Association between large strongyle genera in larval cultures – using rare-event Poisson regression

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(*Received 1 February 2013; revised 6 April 2013; accepted 8 April 2013; first published online 4 June 2013*)

SUMMARY

Decades of intensive anthelmintic treatment has caused equine large strongyles to become quite rare, while the cyathostomins have developed resistance to several drug classes. The larval culture has been associated with low to moderate negative predictive values for detecting *Strongylus vulgaris* infection. It is unknown whether detection of other large strongyle species can be statistically associated with presence of *S. vulgaris*. This remains a statistical challenge because of the rare occurrence of large strongyle species. This study used a modified Poisson regression to analyse a dataset for associations between *S. vulgaris* infection and simultaneous occurrence of *Strongylus edentatus* and *Triodontophorus* spp. In 663 horses on 42 Danish farms, the individual prevalences of *S. vulgaris, S. edentatus* and *Triodontophorus* spp. were 12%, 3% and 12%, respectively. Both *S. edentatus* and *Triodontophorus* spp. were significantly associated with *S. vulgaris* infection with relative risks above 1. Further, *S. edentatus* was associated with use of selective therapy on the farms, as well as negatively associated with anthelmintic treatment carried out within 6 months prior to the study. The findings illustrate that occurrence of *S. vulgaris* in larval cultures can be interpreted as indicative of other large strongyles being likely to be present.

Key words: Large strongyles, *Strongylus*, *Triodontophorus*, larval culture, association, rare event.

INTRODUCTION

The group of equine strongyle parasites consists of about 60 different species described (Lichtenfels *et al.* 2008). They are widely occurring across the world and are practically ubiquitous in grazing horses. Traditionally the group is subdivided into the large (strongylinae) and the small (cyathostominae) strongyles. The small strongyles consist of 50 different species distributed over 14 different genera. During the 1980s, the emphasis was shifted from the pathogenic large strongyles that were declining in prevalence (Herd, 1990) to the small strongyles that were developing resistance to commonly used anthelmintics (Kaplan, 2004), and now also recognized as potentially significant pathogens (Love *et al.* 1999). The large strongyles comprise the three *Strongylus* species *Strongylus vulgaris, Strongylus equinus* and *Strongylus edentatus*, but also *Triodontophorus* spp. as well as *Craterostomum acuticaudatum, Oesophagodontus robustus* and *Bidentostomum ivaschkini* (Lichtenfels *et al.* 2008). Common to the large strongyles is their large buccal capsules, but only larvae of *Strongylus* species migrate outside the intestinal tract.

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The pathogenic impact of *Strongylus* spp. is well described. They all have prepatent periods of 6 months and above (Round, 1969) and spend several months migrating through various tissues and organs of the horse. The life cycle of *S. vulgaris* is classical in veterinary parasitology, with the L3s migrating towards their predilection site at the root of the cranial mesenteric artery. Here, they dwell for several months before they are transported by the bloodstream back to the large intestinal walls (Duncan and Pirie, 1972). Pathological lesions caused by the larvae have been associated with a painful and sometimes fatal colic syndrome named 'thromboembolic colic' (Enigk, 1951). The larvae of *S. edentatus* migrate via the portal system to the liver, from where they continue to the adipose tissue in the ventral abdominal walls and eventually back to the intestinal walls (McCraw and Slocombe, 1978). The prepatent period of *S. edentatus* has been reported to be around 11 months (Wetzel, 1952). *Strongylus equinus* has become very rare in managed horse populations, but has been described to migrate through the peritoneal cavity, passing through the pancreas on the way to the liver and eventually back to the intestine. Larvae of *S. edentatus* and *S. equinus* are described to cause significant lesions, but unlike *S. vulgaris* they have not been associated with any specific clinical syndromes. The life cycles of the remaining large strongyle species strongly resemble

Parasitology (2013), **140**, 1246–1251.
doi:10.1017/S0031182013000589

those of the cyathostomins, without a pronounced phase of tissue migration and with immature stages undergoing encystment in the large intestinal walls. The prepatent period of *Triodontophorus* spp. has been reported to be 2–3 months (Round, 1969). There are no specific clinical syndromes ascribed to *Triodontophorus* spp. infection. It is worth noting, however, that *Triodontophorus tenuicollis* causes characteristic lesions in the dorsal colon consisting of deep ulcers, in which whole colonies of adult parasites of this species are residing (Drudge, 1972). The clinical significance of these lesions is unknown.

