NOTE TO USERS

Gastrointestinal parasites in lactating dairy cattle: Immunological monitoring and its relationship with production.

A Thesis

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Abstract

Gastrointestinal parasitism in cattle, caused mainly by *Ostertagia ostertagi* and several species of *Cooperia*, is an important cause of economic losses worldwide. The most detrimental effects of gastrointestinal nematodes (GIN) are caused by subclinical parasitism. The effect of GIN on milk production was evaluated in a meta-analysis of 75 clinical trials. After controlling for publication bias and/or small study effect, an estimate of 0.35 kg/cow/day was obtained, suggesting that GIN do affect milk production. Historically, the level of GIN has been estimated by using the fecal egg counts (FEC) technique, but this method performs poorly in adult animals. Consequently, other diagnostic techniques have been suggested. One of them is an ELISA using a saline extract of a crude adult *O.ostertagi* antigen. This ELISA showed a good repeatability within plates and between batches of antigen. The ELISA results were not affected by the use of preservatives or by freezing of the milk samples. ELISA optical density ratios (ODR) and total IgG levels were moderately correlated, with both increasing toward the end of the lactation. After controlling for age, season, herd and SCC, an increase in milk production of 10 kg/day was associated with a reduction of 0.04 in ODR. These findings suggested that ODR values were not greatly influenced by production factors but that they might be adjusted for the level of milk production in order to compare ODR values from cows at different stages of lactation. A bulk tank milk survey of all the diary farms in PEI was carried out during the Fall of 2000. Exposure of cows to pasture and whole herd anthelmintic treatment were associated with ODR levels. An increase in ODR levels from the 25th to the 75th percentile was associated with a drop in milk production of 1.2 kg/cow/day. These results indicated that this ELISA is a potentially useful diagnostic technique to measure parasite exposure in adult dairy cows and that GIN have an important impact on milk production.

As part of the evaluation of the ELISA, a longitudinal study was performed where milk, serum and fecal samples were collected from 38 farms. The ODR values increased with cow age and tended to decrease during the housing period and start increasing in the spring before the cows went out to pasture. Individual cow ODR values had very low correlation with FEC but showed a reasonably high correlation when herd averages values were compared. Twenty-eight of the herds participated in a clinical trial of eprinomectin treatment at calving. The cow level ODR values determined late in the previous lactation before treatment had a marginally significant effect on treatment response, suggesting that high ODR cows responded better to the anthelmintic treatment. Similarly, the ability of this ELISA to predict reproductive performance was also evaluated. The hazard of conception was lower for cows having high ODR in the late lactation before treatment compared to low ODR cows, suggesting that higher parasite burdens had an adverse effect on reproductive performance. Finally, the performance of this ELISA was evaluated in a second clinical trial using confined and semi-confined dairy herds. In this trial the anthelmintic treatment did not affect the milk production response and there was no interaction effect between late lactation ODR values and milk yield response. Although this analysis is based on preliminary data, it suggested that GIN nematodes did not affect milk production in semi-confined and confined herds.

In conclusion, the results of this research indicated that this ELISA is a promising tool for monitoring GIN levels in adult animals and that parasites have and adverse effect on performance in dairy herds that utilize pasture.

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Dedicated to:

Gaby, Milagros, Ignacio

and Francisco

Contents

Abbreviations

A	Accuracy parameter (concordance correlation coefficient)
ABTS	2,2'-azino-bis-(3-ethyl-benzth-iazoline-sulfonic acid) (substrate)
ANOVA	Analysis of variance
AR1	First order autoregressive (correlation)
BA	Bland-Altman plot
CCC	Concordance correlation coefficient
CDHMS	Canadian dairy herd management system
CMT	California mastitis test
CV	Coefficient of variation
DIM	Days in milk
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FEC	Fecal egg counts
GEE	General estimation equation
GIN	Gastrointestinal nematodes
GR	Grams
H+L	Heavy and light chains
H_2O_2	Hydrogen peroxide
HR	Hazard ratio
ICC	Intra-class correlation coefficient
Ig	Immunoglobulins
L	Liters
Nt	Average negative controls
OD	Optical density
ODR	Optical density ratio
PBS-T20	Phosphate buffered solution containing Tween 20
Pt	Average positive controls
R/r	Pearson correlation coefficient
R^2	Squared correlation coefficient
RIGLS	Restricted generalized iterative leas-square

SCC	Somatic cell count
SD	Standard deviation
SE	Standard error

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1. General introduction

1.1 Gastrointestinal nematodes in cattle

Ostertagia ostertagi and *Cooperia* spp. are the two most important gastrointestinal nematodes (GINs) infecting cattle worldwide (1). They are an important cause of economic losses (2). In adult cows, clinical signs of GINs are very rare. By far the most important detrimental effects of GINs under modern production systems are caused by subclinical parasitism. These effects include reduced weight gain, decreased milk production (3) and impaired reproductive performance in adult animals (4; 5).

The direct life cycle of *O.ostertagi* and *Cooperia* spp. involves one definitive host and is characterized by a freeliving and a parasitic phase. The freeliving stage takes place in the environment, and contaminated pastures are the main source of infective third stage larvae (L3). Parasite eggs are shed into the feces where first stage larvae (L1) develop within a few days. After two molts they develop into infective larvae (L3), which move from the fecal pat onto the pasture. Once the L3 are ingested by the host, they develop into fourth stage larvae (L4) and then into adults; these developments occur in the abomasum or small intestine, in approximately three weeks. However, if hypobiosis or arrested development occurs at the fourth stage, the L4 may remain in that stage for several months before resumption of development (6).

O.ostertagi is considered the most pathogenic parasite of cattle in North America (1; 7) and causes two clinical conditions: type I and type II ostertagiosis. Type I ostertagiosis is seen in young stock exposed to GINs during the first grazing season; the main clinical signs are anorexia and weight loss (7). Type II ostertagiosis is seen in yearlings and sometimes in adult animals; it is most commonly seen late in the housing period. Type II ostertagiosis

occurs when hypobiotic larvae emerge from the gastric glands following an overwintering period, and is characterized by reduced feed intake, diarrhea and hypoalbuminemia (8). The epidemiological patterns of ostertagiosis have been well defined in most areas of the world. The most important factors controlling the preparasitic phase of the life cycle are temperature and moisture. In Atlantic Canada larvae from GINs were able to survive the winter on pastures and be the source of infection the following spring (9). While weather conditions will mainly determine the larvae's availability on the pasture, other factors related to immune status, nutritional levels and management practices will also influence the level of pasture contamination (9). In temperate areas of the world, hypobiosis is the most significant factor controlling transmission patterns of *O.ostertagi.* In northern North America, hypobiosis begins in the fall, enabling the parasite to survive the long and cold winter. By contrast, in southern North America which is characterized by a warm spring and autumn, a hot summer and a mild winter, hypobiosis begins in the spring and lasts over the summer; development of arrested larvae resumes in late summer and early fall (6).

Young stock are more susceptible to the detrimental effects and clinical disease caused by GINs, but adult cattle can harbor a significant number of GINs associated with potential production losses (10; 11). The effect of GINs in adult cattle has been mainly evaluated by looking at their impact on milk production. Many studies have been carried out to examine whether these parasites do or do not affect milk production levels. Results have varied. Recently, a literature review summarized 87 trials that evaluated the milk production response after anthelmintic treatment in adult dairy cattle (3). The authors reviewed trials involving different protocols and drugs and concluded that a median increase of 0.63 kg/cow/day might be expected after anthelmintic treatment.

While many studies have evaluated the effect of GINs on milk production, very few have been performed to determine the effect on reproductive performance in adult cattle. In beef cattle, there is some evidence that anthelmintic treatment has improved either percentage of pregnancy (12) or calving rates (13). In dairy cattle, days from calving to conception (4; 5) have also been evaluated.

1.2 Diagnostic tests for gastrointestinal nematodes

Eysker and Ploeger (7) suggested that the diagnosis of GINs should be part of any herd health-monitoring program. They pointed out that currently all the parameters needed to monitor GINs in dairy cattle have not yet been established. They recommended a five-point checklist that any diagnostic test should have in order to fill these requirements:

1. the test enables an estimate of nematode exposure;
2. test values should reflect production losses;
3. test values can be used to predict the risks of future production losses and allow recommendation of appropriate preventive measures;
4. test results are easy to assess;
5. the test is inexpensive.

Historically, the level of GINs has been estimated by using the fecal egg count (FEC), which meets some of the above requirements. The method presents a high correlation with parasite levels during the first grazing season (7), but it has a very low correlation with parasite burden (i.e. number of worms) in adult animals (3; 14). Consequently, FECs have not led to a clear understanding of the impact of GINs in adult cattle, and other diagnostic

techniques have been suggested. One of them, which represents the main body of this thesis, is an indirect enzyme-linked immunosorbent assay (ELISA) using the saline extract of a crude adult *O.ostertagi* antigen.

