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EPIDEMIOLOGY AND DIAGNOSIS OF *ANOPLOCEPHALA PERFOLIATA* IN HORSES FROM SOUTHERN ALBERTA, CANADA

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ABSTRACT

The cestode *Anoplocephala perfoliata* is known to cause fatal colic in horses. The epidemiology of the cestode has rarely been evaluated in Canada. I detected *A. perfoliata* eggs in 4-18% of over 1000 faecal samples collected over 2 years. Worm intensity ranged from 1 to >1000 worms. Pastured horses were infected more often than non-pastured horses, especially in western Alberta, likely reflecting their higher rates of exposure to mite intermediate hosts. In a comparison of diagnostic techniques, fecal egg counts were the least accurate. Western blot analysis had the highest sensitivity to detect antibodies to the cestode (100%), but had lower specificity. A serological enzyme-linked immunosorbent assay (ELISA) had a lower sensitivity (70%) for detection of antibodies than described in previous studies. A coproantigen ELISA had 74% sensitivity and 92% specificity, and a positive correlation was found between antigen concentration and cestode intensity. The latter is important because it implicates the utility of this method for accurate clinical diagnosis and epidemiological studies.

PREFACE

"A person who never made a mistake, never tried anything new."

"If we knew what it was we were doing, it would not be called research, would it?"

- Albert Einstein (1879-1955)

who was a lifesaver on so many levels during my final field season. Thanks to Clayton James, my partner in research crime, who was great support, but always there to harass me and make me laugh. Peers Chelsea Matisz, Amie Quinn, Rheanna Flitton and Will Warnock made graduate studies an enjoyable experience.

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Malaspina University-College provided so many unique opportunities, one of which was the chance to carry out, present and write up a year-long independent study. I would like to sincerely thank all of the professors and staff that helped me along during

my time there. I would especially like to thank Dr. Tim Goater, the multifaceted, diverse and extraordinary professor who was the first to "infect" me with a passion for parasites.

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This thesis is dedicated to everyone that believed in me, all the cooperative horse owners in southern Alberta, and to the horses that lost their lives for sustenance but were able to contribute to scientific research in doing so. It is also dedicated to all the horses that have ever been part of my life. To Andy, who taught me more life lessons than I could have ever imagined, in particular how important trust can be, and to Arwin who has reminded me that patience is a virtue.

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Chapter 1. General Introduction

Veterinary parasitologists focus on the prevention, control and treatment of

parasites of companion animals and livestock. From an applied context, helminth and

other parasite infections can cause loss of productivity to livestock and they can reduce

survival rates of pets (Thompson, 1999; Coles, 2001). From a conceptual perspective,

parasites of veterinary importance can offer opportunities to better understand

fundamental problems in transmission biology and epidemiology. In two reviews of the

field, Thompson (1999) and Coles (2001) emphasized future aspects of veterinary

parasitology research that should be addressed. Each author stressed the importance of

emerging diseases, evolution of resistance, global warming and the development of

reliable molecular and immuno-diagnostics as key features. They considered improved

diagnosis of micro- and macro-parasites in livestock to be crucial, because there currently

exists no reliable quantitative technique to estimate parasite burdens for many parasitic

diseases, making treatment challenging (Thompson, 1999; Coles, 2001). Thus, the

diagnosis and characterization of epidemiological patterns of infection in livestock and

companion animals remain important challenges for veterinary parasitologists

(Thompson, 1999).

The Anoplocephalid (Cyclophyllidea: Anoplocephalidae) cestodes are a species-

rich taxa of platyhelminth worms, the members of which are cosmopolitan in their host

and geographic distribution. This group of worms has become increasingly important to

veterinary parasitologists because several species have been implicated to cause problems

in domestic livestock (Hansen *et al*., 1950; Armour, 1980; Colwell and Baron, 1990;

Casaravilla *et al*., 2005). There are four subfamilies that have been described:

Anoplocephalinae, Linstowiinae, Inermicapsiferinae and Thysanosomatinae (Beveridge, 1994) containing a total of 32 genera, each of which includes species that are found within the gastrointestinal tract of mammalian hosts (Denegri *et al*., 1998). One unique feature of this family, in addition to their characteristic unarmed scolex (Schmidt, 1986; Beveridge, 1994), is their incorporation of oribatid soil mites as intermediate hosts (Stunkard, 1937; Bashkirova, 1941; Sengbusch, 1977). The subfamily Anoplocephalinae, Blanchard, 1891, includes tapeworms that infect equines (including horses, mules, and donkeys): *Anoplocephala perfoliata* (Goeze, 1792), *A. magna* (Abildgaard, 1789) and *Anoplocephaloides mamillana* (Mehlis, 1831) (Schmidt, 1986; Denegri *et al*., 1998).

Anoplocephala perfoliata has become increasingly recognized for its potential to cause gastrointestinal problems in equines (Tinker *et al*., 1997; Proudman *et al*., 1998; Edwards, 1999; Gasser *et al*., 2005). Stunkard (1939) was the first to confirm that the related Anoplocephaline tapeworm *Moniezia expansa* required oribatid soil mites (Acari: Oribatida) as intermediate hosts. Shortly thereafter, Bashkirova (1941) completed the developmental life cycle of *A. perfoliata*. Eggs are released in the horses' faeces and are then available for ingestion by soil mites. Once ingested, the oncosphere penetrates the mite's body cavity and goes through 5 stages of development depending on temperature, requiring 8-20 weeks to mature, finally creating a capsule with the fifth-stage infective cysticercoid larva (Stunkard, 1939; Bashkirova, 1941; Gasser *et al*., 2005). Infected mites are accidentally ingested by grazing horses and digested to release the oncosphere, or juvenile tapeworm that can attach to the intestinal mucosa (Sengbusch, 1977; Bowman, 2003). Adult *A. perfoliata* are typically found attached to the caecal wall, around the

ileo-caecal junction, but as intensities increase they can also be found in the distal ileum, and ventral colon where they attach themselves using muscular suckers, mature and begin reproduction (Williamson *et al.*, 1997; Beelitz and Goethe, 2001; Bowman, 2003).

With increasing intensity of *A. perfoliata* there is an associated increase in the degree of damage to the intestinal mucosa (Pearson *et al.*, 1993; Fogarty *et al.*, 1994; Nilsson *et al.*, 1995; Williamson *et al.*, 1997; Rodriguez-Bertos *et al.*, 1999). This parasite is also known to cause enteritis (Tennant, 1972), caeco-caecal intussusception (Tennant, 1972; Barclay *et al.*, 1982; Beroza *et al.*, 1986; Edwards, 1986; Owen *et al.*, 1989; Mezerova *et al.*, 2007), eosinophilic infiltration (Rodriguez-Bertos *et al.*, 1999), caecal perforation leading to peritonitis (Barclay *et al.*, 1982; Beroza *et al.*, 1986; Mezerova *et al.*, 2007), thickening of the ileo-caecal valve and mucosa (Pearson *et al.*, 1993), caecal rupture (Ryu *et al.*, 2001) and intestinal obstruction by masses of worms (Slocombe, 1979; Carmel, 1988).

Most importantly, *A. perfoliata* has recently been implicated as a cause of colic (Proudman and Edwards, 1993; Proudman *et al.*, 1998; Proudman and Trees, 1999; Mezerova *et al.*, 2007). An increased risk of ileo-caecal colic in tapeworm infected horses has been reported (Proudman and Edwards, 1993). Proudman *et al.* (1998) stated that with increasing infection intensities of *A. perfoliata* there was an increasing risk of spasmodic colic. Mezerova *et al.* (2007) reported that 5 horses were found to colic from massive tapeworm infections with intussusceptions and ileal perforation, with only one horse recovering. Boswinkel and van Oldruitenborgh-Oosterbaan (2007) found that horses with colic also had a significantly higher *A. perfoliata* antibody level than controls. Researchers have reported the incidence of any potentially fatal colic to range

from 4.2 to 10.6 colics per 100 horses per year (Tinker *et al.,* 1997; Traub-Dargatz *et al*., 2001). Roughly 10% of horses in North America experience colic and about 3% of these cases are fatal (Traub-Dargatz *et al*., 2001). If colic cases caused by *A. perfoliata* can be decreased or eliminated, benefits would be seen in both animal health and the economy of the industry.

Given the proposed linkage of *A. perfoliata* to intestinal disease, it has become increasingly important to understand general epidemiological patterns in horses. As for many parasites of veterinary importance, a poor or incomplete understanding of epidemiological characteristics can lead to poor, inefficient, and expensive treatment (Meana *et al*., 1998). Further, without an accurate picture of transmission rates and associated risk factors (e.g. temperature, soil conditions, and horse management strategies) we have little understanding of when and where hosts are more likely at risk of infection.

A number of studies have documented general epidemiological patterns of *A. perfoliata* infection in horses. These are summarized in Table 1.1. These studies indicate two important and general shortcomings. First, almost all studies involved horses sampled from various sites in Europe; very few studies have been completed on North American horses, with only two completed in Canada. Second, prevalence of *A. perfoliata* in horses has been reported to range from 0.4-82%, with enormous variation between studies and geographical locations (Bain and Kelly, 1977; Sotiraki *et al*., 1997; Table 1.1). Therefore, one key limitation lies in our inability to understand the magnitude or causes of variation in the prevalence and intensity of *A. perfoliata* in horses, especially in Canada.

The enormous variation in infection characteristics of *A. perfoliata* in horses

sampled throughout the world is almost certainly linked to regional and local variation in

environmental characteristics (Hoglund *et al*., 1998; Meana *et al*., 2005). Very generally,

regions characterized by moist humid conditions year-round (e.g. New Zealand) tend to

have high prevalence (Bain and Kelly, 1977), whereas areas with more pronounced

seasonality (e.g. Ontario, Canada) tend to have lower prevalence (Slocombe, 1979).

However, inaccurate diagnosis likely also contributes to the magnitude of variation seen

in the studies listed in Table 1.1. Inaccuracies associated with detection of eggs in faeces

are very well known for many parasites of veterinary importance (Ward *et al*., 1997;

Cringoli *et al*., 2004), and results of studies involving *A. perfoliata* are no exception

(Agnessens *et al*., 1998; Meana *et al*., 1998; Beelitz and Gothe, 2001; Trotz-Williams *et al*., 2008). Beroza *et al*. (1986) found *A. perfoliata* eggs in the faeces of only 50% of

horses that were known to be infected. Similarly, Beelitz and Goethe (2001) and Meana

et al. (1998) showed that eggs were only detectable in 45% and 54% of horses,

respectively, when samples from horses of known infection status were examined. These

results indicate that faecal egg counts may diagnose only about 50% of infected horses.

Given the notoriously poor accuracy of faecal egg detection and the association of

infection with intestinal problems, the development of accurate diagnostic tools for the

detection of *A. perfoliata* in horses has become increasingly important. Modern

immunological and molecular tools have played an important role (Hoglund *et al*., 1995;

Proudman and Trees, 1996a, b; Drogemuller *et al*., 2004; Gasser *et al*., 2005; Traversa *et al*., 2008). Hoglund *et al*. (1995) evaluated *A. perfoliata* scolex antigens to detect

tapeworm antibodies in horse sera using an enzyme-linked immunosorbent assay

(ELISA), but high variation in readings were found in horses with similar worm intensities, indicating that the technique was not reliable. Proudman and Trees (1996a) found that tapeworm excretory/secretory (E/S) antigens gave the best differentiation in an ELISA, suggesting the E/S antigens were the most specific and accurate for diagnostic use. A 12/13kDa E/S protein was purified and antibodies were produced and used in a serological ELISA, which had a higher specificity and sensitivity than other techniques (Proudman and Trees, 1996b). Recently, Kania and Reinemeyer (2005) developed a coproantigen ELISA to detect E/S products released in the faeces of infected horses, and with a small sample size were able to accurately identify infected and uninfected horses, although worm intensity was unknown.

Polymerase chain reaction (PCR) methods have also been established for diagnosing parasitic infection in an attempt to overcome the limitations of other diagnostic techniques. Internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA) have become popular for investigating inter- and intraspecific variation in parasitic groups because of their high nucleotide substitution rate (van Herwerden *et al*., 2000; Verweij *et al*., 2000; Drogemuller *et al*., 2004). Species-specific amplification of strongyle nematode DNA from faecal samples of horses was successful, signifying potential for the diagnosis of parasite infection (Hung *et al*., 1999). Drogemuller *et al*. (2004) developed a PCR technique using *A. perfoliata* rDNA sequences, and was able to amplify fragments of parasite DNA in horse faeces. A nested PCR technique was found to correctly identify all infected horses, but it was not useful in estimating the parasite intensity of infected animals (Traversa *et al*., 2008).

Only a small number of studies have compared diagnostic techniques for their accuracy and sensitivity in detecting the occurrence and especially the intensity of *A. perfoliata*. One key weakness is that comparative techniques only rarely include hosts of known infection status. Coprological diagnosis in conjunction with necropsy has been well documented, and faecal egg counts are not considered to diagnose horse tapeworm infection accurately (Beroza *et al*., 1986; Nilsson *et al*., 1995; Agnessens *et al*., 1998; Meana *et al*., 1998; Beelitz and Gothe, 2001). Serum antibody levels and tapeworm intensity have been positively associated in some studies (Proudman and Trees, 1996b), and not related in others (Boswinkel and van Oldruitenborgh-Oosterbaan, 2007). Traversa *et al*. (2008) were the first to compare coprological, serological and molecular methods for diagnosing *A. perfoliata*, but worm intensity was unknown. Multiple diagnostic assays have been created, but no studies have evaluated current coprological, serological (both Western blot and ELISA), and coproantigen techniques for the diagnosis of *A. perfoliata* with respect to infection status and worm intensity.