Compared with the cyathostomins, large strongyle species are generally rare in occurrence and less abundant, when present (Lyons *et al*. 2006). To date, there have been no convincing reports of anthelmintic resistance in any large strongyle species, and this appears to largely explain the prevalences of these observed in managed horse populations (Lyons *et al*. 2000). We recently published a study illustrating that on Danish farms adopting the widely recommended selective therapy principle, where horses are treated based on their fecal egg count level, *S. vulgaris* had a significantly higher occurrence compared with farms that did not use selective therapy (Nielsen *et al*. 2012). This prompts for implementing routine surveillance of *S. vulgaris* on farms using similar parasite control programmes, and one survey documented that larval cultures are widely used by Danish equine practitioners (Nielsen *et al*. 2006). However, the negative predictive value for diagnosing *S. vulgaris* infection in individual horses has been found to be 0·37, which allows for false negative results (Nielsen *et al*. 2010*a*). Other large strongyle species can be identified in the larval cultures as well, but it remains unknown if these can be interpreted as indicative of *S. vulgaris* being potentially also present. In the dataset generated in our recent publication (Nielsen *et al*. 2012), large strongyle parasites besides *S. vulgaris* were identified in the larval cultures, but they were sparse in occurrence. While it can be hypothesized that there is an association between the occurrences of these large strongyle species, it remains a statistical challenge to analyse such data given the relatively rare occurrence of these species.

The aim of the present study was to adopt a rare-event Poisson regression to evaluate whether the presence of *S. vulgaris* in individual horses was associated with presence of *S. edentatus* and *Triodontophorus* spp. also recorded in the dataset mentioned above.

MATERIALS AND METHODS

The study design has previously been described (Nielsen *et al*. 2012), but will be briefly outlined below.

Farms

Veterinary practitioners were contacted in all regions of Denmark, and asked to enrol farms in the study in the spring months of 2010 (March–May). Inclusion criteria were as follows: farm sizes should be of 6 horses or more, anthelmintic treatments should not have been prescribed on the farm for at least 2 months prior to the onset of the study, and anthelmintic strategies should fall into one of two broad categories: (1) anthelmintic treatments were based on routine fecal egg counts performed from all horses, or (2) anthelmintic treatments were applied to all horses without performing fecal egg counts. As a guideline, each veterinarian was asked to identify one farm in each of these categories for the study. For each farm, information about the time of the most recent anthelmintic treatment was recorded for every horse.

A total of 663 horses from 42 different farms entered the study. For a detailed description of the participating farms and horses, the readers are referred to our recent publication (Nielsen *et al*. 2012). The principles defined in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes were followed, and the study was designed to collect data from the minimum number of horses needed to produce experimentally reproducible results.

Parasitological procedures

Fecal samples. Samples were collected by the participating veterinarians following published recommendations (Nielsen *et al*. 2010*b*). Samples were collected fresh, packed airtight in plastic bags and shipped overnight to the parasitology laboratory at the Department of Large Animal Sciences, University of Copenhagen. Here, samples were kept refrigerated and cultures were set up within 5 days.

