortion time data by the AFT model provides a solid statistical basis for integration and interpretation of experimental and field MRLS data. Early in the exposure period, the lag time represents the period during which the likelihood of an abortion occurring remains close to zero. Following the lag time, the rate at which abortions occur accelerates to the peak abortion rate. The abortions then proceed until either all of the exposed mares abort or until some time, currently unknown, after exposure to ETC has ceased.

This analysis makes clear that it is the *rate* at which abortions occur, rather than the absolute number of abortions, that establishes the relative abortigenic efficacy of any ETC treatment. The AFT model predicts that ETC doses anywhere between 5 and 500 g/day of ETC will, if maintained, sooner or later abort 100% of the exposed mares.

The decreased rate of abortions for irradiated ETC suggests that the efficacy of the ETC abortion-inducing factor was substantially reduced by the irradiation treatment, or something associated with this treatment, since the first abortion did not occur until 12 days after the first ETC administration. The abortions then proceeded slowly and, according to AFT analysis, the time to the last abortion (100%) will be about 206 days, assuming continuous dosing. Based on the AFT model, the apparent efficacy of the abortigenic factor in irradiated ETC is less than 1 g/day of untreated ETC, for an apparent loss of >99% of the ETC-related abortigenic activity.

The mathematical relationships described by the AFT model explain many aspects of the epidemiology/clinical presentations of MRLS. If the exposure to ETC is high, all susceptible exposed mares are likely to abort within a relatively short period. Of critical importance during the 2001 investigation of MRLS, when the dose of ETC is high and the abortions occur rapidly, there will be an obvious temporal relationship between the presence of caterpillars and the abortions, as occurred during 2001. In point of fact, it was the close spatial and temporal relationships between the presence of the ETC and the appearance of MRLS (J. Henning, personal communication), as well as the lack of any other convincing hypothesis, that led to the early conclusion that ETC were most likely associated with MRLS (Tobin 2003a,b; Tobin *et al.* 2004).

While the AFT model predicts that continuous exposure to low doses of ETC will eventually abort all exposed susceptible mares, this is not likely to happen in the field, where exposure is transient. Once ETC exposure stops, the abortion process eventually ceases, and the full abortion potential of a low dose is never achieved. As such, with lower doses of ETC, the lag-time is longer, and the actual fraction of possible abortions likely to occur is smaller.

The critical factor in the identification of MRLS in 2001 was the high concentration of pregnancies closely monitored first via ultrasound by highly skilled practitioners, and shortly thereafter by skilled pathologists and veterinary scientists. EFL was recognised within hours of its first appearance, infectious causes were very rapidly ruled out, and the syndrome was defined and described within weeks. The caterpillars also were rapidly pinpointed and an intensive search for a caterpillar-associated toxin or factor was underway by Week 3.

The conclusions drawn from this analysis are valid for LFL and throw much light on the epidemiology and clinical presentation of the 2001 MRLS outbreak. The analysis also carries some clear messages for further work. In the first place, any attempts to quantitatively relate experimental ETC exposure to abortion responses need to ensure that the database includes abortions of all exposed mares. This is because it is difficult to estimate the rates of abortion if only a small fraction of the exposed mares have aborted and, as this analysis shows, it is the rate at which the abortions occur that relates directly to the dose of ETC abortigenic activity.

The second factor, and the one that is not addressed in this analysis, is the apparent ability of horses and also late-term fetuses *in utero* to resist the mechanism of ETC-induced abortions and the other syndromes associated with MRLS. The birth of a large number of weak foals with high mortality during the 2001 ETC season is consistent with an MRLS related subclinical disease that failed to produce 'dead foal' cases of LFL. These cases and the small number of pericarditis and uveitis cases illustrate a substantial resistance to the pathogenic mechanism of MRLS in late-term fetuses and in horses in general, which need to be taken into account in any model of the pathogenesis of MRLS.

Acknowledgements

Published as #338 from the Equine Pharmacology and Experimental Therapeutics Program at the Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky.

Published as Kentucky Agricultural Experiment Station Article 04-14-020 with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station.

Supported by grants from the USDA Agricultural Research Service Specific Cooperative Agreement for Forage-Animal Production Research, the Kentucky Department of Agriculture and the Kentucky Owners and Breeders Association.

The data presented in this paper, in part, also appeared in *Veterinary Therapeutics* **4**, 324-339, 2003.

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