1.1 Thesis Objectives

The purpose of Chapter 2 is to characterize the prevalence of *A. perfoliata* in privately owned horses sampled across southern Alberta. This was the first study to monitor horses kept for pleasure or competitive purposes in realistic management settings in Canada. The overall aim was to evaluate spatial and temporal variation in prevalence of *A. perfoliata* in horses sampled over two years from three selected regions that spanned an East-West gradient of climate in southern Alberta, Canada, using faecal analysis.

In Chapter 3, the relative efficiency and accuracy of four diagnostic techniques for the detection of *A. perfoliata* in horses was evaluated. Specifically faecal, blood, infection status and worm intensity data were collected from individual horses over the course of one year. Access to a local abattoir located in Fort Macleod, Alberta provided a unique opportunity to address the shortcomings of other studies for diagnosis of *A. perfoliata*. Horses of known infection status allowed me to evaluate efficiency of different diagnostic techniques. I was able to evaluate blood samples using (1) western blot analysis and (2) a serological ELISA, to determine whether horses possessed circulating antibodies against tapeworm antigens. Further, I was able to evaluate faeces of individual hosts of known infection status for both (3) tapeworm egg counts and (4) the development of a coproantigen ELISA, which identified small amounts of worm E/S products released in the horse faeces. I was then able to compare each diagnostic technique of infected horses with known tapeworm intensities. I was also able to compare the results to a sub-sample of uninfected horses with the western blot, serological and coproantigen ELISAs.

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Table 1.1: Worldwide prevalence and diagnosis studies of *A. perfoliata*.

Location	Method	Sample Size	Prevalence (%)	Mean Worm Intensity or Range	Author(s)	Comments
Belgium	Necropsy Coprological	270 66	28.9 4	13, 1-370	Agneessens et al., 1998	Only 13 of the 66 necropsy confirmed positives were correctly identified by faecal egg counts. It was concluded that faecal eggs counts were an unreliable method for diagnosis of cestode infection.
New Zealand	Necropsy	65	82	50	Bain and Kelly, 1977	Survey was completed over one year. There was an association of excessive pathological change with infection.
Upper Bavaria	Necropsy Coprological	100 47	38 17	1-1066	Beelitz and Gothe, 2001	Reported that faecal egg counts greatly underestimated prevalence.
Kentucky	Necropsy	118	61	84 1-853	Benton and Lyons, 1992	Horses were submitted for necropsy to the Livestock Disease Diagnostic Center between August and December.
Massachusetts	Necropsy Coprological	28	32 1-10	n/a	Beroza et al.,1986	Adult tapeworms were crushed and known numbers of eggs were added to faecal samples which yielded 10% recovery using centrifugation and 1% recovery using flotation of spiked samples. Eggs were found in half of the necropsy positive horses.
Australia	Necropsy	150	29	99	Bucknell et al., 1995	Necropsies competed between January and August, 1993. *A. perfoliata* most prevalent in winter and in horses from uniform to high rainfall areas.

Table 1.1 Continued

Louisiana	Necropsy	117	52	10	Chapman et al., 2002	*A. perfoliata* prevalence increased slightly, but intensity did not differ between 1986 and 2000.
Ireland	Necropsy	363	51	1- >100	Fogarty et al., 1994	Necropsies were completed from May through September and attachment site was examined.
Sweden	Necropsy	426	n/a	n/a	Hoglund et al., 1998	Reported that antibody response is related to establishment of new infections, and horses are at risk of infection during the first part of the grazing season.
Kentucky	Necropsy	268	52	84 1-2206	Lyons et al., 2000	Necropsies were performed over 5 years.
Spain	Necropsy McMaster Zinc sulphate Sucrose	107	22 1.8 9 8	1-248	Meana et al., 1998	No relationship could be established between tapeworm burden and egg detection. Horses with burdens of less than 100 worms had a much lower probability of being correctly diagnosed by faecal analysis.
Spain	Necropsy	372	24	1-491	Meana et al., 2005	Suggest one generation of worms per year, and results indicate dependence on environmental conditions. Reported seasonality of *A.perfoliata* to depend on moisture conditions, from 2001-2004.
England	Necropsy	81	52	62 1-1849	Morgan et al., 2005	Necropsies were performed over one winter. It was reported that as the population of worms increases, individual worms are smaller.

Table 1.1 Continued

Sweden	Necropsy Coprological	470	65 23	79 1-912	Nilsson et al., 1995	Samples were collected during one year, 11% had severe mucosal changes in the caecum suggestive of *A. perfoliata* lesions. Discharge of tapeworm segments is sporadic and eggs not evenly distributed in faeces.
Spain	Necropsy	121	23	1-248	Rodriguez-Bertos et al., 1999	Pathological changes in the ileocaecal junction were evaluated. Lesion severity was clearly related to infection intensity.
Ontario	Coprological	580	13.6	n/a	Slocombe, 1979	Horses were from 25 locations, and following treatment with menbendazole, niclosamide or pyrantel base, segments and worms were recovered. The author cautions that reported prevalence must be regarded as a minimum because of poor levitation of eggs in flotation solution.
Greece	Coprological	226	0.4	n/a	Sotiraki et. al., 1997	Samples were taken directly from the rectum of each animal, and each sample was evaluated by two techniques.
Ontario	Coprological Serological	234	6 56	n/a	Trotz-Williams et al., 2008	Horses that were on pasture for a significant part of their diet were over six times more likely to be seropositive for *A. perfoliata*. There was no evidence of association between seropositivity and risk of colic. Serological ELISA is more sensitive in detecting tapeworm exposure of horses than faecal egg counts.

Chapter 2. Prevalence and distribution of the cestode *Anoplocephala perfoliata* in southern Alberta horses

2.1 INTRODUCTION

The cestode *Anoplocephala perfoliata* is a well known parasite of the gastrointestinal tract of equines (review by Gasser *et al*., 2005). It is cosmopolitan in its distribution, apparently occurring wherever soil and moisture conditions are appropriate for the survival of its mite intermediate host (Meana *et al*., 2005; Tsiafouli *et al*., 2005). Recently, evidence has been accumulating that there is a link between high *A. perfoliata* intensities and gastrointestinal diseases, especially colic (reviewed in Chapter 1; Ryu *et al*., 2001; Boswinkel and van Oldruitenborgh-Oosterbaan, 2007). The incidence of colic in horses in the USA has been estimated at 4.2 cases per 100 horses per year, with 11% being fatal (Traub-Dargatz *et al*., 2001). The extent to which the incidence of fatal colic can be attributed to complications arising from *A. perfoliata* infection is unknown, but concern within the veterinary parasitology and equine health community has increased over the past decade (Proudman and Edwards, 1993; Proudman and Trees, 1998; Rodriguez-Bertos *et al*., 1999; Meana *et al*., 2005).

The concern over potential *A. perfoliata* induced pathology in horses has led to increased efforts in characterizing basic epidemiological characteristics. Survey studies in Spain (Meana *et al*., 2005) and Sweden (Hoglund *et al*., 1998) indicate an approximate annual cycle of infection. In general, encysted larval stages in mites are ingested during mid- to late-summer and require approximately 8-10 weeks to mature to the egg-producing stage. Transmission rates from egg to mite are unknown, but in temperate habitats, transmission likely occurs in spring/early summer when mites emerge from winter dormancy. Thus, the annual cycle repeats when fully-developed cysticercoids are

available for ingestion in mid- to late summer. The rates of transmission of eggs to mites, the development of cysticercoids, and the rates they are ingested by horses, have been shown to be influenced by soil moisture conditions (Tsiafouli *et al*., 2005) and climate (Hoglund *et al*., 1998; Meana *et al*., 2005). Previous surveys indicate enormous variation in infection characteristics of *A. perfoliata* in horses (reviewed in Chapter 1).

Characterizing this variation and understanding its underlying causes is an important feature in the control, diagnosis and management of this tapeworm. The distribution of this tapeworm in Canada is unknown apart from one study completed in Ontario (Slocombe, 1979). Anecdotal reports confirm its presence in the prairie regions of Canada (Colwell personal communication), but no information is available regarding seasonal and spatial variation in prevalence or intensity. The purpose of this study was to determine *A. perfoliata* prevalence in horses from selected regions in southern Alberta and to evaluate whether factors such as season, management regime and habitat could explain variation in prevalence within selected regions. The prevalence and intensity of *A. perfoliata* infection in horses from a local abattoir was also investigated.

2.2 METHODS

2.2.1 Seasonal and spatial variation in A. perfoliata prevalence

Faecal samples were collected from privately-owned horses in three southern Alberta regions (Figure 2.1). Regions were classified based on physical habitat designations described by the Alberta Government Natural Subregions (Adams *et al*., 2007). The 'western' sites included samples collected in the foothills fescue subregion within 50km of the towns of Pincher Creek and Fort Macleod. The 'central' sites were in the mixed grass subregion and included ranches within 60km of the city of Lethbridge.

The 'eastern' sites were in the dry mixed grass subregion, from ranches within 15km of Medicine Hat and Redcliff, with one site at the far south-east corner of Alberta at the Onefour site of Agriculture and Agri-Food Canada Research Centre.

Fresh faecal samples were collected during the months of January and June 2006. The same ranches and if possible the same individual horses were re-sampled in January and June 2007. Individual horses that were in pasture groups or group corrals were watched until they passed faeces to identify each horse to a fresh sample. For those horses that were kept in individual corrals or stalls, the freshest faecal sample was collected. Samples were collected into re-sealable plastic bags, labelled and frozen at -20°C until analyzed.

Standard coprological centrifugation/flotation analyses were carried out as described by Proudman and Edwards (1992), with minor modifications. Briefly, 10g of well-mixed faecal material was homogenized with 2.5% potassium dichromate solution and strained through 2 layers of cheese cloth. The liquid portion was placed into 15ml tubes and centrifuged at 1200xg for 5 minutes. The remaining pellet was re-suspended by vortexing with potassium dichromate solution and centrifuged. The supernatant was discarded and the pellet was re-suspended by vortexing with a small amount of saturated sucrose solution (with 1% phenol) then filled to the brim of the 15 ml tube. A coverslip was placed on the top of the tube and was centrifuged at 1200 g for 5 minutes. The coverslip was removed and placed on a slide for viewing with a compound light microscope at 63X magnification, *A. perfoliata* eggs are easily identifiable (Bowman, 2003). All eggs were counted and expressed as eggs per gram (EPG) of faeces collected.

2.2.2 Factors affecting the distribution of A. perfoliata

Horses were further categorized according to their history of pasture use. Pasture horses were defined as those that had access to pasture within the previous year. Non-pasture horses were those that were stabled or kept in dirt corrals for at least 12 months prior to sampling. Horse owners indicated the management regime of the animal when the description of the horse was taken. Deworming regime was collected from owners for each horse. Until the launch of Equimax™ by Pfizer Animal Health in March of 2006, there was no anthelmintic available in Canada that was effective in eliminating this tapeworm. One site reported using this product to control tapeworm infection.

2.2.3 Prevalence and intensity of A. perfoliata in slaughter horses

Ideally, data was obtained on the intensity of *A. perfoliata* corresponding to the data on faecal egg counts. This was not possible due to the constraints of obtaining worm counts from individual horses. However, a local abattoir in Fort Macleod, south-central Alberta, processes horses primarily from western Canada. Although the abattoir does not provide information on the origin of the individual horses, I used necropsy data from a sample of these horses to indicate broad patterns in cestode intensity in horses from western Canada. A total of 434 horses were necropsied between March 2006 and April 2007, representing 24-45 horses sampled per month. The caecum of each horse was opened and carefully inspected for the presence of adult *A. perfoliata*. This method provided absolute counts of all tapeworms greater than approximately 3mm in total length. If tapeworms were present, they were counted and maintained in phosphate buffered saline (PBS) and then preserved in 90% ethanol. Descriptions in Bone (1982)

were used to determine the age of infected horses via the wear pattern, number and angle of teeth in the lower jaw.

2.2.4 Data Analyses

The terms prevalence (the proportion of horses infected with *A. perfoliata*) and intensity (the number of worms in an individual infected host) as defined by Margolis *et. al*., (1982) were used to describe infection patterns in horses. Contingency table analyses and pairwise combinations were evaluated with a Chi-squared (χ^2) comparison for proportions (prevalence in this case) as described in Zar (1999). Differences in mean adult *A. perfoliata* intensity between horses collected from different seasons (summer (May, June, July 2006; n = 6), fall (August, September, October 2006; n=8), winter (November, December 2006, January 2007; n = 9) and spring (February, March, April 2007; n = 8)) from the abattoir were evaluated using non-parametric Kruskal-Wallace tests (Zar, 1999).

2.3 RESULTS

2.3.1 Seasonal and spatial variation in A. perfoliata prevalence

The estimated prevalence of adult *A. perfoliata,* based upon the presence of eggs in a total of 1094 faecal samples was 17%. Overall, samples evaluated from horses collected in winter 2006 had significantly higher prevalence (18%) than samples collected during any other sampling season (Figure 2.2; $p < 0.05$). Pairwise comparisons also showed that there were no significant differences between summer 2006 (7.8%) and winter 2007 (8.5%, Figure 2.2, $p > 0.05$). Prevalence in the summer 2006 sample (7.8%) was significantly higher than prevalence in summer 2007 (4%, Figure 2.2, $p < 0.05$). However, the estimated prevalence of *A. perfoliata* in winter 2007 samples (8.5%) was

significantly higher than for samples collected in summer 2007 (4%, Figure 2.2; $p <$ 0.05).

Over the 4 sampling periods, 10 horses from the west and central regions were found to be infected with *A. perfoliata* on more than one sampling occasion (Table 2.2). Of those 10 horses, 9 were found to be infected on two occasions, and 1 on three. Of the 9 horses infected during two occasions, 3 were found to be infected consecutively. Of the 10 horses infected on more than one occasion, 6 were infected only during the winter.