Larval cultures. Larval cultures for detecting large strongyle parasites were performed as follows: Ten grams of feces were weighed and mixed with an equal volume of vermiculite. Tap water was added to yield a moist texture of the feces. Samples were cultured in individual humidity chambers created as described by Henriksen and Korsholm (1983). Incubation occurred at room temperature (20–24 °C) for 14 days, during which time samples were regularly checked for desiccation, and more water added if necessary. Upon incubation, third-stage larvae were harvested after 24 h of sedimentation in a Baermann glass. All larvae were examined and identified under the microscope using morphological criteria (Russell, 1948). All cultures were examined by the first author. Results were recorded as total number of larvae harvested, and total number of *S. vulgaris*,

https://doi.org/10.1017/S0031182013000589 Published online by Cambridge University Press

S. edentatus and *Triodontophorus* spp. larvae identified.

Statistical analyses

Development of statistical framework for rare-event analysis. To develop the statistical frame work, we express the data as a 2 × 2 table involving two levels of two factors: Factor 1 concerns occurrence or non-occurrence of *S. vulgaris* in each horse; and factor 2 concerns occurrence or non-occurrence of *S. edentatus* (or *Triodontophorus* spp.) in each horse. When relative risk is our primary interest in the 2 × 2 table, binomial regression is usually recommended since the data in each cell of the table represent counts. However, if the probability of some cells are very small (referred to as a rare event), convergence problems arise with binomial regression models; in this case, they may fail to provide an accurate estimate of the relative risk. Under these circumstances, Poisson regression has been known to be a better choice (Wallenstein and Bodian, 1987; Zou, 2004).

Returning to our problem, let n be the total number of horses that are positive for *S. vulgaris* and let X denote the number amongst these horses that are also positive for *S. edentatus*; let p denote the probability that a horse that is positive to *S. vulgaris* is also positive for *S. edentatus*. The probability distribution of number of horses that are positive for both *S. vulgaris* and *S. edentatus* (X) is given by the following binomial distribution:

$$P(X = x) = \frac{n!}{(n-x)!x!} p^x (1-p)^{n-x}$$

If the event is rare, and p is very small, then the binomial distribution can be approximated by the Poisson distribution. Hence, if $\lambda = np$, the distribution for X is given by

$$P(X = x) \approx \frac{e^{\lambda}\lambda^x}{x!}$$

This is the probability mass function for the Poisson λ distribution. However, when Poisson regression is applied to binomial data, the error for the estimated relative risk will be overestimated, leading to reduced statistical power (Zocchetti *et al.* 1995). This problem may be addressed by using a robust error variance procedure, referred to as modified Poisson regression (Zou, 2004). Following the work in Zou (2004), one can show that

$$\widehat{RR} = \frac{an_0}{cn_1},$$

where a and c correspond to the number of events with and without occurrence, respectively, which refers to the classical positions in the 2 × 2 table. Further, n_0 is the total number of horses with larval cultures negative for *S. vulgaris* and n_1 represents the total number of horses with *S. vulgaris* infection detected. The estimated variance is given by:

$$\mathrm{Var}(\widehat{RR}) = \frac{1}{a} + \frac{1}{c}$$

And its robust version is given by:

$$\mathrm{Var}(\widehat{RR}) = \frac{1}{a} + \frac{1}{c} - \frac{1}{n_0} - \frac{1}{n_1}$$

The robust error estimation can be implemented by using the genmod procedure with repeated statements in SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

Similar analyses were performed for other occurrence variables in the dataset: (i) usage of selective therapy on the farm, and (ii) anthelmintic treatment within the last 6 months prior to the study.

Validation by computer simulation

To validate the significance of the robust error modification, simulations were performed comparing the percentage of confidence interval (CI) coverage with the modified *vs* the unmodified Poisson regression. The field dataset had 663 observations, which can only allow for relatively small sample sizes with resulting wide CIs. Therefore, for the simulations, total sample sizes N were considered as 500 and 1000, with relative risk values of 1, 2 and 5. In each of the 1000 simulated datasets, n subjects were randomly assigned to the *S. vulgaris* positive and negative groups with the same probability of 0·5. The probability of getting an event (i.e. infection with *S. edentatus* or *Triodontophorus* spp.) in the occurrence group was set to be 0·2, while it was 0·2, 0·1 and 0·04, respectively, in the non-occurrence group. These values were chosen to reflect a range of realistic probabilities as observed in the field dataset reported in this article.