1.3 The indirect ELISA

This ELISA was originally developed in the Netherlands by Keus et al. (15) in the early 1980s. The test detects antibody levels against *O.ostertagi* and *Cooperia* spp. and provided a new alternative for monitoring GINs in cattle. However, cross-reaction between parasite species (1 1; 16-19) and the lack of standardized protocols have been reported as drawbacks of the test (20) and have limited its adoption.

High cross-reactivity is attributable to the source of the ELISA antigen. Crude worms extracts are the usual source (10; 21-23), but the associated antigens are not species-specific and appear to be shared between closely related species. The importance of parasite species specificity has been challenged because the gastrointestinal parasitism is not only related to *O.ostertagi* but also to other nematodes present on the pasture such as *Cooperia* spp. (24). Consequently, the ELISA results should be able to correlate with total parasite burden and not only with one parasite species. Furthermore, the technique should be targeted to determine the relative level of infection rather than the mere presence of the GINs, because infections with these nematodes are present in all pastured animals.

The lack of standardized protocols relates to the difficulty of obtaining high-quality antigens. Although, several papers (21; 24;25) refer to Keus's work (15) when describing the methodology of the immunoassay, they do not contain a clear description of the ELISA protocol; this makes comparison of ELISA results difficult. In addition, different methods

have been used to express the ELISA results. These include adjusting the OD of the sample to OD values of the negative and positive controls in each plate (10; 26), and calculating the standard curve with a logit transformation and applying that to adjust the sample OD values (27). However, none of the mentioned studies made reference to the performance of the immunoassay used.

The above concerns might be addressed by specifying a clear test protocol, obtaining purer antigen and evaluating the repeatability of the test. Nevertheless, ELISA antibody titers using crude antigens have shown significant between herd variation and are correlated with production response after anthelmintic treatment (21; 25). In addition, an experimental study showed that antibody titers reflected the level of parasite exposure in first year calves (22) and later it was shown that antibody titers in adult cows correlated with GIN infection levels on the pasture at the end of grazing season (28).

1.4 Aim and scope of thesis

The overall objective of this research program was to evaluate the use of a crude antigen, indirect, adult *O.ostertagi* ELISA to monitor the GIN parasitism in adult dairy cattle using milk samples. This work had the following three components.

1. Evaluation of the expected impact of GINs on productivity in dairy cattle. This was achieved by conducting a meta-analysis of all previous research into the effects of anthelmintic treatment on milk production in dairy cattle (Chapter 2).

2. Evaluation of the laboratory characteristics of the ELISA and determination of how intrinsic factors (e.g. level of milk production, stage of lactation) affect those findings. This component involved determining the optimal method for standardising ELISA results, and assessing the repeatability of the ELISA (Chapter 3). This was followed by an assessment of how factors such as age and stage of lactation affect ELISA results (Chapters 4 and 6).

3. Evaluation of the ELISA as a tool for monitoring GIN parasite burdens and their potential impact on productivity. This task was difficult since there is no reliable measure of GIN burdens or their impact. While slaughterhouse studies can be used to determine levels of adult GINs in culled cows, even these may not correlate well with the impact of these parasites, as larval stages may play an important role in reducing productivity. Also, slaughterhouse studies are extremely expensive to carry out and consequently have very small sample sizes. Thus, the value of the ELISA can only be assessed indirectly and this was done by answering the following questions:

   a. Do ELISA values vary with management practices that are expected to influence GIN parasite levels in a predictable manner? For example, does increasing exposure to pasture result in higher ELISA values for a herd? Previous work suggested that ELISA optical densities are related to the level of pasture exposure, anthelmintic treatment and spread of manure (29). Moreover, a negative relationship with milk production has also been found (29; 30). These relationships were investigated using bulk milk samples (Chapter 5), and, to a limited extent, individual cow samples (Chapter 6).

b. Are high ELISA values associated with reduced levels of milk production? This was evaluated using bulk milk samples (Chapter 5) and individual cow samples (Chapter 6 - impact on milk production). The relationship between reproductive performance and ELISA values was also assessed using individual cow samples (Chapter 7).

c. Are ELISA values predictive of the response to treatment with anthelmintics? The ability of the ELISA to predict response to anthelmintic treatment would provide the most compelling evidence that the ELISA is a useful tool for monitoring GIN parasite burdens. This would allow use of the ELISA in devising more rational anthelmintic strategies. The test's potential in this regard was indicated by Ploeger at al. (21) who found a positive correlation with milk production response after anthelmintic treatment, using serum samples in a small number of animals. However, in a later study using a similar approach, they could not find a significant correlation (25). The relationship between ELISA results and response to anthelmintic treatments requires more study, and the ability of the ELISA to predict milk production response was evaluated in the present programme (Chapter 6 - pastured herds; Chapter 8 - non-pastured herds). The predictive ability in terms of reproductive performance was also examined (Chapter 7).

The above components were broken down into six specific objectives:

1. conduct a meta-analysis review of the effect of GIN on milk production;
2. standardize and evaluate the repeatability of the crude antigen, adult, *O.ostertagi* ELISA;

3. investigate the relationship between total IgG and production parameters with ELISA results;
4. evaluate the associations between ELISA results and milk production and management practices known to be related to GIN parasitism;
5. evaluate the ELISA as a predictor of milk production and reproductive performance after anthelmintic treatment in pastured adult dairy cattle; and
6. evaluate the ELISA as a predictor of milk production response after anthelmintic treatment in confined and semi-confined adult dairy cattle.

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2. A meta-analysis of the milk production response after anthelmintic treatment in adult dairy cattle

2.1 Abstract

This manuscript presents the results of a meta-analysis to estimate the effect of anthelmintic treatment on milk production in dairy cattle. The literature search included peer reviewed journals (both full articles and abstracts), conference proceedings and theses, and included documents written in English, Spanish, French, Portuguese or Italian. The study outcome was defined as the difference in milk production (kg/cow/day) between treated and untreated cows. Fixed and random effect meta-analyses were performed on 75 trials published between 1972 and 2002. The combined estimate after controlling for publication bias and/or small study effect was of +0.35 kg/cow/day. Significant variation among studies was detected and although several variables were found to be associated with the study outcome, they did not significantly reduce the unexplained variability among trials. Trials reporting the use of endectocides had higher milk production response compared with trials using older anthelmintics. Similarly, whole herd treatment trials or trials which applied the treatment in mid lactation or strategically had higher milk response compared with calving or dry period treatment trials. Trials reporting the results as total 305 days milk production had lower response compared with trials that measured production as daily weight. Primiparous cow trials and trials carried out in southern countries had lower responses compared with multiparous cows trials and trials carried out in northern countries, respectively.

2.2 Introduction

The milk production response results obtained from field trials of anthelmintic treatments in adult dairy cattle have been equivocal and consequently clear guidelines as to when anthelmintic treatments should be applied have not been available. While it has been shown that adult dairy cattle can harbour an important number of gastrointestinal parasites, mainly *O.ostertagi* (1; 2), the lack of a reliable diagnostic test for use in this group of animals (3; 4) makes it difficult to establish a threshold value that justifies anthelmintic treatment (5).

In an attempt to obtain an overall estimate of the effect of deworming adult dairy cattle on milk production, Gross et al (3) performed a narrative review of more than 80 trials in dairy cattle and concluded that a median increase in milk production of 0.63 kg/cow/day might be expected after anthelmintic treatment. Although traditional narrative reviews have been widely used in veterinary literature, they are somewhat subjective in nature and therefore prone to a reviewer bias (6). They also do not easily take into account the precision of the observed effects and consequently studies tend to be weighted equally.

On the other hand, a meta-analysis allows a reviewer to arrive at conclusions that may be more accurate than can be obtained from a non-quantitative, narrative review (7). A meta-analysis is a systematic review of the literature followed by a quantitative compilation of all relevant results in which the precision of each individual study is taken into account. A meta-analysis may be biased by the exclusion or inclusion criteria used in the study selection process or by the methods chosen to combine the selected studies (6). However these biases can be minimized when a detailed protocol specifying the selection of the studies and collection and analysis of the data is followed.

The objective of this paper was to use a meta-analysis to estimate the extent to which anthelmintic treatment, with a variety of drugs and treatment protocols, influenced milk production in dairy cattle.

2.3 Material and Methods

2.3.1 Literature review

The literature review was based on the following databases: Parasite CD (1973 – 2002), CAB Abstracts (1972 – 2002), Medline (1966 – 2001). The keywords utilized were "anthelmintic dairy cattle"; "milk production nematodes"; "milk production anthelmintic"; "dairy cows dairy herds anthelmintics". A total of 416 references related to parasitism in cattle were identified. References were removed if the study pertained to species other than dairy cattle, pertained to the use of anthelmintics in ages other than lactating age dairy cattle, did not measure milk production and were not written in English, Spanish, French, Portuguese or Italian. The search was not restricted to peer reviewed journals and it included abstracts, conference proceedings and theses. In addition, all the references related to milk production trials cited in a recent review paper (3) were also identified. A total of 83 potential articles were identified for the meta-analysis. Bibliographies of retrieved articles were examined for further references.