The prevalence of *A. perfoliata* in pastured horses was significantly higher in samples collected from western and central Alberta and lowest in those collected from eastern Alberta in winter 2006 (Figure 2.3, $p < 0.05$). There were no significant differences in prevalence between the three regions during the other sampling periods. Prevalence was significantly higher in horses collected from pastures in winter 2006 than any other sampling season or site (Figure 2.3, $p < 0.05$). The prevalence of *A. perfoliata* on eastern pastures was not significantly different between sampling occasions (Figure 2.3, $p > 0.05$).

2.3.2 Factors affecting the distribution of A. perfoliata

Climate

The annual precipitation patterns in southern Alberta regions that occurred during this study provided general insight into the potential linkage between soil moisture conditions and *A. perfoliata* transmission. In general, precipitation was highest in the western region during each sampling season (Figure 2.4a). Prior to the high prevalence found in the west and central regions there was high precipitation during the summer grazing season of 2005 (Figure 2.4A, B). Precipitation was lowest during the winter in

all regions. The highest prevalence in all regions was observed following the summer 2005 grazing season, which also had the highest precipitation of all years (Figure 2.4).

Management

Only 2 out of the 314 fecal samples collected from non-pasture horses were found to be positive for *A. perfoliata* (0.6%). In the overall data set, prevalence of infection was significantly higher in pastured vs. non-pastured horses (z_c= 5.869 regions and seasons pooled). One of the non-pasture positive horses was from the East, and the other from the Central region. Both animals were positive in the winter of 2006. Both animals had only been dewormed with Ivermectin products.

2.3.3 Prevalence and intensity of A. perfoliata in slaughter horses

Thirty one of 434 horses were infected with *A. perfoliata* at necropsy, and prevalence ranged from 0-13% over the 12 month sampling period. Worm intensity varied from 1 to approximately 1000 in horses sampled from the abattoir (Figure 2.5). The shape of the frequency distribution was strongly affected by one heavily infected horse. Most horses (81%) had fewer than 10 tapeworms, whereas 6% of horses had greater than 100. Worm intensity ranged over 3 orders of magnitude (Figure 2.5). Only 5 of the 31 infected horses had more than 10 worms. There was no significant difference in median worm intensity between the 4 sampling periods (Figure 2.6; p > 0.05).

2.4 DISCUSSION

The combined survey and necropsy results of my 2-year study indicate that between 4-18% of horses in southern Alberta were infected with gravid cestodes. This result approximates prevalence estimates reported in a related study completed in Ontario (Slocombe, 1979), but is lower than most other studies completed in North America and

elsewhere. However, there are no comparable studies that have been completed in western Canada, making it difficult to generalize these results. The epidemiology of *A. perfoliata* in southern Alberta appears to be strongly tied to local moisture conditions and to horse management.

Despite the relatively low prevalence of cestodes in horses from southern Alberta, the results of this study indicate significant variation between regions, with a general decrease in prevalence from west to east. This result is not surprising. The western regions of Alberta are higher in elevation, have a lower average temperature and receive more precipitation (Adams *et al*., 2007). Rates of transmission of *A. perfoliata* between eggs and mites and between mites and horses are known to be linked to soil moisture conditions (Meana *et al*., 2005). For example, Tsiafouli *et al*. (2005) found that oribatid soil mites were significantly more common in irrigated plots than in dry plots. The positive association between moisture conditions and transmission success is typical of many other helminth and nematode parasites of grazing animals (Stromberg, 1997; Baudena *et al*., 2000; Roepstorff *et al*., 2001). Therefore the observation of moderate prevalence of *A. perfoliata* in western Alberta is probably typical of other regions that experience broadly similar patterns of precipitation. Likewise, the atypically low prevalence that was observed in eastern horses is best explained by reduced rates of transmission within the semi-arid habitat that is typical of south-eastern Alberta.

Results from the seasonal survey indicate that the transmission of *A. perfoliata* in southern Alberta varies over the course of the year. Prevalence was generally higher during the winter months, supporting the results of similar studies in Spain (Meana *et al*., 2005) and Sweden (Hoglund *et al*., 1998). I interpret the seasonal patterns of infection as

follows. Horse owners in Alberta, as in other temperate areas, tend to put their horses out to pasture in May/June for the summer, which corresponds with the period of peak annual rainfall (Agriculture and Agri-Food Canada, 2007). Following the period of *A. perfoliata* maturation during the fall months, the maximum number of eggs seeded onto pasture likely occurs over winter and spring.

Results of this study support other reports that indicate an annual cycle of transmission of *A. perfoliata* that parallels the patterns observed in related species such as *Moniezia expansa* (Worley *et al*., 1974; Hoglund *et al*., 1998; Meana *et al*., 2005). Hoglund *et al*. (1998) and Meana *et al*. (2005) showed that ingestion of infected mites occurs during summer grazing, followed by deposition of eggs onto pasture when worms mature in winter. It is likely that eggs would not be detected in the faeces until adult worms had developed over a period of 2-3 months (Worley *et al*., 1974; Hoglund *et al*., 1998). This lag period associated with the worm maturation explains the decrease in prevalence that I observed during summer at all sites. A similar trend was also observed in the abattoir samples, with consecutive winter months having consistently higher prevalence than summer, with the exception of August which had a higher prevalence.

The observation that prevalence was significantly higher in pastured horses is not surprising. Rates of transmission for indirectly transmitted helminths and nematodes are often linked to intermediate host densities (Stunkard, 1937; Marcogliese *et al*., 2001; Otranto and Traversa, 2002). In the case of *A. perfoliata*, mite densities are likely to be much lower within stables and corrals compared to adjacent pasture (Proudman *et al*., 1998; Trotz-Williams *et al*., 2008). Grazing horses would therefore have a much greater probability of encountering infected mites. One implication of this result, from a cestode

control perspective, is that treatment of horses during peak exposure in late summer might be a realistic goal, and important to reduce infection before worms are able to mature and release eggs.

The shortcomings associated with inferring epidemiological patterns from egg count data alone are well known (Proudman and Edwards, 1992; Meana *et al*., 1998; Trotz-Williams *et al*., 2008). Even in cases where hosts of equivalent age, gender and size are infected with known numbers of worms, faecal egg counts varied greatly according to the time of day the faeces were sampled and in the distribution of eggs in the faecal mass. For *A. perfoliata* Proudman and Edwards (1992) showed that the sensitivity of detection of eggs from horses of known infection status was only 61%. Further, Nilsson *et al*. (1995) indicated that gravid tapeworm segments were sporadically discharged and were unequally distributed in the faecal mass, which could explain the lack of relationship between tapeworm intensities and egg detection by Proudman and Edwards (1992) and Meana *et al*. (1998). Thus prevalence values observed in my study likely underestimate the true prevalence of *A. perfoliata* in southern Alberta horses. In resident horses that were sampled from the same pastures over the two years, infected and uninfected horses co-occurred. It is likely that some proportion of the latter were infected on each pasture that contained an infected horse, but may have had low worm burdens that could have led to misdiagnosis. This shortcoming emphasizes the need for more accurate diagnostic techniques.

2.5 LITERATURE CITED

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Table 2.1. Summary of biotic and abiotic features of southern Alberta sampling areas.

Region	Vegetation	Average annual precipitation (mm)	Altitude (m)	Average annual temperature (°C)
West*	Rough fescue grass	750	1190	19.1
Central*	Moist mixed short grass	500	914	20.8
East*	Short/mixed grass	300	721	23.2

*Information from: Environment Canada, accessed August 2007
(http://www.climate.weatheroffice.ec.gc.ca); Adams et. al., 2007; Agriculture and Agri-food Canada, accessed August 2007 (http://agrisource1.agr.gc.ca).

Table 2.2. Changes in the presence of *A. perfoliata* eggs in the faeces of 10 individual horses sampled between 2006-2007.

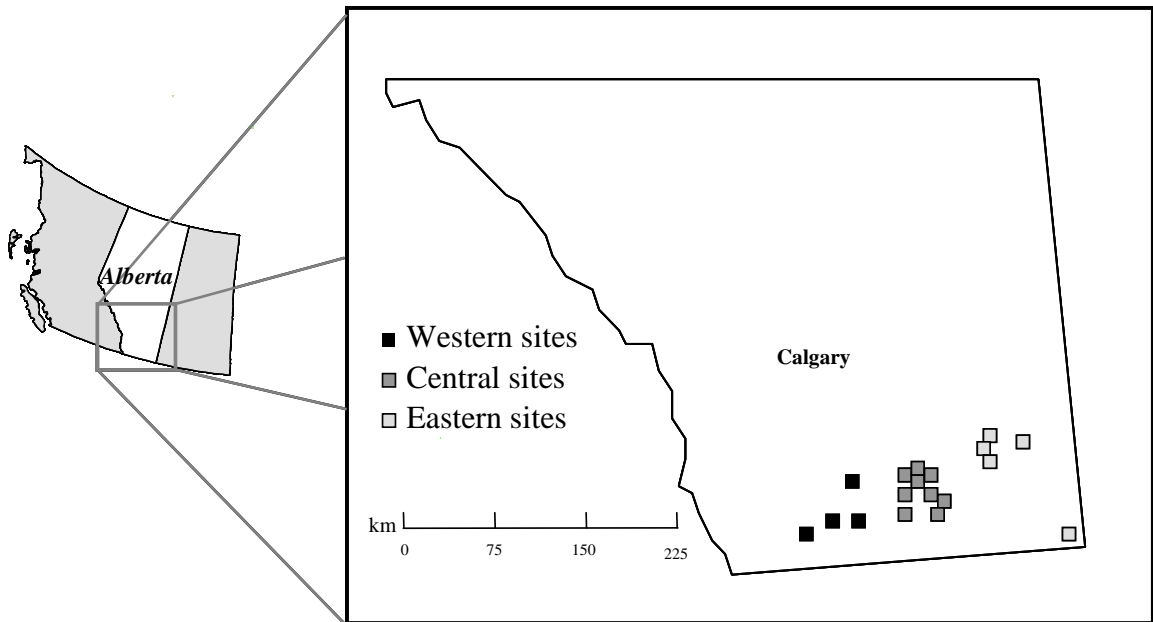| Horse	Winter 06	Summer 06	Winter 07	Summer 07
WP22	+	+	-	-
WP25	+	+	-	-
WP28	+	-	+	-
WP48	-	+	-	+
CP2	+	-	+	-
CP3	+	-	+	-
CP10	+	-	+	-
CP12	+	-	+	-
CP14	+	-	+	+
CP54	-	-	+	+

Figure 2.1. The approximate locations of privately owned sample sites found across southern Alberta regions. Sites not to scale.
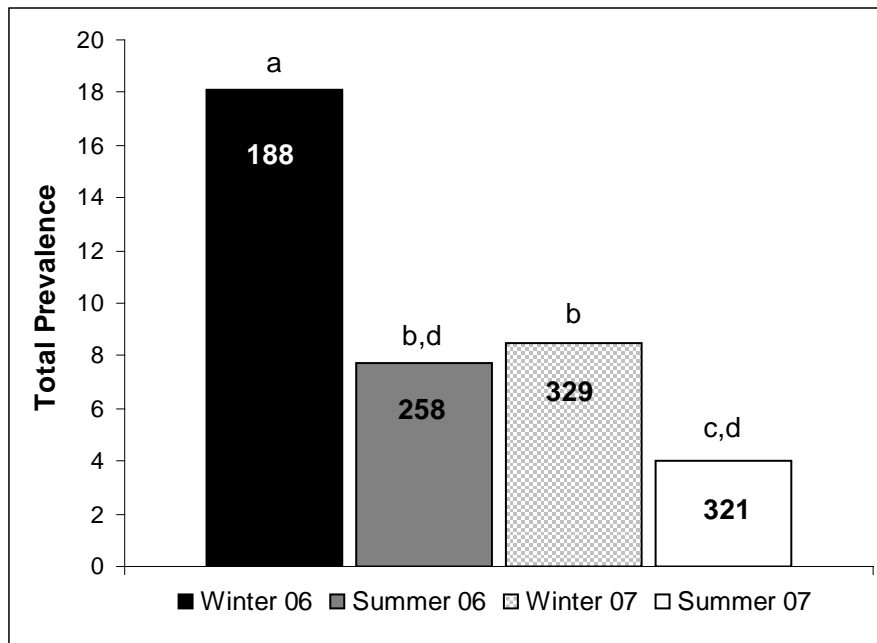

Figure 2.2. Prevalence of *A. perfoliata* based on faecal analysis of horses in southern Alberta. Letters represent significant differences and numbers in bars represent sample size.