Poisson regression analyses were performed both with and without the robust error modification described in the previous section, and 95% CIs are constructed using the original standard error (referred to unmodified in the tables) and the robust version (referred to as modified in the tables). The procedure was repeated 1000 times for each setting of N and RR, and the analysis yielding the empirical CI coverage closest to 95% was considered the more accurate approach.

Relative risks of S. edentatus *and* Triodontophorus *spp.*

Using the modified Poisson regression described above, the risks of occurrence of *S. edentatus* and *Triodontophorus* spp. were evaluated relative to the presence of *S. vulgaris*, recent anthelmintic treatment and use of selective therapy. When the 95% CI for the generated RR included 1, results were considered non-significant.

https://doi.org/10.1017/S0031182013000589 Published online by Cambridge University Press

Table 1. Empirical coverage of the 95% confidence intervals generated by the modified Poisson regression compared with the unmodified version of the same analysis

Relative risk	Sample size	Risk (exposed/ unexposed)	Poisson regression	
			Unmodified	Modified
1	500	0·2/0·2	97·1%	94·9%
	1000	0·2/0·2	96·5%	94·9%
2	500	0·2/0·1	96·2%	94·6%
	1000	0·2/0·1	96·8%	94·9%
5	500	0·2/0·04	97·0%	95·6%
	1000	0·2/0·04	96·1%	95·4%

Table 2. Risk of occurrence of *Strongylus edentatus* relative to presence of *Strongylus vulgaris* in individual horses, recent anthelmintic treatment and use of selective therapy on the farm

	Relative risk	95% confidence intervals
Presence of *S. vulgaris*	7·19	3·07–16·73
Recent anthelmintic treatment[a]	0·24	0·07–0·82
Selective therapy	13·30	1·79–98·76

[a] Defined as treatment within 6 months prior to the study.

Table 3. Risk of occurrence of *Triodontophorus* spp. relative to presence of *Strongylus vulgaris* in individual horses, recent anthelmintic treatment and use of selective therapy on the farm

	Relative risk	95% confidence intervals
Presence of *S. vulgaris*	2·19	1·37–3·52
Recent anthelmintic treatment[a]	0·93	0·60–1·430
Selective therapy	1·30	0·88–2·02

[a] Defined as treatment within 6 months prior to the study.

RESULTS

Of the participating horses, 211 (32%) had been treated within the last 6 months prior to the study. Of these, only 14 (2%) were treated within the last 2 months. As previously reported, the prevalence of *S. vulgaris* in this dataset was 12% on the individual level, and 64% on the farm level (Nielsen *et al*. 2012). The corresponding prevalences reported in this paper for *S. edentatus* were 3% and 14%, respectively, while the prevalences were 12% and 58% for *Triodontophorus* spp. *Strongylus equinus* was not found in this study.

Table 1 presents the results of the computer simulations evaluating the coverage of the 95% CIs generated with the modified compared with the unmodified Poisson regressions. It can be seen that the modified analysis consistently yielded coverage closer to the 95% compared with the unmodified Poisson regression. Tables 2 and 3 present the outcomes of the modified Poisson regressions analysing the risks of the occurrence of *S. edentatus* and *Triodontophorus* spp., respectively, relative to the presence of *S. vulgaris*, recent anthelmintic treatment and use of selective therapy in the study population.

DISCUSSION

We have evaluated and illustrated the usefulness of a modified Poisson regression for analysing data for associations between sparsely occurring parasites in animal populations. Our data illustrate some of the possible associations between large strongyle species and the deworming regimens carried out on horse farms, and provide useful insight into the interpretation of larval culture results.