2.3.2 Outcome evaluated / Data extraction

The mean difference in milk production between treated and control groups in kg/cow/day was used as the outcome. If the study reported this outcome using any other time

frame (e.g. actual 305-day milk yield, projected 305-day milk yield) or measurement (e.g. liters, pounds), the outcome was transformed to kg/cow/day.

The precision of the estimate reported was based on the standard errors (SE) or standard deviations (SD) of the treatment and control groups. If the paper reported separate estimates for each group, they were recorded as such. If the paper reported a common SE (or SD), that estimate was used for both groups. If the paper only reported a Z statistic or P-value, an estimate of a common SE was computed. For papers that only reported a P-value less than or equal to a given value (e.g. <0.05), then that given value was taken and the P-value and SE computed as above. Finally, for studies that simply reported a non-significant effect, an arbitrary P-value of 0.15 was assumed and used in the calculation of the SE.

Data were only extracted from clinical trials although the studies need not have been conducted in either a randomized or blinded manner.

In addition to the outcome of interest, the information described in Table 2.1 was also extracted. All this information was extracted from the articles independently by two investigators using a structured data collection form. The two datasets were then compared and all the disagreements were resolved by the senior author (Sanchez) re-reviewing the source paper.

2.3.3 Meta-analyses

Fixed and random effects meta-analyses were carried out to evaluate the effect of anthelmintic treatment on milk production. A fixed effect meta-analysis assumes that the treatment effect is constant across trials and that the variability between studies is only due to chance. The fixed effect meta-analysis weighted each study by the inverse of the variance of

the parameter estimate. On the other hand, a random effects meta-analysis assumes that there is a normal distribution of the study effects and the variance of the distribution is estimated from the data. The method of DerSimonian and Laird (8) was used to estimate the variance for the random effects model. The heterogeneity statistic Q (8) was used to evaluate if there was significant variability between studies. Under the null hypothesis of a common treatment effect among trials, these Q statistics follow a chi-squared distribution with K-1 degrees of freedom, where K is the number of trials. If a significant P value (i.e. <0.05) for the Q statistic was observed, the results from the random effects model were presented.

Because several biases might influence the results of a meta-analysis, the following procedures were performed in order to detect and, if needed, to correct for possible publication bias or other small study effects. First the Begg's (9) and Egger's (10) tests were used in combination with a funnel plot (11). If there was any evidence of publication bias, from either of these tests or the funnel plot, the "trim and fill" method suggested by Duval and Tweedy (12) was used to estimate and correct for this publication bias. This method works by omitting small studies until the funnel plot is symmetrical. Then, using the trimmed funnel, the center of the plot is estimated and the omitted studies are replaced along with their hypothetical "missing counterparts" around the center (11).

2.3.4 Meta-regression

2.3.4.1 Study quality characteristics

In order to investigate factors which may have influenced study results, weighted regression analyses (meta-regression) between the study effect and trial quality characteristics (including precision of estimate) were performed. This was done in two steps; first an

unconditional analyses were carried out between the study outcome and the following trial characteristics: precision, randomization, blinding, control for confounders in the analysis and publication type. Subsequently, all unconditionally significant variables (P value ≤ 0.15) were retained and evaluated in a multivariable analysis.

2.3.4.2 Other study characteristics

Meta-regression analyses were also used to evaluate the effects of: product formulation (endectocides or other drugs), parity of cows (primiparous, multiparous or all combined), time of treatment (dry off, calving, mid lactation or strategic treatment), time after treatment (days after treatment), individual treatment (vs. whole herd treatment), geographic location where the trial was performed and pasture exposure on the study outcome.

2.3.5 Cumulative meta-analysis

A random effect cumulative meta-analysis was performed using the 75 trials. This methodology computed an overall estimate of treatment effect at the time each study was published. A cumulative meta-analysis may be used to identify, retrospectively, when a treatment effect reached conventional levels of statistical significance. However, it was used in this study to identify possible temporal patterns in the study results.

A moment estimator of the between-study variance was used in all of these analyses and no adjustment for clustering of results within author was carried out, since the number of reports per author was low. All analyses were carried out using the statistical program Stata, Version 8 (13).

2.4 Results

2.4.1 Literature review

From the 82 articles identified by the literature review, 7 of them could not be retrieved (6 English and 1 Italian). Of the remaining 75, 11 articles were not used in the analyses for the following reasons: 4 were review articles with no original data (14-17), 2 were duplicates (18; 19), 4 were trials in which the animals were artificially challenged (20-23), and 1 only evaluated the effects of flukes on milk production (24).

The remaining 64 articles described 97 anthelmintic field trials. Out of these, 8 articles (9 trials) did not contain data on the study outcome (25-32), 11 articles (13 trials) presented data in a manner that were not usable in the meta-analyses (usually no estimate of the precision of the results was available) (30; 33-42). Consequently 48 articles with results from 75 trials were used for the meta-analysis (articles presented in Table 2.3 and listed in the references). Forty-five of these articles were written in English, two were in French and own was in Spanish. Summaries of the main study characteristics of the trials not used and used in the meta-analysis are presented in Tables 2.2 and 2.3, respectively.

From the 9 trials that did not report the outcome of interest, 6 reported a non-significant effect of treatment on milk production while the other 3 did not report any value. Out of the 13 trials not used in the meta-analysis, 3 did not report the length of the milk production measurement so the outcome could not be computed, 1 reported a negative effect and 9 reported a positive effect of treatment on milk production (2 were significant, 7 did not report the significance). The mean number of cows used in these trials was 241 (range: 20-1643).

Out of the 75 trials used in the meta-analysis, 16 reported a negative effect and the other 59 reported a positive response. The mean number of cows used in these trials was 535

(range: 12-4500). The descriptive statistics are presented in Table 2.4. Overall, the 75 trials had a median increase in milk production after anthelmintic treatment of 0.64 kg/cow/day (mean: 0.52, 95% C.I.: 0.35; 0.70) (Table 2.4).

2.4.2 Meta-analysis methods

The heterogeneity test was significant (P<0.001) so the results from the random effects model are presented. The DerSimonian and Laid pooled estimate of the mean difference in milk production was of 0.46 kg/cow/day (95% C.I. 0.36; 0.56). A forest plot presenting the results from each trial as well as the combined effect is shown in Figure 2.1. Each line represents the results from a single study. Each line is labeled with a unique label which identifies the study and groups of cows represented. The length of the line represent the 95% confidence interval for the study outcome from the study. The center of the shaded box on each line marks the point estimate of the outcome, and the area of the box is proportional to the weight assigned to the study in the meta-analysis. The dashed vertical line marks the overall effect estimate. The <> at the bottom of the dashed line shows the confidence interval for the overall effect. The solid vertical line marks the value where anthelmintic treatment would have no effect.

The statistical approaches for the detection of publication bias or small study effect showed different results. While the Begg's test reported a non-significant bias (P = 0.73), the Egger's test reported a highly significant value (P < 0.001) and a visual inspection of the funnel plot suggested that publication bias may have been present (Figure 2.2). In addition, the random effects "trim and fill" method reduced the combined pooled estimate from 0.46 to 0.35 (95% C.I. 0.25; 0.45). This method also indicated that an additional 12 trials would have

been necessary in order to remove this publication bias or other small study effects. A funnel plot is presented in Figure 2.2 showing the 12 "filled" studies in addition to the 75 original trials used in the meta-analysis.

Although only 11 trials reported both a formal randomization procedure and a blinded treatment allocation, a similar pooled estimate (0.33 kg/cow/day) to that reported by the "trim and fill" method was obtained when considering only these trials, suggesting some association between study quality and effect estimate.

2.4.3 Meta regression analyses

Table 2.5 shows the results obtained from the meta-regression analyses of the associations between study effect and trial quality characteristics. Both the unconditional and multivariable analyses showed an association between study effect and precision (as would have been expected based on the previous assessment of publication bias). Similarly, the study outcome was associated with publication type and control for confounders. If control for other confounders was used in the statistical analysis the mean difference in milk production was approximately 0.25 kg/cow/day lower than in trials that did not control for confounders in the analysis.

Because trials reporting anthelmintic treatment during the dry-off period were not statistically different from trials reporting treatment at calving, time of treatment for these two groups was combined into one category (dry-off/calving). The results from the meta-regression analyses performed between the study outcome and variables reflecting other trial characteristics are presented in Table 2.6. Although the variables evaluated in this analysis did not substantially reduce the variance between studies, three of them (time of treatment, milk

measure and individual treatment) were significantly associated with the study effect. For example, studies that applied the anthelmintic treatment to mid-lactation cows or strategically throughout the year had an average production response of 0.40 kg/cow/day higher compared with trials where the cows were treated either during the dry period or at calving. On the other hand, trials in which individuals were assigned to treatment groups (vs. whole herd treatment) had a substantially lower production response. These two study characteristics were highly correlated as studies in which individuals were treated generally applying the treatment at the time of calving, while whole herd treatment encompassed all stages of lactation.