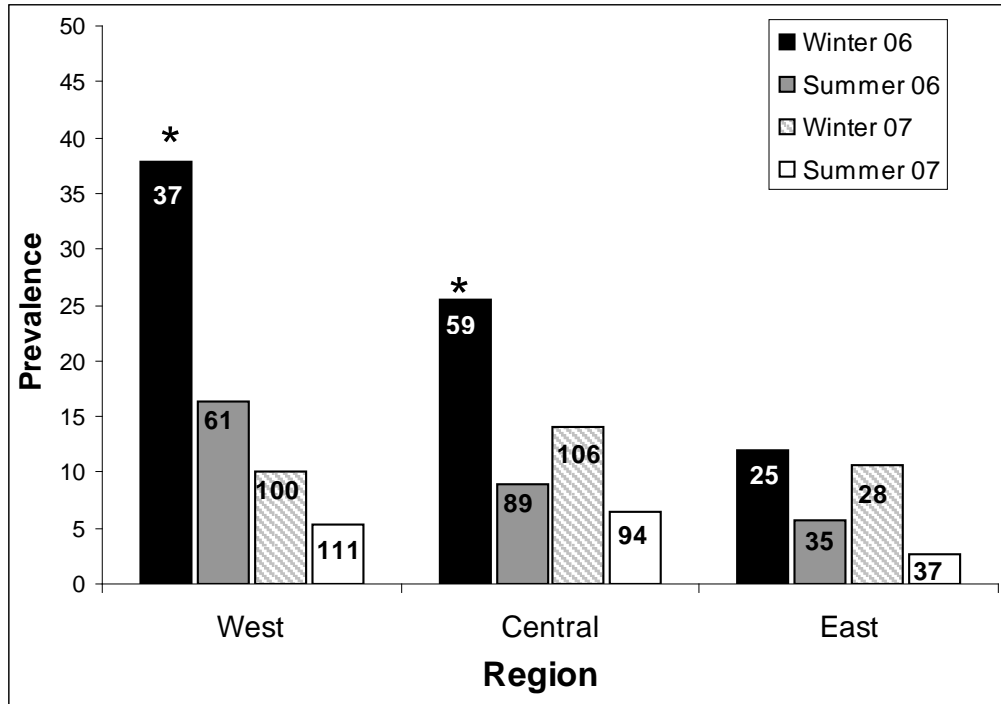**Figure 2.3.** Seasonal and regional prevalence of *A. perfoliata* based on faecal analysis of pastured horses in southern Alberta. Asterisks indicate significant differences in prevalence between pairwise samples. Numbers in bars indicate sample size.
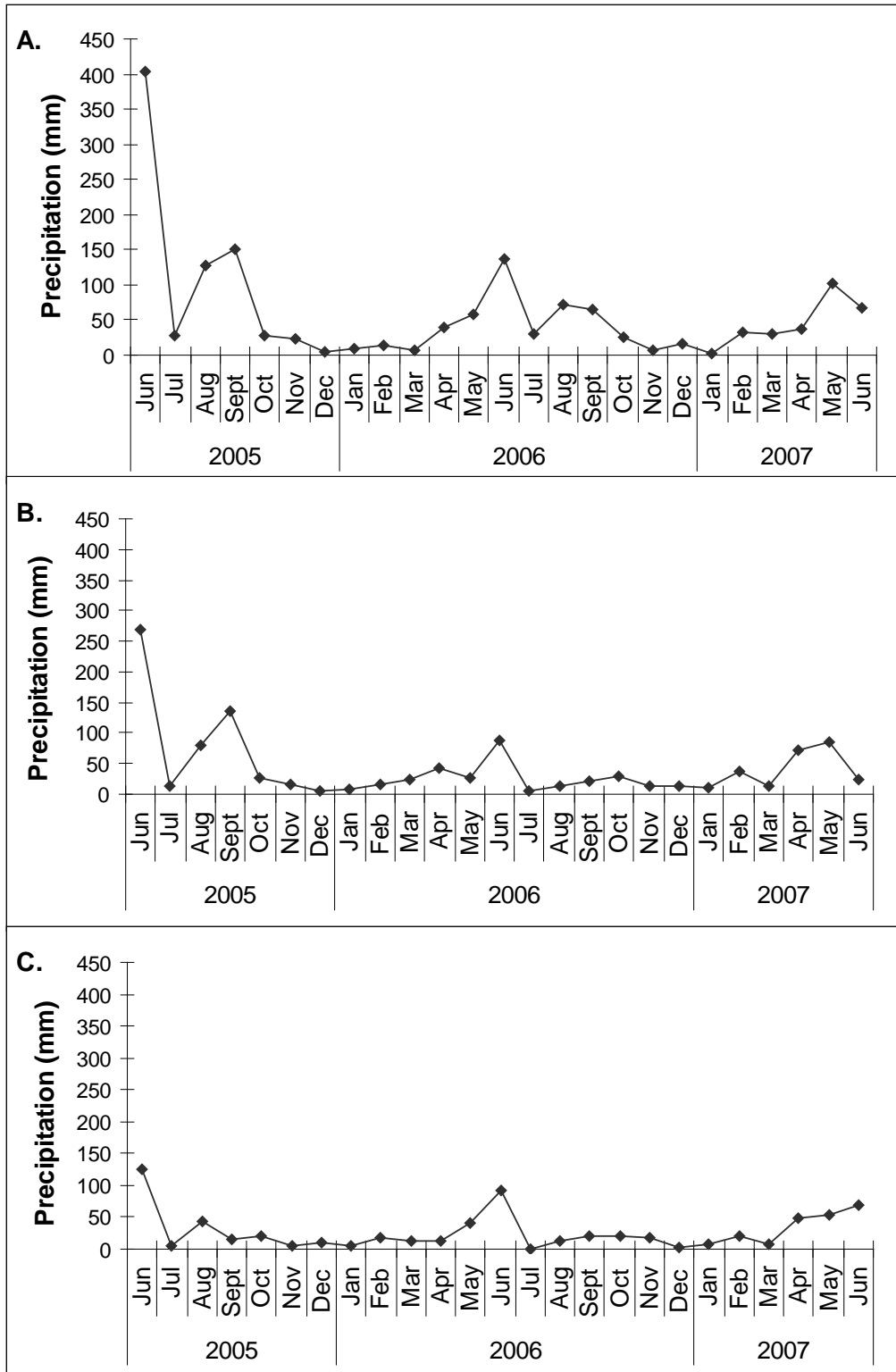
Figure 2.4. Annual changes in precipitation from A) western B) central and C) eastern Alberta. Mean monthly precipitation data from Agriculture and Agri-Food Canada.

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Figure 2.5. Frequency distribution of *A. perfoliata* in western Canadian horses at necropsy (n=31 horses).

Figure 2.6. Seasonal changes in median intensities of *A. perfoliata* in horses necropsied from an abattoir in southern Alberta, Canada. Boxes encompass the 25th to 75th interquartile ranges in intensity; extensions represent range. Horizontal lines within the boxes represent median intensity.

Chapter 3. Evaluation of diagnostic techniques for *Anoplocephala perfoliata* in horses.

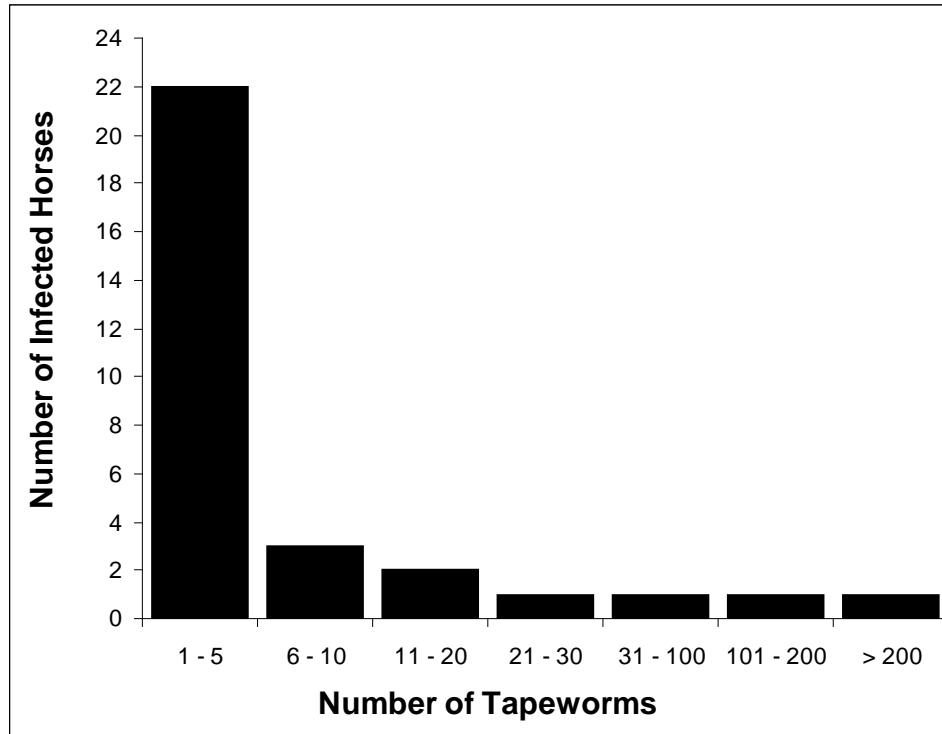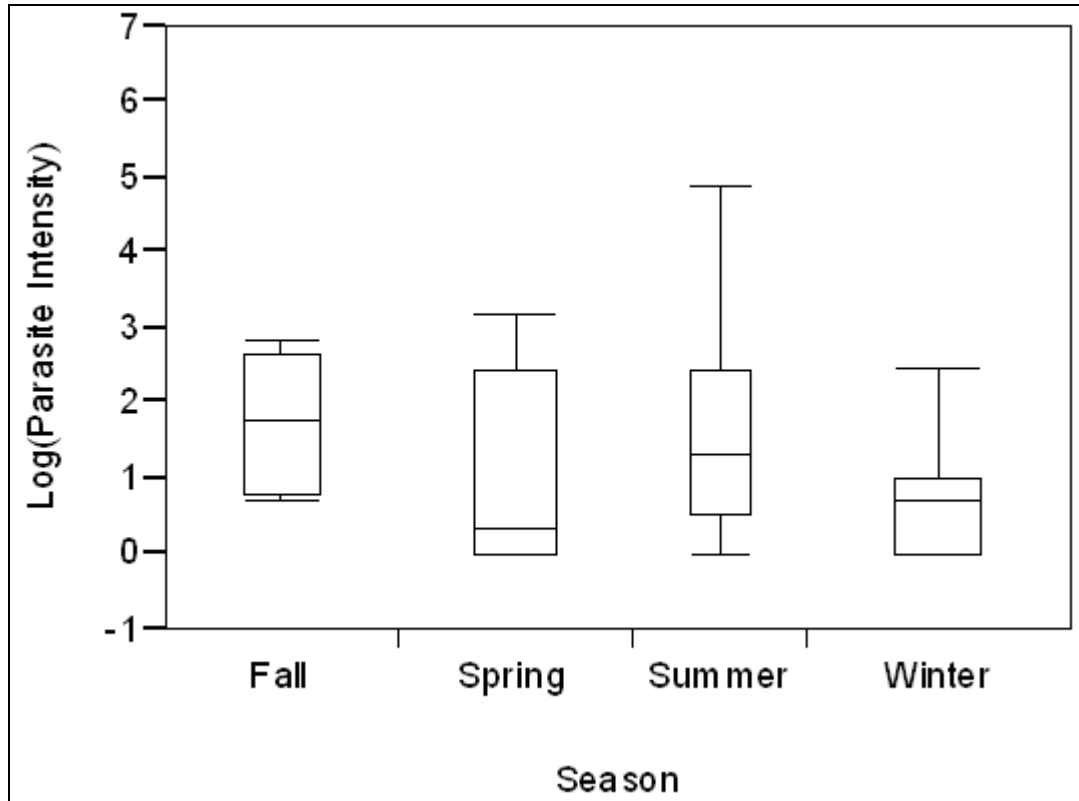## 3.1 INTRODUCTION

Recognition of disease associated with helminth infection leads to the need for accurate diagnosis and determination of worm intensity. Many helminth parasites are found to cause pathology in their hosts such as: *Strongylus vulgaris* (Nematoda) infections in horses (Bailey *et al*., 1989), *Ostertagia* (Nematoda) in cattle (Agneessens *et al*., 2001), and *Dicrocoelium dendriticum* (Trematoda) in cattle, sheep and grazing ungulates (Otranto and Traversa, 2002), *Echinococcus* (Cestoda) in dogs (Benito *et al*., 2006), and *Fasciola hepatica* (Trematoda) in cattle (review by Charlier *et al*., 2008). If individual infected animals can be identified, they can be treated on a case by case basis. Also, if parasite intensity can be evaluated, precautionary measures can be made to ensure gastrointestinal disease can be treated before large worm burdens occur. Correct diagnosis also means parasite infection can potentially be controlled by both treating infection and decreasing transmission. As with well-known helminth species such as *Ostertagia* (Nematoda) and *Echinococcus* (Cestoda), parasitological examination of the gastrointestinal tract at necropsy is still considered the best method for diagnosis, since no other method for determining presence and abundance of worms is available (Agneessens *et al*., 2001; Benito *et al*., 2006).

Anoplocephala perfoliata is a common tapeworm found in horses all over the world. Mature worms are found attached to the intestinal mucosa, most often around the ileocaecal junction (Tinker *et al*., 1997; Williamson *et al*., 1997). This tapeworm was once believed to be non-pathogenic in horses, but is now known to cause severe

gastrointestinal disease at high intensities, causing death in horses and great economic

loss (Proudman and Edwards, 1993; Tinker *et al*., 1997; Proudman *et al*., 1998; Edwards,

1999; Rodriguez-Bertos *et al*., 1999; Ryu *et al*., 2001; Traub-Dargatz *et al*., 2001). Since

accurate diagnosis currently presents significant challenges and this tapeworm is known

to have a role in gastrointestinal disease, it reinforces the importance of continued

research.

Unfortunately the lack of an accurate method for diagnosis of infection *in vivo*

means horses may be at risk of clinical disease. Since *A. perfoliata* infection has been

linked to gastrointestinal disease (reviewed in Chapter 1), an accurate diagnostic

technique would mean that horses could be treated on a case by case basis, before

pathology associated with high worm intensities could occur. Diagnosis followed by

treatment would also limit the number of tapeworm eggs shed onto the pasture, reducing

further infection of other pastured horses.