Both *S. edentatus* and *Triodontophorus* spp. were associated with presence of *S. vulgaris*, but only *S. edentatus* was statistically associated with selective therapy and anthelmintic treatment within the last 6 months. The reason for this is likely to be the large difference in prepatent periods between *S. edentatus* and *Triodontophorus* spp. The large majority of anthelmintic treatments carried out on the studied farms were ivermectin (Nielsen *et al*. 2012), which can be expected to have good larvicidal efficacy against migrating larvae of *S. edentatus*. With the very long prepatent period, just one yearly treatment would be enough to break the life cycle of *S. edentatus*, which can explain the much lower prevalence observed for this parasite. For *Triodontophorus* spp., however, the much shorter life cycle makes it unlikely to see any significant responses to the most recent treatment. This is simply because treatment intensities on the studied farms were too sparse to significantly impact the occurrence of this parasite. Thus, anthelmintic treatment within the past 6 months is unlikely to affect the occurrence of this parasite, while treatment within the past 2 months would be expected to have

https://doi.org/10.1017/S0031182013000589 Published online by Cambridge University Press

an effect. This study was carried out in the spring in Denmark after a winter period, where very few anthelmintic treatments are usually administered, which was illustrated by the fact that only 2% of horses had received anthelmintic treatment within the 2 months prior to the study.

These data illustrate that despite *Triodontophorus* spp. being a large strongyle, it epidemiologically behaves much like the cyathostomins. It is interesting, however, that it was associated with *S. vulgaris* without being related to the use of selective therapy. The scientific literature contains no reports of anthelmintic resistance in *Triodontophorus* spp., and there is no reason to speculate it playing a role in this dataset, as anthelmintic treatment intensities were very low. Of course, our findings could represent a chance association between the species, but there may be other explanations. For instance, horses harbouring *S. vulgaris* may exhibit a larger strongyle species diversity allowing for a higher abundance of *Triodontophorus* spp. The results may also represent a bias occurring when reading the larval cultures under the microscope. The identification of *S. vulgaris* larvae in a sample could have caused the operator to more critically scan the slide for other non-cyathostomin larvae leading to an apparent association between the species.

One practical implication of the results is that whenever *S. vulgaris* is identified on a farm, there is an increased likelihood to also find *S. edentatus*. As recently reported, the larval cultures for identifying either of these two species is characterized by high positive predictive values, but low to moderate negative predictive values (Nielsen *et al.* 2010*a*). This means that false negatives are likely to occur. However, if a finding of one *Strongylus* species can be interpreted as indicative of other *Strongylus* spp. being also present, the negative predictive value of the larval culture can be improved considerably for herd diagnosis.

As with the other large strongyle species, *S. edentatus* has not been convincingly reported resistant to anthelmintic treatment. One study illustrated an apparent loss of pyrantel efficacy (Coles *et al.* 1999), but it should be borne in mind that pyrantel efficacy always had significant variability (Lyons *et al.* 1975) and that it has no activity against migrating stages. Therefore, treatment with a larvicidal drug should still be expected to effectively interrupt the life cycle of *S. edentatus*, and it can be argued that if a horse is positive for *S. edentatus*, it has been exposed to a deworming regimen that also allows for *S. vulgaris* to be present. Given the pathogenic potential and clinical implication of *S. vulgaris*, the primary reason for veterinary practitioners to perform larval cultures is to screen for presence of this parasite (Nielsen *et al.* 2006), and a positive finding is likely to make the veterinarian adjust the deworming regimen on that particular farm. As other large strongyles have not been associated with specific severe parasitic disease complexes to the same extent as *S. vulgaris*, their mere finding in a larval culture is unlikely to lead to major changes in the parasite control programmes. Through the significant associations with *S. vulgaris* found in this study, there is now reason to consider findings of other large strongyles to be of potential clinical importance. Although similar larval culture-based surveys have not recently been performed in regions outside Denmark, results are likely to have relevance elsewhere as surveillance-based parasite control programmes are widely recommended with resulting lower anthelmintic treatment intensities (Kaplan and Nielsen, 2010).

In summary, this study made use of a modified Poisson regression analysis for rare-event data to analyse the association between *S. vulgaris* and other large strongyle species in the field. The results illustrated a strong association between the occurrence of *S. edentatus* and *S. vulgaris*, which can be helpful for interpreting routine larval culture results on farms.

FINANCIAL SUPPORT

This study was funded by the Danish Research Council's Agency for Science, Technology and Production, grant number 274-08-0081.

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