In relation to geographic location, trials were categorized as northern and southern: Northern trials were those carried out in Canada, Northern United States, north-west Europe. Southern trials were those carried out in the southern United States, New Zealand, Australia, Argentina, India and Sri Lanka. Northern trials tended to have higher milk response compared with southern country trials, but this difference was not significant. Pasture exposure was classified as pasture-seasonal and pasture-year-round. Out of the 75 trials, only 59 reported information on pasture exposure (36 trials were pasture-seasonal trials and the remaining 23 were pasture-year-round trials). No statistical significant difference was found between level of pasture exposure and the study outcome ($\beta = 0.11$, $P = 0.4$).

2.4.4 Cumulative meta-analyses

The results of the cumulative meta-analysis showed a significant effect after the first trial used in this analysis. However, a pronounced pattern was observed for the overall estimate between 1972 until 2002 (Figure 2.3). During the 70s the trials had the highest treatment response. This estimate tended to decline during the 80s and start increasing again

during the 90s, but without reaching the values observed initially. Control for confounders and especially controlling for farm effect was related to publication year, studies carried out during the 70s were less likely to control for farm effect, so larger responses with significant effects were more likely to be reported (data not shown). Moreover, the type of drug used was related to the publication year. Older drugs (eg. thiabendazole, morantel, levamisol) were more likely to be used during the 70s, newer benzimidazole drugs were more likely to be used during the 80s and trials using endectocides (eg. ivermectin) were more likely to be performed during the 90s.

2.5 Discussion

The combined unadjusted and adjusted estimates of 0.46 and 0.35 kg/cow/day, respectively, obtained from the 75 studies were smaller than the 0.63 reported by Gross et al. (3). Although not all the studies used in that review were used in the present meta-analysis, a similar median increase in milk production was obtained in this study (Table 2.4) which suggests some similarity between these two reviews. Only 57 trials used in this meta-analysis matched with those evaluated by Gross et al. (3) (n=87) (the other 30 studies did not have data suitable for the meta-analyses, did not meet the inclusion criteria or were not retrieved). They had a median increase in milk production of 0.54 kg/cow/day (data not shown). Using these 57 studies, the combined estimate derived from the random effects model after correcting for a possible publication bias was 0.32 kg/cow/day (95% CI 0.21; 0.43), which was similar to that obtained from the full dataset.

The significant heterogeneity found in this analysis was expected because the differences in study designs, treatment protocols, drugs, geographic locations and age groups would have influenced the treatment response.

The visual assessment of publication bias based on the funnel plot (Figure 2.2) as well as the results from the Begg's test suggested that there was not a large publication bias or other small study effects in this study. On the other hand the Egger's test showed a highly significant association between study effect and precision of the study. However, both tests have been reported to produce false-positive results (i.e. they may suggest the presence of bias when in fact none is present) with the regression approach (Egger's test) more sensitive than the rank correlation test (Begg's test) (43) to this possibility. Moreover, funnel plot asymmetries have been related not only to publication bias but also to inclusion of trials of lower quality (i.e. studies which are not double blind, studies with inadequate allocation of animals to the treatment group). Lower quality trials tend to overestimate the true treatment effect (44). However, collectively, this was substantial evidence of bias due either to publication bias or other study quality effects.

When variables accounting for trial quality were evaluated, a number of them were associated with treatment effect and they showed similar trends to those reported by Moher et al. (44). Two variables not associated with treatment effect were the use of a formal randomization procedure and blinding of treatment allocation. While those studies reporting a blinded treatment assignment tended to have a lower effect, those reporting a formal randomized procedure had a higher response, which was not expected. The finding must be interpreted with caution because these variables were recorded as reported in the paper, which may not reflect the way that the trial was conducted in all cases. In relation to that, Thompson

and Higgins (45) pointed out that the results obtained from the meta-regression analysis should be interpreted with some caution, especially when the trial characteristics have low variability across studies, because analysis may be biased by unmeasured confounders. On the other hand, trials published in indexed journals or trials that used better statistical methodologies, which might reflect the quality of the published study, were associated with lower production response. These analyses suggested that the overall estimate of 0.35 kg/cow/day would be less biased and so more appropriate to report as the overall pooled estimate.

The results from the meta-regression analyses of study design characteristics are presented in Table 2.6. Trials using macrocyclic lactone endectocides (i.e. ivermectin, moxidectin and eprinomectin) had a higher milk response compared with those using either benzidmizadoles or older anthelmintics (i.e. coumaphos, thiabendazole). In contrast, Gross et al. (3) found the same median increase between new and old anthelmintics. The results of this meta-regression analysis support the theory that the new generation of anthelmintics is more effective, especially against immature stages, including *O.ostertagi* (46-48), so a higher response might be expected.

Trials where animals were treated either in mid-lactation or strategically (several times during the year) had higher response compared with trials where treatment was during the dry-off period or around calving. Gross et al. (3) found a similar effect; animals treated in mid lactation had twice the production response compared with those treated during the dry period or around calving. The larger production response in whole herd treatment trials might be related to the elimination of the parasites at one point with a more pronounced decrease in the

pasture contamination, and consequently less re-exposure to parasites or to more frequent treatments (strategic anthelmintic treatment).

Production response declined by 2g for each additional day in the study follow-up period. Similarly when the outcome was reported as either 305-actual or 305-projected milk production the response was lower compared with daily weight trials that reported effect on a per day basis. In relation to that, daily weight trials tend to measure milk production during a shorter period of time.

Primiparous cow trials had a lower milk response compared with multiparous cow trials. This might reflect different susceptibilities to gastrointestinal parasites between these age groups or the high production capacity of older cows. Agneessens et al. (1) found a significant number of parasites in adult cattle with the higher worm counts in cows less than 3 years old and greater than 10 years old. Moreover, Nødvedt et al. (49) reported that first lactation animals had statistically significant higher FEC than did second or greater lactation animals. On the other hand, Sanchez et al. (50) found that first lactation animals had lower optical densities from a crude indirect ELISA compared with second or greater lactation animals. This suggests that first lactation animals might be more susceptible to gastrointestinal nematodes and be re-infested soon after anthelmintic treatment resulting in a lower milk production response.

Southern countries are associated with better weather conditions with year-round pasture grazing season. Consequently, conditions in typical warm temperate regions, are more favorable for parasite development and survival resulting in a higher transmission to hosts throughout much of the year (51). Although not significant, southern countries tended to have a lower milk response compared with northern countries, which might reflect either a higher

rate of parasite re-exposure after treatment or lower milk production in countries where cows are on pasture year-round. However, a positive trend between milk response and level of pasture exposure was observed suggesting that cattle under grazing conditions are more likely to suffer the detrimental effect of GINs.

The distinct pattern observed in the cumulative meta-analysis (Figure 2.3) might be related to the combined effect of improvement in the statistical analysis and /or changes in efficacy of the anthelmintic used. The decline in the effect from 1972 to 1985 may have been due to the use of better study designs and analytic methods. Although controlling for a farm effect will have a bigger impact on the precision of the estimate, trials which did control for herd effect also tended to control for other variables in the analysis. Controlling for confounder was associated with lower milk response (Table 2.5). The increased response through the 90s might reflect the greater efficacy of the anthelmintics used (e.g. endectocides).

2.6 Conclusions

In conclusion, the results of this meta-analysis showed that, on average, an increase of milk production of approximately 0.35 kg/cow/day might be expected after anthelmintic treatment. There was evidence of publication bias and small study effect in the published literature, mainly related to studies of lower quality. Variables such as formulation type, time of treatment, time after treatment, outcome measure recorded, parity and geographic location were associated with the study outcome, but only had a small effect in terms of reducing the unexplained variance between studies.

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Table 2.1. Additional information extracted from the studies considered in the review of anthelmintic treatment and milk production response in adult dairy cattle.

Variable		Description
Trial quality		
	Publication type	Journal indexed in *Index Medicus*, journal not indexed, abstracts/ paper proceedings
	Randomization	If a method of randomization was reported
	Treatment blind	Blinded treatment administration reported
	Control confounders	Confounders controlled for in the analysis (i.e. age, farm, season, previous milk production, etc)
Trial design		
	Publication year	Year when the trial was published
	Formulation	If endectocide was used vs others
	Time of Treatment	Dry off, calving, mid lactation or strategic
	Individual treatment	If the treatment was not applied to the whole herd at once
	Milk length	Period of time (days) milk production was measured.
	Milk measure	Milk production measure (daily weight, 305 actual, 305 projected, etc)
	Location	Country where the trial was carried out
	Parity	Primiparous, Multiparous, All combined
	Pasture exposure	If the cows were on pasture year round, pasture seasonal or partially confined

Table 2.2. Summary of the 13 studies not usable in the meta-analysis.