Coprological analysis to determine presence of parasite eggs in faeces,

consistently underestimates infection status and prevalence, but the method can be a

useful tool for estimating general epidemiological patterns (reviewed in Chapter 1; see

also Chapter 2). Faecal analysis is also straightforward and inexpensive, although

positive diagnosis can only be made if adult worms are shedding eggs (*i.e.* the infection is

patent). Sporadic release of tapeworm proglottids in the faeces can result in faecal

samples lacking tapeworm eggs, and can be misdiagnosed (Nilsson *et al*., 1995). Meana

et al. (1998) found that only half of the necropsy confirmed animals infected with *A.

perfoliata* were detected using faecal egg counts. Further, the probability of diagnosis via

egg counts was markedly reduced when horses were infected with fewer than 100 worms, emphasizing further the need for a more accurate and reliable diagnostic technique.

Parasites stimulate many host immune responses, among which is the development of often highly specific antibodies (reviewed by Wakelin, 1996; Wakelin, 1997). Numerous studies have evaluated the reliability of the detection of these antibodies as a diagnostic tool, including examples from the Veterinary literature such as: *Taenia* (Yong and Heath, 1984)*, Echinococcus* (Yong and Heath, 1984; Benito *et al*., 2006) and *Hypoderma* (Colwell and Baron, 1990; Panadero-Fontan *et al*., 2002). By evaluating circulating antibodies, it is possible to determine whether the animal has produced an immunological response to parasite infection, and also the presence of antibody indicates exposure. In some cases, the amount of circulating antibody detected can be correlated with parasite infection intensity (Hoglund *et al*., 1995; Proudman and Trees, 1996b), however in many instances this is not the case (Yong and Heath, 1984; Casaravilla *et al*., 2005; Benito *et al*., 2006).

In an effort to overcome restrictions associated with faecal egg counts, improved diagnostic methods have led to the examination of immunodiagnosis of *A. perfoliata*. Hoglund *et al*. (1995) evaluated scolex antigens from *A. perfoliata* in an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to the tapeworm in equine sera. Proudman and Trees (1996a, b) further refined the technique, and found that the 12/13kDa excretory/secretory (E/S) antigens of *A. perfoliata* in an ELISA gave the best differentiation between tapeworm-positive and negative sera from horses, with a diagnostic sensitivity of 68%. The strength of association of anti-12/13 IgG(T) was not sufficient to predict infection intensity, but it was possible to classify samples into low,

moderate or high intensity (Proudman and Trees, 1996b); therefore it was concluded that detection of antibodies to *A. perfoliata* could be useful as an epidemiological tool (Proudman and Trees, 1996a, b). However, serodiagnosis of *A. perfoliata* may not be useful at the level of the individual horse, because of variability in host immune response, and also because the decay rate of tapeworm-specific antibodies is unknown, making it difficult to distinguish current from past infection (Hoglund *et al*., 1995; Proudman and Trees, 1996a, b).

Coproantigen ELISA detection has been developed to recognize specific parasite antigens released in the faeces of infected hosts. Craig *et al*. (1995) developed a coproantigen ELISA for *Echinococcus*, and found a sensitivity of 77% when compared to 34% for the serum antibody ELISA previously developed. Mezo *et al*. (2004) developed an antigen capture ELISA to detect *Fasciola hepatica* coproantigens in sheep and cattle, and Casaravilla *et al*. (2005) developed an ELISA for detecting *Echinococcus granulosus* coproantigens. Kania and Reinemeyer (2005) developed a coproantigen ELISA to detect *A. perfoliata* E/S products in horse faeces and with a small sample size, were able to detect antigens at low concentrations and accurately identified infected horses. This technique may be useful in accurately identifying currently infected horses since it characterizes antigen released from tapeworms into horse's faeces. The coproantigen ELISA could be more accurate than a serological ELISA, because serum has the added complexity of circulating antibodies, which can be present even after infection is cleared.

Molecular methods of *A. perfoliata* diagnosis have recently been explored. Drogemuller *et al*. (2004) created a polymerase chain reaction (PCR) technique using the partial ITS-2 region of r-DNA from *A. perfoliata*, and were successful in detecting

fragments of tapeworm DNA within horse faeces. Traversa *et al*. (2008) used a nested

PCR technique to correctly identify all infected horses, and they were able to determine

that there was no cross-reactivity from horses infected with other parasites. The PCR

approach was not useful in estimating the parasite intensity (Traversa *et al*., 2008).

The objective of this study was to evaluate current diagnostic techniques using

naturally parasitized horses at necropsy, so that infection status and tapeworm intensity

were known and used as a baseline for comparison of analyses. The techniques were (1)

coprological analysis, (2) antibody detection in serum by ELISA and Western blot, and

(3) a new copro-antigen detection using sandwich ELISA.

3.2 METHODS

3.2.1 Abattoir Sampling

Horses destined for slaughter were processed at a local abattoir (Fort Macleod,

south-central Alberta). The sampling protocol was described in Chapter 2. The abattoir

does not provide detailed information on the origin of the individual horses. Each month

between March, 2006 and April, 2007, 24-45 horses were evaluated for *A. perfoliata*

intensity. At each collection period, every second horse on the production line was

selected for examination, to allow time for data and sample collection. For each horse

selected faeces were collected from the rectum into re-sealable plastic bags and frozen at

-20°C. Blood was collected into sterile Vacutainers$^{®}$ (Becton Dickinson Systems, NJ)

held at 4°C until processed to recover the serum, then stored at -20°C. Examination of

the caecum for presence of tapeworms was carried out by making a 20-30cm incision at

the ileo-caecal junction. The interior surface of the caecum was inspected, worms were

counted, removed and held in phosphate buffered saline (PBS). In addition the lower jaw

was collected for aging of positive animals as described by Bone (1982). Each horse was identified by the abattoir's slaughter number.

3.2.2 Coprological Analysis

Tapeworm eggs were quantified by modification of the technique described by Proudman and Edwards (1992) and described also in Chapter 2. Briefly, 10 grams of faeces was diluted in 40ml of 2.5% potassium dichromate solution mixed thoroughly and strained through two layers of cheesecloth. The resulting sample was centrifuged at 1200g, decanted and the remaining pellet re-suspended in 10ml of potassium dichromate solution and centrifuged at 1200g. The second supernatant was discarded and the pellet re-suspended in saturated sucrose with 1% phenol. The sucrose solution was added to each tube until it formed a convex meniscus. A 22 x 22 mm coverslip was placed on top of the tube. Following centrifugation the coverslip was placed on a slide for viewing. A compound light microscope (Leitz Dialux 22, Ernst Leitz Wetzlar GMBH #512742, Germany) was used to scan the slide at low power (63X) for the presence of parasite eggs. The presence or absence of all parasites eggs was recorded, but only tapeworm eggs were enumerated. Descriptions provided by Bowman (2003) were used as reference for morphological identification of parasite eggs.

3.2.3 E/S Antigen Preparation

Tapeworms recovered from the caecum were placed in PBS for transport to the laboratory. Subsequently, the worms were placed in 100ml tissue culture flasks and maintained in roughly 80ml RPMI 1640 medium at 37°C (lot #10601118, ICN #1060126). The medium was decanted daily, refreshed, and after 48 hours the worms were removed and all medium containing excretory/secretory (E/S) products was kept.

The medium was dialyzed, then freeze-dried and re-suspended in a small amount of double distilled water, to yield the E/S antigen. Protein concentration was quantified using BioRad *DC* Protein Assay (Mississauga, ON, Canada), based on the well documented Lowry assay.

3.2.4 Western Blot Analysis – SDS-PAGE and Immunoblotting

The E/S antigen, at a protein concentration of 146.5μg/ml, was used for Western blot analysis as described by Proudman and Trees (1996a). The proteins in the E/S antigen were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a BioRad Mini Protean® 3 Cell System (Mississauga, ON, Canada). Samples were reduced by boiling in 5% mercaptoethanol and electrophoresed in 18% separating gels with a 4% stacking gel. E/S antigen was loaded at approximately 30μg per well. Broad range molecular weight markers (BioRad, # 161-037, LS1610317) were run adjacent to E/S antigen on each gel.

The separated proteins were then transferred onto nitrocellulose membrane electrophoretically. E/S antigens and markers were visualized using Coomassie blue stain. Nitrocellulose membrane was blocked with SuperBlock[TM] dry blend buffer in TBS (Pierce Biotechnology, Rockford, IL #37545). The nitrocellulose was cut in strips and incubated with individual horse serum diluted 1:500 in PBS with 0.05% Tween. Washes were done with Tris-buffered saline with 0.0005% tween-20 (TTBS, pH 7.2). Peroxidase-conjugated AffiniPur F(ab')2 fragment goat anti-horse IgG (H+L) (Jackson Immuno-Research Lot #6400) was the detector or secondary antibody used, diluted 1:500 in PBS with 0.05% Tween (pH 7.4). HRP colour development buffer (Opti 4CN; BioRad Cat. #170-8235) was used for colour development. Strips were washed with distilled

water, air-dried and scanned. All of the necropsy positive (n=31) and a random selection of sera from uninfected horses (n=77) were analyzed.

3.2.5 Serological ELISA

The antibody ELISA serological diagnosis protocol was modified from Proudman and Trees (1996a, b). E/S antigen was used as the capture antigen at a 1:250 dilution in wash buffer (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) on 96 Well Flat Bottom Immuno Plates (Nalge Nunc International, NY; Maxisorp Non-sterile PS lot #613251) and incubated for 45 minutes at room temperature. The plates were washed with wash buffer using an electronic plate washer (Model 1575 ImmunoWash^{TM}, BioRad, Mississauga, Ont., Canada). Plates were then blocked with SuperBlock^{TM} dry blend buffer in TBS (Pierce Cat. #37545; Rockford, IL, USA) for 45 minutes at room temperature and washed twice. Following washes, test serum was diluted 1:28 in wash buffer and 100 μl was added to duplicate wells. Plates were incubated for 45 minutes at room temperature then washed twice. Peroxidase-conjugated AffiniPur F(ab')2 fragment goat anti-horse IgG (H+L chains, Jackson Immuno-Research, ON, Canada, Lot #6400) was the detector antibody used diluted 1:1000 in wash buffer. Finally, plates were washed twice and 100μl of colour development substrate consisting of 0.004g ABTS (2-2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid diammonium salt, Sigma Chemical Co., lot #67H1610) in 6.2ml citrate buffer and 3.8ml phosphate buffer with 1ml of 0.1% H_2O_2 was added to each well as described by Panadero-Fontan et al. (2002). Optical density (OD) was read using a spectrophotometer at 410nm (Spectra Max 190 plate reader, Molecular Devices Co. CA, USA).

Each ELISA plate contained sera from a known positive horse serially diluted in one column, 8 wells (horse #4064, intensity >1000 worms, Western blot positive); a column of blank wells containing only wash buffer (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) and a selection of sera from known negative samples (both necropsy negative and Western blot analysis negative). Test serum was added to duplicate wells on a randomly generated template. Once the plate was read, the mean OD of the blank wells was subtracted from each of the test serum OD readings, to control for variability between different batches of plates.

3.2.6 Coproantigen ELISA

The coproantigen ELISA was developed using Kania and Reinemeyer (2005) and Panadero-Fontan *et al*. (2002) to detect 12/13kDa E/S antigen shed in faeces of horses. Faecal samples were prepared in PBS (phosphate buffered saline pH 7.4) as described by Kania and Reinemeyer (2005), but were not diluted in wash buffer containing foetal bovine serum. Samples were stored at -20°C. E/S antigen was prepared as described above. In brief, E/S antigen was freeze dried, reconstituted in a small amount of molecular quality water and had a protein concentration of 241μg/ml. E/S antigen (30-40μg per well) was resolved by SDS-PAGE. Multiple gels were run and bands corresponding to the 12/13kDa protein were cut out and stored in PBS at -20°C until they were sent to GenWay Biotech Inc., to produce rabbit anti-tapeworm E/S antigen polyclonal antibodies. Affinity purified rabbit anti-tapeworm E/S antigen 12/13kDa IgG polyclonal antibodies produced by GenWay Biotech Inc. were verified by running Western blot analysis as described above, with the antibody replacing the horse serum

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step. The affinity purified rabbit anti-tapeworm 12/13kDa IgG was found to recognize

and bind the specific 12/13kDa antigen as evidenced by a strong band.

Verification of antigen capture ELISA was done using spiked samples with

known concentrations of antigen, starting with 100μg/ml protein concentration. The

assay was optimized using checkerboard titration. Starting antigen concentration of

2μg/ml and antibody concentration of 38-44μg/ml were found to work the best. On the

basis of these results, and the integration of the protocol by Kania and Reinemeyer

(2005), the following coproantigen protocol was developed.

Affinity purified rabbit anti-tapeworm E/S antigen IgG polyclonal antibodies

recognizing the 12/13kDa proteins were used as the capturing antibody (prepared by

GenWay Biotech Inc., lot #537 cat # 17-288-23282). The antibody was diluted 1:50 in

wash buffer (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) and bound,

at a protein concentration of 38-44μg/ml, to 96 Well Flat Bottom Immuno Plates (Nalge

Nunc International, NY; Maxisorp Non-sterile PS lot #613251) for 45 minutes at 37°C.

Plates were washed twice using an electronic plate washer (Model 1575 ImmunoWash^{TM},

BioRad, ON, Canada). Goat milk diluted 1:1 in double distilled water was used as the

blocking agent, added at 200μl per well and incubated at room temperature on a shaker

for 15 min. Plates were washed twice. Samples were plated at 100μl per well. E/S

antigen at a starting concentration of 2μg/ml was added in serial dilutions to one column

as the standard positive. Samples were incubated for 1 hour at room temperature on a

shaker, and 1 hour at 37°C. Plates were washed twice then antibody diluted 1:50 in wash

buffer was added again and incubated for 45 minutes at 37°C. Plates were washed twice

and 50μl of peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L chains, Jackson

Immuno-Research, ON, lot #34611) was the detector or secondary antibody diluted

1:1000 in wash buffer and added to each well. Finally, plates were washed twice and

100μl of colour development substrate consisting of 0.006g ABTS (2-2'-azino-bis(3-

ethylbenz-thiazoline-6-sulfonic acid diammonium salt, Sigma Chemical Co., lot

#67H1610) in 6.2ml citrate buffer and 3.8ml phosphate buffer with 1ml of 0.1% H_2O_2

was added to each well. Absorbance was read using a spectrophotometer at 410nm. All

of the necropsy positive (n=31) and the same random selection of sera from uninfected

horses used in the Western blot and serological ELISA analyses (n=77) were analyzed.