Publication year	First author last name	Drug	Parity	Number of cows	Control conf.[1]	Milk Measure[2]	Mean diff.	Sig.[3]	Reason not used[4]
1974	Brown	Coumaphos	2nd +	36	No	DW	-0.54	NR	A
1976	Harris	Coumaphos	2nd +	85	Yes	NR	-	NS	B
1978	Todd	Coumaphos	2nd +	175	No	305	1.14	NR	A
1978	Todd	Coumaphos	2nd +	157	No	305	0.11	NR	A
1978	Pouplard	Thiabendazole	2nd +	190	No	305	1.31	NR	A
1978	Pouplard	Thiabendazole	1st	47	No	305	3.52	NR	A
1979	Mcbeath	Fenbendazole	All	174	Yes	305	0.57	NR	A
1980	Corba	Thiabendazole	2nd +	84	No	305	0.92	NR	A
1981	Gremillet	Thiabendazole	All	46	No	NR	-	NR	B
1982	Kloosterman	NR	2nd +	NR	Yes	305	0.67	S	C
1983	Mathews	Fenbendazole	2nd +	NR	Yes	140	1.10	S	C
1984	Thomas	Fenbendazole	NR	1643	Yes	NR	-	NS	B
1999	Yazwinski	Moxidectin	NR	20	No	DW	0.36	NR	A

[1] Control for confounding (i.e. previous lactation, age, season, farm) in the analysis

[2] DW = daily weight, 305 = 305 total milk production (actual or projected), 140 = total milk production at 140 days in milk.

[3] Statistical significance reported: NR = not reported, NS = not significant, S = significant

[4] Reason not being used: A: no precision or P-value reported, B: no measure of milk production reported; C: no sample size reported

Table 2.3. Summary of the 75 studies used in the meta-analysis.

Study ID	Publication year	First author last name	Drug	Parity	Number of cows	Control conf.[1]	Milk Measure[2]	Mean difference	Significance [3]
1	1972	Todd	Copper sulfate	2nd +	692	No	DW	0.94	< 0.01
2	1972	Todd	Phenothiazine	2nd +	427	No	DW	1.10	< 0.01
3	1972	Todd	Thiabendazole	2nd +	397	No	DW	1.02	< 0.01
4	1973	Bliss	Coumaphos	2nd +	1003	No	DW	0.54	< 0.01
5	1974	Bliss	Thiabendazole	2nd +	488	Yes	305	0.63	<0.1
6	1976	Bliss	Thiabendazole	2nd +	267	Yes	305	0.79	<0.05
7	1976	Harris	Thiabendazole	2nd +	315	Yes	305	-0.93	NS
8	1977	McQueen	Levamisole	2nd +	48	Yes	220	1.12	< 0.05
9	1977	Mcqueen	Levamisole	2nd +	48	Yes	220	0.75	<0.05
10	1977	Mcqueen	Levamisole	2nd +	48	Yes	220	1.23	< 0.05
11	1977	van Adrichem	Cambendazole	1st	48	No	287	0.67	< 0.05
12	1979	Barger	Fenbendazole	2nd +	335	No	DW	-0.25	NS
13	1979	Pluimers	Thiabendazole	2nd +	542	Yes	305	0.75	< 0.01
14	1980	Gibbs	Thiabendazole	2nd +	212	No	305	-0.61	NS
16	1980	Heider	Thiabendazole	1st	28	No	305	0.05	NS
15	1980	Heider	Thiabendazole	2nd +	84	No	305	0.43	NS
17	1980	Wilk	Thiabendazole	All	1180	Yes	305	0.31	< 0.01
18	1980	Wilk	Thiabendazole	All	1520	Yes	305	0.44	< 0.01
19	1981	Frechette	Morantel	2nd +	217	Yes	305	0.84	< 0.05
20	1981	Morhain	Thiabendazole	2nd +	12	Yes	DW	0.71	NS
21	1981	Morhain	Thiabendazole	2nd +	12	Yes	DW	-0.26	NS
22	1981	Thomas	Thiabendazole	2nd +	96	Yes	305	-0.53	NS
23	1982	Barger	Fenbendazole	2nd +	316	Yes	305	-0.14	NS

Study ID	Publication year	First author last name	Drug	Parity	Number of cows	Control conf.[1]	Milk Measure[2]	Mean difference	Significance [3]
24	1982	Bliss	Morantel	2nd +	210	Yes	305	1.23	< 0.05
25	1982	Fisher	Levamisole	All	116	Yes	305	-0.36	NS
26	1982	Fisher	Levamisole	All	42	Yes	305	3.16	< 0.05
27	1982	Michel	Levamisole	All	3660	Yes	305	0.19	NS
28	1982	Michel	Thiabendazole	All	3660	Yes	305	0.17	NS
29	1982	Michel	Fenbendazole	All	3660	Yes	305	0.21	NS
30	1984	Fox	Levamisole	2nd +	343	Yes	305	0.05	NS
31	1984	Gouffe	Albendazole	All	341	Yes	DW	1.10	< 0.05
32	1985	Fetrow	Thiabendazole	1st	218	Yes	305	0.83	NS
33	1985	Fetrow	Thiabendazole	2nd +	486	Yes	305	-0.34	0.73
34	1986	Block	Morantel	2nd +	2660	Yes	DW	1.20	< 0.05
35	1986	Miller	Coumaphos	1st	80	Yes	305	-0.26	> 0.05
36	1986	Miller	Thiabendazole	1st	25	Yes	305	-2.17	> 0.05
37	1986	Miller	Thiabendazole	1st	30	Yes	305	1.38	> 0.05
38	1986	Miller	Coumaphos	2nd +	242	Yes	305	0.88	> 0.05
39	1986	Miller	Thiabendazole	2nd +	78	Yes	305	-0.31	> 0.05
40	1986	Miller	Thiabendazole	2nd +	57	Yes	305	0.43	> 0.05
41	1986	O'farrell	Febantel	All	807	Yes	305	0.32	< 0.05
42	1986	Sommerfeldt	Thiabendazole	1st	68	No	DW	-1.10	> 0.05
43	1986	Takagi	Coumaphos	2nd +	28	No	DW	1.02	< 0.09
44	1987	Bisset	Oxfendazole	2nd +	4500	Yes	251	0.21	< 0.01
45	1987	Block	Levamisole	2nd +	1296	No	DW	1.24	< 0.05
46	1988	Biondani	Fenbendazole	2nd +	530	No	305	0.66	< 0.05
47	1989	Ploeger	Ivermectin	2nd +	469	Yes	305	0.67	< 0.01
48	1989	Tharaldsen	Fenbendazole	1st	184	Yes	305	-0.32	> 0.05

Study ID	Publication year	First author last name	Drug	Parity	Number of cows	Control conf.[1]	Milk Measure[2]	Mean difference	Significance [3]
49	1989	Tharaldsen	Fenbendazole	2nd +	232	Yes	305	-0.72	< 0.05
50	1990	De Rond	Ivermectin	2nd +	20	Yes	133	0.84	< 0.10
51	1990	De Rond	Febantel	2nd +	20	Yes	133	0.89	< 0.05
52	1990	Ploeger	Albendazole	1st	347	Yes	305	0.64	< 0.01
53	1990	Ploeger	Albendazole	2nd +	1385	Yes	305	0.44	<0.01
54	1992	Sanyal	Fenbendazole	2nd +	96	Yes	DW	1.42	0.02
55	1992	Spence	Fenbendazole	2nd +	779	Yes	DW	0.60	< 0.05
56	1993	Bhongade	Albendazole	2nd +	50	Yes	DW	0.65	< 0.05
57	1993	Bhongade	Albendazole	2nd +	50	Yes	DW	0.71	< 0.05
58	1993	Bhongade	Albendazole	2nd +	50	Yes	DW	0.66	< 0.05
59	1993	Bhongade	Albendazole	2nd +	50	Yes	DW	0.67	< 0.05
60	1995	Sanyal	Fenbendazole	2nd +	47	No	DW	1.96	< 0.05
61	1995	Walsh	Ivermectin	2nd +	498	Yes	100	0.74	< 0.01
62	1996	Kloosterman	Ivermectin	1st	116	Yes	305	0.41	0.38
63	1996	Kloosterman	Ivermectin	2nd +	262	Yes	305	0.49	0.08
64	1996	Spence	Oxfendazole	2nd +	460	Yes	DW	0.50	< 0.05
65	1998	Murphy	Moxidectin	All	137	No	140	0.75	NS
66	1998	Murphy	Moxidectin	All	325	No	320	0.53	< 0.01
67	1998	Murphy	Moxidectin	All	200	No	125	0.37	0.08
68	1999	Carrier	Eprinomectin	1st	61	Yes	305	0.83	NS
69	1999	Carrier	Eprinomectin	2nd +	229	Yes	305	-0.24	NS
70	2001	Descoteaux	Ivermectin	1st	67	Yes	305	1.14	< 0.05
71	2001	McPherson	Eprinomectin	1st	182	Yes	DW	-0.10	0.78
72	2001	McPherson	Eprinomectin	2nd +	560	Yes	DW	0.60	0.005
73	2001	Pfister	Eprinomectin	2nd +	490	No	305	2.14	< 0.001

Study ID	Publication year	First author last name	Drug	Parity	Number of cows	Control conf.[1]	Milk Measure[2]	Mean difference	Significance [3]
74	2001	Pfister	Trichlorfon	2nd +	385	No	305	1.88	< 0.001
75	2002	Nødtvedt	Eprinomectin	All	901	Yes	DW	0.94	0.002

[1] Control for confounding (i.e. previous lactation, age, season, farm) in the analysis

[2] DW = daily weight, 305 = 305 total milk production (actual or projected), other figures mean days in milk when total milk production was measured.

[3] Statistical significance reported: NR = not reported, NS = not significant, S = significant

Table 2.4. Median, mean, 95 % C.I. of the study effect (mean difference kg/cow/day) , sample size and number of trials used in the meta-analysis stratified by the statistical significance reported in the article.

Significance	Median	Mean	95 % C.I.	Total Sample size	Number of studies
<= 0.05	0.74	0.89	0.70; 1.09	24084	41
> 0.05	0.11	0.08	-0.16; 0.32	16040	34
Overall	0.64	0.52	0.35; 0.70	40124	75

Table 2.5. Meta-regression analysis of the association between study effect, precision and methodological quality of the trial. Table presents coefficients, 95 % C.I. and P values from the unconditional as well as the multivariable (controlling for all variables) analyses.

Factor	Unconditional analysis		Controlling for all variables	
	Effect on overall estimate (95% CI)	P	Effect on overall estimate (95% CI)	P
Unit increase in standard error	1.06 (0.52; 1.61)	0.000	0.86 (0.28; 1.44)	0.003
Control confounding	-0.24 (-0.48; 0.01)	0.06	-0.25 (-0.51; 0.01)	0.06
Randomization	0.14 (-0.06; 0.35)	0.17	-	NS
Treatment blinded	-0.17 (-0.41; 0.06)	0.15	-	NS
Publication type		0.005		0.03
Journal indexed	Baseline		Baseline	
Journal not indexed	0.37 (0.14; 0.60)	0.000	0.23 (-0.005; 0.47)	0.06
Abstracts	0.03 (-0.24; 0.31)	0.808	-0.13 (-0.44; 0.18)	0.42

Table 2.6. Unconditional meta-regression analyses based on 75 trials of anthelmintic treatment in lactating dairy cattle. Table presents coefficients, standard error, *P* values and the moment estimator of the between study variance (τ^2).

Factor	Coefficient	P	τ^2
Null	0.46 (0.36; 0.56)	0.000	0.10
Formulation [1]	0.21 (-0.02; 0.44)	0.08	0.09
Time of treatment		0.000	0.09
Dry off /calving	baseline	-	-
Mid lactation	0.44 (0.17; 0.71)	0.001	-
Strategic	0.40 (0.14; 0.65)	0.002	-
Individual treatment [2]	-0.40 (-0.61; -0.20)	0.000	0.09
Milk length	-0.002 (-0.002; -0.001)	0.002	0.08
Milk measure	-	0.000	0.08
DW	Baseline	-	-
305 day [3]	-0.44 (-0.66; -0.21)	0.000	-
Other [4]	-0.10 (-0.40; 0.21)	0.530	-
Location	-		0.12
Northern	Baseline	-	-
Southern	-0.10 (-0.31; 0.11)	0.37	
Parity	-	0.13	0.12
Multiparous	Baseline	-	-
Primiparous	-0.31 (-0.61; -0.01)	0.04	-
All combined	-0.08 (-0.34; 0.17)	0.52	-

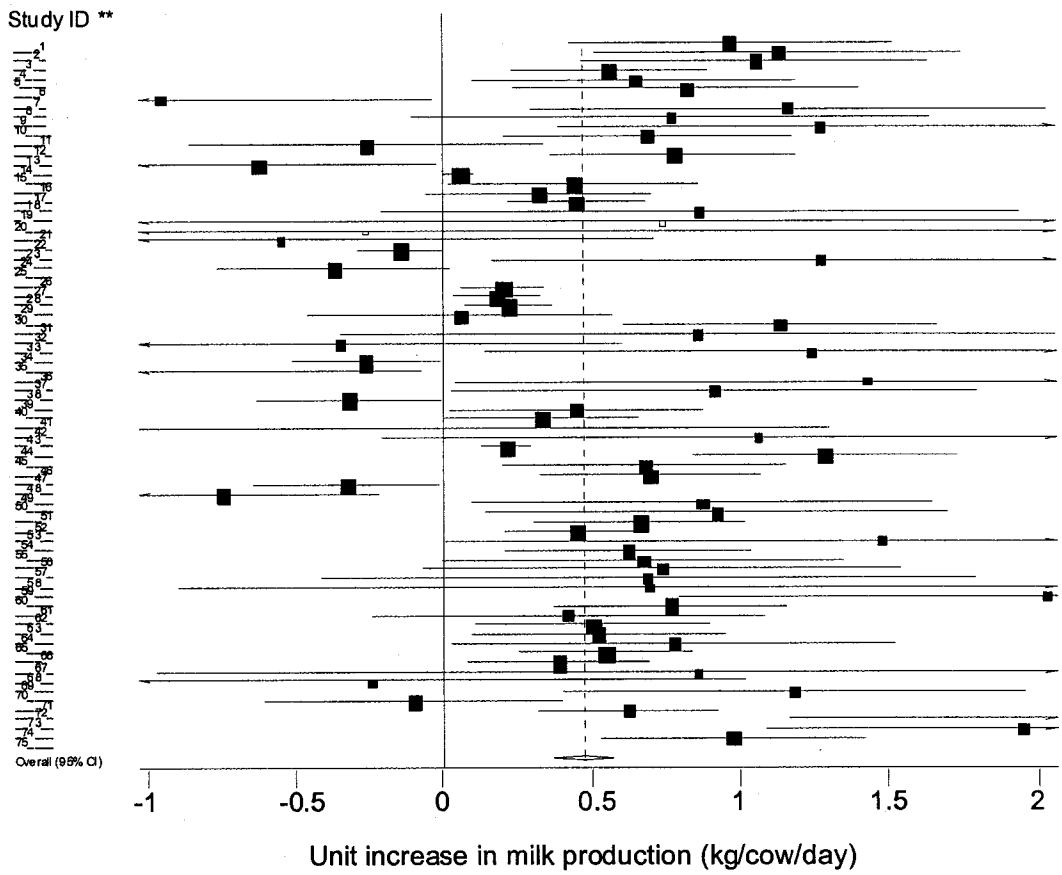
[1]Endectocides versus others

[2] vs. whole herd treatment

[3] Actual or projected

[4] i.e. total production at 100 days in milk

Figure 2.1. Forest plot of the effects of anthelmintic treatment on milk production response (kg/cow/day). The overall estimate was derived from the random effect meta-analysis (see text for details).



* lines with arrows are truncated

** Study ID: Study id number as presented in Table 2.3.

Figure 2.2. Funnel plot of the point estimates of the effect of anthelmintic treatment on milk production response (kg/cow/day). Square points were added by the "trim and fill" procedure to correct for publication bias or small study effect (see explanation in the text).