Plates were set up with serially diluted known positives down one column, a

column of blank wells were incubated with PBS (pH 7.4) only. Test faecal preparation

was added to duplicate wells on a randomly generated template. Once the plate was read,

the mean OD of the blank wells was subtracted from each of the test serum OD readings,

to control for variability between different batches of plates.

3.2.7 Data Analysis

All positive horses and a random selection of necropsy-negative horses were

tested with Western blot analysis, serological ELISA and coproantigen ELISA. Linear

regression analysis of anti-12/13kDa antibody against infection intensity and 12/13kDa

antigen against infection intensity was completed using Microsoft® Office Excel (2003)

as described by Zar (1999).

Sensitivity or detectability indices were calculated for all techniques, using the

formula DI(+) = (true positives X 100) / (true positives + false negatives) (Colwell and

Baron, 1990). Specificity of analyses was calculated using the formula DI(-) = (true

negatives X 100) / (true negatives + false positives) (Colwell and Baron, 1990). In this

study sensitivity defines how well a technique correctly identifies horses infected with *A. perfoliata*, and specificity is how well a technique identifies uninfected horses. True positives were those animals found to have tapeworms present in the caecum. True negatives are those animals that did not contain tapeworms in the caecum at necropsy. False positives were those without tapeworms in the caecum, but which were positively detected by one of the diagnostic techniques. False negatives were those animals with tapeworms present in the gut, but not detected by analysis.

3.3 RESULTS

3.3.1 A. perfoliata Intensity

A. perfoliata were found at the ileo-caecal junction and in the caecum of 7% of the horses (31/434). Infection intensity ranged from 1 to greater than 1000 worms per animal, and mean intensity (± standard deviation) was 41 ± 179. One horse had a severe infection of greater than 1000 worms, and many juvenile worms may have been undetected. Horse age was not correlated with infection intensity (Figure 3.1; n = 31, r = 0.166, p > 0.05).

3.3.2 Coprological Analysis

Coprological analysis detected *A. perfoliata* eggs in 4 out of 29 known necropsy positive horses (Figure 3.2). Faecal samples were not available for two of the infected horses. Tapeworm eggs were detected in horses with worm burdens of 2, 5, 7 and >1000 worms. Sample size was not large enough to determine whether there was a relationship between tapeworm EPG and infection intensity. Sensitivity of the coprological analysis was only 54%, specificity was 100% (Table 3.1). Faeces of necropsy negative horses were not examined for eggs.

3.3.3 Western Blot Analysis

Western blot analysis of the sera from necropsy positive horses (n=31) showed all were positive for antibodies, particularly to the 12/13kDa antigen (Figure 3.2). Sensitivity of western blot was 100% and specificity was 87%, as 12 false positives were found (Table 3.1). Of the randomly selected sub-sample of necropsy negative horses, 15% were found to have antibodies to the 12/13kDa antigen (n=12). Figure 3.3 shows an example of the Western blot analysis. Horse number 2119 was negative for worms in the caecum at necropsy, but was found by Western blot analysis to be positive, and therefore was considered a false positive.

3.3.4 Serological ELISA

The serological ELISA identified 18 of the 31 necropsy positive horses to have antibodies that recognized the crude E/S antigens (Figure 3.2). The negative cut-off OD for the ELISA was determined to be the mean of horses positively identified by Western analysis of 0.333 (n = 43). There were 22 horses detected as false positives and 13 infected horses detected as false negatives. No correlation was found between *A. perfoliata* infection intensity and the OD (Figure 3.4; $r = 0.051$; $p > 0.05$). The sensitivity was 70% and the specificity was 78% (Table 3.1).

3.3.5 Coproantigen ELISA

The coproantigen ELISA identified 19 of the 29 necropsy positive horses to have 12/13kDa E/S antigen in the faeces (Figure 3.5). The coproantigen ELISA results from the necropsy confirmed positive and negative horses were compared and indicated that uninfected horses had a lower average OD (Figure 3.5). Horses with fewer than 5 worms were excluded and a negative cutoff was established at 0.07 (Figure 3.6). Infection

intensity was found to be positively correlated with 12/13kDa E/S antigen OD (Figure 3.7; $r = 0.572$, $p < 0.05$). The sensitivity of this assay was 74% and the specificity 92%, with only 6 found as false positive (Table 3.1).

3.3.6 Comparison of Analyses

Only three horses were positively identified as being infected with *A. perfoliata* by all four techniques (Table 3.2). Twelve horses were correctly identified as being infected by Western blot analysis, serological ELISA and coproantigen ELISA combined (Table 3.2). Coproantigen ELISA detected all 11 horses found to have 5 or more tapeworms in the caecum, whereas the serological ELISA detected only 7 of these animals (Table 3.2).

3.4 DISCUSSION

Cestode intensity varied considerably in horses from the abattoir. Overall prevalence was found to be 7% (see Chapter 2). The majority of horses (n=29) had fewer than 30 tapeworms, and only two horses had severe infections of 132 and greater than 1000 worms respectively. A distribution of this sort within a sample of necropsied horses has also been reported by Meana *et al*. (2005). High intensities of *A. perfoliata* are known to cause severe intestinal lesions (Williamson *et al*., 1997), intussusception (Barclay, 1982), caecal rupture leading to death (Ryu *et al*., 2001) and colic (Proudman and Edwards, 1993; Proudman and Trees, 1998; Proudman *et al*., 1998). Only one highly infected horse in this study showed any form of pathology associated with infection, and had lesions at the tapeworm attachment sites.

The age of infected horses ranged from 4-16 years old, and was not related to the infection intensity. Under the management system used at this facility, the majority of

horses raised for slaughter are grazed throughout their lives, before coming into the feed-lot just prior to slaughter, so they may have an equal probability of becoming infected throughout their lives (Personal communication, Martin Conrad, 2006). The range of infection intensities was useful as a baseline for evaluating the different techniques in this study.

Diagnostic analyses used in this study varied in their sensitivity and specificity, as expected. Coprological analysis results are in agreement with those of several other studies (Proudman and Edwards, 1992; Nilsson *et al*., 1995; Meana *et al*., 1998; Traversa *et al*., 2008) indicating that coprological techniques have low sensitivity. Eggs were detected in the faeces of horses harbouring from 2-1000 worms. Nilsson *et al*. (1995) stated that the probability of accurately diagnosing a horse with fewer than 100 tapeworms is very low, which could explain the low sensitivity in this study as only 2 out of 31 horses had more than 100 worms. The random release of gravid tapeworm segments and unequal distribution in faeces as described by Nilsson *et al*. (1995) may explain the lack of a relationship between EPG and infection intensity found in this study. Faecal analysis had high specificity, because no false positive samples were found.

Western blot analysis was the most sensitive and reliable technique for identifying infected horses. All horses with tapeworms in the caecum were positively diagnosed with this method. Sensitivity of 70% and specificity of 78% were found for the serological ELISA used in this study, which was similar to the sensitivity of 68% and specificity of 95% reported by Proudman and Trees (1996a). Since western blot and serological ELISA analyses detect presence or absence of antibodies to the 12/13kDa antigen, it was assumed that the serological ELISA results would be very similar.

However, the serological ELISA had much lower specificity because 22 horses without tapeworms in the caecum were found to have circulating antibodies to the crude E/S antigens. This could indicate that these horses had been previously exposed to *A. perfoliata* and may have continued to produce antibodies in the absence of an active infection (Proudman and Trees, 1996b). Proudman and Trees (1996b) also caution that there is variability in parasite-induced immune responses of the host and rate of decay of circulating antibody which could alter the sensitivity of diagnosis.

There was no correlation between the anti-12/13 kDa IgG antibody and infection intensity using the serological ELISA in this study (Figure 3.3), which contradicts the findings of Proudman and Trees (1996b). This indicates that infection intensity was not related to the concentration of circulating antibody in infected horses. There are several factors that can affect the results of the serological analyses, such as variability in host immune response, rate of antibody decay levels post-treatment, re-infection after treatment, and modification of the host immune system by the parasite (Hoglund *et al.*, 1995; Proudman and Trees, 1996a, b). There is not enough known about the timing and duration of the horse immune response to tapeworm infection to draw conclusions.

This study documents for the first time a positive correlation between the 12/13 kDa *A. perfoliata* antigen and infection intensity using the coproantigen ELISA. Kania and Reinemeyer (2005) were the first to use a coproantigen ELISA for the detection of *A. perfoliata* antigens in horse faeces. With a limited sample size and unknown infection intensity of positive horses, they found no overlap between positive and negative samples (Kania and Reinemeyer, 2005). In other systems, correlation between parasite intensity and antigen detection has been reported. Mezo *et al.* (2004) found a positive correlation

between *Fasciola hepatica* antigen concentrations using coproantigen ELISA and infection intensity of lambs. Casaravilla *et al*. (2005) found increasing OD values of E/S antigen detection with increasing *Echinococcus granulosus* intensity. The coproantigen ELISA was able to detect horses with *A. perfoliata* intensities as low as 5 tapeworms. Sensitivity of the coproantigen ELISA was found to be 74%, and specificity was 92%. There were 6 false positives detected, but there is a possibility that infected horses were undetected at necropsy because only the caecum was inspected. Tapeworms have been found in the ventral colon in very few cases (Williamson *et al*., 1997). Since the ability of this coproantigen ELISA to correctly identify uninfected horses was very high, this assay could be a potentially useful diagnostic tool for assessing *A. perfoliata* infection.

Colic is considered a major threat to equine welfare, with roughly 11% of cases per year being fatal in U.S.A. (Traub-Dargatz *et al*., 2001). Any abdominal pain experienced by horses, caused by a variety of problems, may be termed colic. An estimate of 115 million dollars was spent on colic-related services in 1999 (Traub-Dargatz *et al*., 2001). If the number of colic cases caused by tapeworm infection could be reduced by properly diagnosing horses infected with tapeworms, hundreds of thousands of dollars and many horses' lives could be saved. A more reliable diagnostic technique to determine equine infection status would mean that horses could be treated on a case by case basis to both eliminate worms and prevent the spread of tapeworms to pasture mates. The findings suggest that the coproantigen ELISA used in this study could more accurately estimate infection intensity. This would facilitate the interpretation of the parasitological status of the infected animals to identify horses at risk of gastrointestinal disease and colic, which the serological analyses and PCR techniques do

not.   It is possible that the coproantigen ELISA assay used in this study may be further validated and used for diagnosis of infected individuals and also infection intensity.

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Table 3.1. Sensitivity and specificity of current diagnostic techniques for detecting *A. perfoliata* infection.

Technique	Sensitivity (%)	Specificity (%)
Coprological	54	100
Western Blot	100	87
Serological ELISA	70	78
Coproantigen ELISA	74	92

Table 3.2. Summary of results from diagnostic analyses used to identify horses infected with *A. perfoliata* at necropsy (n = 31). Asterisk represents horses accurately identified by all four techniques.

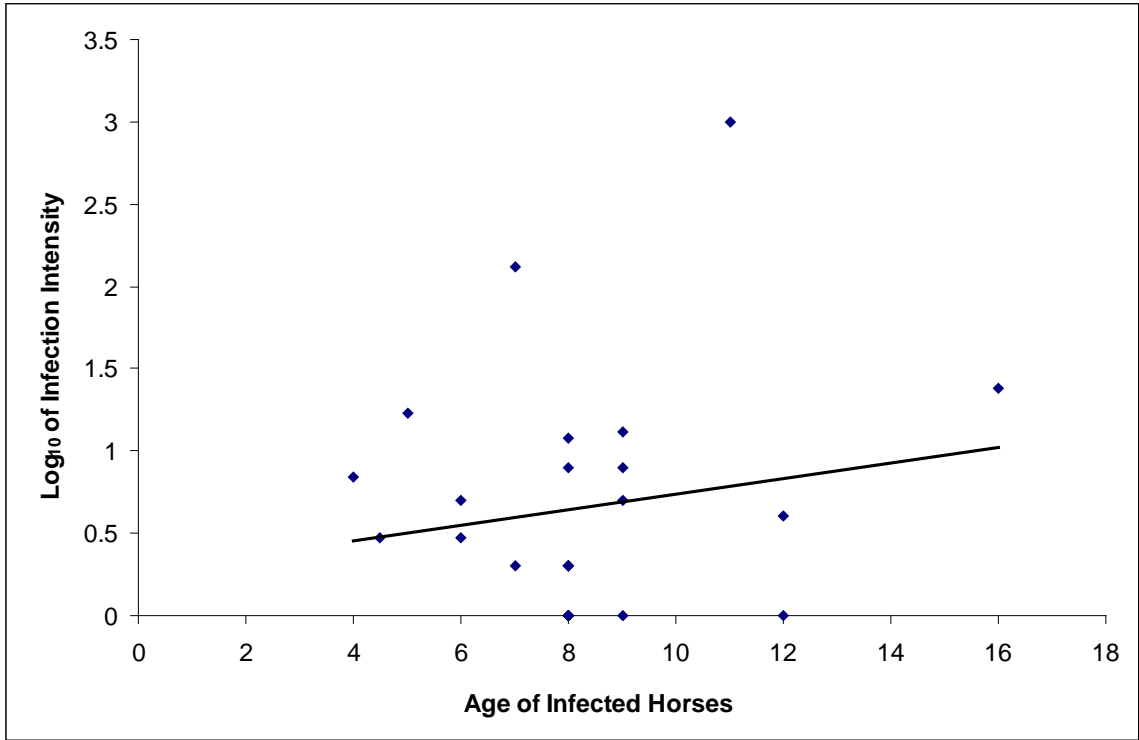| Carcass #	Tapeworm Intensity	Western Blot	Coprological (Eggs per 10 grams)	Serological ELISA	Coproantigen ELISA
2087a	132	+		+	+
2109	3	+		+	
2071a	5	+	+ (1)		+
2117	1	+			
2137	2	+		+	+
2066	5	+			+
4064 *	1000	+	+ (14)	+	+
4082	17	+			+
4098	3	+			+
4106 *	5	+		+	+
4110	7	+	+ (37)	+	+
4124 *	2	+	+ (2)	+	+
2148	8	+	n/a	+	n/a
2128	2	+			
2079a	4	+			+
2131	2	+			
2073	1	+			+
2077	2	+			
2087b	1	+			
2095	1	+			
2075	2	+		+	
2089	12	+		+	+
2115	1	+	n/a	+	n/a
2079b	1	+		+	
2083	1	+		+	+
2087c	13	+		+	+
5206	2	+		+	+
2058	1	+		+	
2060	1	+		+	+
2069	8	+		+	+
2071b	24	+			+