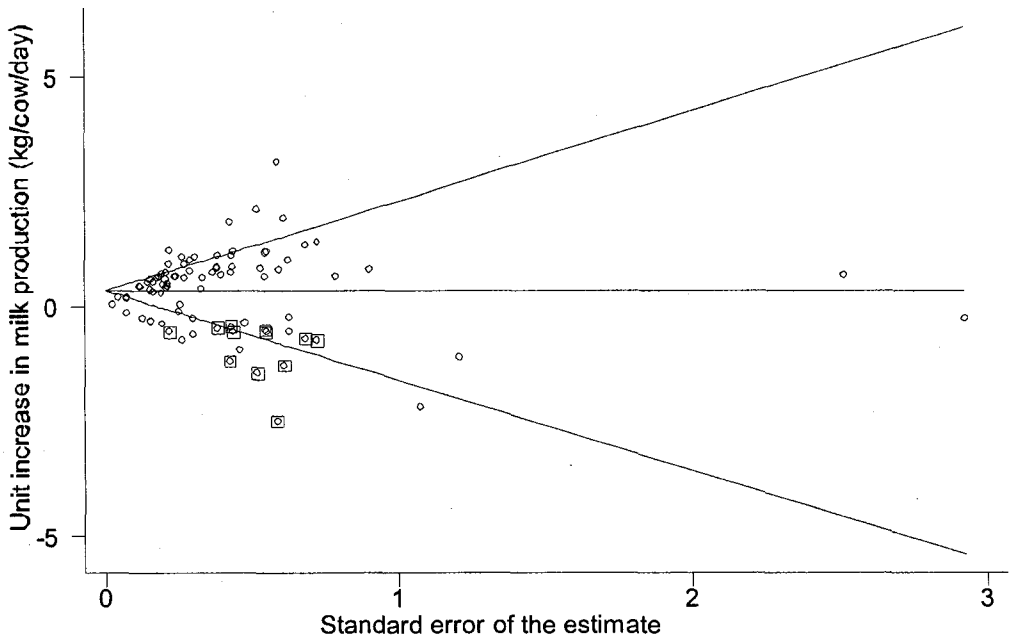
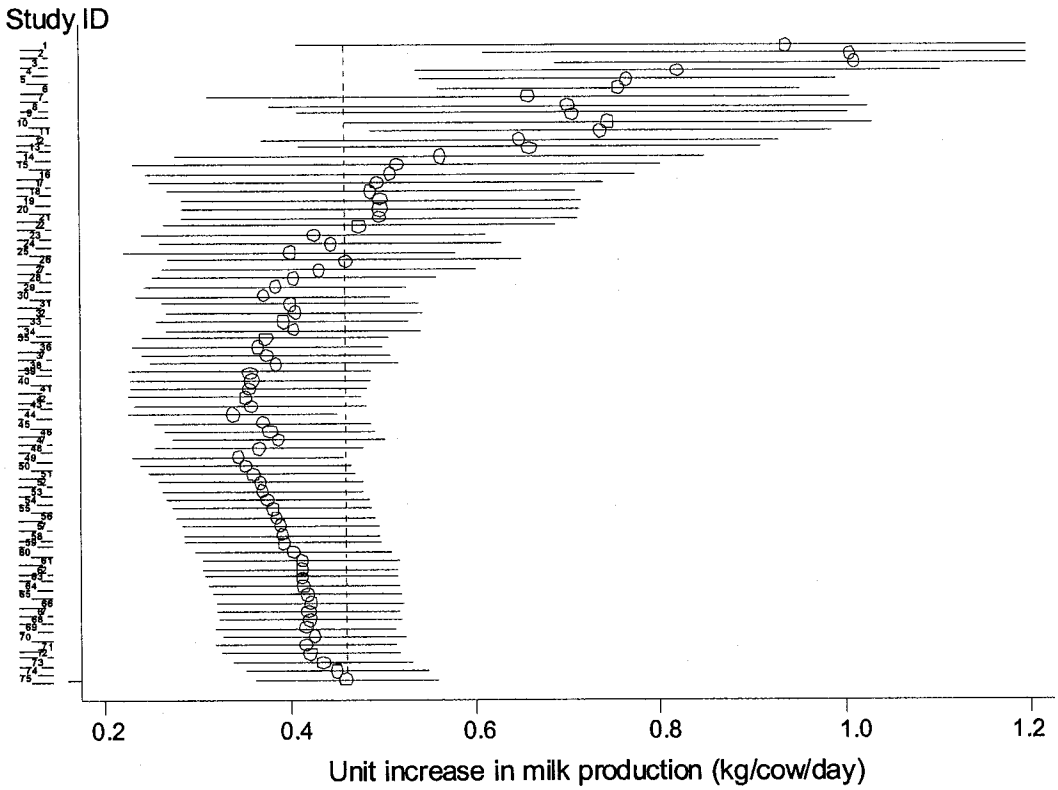


Figure 2.3. Cumulative random effect meta-analysis of 75 trials, to assess change in the effect on milk production response after anthelmintic treatment.



3. Evaluation of the repeatability of a crude adult indirect *Ostertagia ostertagi* ELISA and methods of expressing test results

3.1 Abstract

An indirect enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Ostertagia ostertagi* using a crude adult worm antigen was evaluated using serum and milk samples from adult cows, as well as from bulk tank milk. Within and between plate repeatabilities were determined. In addition, the effects of factors such as antigen batch, freezing, preserving of the samples and somatic cell counts (SCC) of the samples were evaluated. Raw optical densities (OD) and normalized values were compared using the concordance correlation coefficient (CCC), the coefficient of variation (CV), Bland-Altman plots (BA). Based on raw optical density (OD) values, there was a high repeatability within a plate (CCC $\approx$ 0.96 and CV < 10%). Repeatability between plates was evaluated following normalization of OD values by four methods. Computing normalized values as (OD - Nt)/(Pt – Nt), gave the most repeatable results, with the CCC being approximately 0.95 and the CV $\approx$ 11%. When the OD values were higher than 1.2 and 0.3 for the positive and the negative controls, respectively, none of the normalization methods evaluated provided highly repeatable results and it was necessary to repeat the test. Two batches of the crude antigen preparation were evaluated for repeatability, and no difference was found (CCC=0.96). The use of preservative (bronopol) did not affect test results, nor did freezing the samples for up to 8 months. A significant positive relationship between ELISA OD for milk samples and SCC score was found. Therefore, the use of composite milk samples, which have less variable SCC

than samples taken from each quarter, would be more suitable when the udder health status is unknown. The analytical methods used to evaluate repeatability provided a practical way to select among normalization procedures.

3.2 Introduction

Over the last decade, the use of alternative diagnostic techniques for gastrointestinal parasitism in cattle has been extensively investigated. Traditionally, fecal egg counts have been the only available technique used for routine monitoring of parasite burdens. However, this technique has been shown to be of use only in the first grazing season in young animals (1; 2). Of the alternative methods that have been evaluated, pepsinogen assays and immunological methods have been the most studied. The ELISA technique has been recognized as a promising alternative for diagnosis of gastrointestinal parasitism in herd health monitoring programs in cattle (3).

An ELISA using a crude adult *Ostertagia ostertagi* antigen developed by Keus (4) has been evaluated in a number of research programs. The results of these ELISAs have been presented in a variety of ways. Studies in first year calves reported results as titres using a logit transformation (5; 6). Guitian et al. (7) reported raw optical density values using bulk tank milk samples. Recently two studies on adult dairy cows in Belgium and The Netherlands reported them as optical density ratios (8; 9) that adjusted the raw values based on the positive and negative control values. Problems that have been reported are the low species specificity (cross-reaction with other parasite antigens) and the difficulty to obtain this crude antigen in highly standardized preparations (3). In addition to these issues, the lack of consistent standardization of the test results makes it more difficult to interpret and to compare results from different studies.

3.2.1 Methods for evaluating repeatability

One of the main goals in the process of developing an assay method is to minimize the variability in test results by looking at different ways that maximize the repeatability and reproducibility of the test (10). They express the agreement between multiple tests on the same sample carried out in either the same or different laboratories. Traditionally, for continuous data (i.e. ELISA OD values), repeatability and reproducibility have been assessed using the Pearson correlation coefficient (R) and coefficient of variation (CV) and, to a lesser extent, paired t-tests and intraclass correlation coefficients (ICC). The drawbacks of these methods have been discussed in some detail (11). Briefly, it was concluded that R measures the strength of the relation between two variables but it fails to measure a change in scale of the measurements. The paired t-test only looks for the differences in the means of the two readings. The CV and ICC consider duplicate readings as replicates rather than two distinct readings. Consequently, none of these approaches alone can fully assess the reproducibility or repeatability characteristics of the test.

Based on this, Lin (11) created a reproducibility index called the concordance correlation coefficient (CCC). The CCC computes the agreement between two continuous measures. This coefficient is formed from three parameters. The location shift parameter measures how far the data are from the 45 degree line (equality line) in a scatter plot, the scale shift parameter measures the change in the slope, and R measures how tightly clustered the data are around the best fit line. The accuracy parameter (A) is computed from the location and scale shift parameters to measure how far the best-fit line deviates from the equality line. Finally the CCC is the product of A times R. The interpretation of these parameters is as

follows. A value of zero for the location shift parameter is desirable, as is a value of 1 for the scale shift, A, R, and CCC.

An alternative approach to evaluating agreement between 2 continuous measures was first proposed by Bland and Altman (12). A Bland-Altman (BA) plot is a graphical procedure that plots the average values of the two determinations against the mean difference between them. From this plot the 95% limits of agreement are set at 2 standard deviations of the difference above and below their mean value. It indicates the range of differences expected to include 95% of the observations (12) . This approach is also useful to evaluate relationships between mean values and the differences, as well as to identify the presence of outlying observations.

3.2.2 Factors affecting ELISA results

General factors affecting ELISA response variability have been discussed by several authors (13-15) who also suggested methods to reduce this variation. In the process of validation, it is also important to quantify (and take into account) how external factors such as sampling method, handling and preserving the samples and the presence of the other milk components influence ELISA results. Each of these factors has been reported as source of variation in different tests using milk samples (16-18).