Figure 3.1. No correlation was found between age of infected horses and infection intensity. Linear regression analysis indicated no relationship (n = 31, r = 0.166, p > 0.05). Equation of the regression line is $y = 0.0478x + 0.2608$, adjusted $R^2 = 0.0277$.
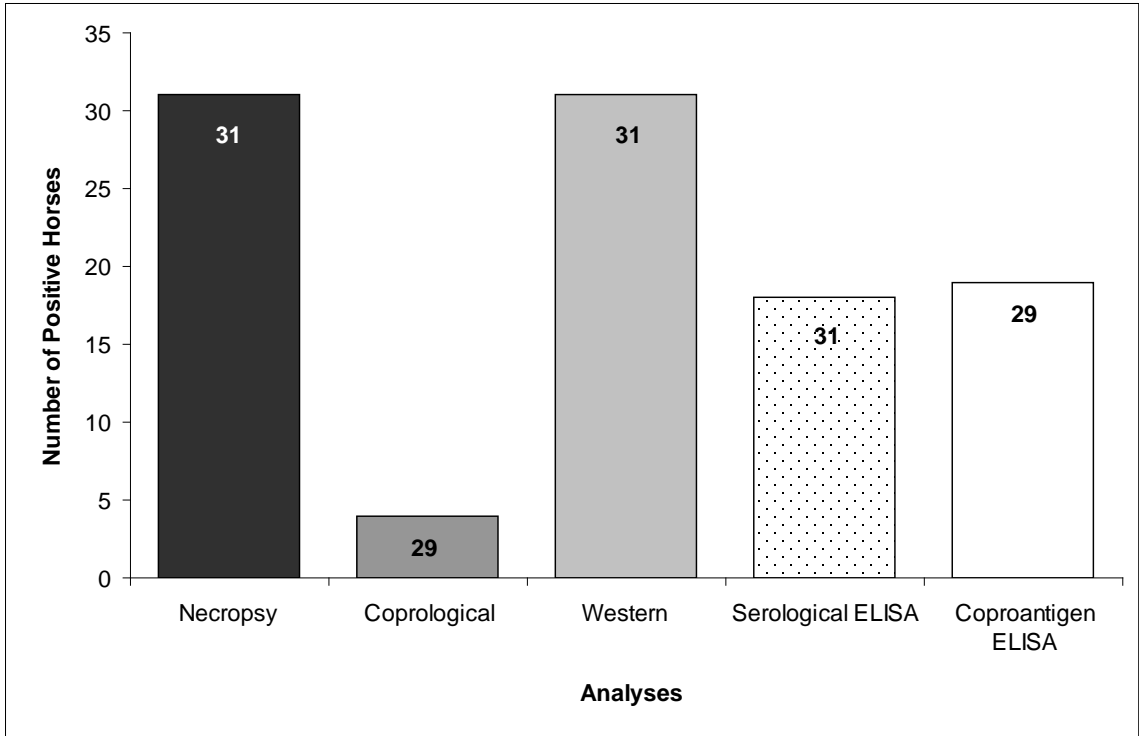
Figure 3.2. A comparison of the different diagnostic techniques in positively identifying *A. perfoliata* infection of necropsied horses. Data presented is from the 31 necropsy positive horses. Numbers in bars indicate sample sizes.
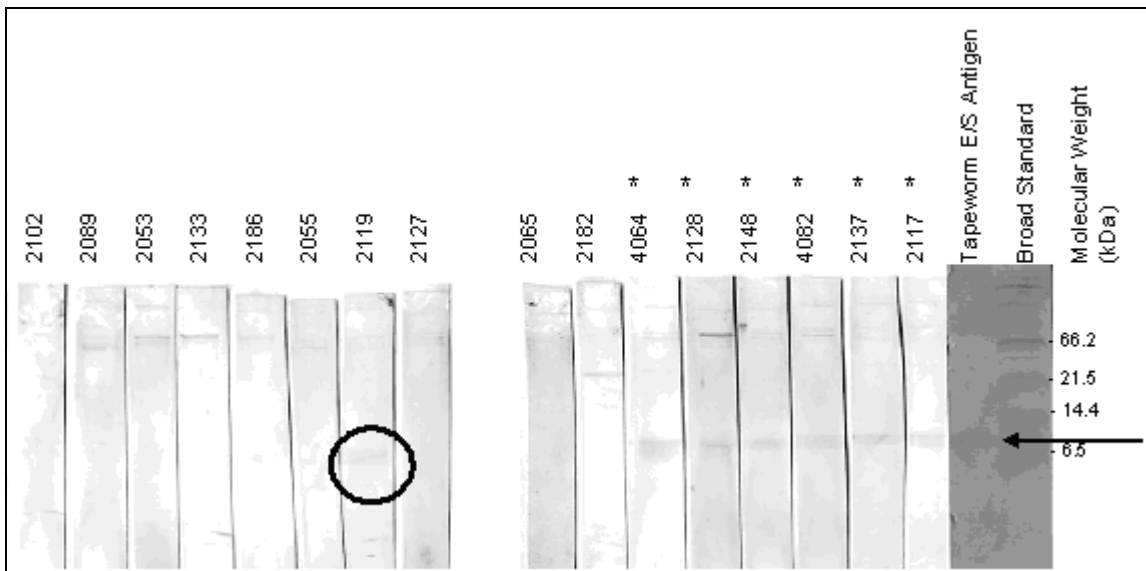

Figure 3.3. Examples of Western blot analysis. Arrow indicates the 12/13 kDa antigen. Numbers indicate individual horses. Necropsy-positive animals are indicated with an asterisk. The circle around horse # 2119 indicates that although it was necropsy negative, it was found to be Western blot positive.
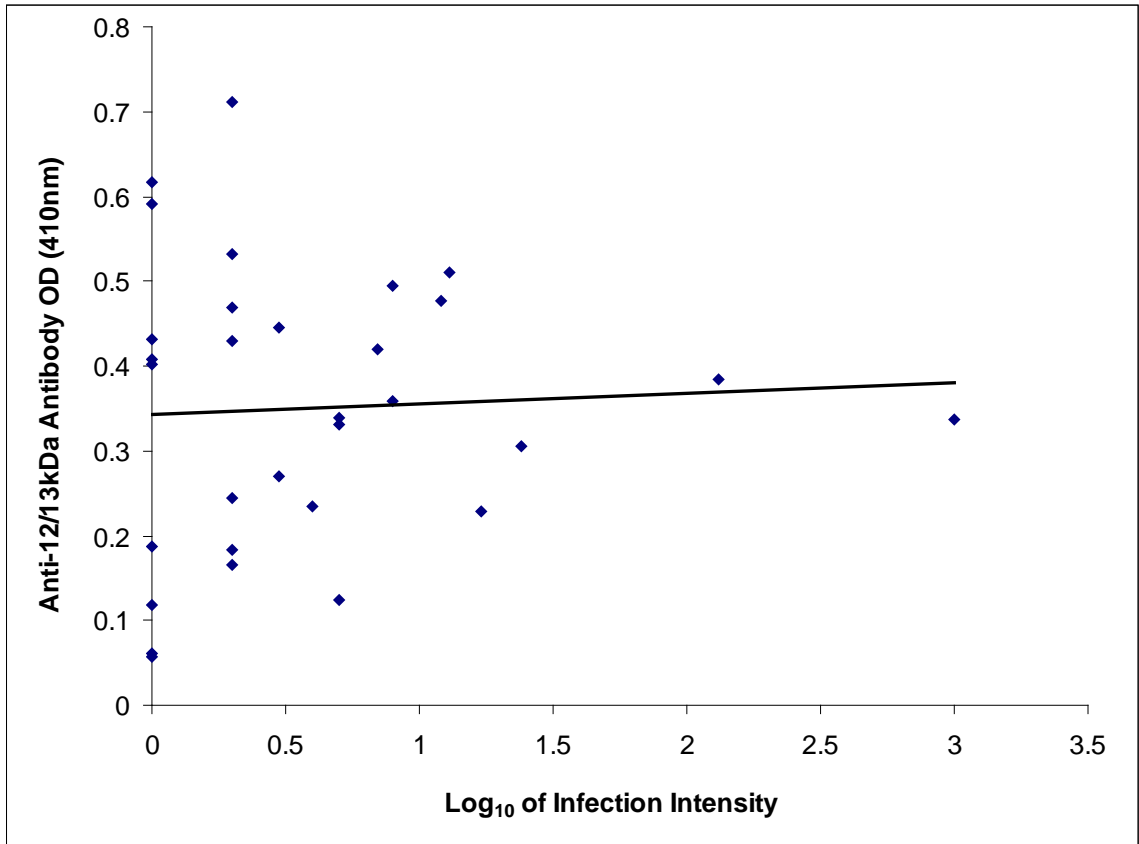
Figure 3.4. Scatter plot showing the relationship between anti-12/13 kDa antibody and *A. perfoliata* infection intensity as evaluated using the serological ELISA. Linear regression analysis indicated there was no relationship (n = 31, r = 0.150, p > 0.05). The equation of the regression line is $y = 0.0123x + 0.3432$; adjusted R^2 = 0.0026; p > 0.05.
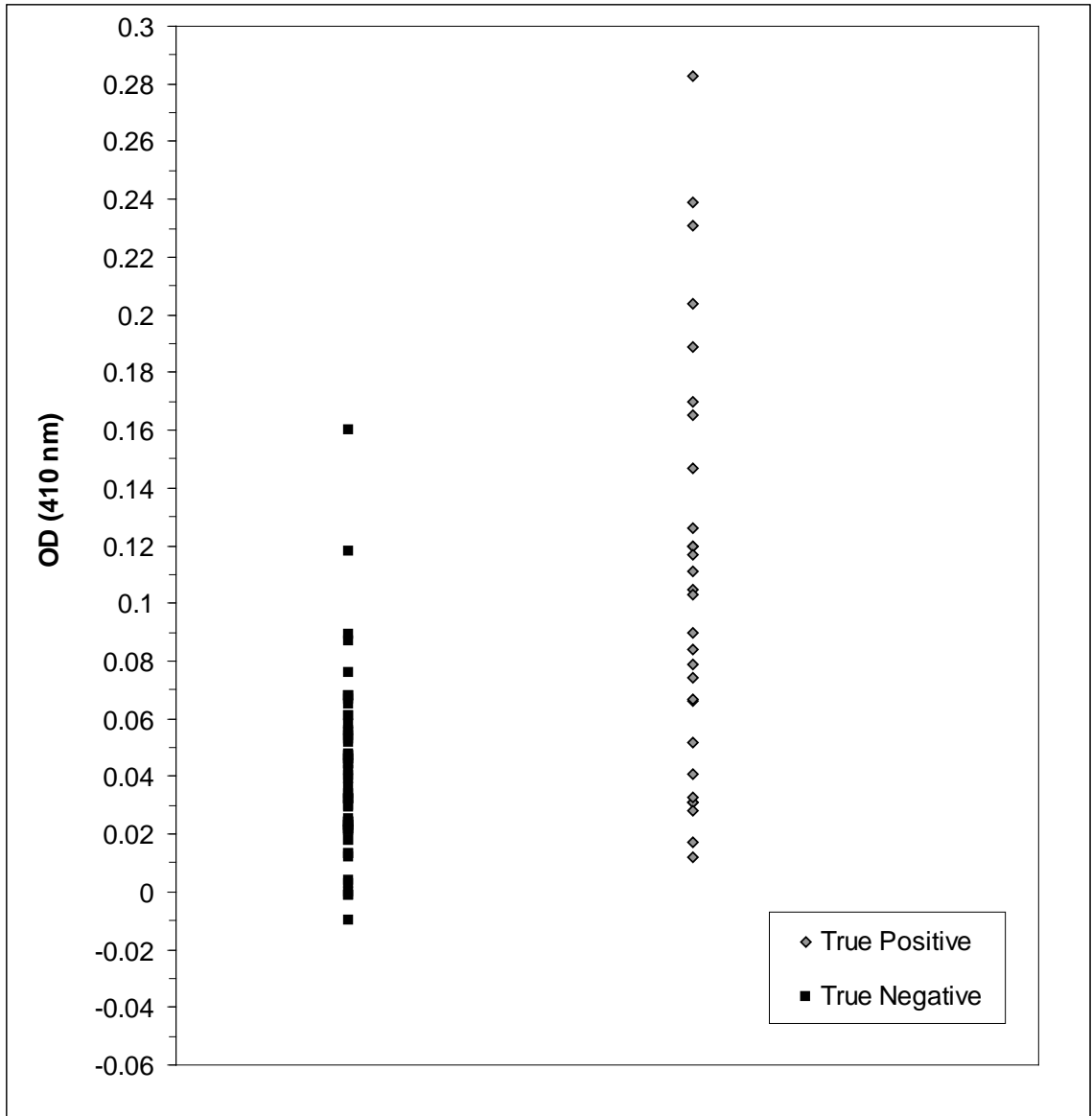
Figure 3.5. Scatter plot showing the OD_{410nm} values from confirmed positive (n = 29) and negative (n = 69) horses using coproantigen ELISA.
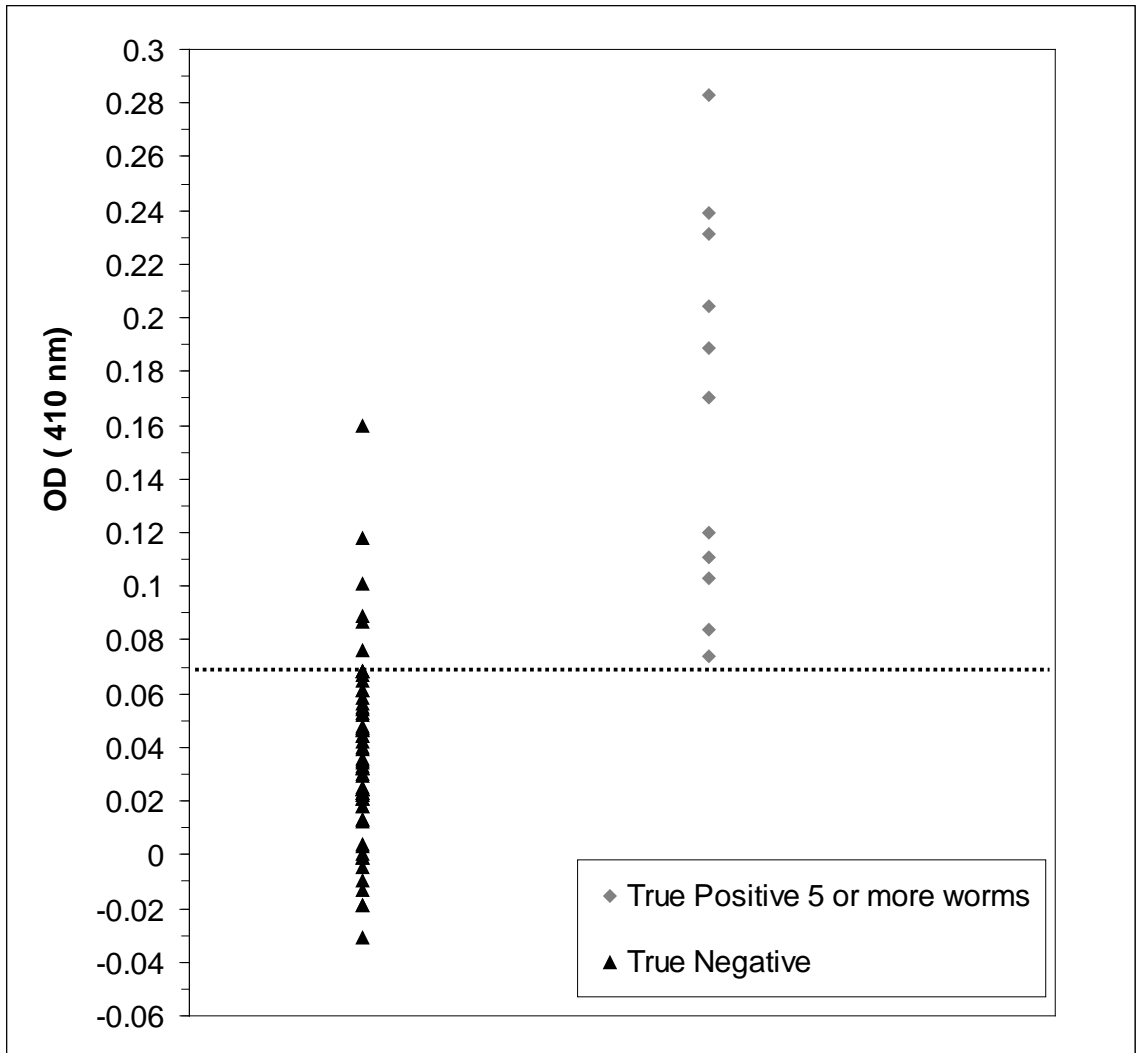
Figure 3.6. Scatter plot showing the OD values from coproantigen ELISA analysis of necropsy-confirmed positive horses with 5 or more worms (n = 11) in the caecum compared to negatives (n = 69). Dashed line is at 0.07 OD the negative cut-off point for this assay.
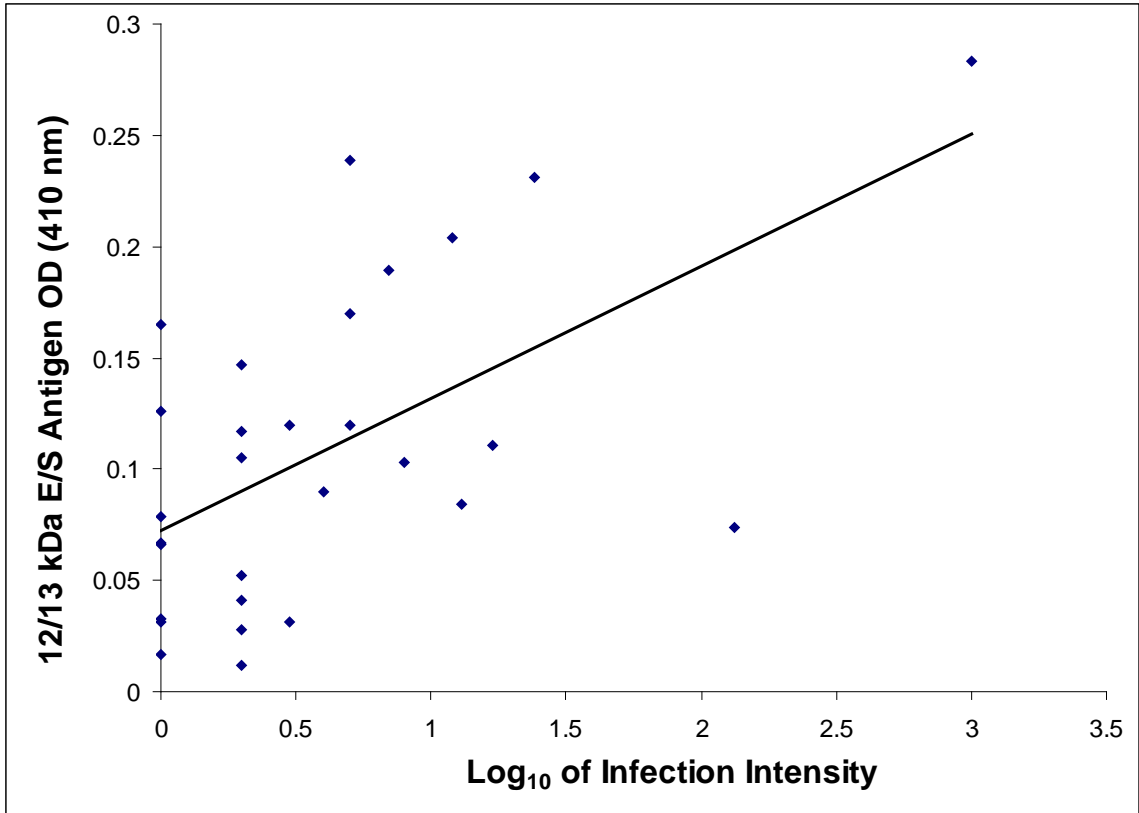
Figure 3.7. Scatter plot showing the relationship between the infection intensity and amount of 12/13 kDa E/S antigen detected in faeces using coproantigen ELISA analysis. Infection intensity and 12/13 kDa E/S antigen were found to be positively correlated (n = 29, $r = 0.572$, p < 0.05). The equation of the line is $y = 0.0595x + 0.0724$, adjusted $R^2 = 0.3278$.

Chapter 4. General Conclusions

Results from the epidemiological study reported in Chapter 2 are the first to confirm the presence of the tapeworm *Anoplocephala perfoliata* in horses of southern Alberta, Canada.   Prevalence of cestode eggs ranged between 4-18% in over 1000 faecal samples collected over 24 months from selected sites across southern Alberta. Data obtained from necropsies of individual horses sampled over one of these years confirmed the generally low prevalence in horses from this region, although intensities of infection varied greatly between individual horses. Overall, results from the faecal survey indicated that seasonal and spatial variation in prevalence tended to parallel results reported outside North America (Meana *et al*., 2005). Thus, prevalence was higher in faeces collected from habitats with higher precipitation and during the winter.

One important result from the epidemiological survey is the identification of general environmental characteristics (probably linked to soil moisture) and pasture management as risk factors. Management practices appear to be very important in transmission of this parasite, because horses on pasture had a significantly higher prevalence of infection than those kept in corrals or stables (Chapter 2). It would follow that horses exposed to pasture likely have a higher probability of coming into contact with infected mites. Precipitation and temperature differences are well known to exist between the eastern and western regions of southern Alberta (Adams *et al*., 2007); both of these factors are recognized as important determinants of parasite transmission on pastures. One important direction for future studies is to complete experiments that are designed to evaluate the biology and distribution of the oribatid soil mites that act as intermediate hosts. Few studies have been completed at the level of the intermediate

hosts for Anoplocephaline cestodes, except to confirm their role in the life cycle (Stunkard, 1939; Bashkirova, 1941). The identification of key factors in determining rates of transmission is still a significant shortcoming. Therefore, the precise roles of environmental characteristics (especially precipitation and temperature) and management strategies in the transmission of *A. perfoliata* are speculative. More information needs to be collected about the transmission rates of tapeworm eggs to mites and mites to horses. Mites could be collected from pastures with resident infected horses to determine mite infection status, prevalence, and density, which would give clues on the probability of ingestion by grazing horses.

The lack of an accurate method of *A. perfoliata* diagnosis has lead to the difficulty associated with determining prevalence and intensity in horses. Based on the results of previous studies summarized in Chapter 1 (Table 1.1) and the results of this study, faecal analysis for *A. perfoliata* is easy and inexpensive, yet it may underestimate prevalence by up to 50% (Meana et al., 1998; Beelitz and Gothe, 2001). Given that only 4 of the 29 known infected horses from the abattoir were detected as egg-positive (Chapter 3), the sensitivity of this technique was only 54%. It is very likely that the prevalence of *A. perfoliata* in southern Alberta horses is higher than observed. It is possible that on an individual ranch, especially in western regions, most co-grazing horses are likely to be infected. However, coprological analysis identified only some of the horses from the same pasture as being infected, while others found to be infected on previous sampling occasions were not. On the other hand, it is possible that patterns of worm intensity on a ranch are highly variable between individual horses, as indicated by necropsy results from the abattoir. It is these results that further emphasize the need for refined diagnostic

methods, either using a technique other than faecal egg counts, or using it in conjunction with another method.

Accurate diagnostic techniques for detecting *A. perfoliata* are still a challenge facing veterinary parasitologists. One key strength of this study was access to necropsies at a local abattoir, where *A. perfoliata* infection status and intensity in horses was determined and used as a baseline for evaluating current diagnostic techniques. Serological analyses were the first to be explored, and were more reliable at diagnosing infection than faecal analyses (Proudman and Trees, 1996a, b). Comparison of current serological techniques in this study revealed that Western blot analysis had the highest sensitivity and specificity, but false positives were still detected (Chapter 3). Proudman and Trees (1996b) found a significant positive correlation between worm intensity and amount of antibody in a serological ELISA, a result which was not supported by this study (Chapter 3). Specificity analysis in the serological ELISA is hindered by the inability to define horses as tapeworm-negative. Another problem with serological analyses is that the persistence of antibodies following elimination of parasites is still unknown, and individual horses likely have different responses to infection (Proudman and Trees, 1996b). Without knowing the antibody half life in circulation, it is impossible to identify currently infected horses, but does indicate if they have been exposed to the parasite.

To overcome the confounding problems associated with serology, Kania and Reinemeyer (2005) developed a coproantigen ELISA that detected small amounts of *A. perfoliata* E/S antigens excreted in horse faeces. The coproantigen ELISA used in this study had a higher sensitivity and specificity than the serological ELISA used by

Proudman (1996a, b). Importantly, this study is the first to report a positive correlation

between *A. perfoliata* antigens released in the faeces and tapeworm infection intensity

(Chapter 3, Figure 3.7). This finding has considerable implications because the

coproantigen ELISA is fairly inexpensive to run, it has high specificity (94%), and seems

to accurately assess worm intensity. Also, the ability to predict infection intensity has

considerable clinical importance, since tapeworm infection has clearly been linked to

gastrointestinal disease and colic in horses (Pearson *et al*., 1993; Proudman and Trees,

1999; Mezerova *et al*., 2007). This study had a limited sample size of known positive

horses, yet even horses with low tapeworm intensities (<5 worms per host) were correctly

identified (Chapter 3). Thus, the coproantigen ELISA technique has the potential to more

closely identify tapeworm intensity in horses, which appears to offer an improvement on

the results of Proudman and Trees (1996b) that classified horses into low, moderate and

high infections.   Therefore, the coproantigen ELISA needs to be further verified with

more horses of known infection status and intensity. In the future, the coproantigen

ELISA may prove to be useful both as a clinical diagnostic tool, and in epidemiological

studies especially to further examine the role of tapeworm infection with equine colic.

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