3.2.3 Objective

The overall objective of the research program, of which this study was one component, was to evaluate the ELISA as a tool for quantifying parasite burdens in dairy

cattle. As part of this research program it was necessary to determine if an ELISA based on a crude *O.ostertagi* antigen was able to consistently measure levels of antibodies in serum and milk. The specific objectives of this study were: to evaluate the within and the between plate repeatability; to identify normalization methods that maximize the between test repeatability; and to determine how factors such as batches of antigen, sample storage time, use of preservatives, somatic cell counts and variation among mammary gland quarters influenced the repeatability of the ELISA test results.

3.3 Materials and Methods

3.3.1 Samples

Milk and blood samples were collected from dairy herds in two provinces of Canada (Prince Edward Island and Nova Scotia). The milk samples were obtained from 369 bulk milk tanks and 466 milking cows. The blood samples were taken from 122 adult cows and 46 bred heifers.

Serum samples were obtained by centrifuging the blood at 850 x g for 10 min. Milk samples were prepared from whole milk by centrifugation at 16000 x g for 4 min. The fat was removed and the underlying supernatant was obtained and frozen. The samples were frozen (-20 °C). All samples were processed and stored according to this standard procedure until they were tested.

3.3.2 ELISA procedure

An indirect enzyme-linked immunosorbent assay was performed, using a crude saline-extract, whole worm *O.ostertagi* antigen. The adult worm antigen was prepared according to the procedure described by Keus (4). Worms were homogenized in a tissue grinder and the preparation was centrifuged at 16000 x g. The supernatant was collected and protein content determined. Flat-bottom, 96 well microplates[1] were coated with 0.1 ml of antigen per well (1ug/ml in carbonate-bicarbonate buffer, pH 9.6). Plates were incubated for 24 hours at room temperature. Plates were washed 3 times with 0.4 ml per well of phosphate buffered saline solution containing Tween 20 (PBS-T20), using an automated plate washer[2], and were subsequently blocked using 0.2 ml of PBS-T20 with 3% fetal calf serum[3] (FCS). Plates were left at room temperature for 1 hour, then frozen at -20 °C until needed. After thawing at room temperature, plates were washed as before. Negative control serum was obtained from pooled samples from 3-month-old helminth-naïve calves. A positive control serum was obtained from a hyperimmune calf after repeated artificial infections with L3 of *O.ostertagi*. Test and control sera were diluted 1:140 in PBS-T20 containing 1% of FCS. Diluted sera (0.1 ml) were added to test wells and 1 positive serum, 1 negative serum and 1 blank (PBS-T20 in 1% FCS) was run in quadruplicate on each plate. Plates were incubated at room temperature for 1 hour, and washed 3 times as before. After washing, 0.1 ml of a 1:500 dilution (in PBS-T20 containing 1% of FCS) of rabbit anti-bovine IgG conjugated to horseradish peroxidase[4] was

[1] Dynex Tecnologies, Immulon II HB

[2] Tecan US Inc., USA

[3] Cansera, Rexdale, Ontario, Canada

[4] BioCan Scientific, Canada

added to each plate, incubated at room temperature for 1 hour, and washed as before. ABTS (5.5 mg) substrate [2,2'-azino-bis-(3-ethyl-benzth-iazoline-sulfonic acid)][5] was diluted in 15.5 ml of citrate acid buffer (0.1 M) plus 9.5 ml of sodium phosphate (0.2 M) and 23ul of 30% H_2O_2.. Plates were incubated at room temperature for 35 minutes. Absorbance was read at 405/490 nm, using an ELISA reader[6]. Milk samples were tested, using the same procedure, except samples were used undiluted. The optical density (OD) values were recorded along with the values of the blanks and positive and negative controls.

3.3.3 Statistical analysis

Descriptive statistics and measurements of repeatability (including CCC and BA plots) were computed. A regression model using a generalized estimated equation algorithm was fit to evaluate the relationship between cow factors and ELISA test results. All the analysis were carried out using Version 7.0 of the statistical package Stata (19).

3.3.3.1 Within plate repeatability

Four plates containing duplicates of 40 milk bulk tank samples were used. CCC, CV and BA plots were used on raw optical densities to measure repeatability within a plate.

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[5] Boehringer Mannhein, Germany

[6] Spectra Max 340, Fisher Canada

3.3.3.2 Between plates repeatability

Two trials were carried out to evaluate the repeatability between plates. In Trial 1; 963 samples (329 bulk tank milk samples, 466 cow milk samples, 122 cow serum samples and 46 bred heifer serum samples) were tested twice (on different days). Fourteen plates were used on each of the 2 test days. In Trial 2; 40 bulk tank milk samples were tested 6 times using different plates.

For each sample, 4 methods of normalizing ELISA test results were used:

$$OD_A = OD$$

$$OD_B = \frac{OD}{Pst}$$

$$OD_C = \frac{OD}{(Pst - Nt)}$$

$$OD_D = \frac{(OD - Nt)}{(Pst - Nt)}$$

Where OD is the absorbance value of the sample, Pt and Nt are the mean absorbance values of the 4 positive and 4 negative controls on each plate, respectively.

For each normalization method, CCC, CV and BA plots were computed within each trial.

3.3.3.3 Comparison of batches of antigens

Crude adult *O.ostertagi* antigen prepared in September (old) and December (new) of 1999 were coated, separately, in 4 ELISA plates on the same date (two plates for each batch of antigen). Each batch was tested on two different dates (December 1999 and January 2000).

Forty bulk tank milk samples were compared for each antigen preparation and test day. The repeatability of OD_D values between batches of antigen was evaluated using the CCC, CV and the BA plots.

3.3.3.4 Preserving and freezing trial

Milk samples from 37 cows were collected on the same day and divided into separate samples to be handled as follows:

a) Stored at 4°C with no preservative and tested at 1 day after collection.

b) Stored at 4°C and preserved with bronopol and tested at 1, 7 and 42 days after collection.

c) Stored at –20°C with no preservative and tested at 7, 42 and 224 days after collection.

All the OD values were expressed by OD_D. The repeatability of the test between preservation status and storage time was evaluated using the CCC and BA plots.

3.3.3.5 Variation among quarters and influence of the SCC of the sample

Quarter milk samples were taken from 18 cows. One day after collection the diagnostic laboratory at the Atlantic Veterinary College performed bacteriological cultures and a California Mastitis Test (CMT) on all samples. The CMT values were recorded according to the scale 0, T (trace), 1, 2, and 3 (20). Observed ODs were normalized by OD_D. The association between quarter location, CMT score and OD_D was evaluated using a generalized estimating equation (GEE) algorithm (21) assuming an exchangeable correlation

structure. Since observations were clustered within a cow, the standard errors were obtained using the robust (sandwich) estimator of variance (22).

3.4 Results

3.4.1 Within plate repeatability

The CCC and CV for all plates ranged between 0.94 and 0.97 and between 3.0% and 5.0 %, respectively. BA plots (not shown) indicated the 95% limits of agreement ranged from -0.136 to 0.133.

3.4.2 Between plate repeatability

In order to investigate the variability between ELISA plates, 2 separate trials were conducted. In Trial 1, 14 pairs of plates were read. Four pairs had higher than expected (abnormal plates) OD values for the control sera. They ranged between 1.21 and 1.35 and between 0.20 and 0.25 for positive and negative controls, respectively. The other 10 pairs (677 samples) had values between 0.58-1.04 and 0.02-0.24 for positive and negative controls, respectively. Table 3.1 shows the average and range values of the CCC and the CV of the 10 normal plates for each of the 4 normalization methods. Figure 3.1 presents the data from one normal plate with OD_D values obtained from the first test plotted against the OD_D values of the same sample tested later. The dotted line would represent perfect agreement between the two readings. Figure 3.2 shows the BA plot for the same plate, the limits of agreement were from -0.089 to 0.129 with a mean difference of 0.02.

Normalization method OD_D produced the highest CCCs and was selected for the presentation of all subsequent results. The CCC obtained from each sample type according to OD_D was 0.95, 0.92 and 0.96 for cow milk samples, bulk tank samples and serum samples, respectively. When the control sera had a higher value than was expected, (OD > 1.2 for the positive controls and OD > 0.3 for the negative controls), none of the normalization methods provided highly repeatable results. Although method D had the highest CCC values, it ranged from 0.50 to 0.71. Even though the CCC was low for these plates, the Pearson correlation coefficient for all methods was 0.96.

The results of Trial 2, in which the CCC and the CV from the same 40 bulk milk samples were tested on 6 different plates are presented in Table 3.2. As in Trial 1, method D presented the highest CCC and followed by method B. Both methods presented the same spread between maximum and minimum CCC values.

3.4.3 Comparison of antigens

The CCC and CV of the comparison of 2 batches of antigen are presented in Table 3.3. It can be seen that for each test day as well as the average of 2 test days high CCCs and CVs within expected values were observed. The BA plots in Figure 3.3 shows that most of the observations were approximately between –0.10 and 0.18.

3.4.4 Preserving and freezing trial

The group mean differences and CCCs of the test results expressed by OD_D are presented in Table 3.4 and the BA plots are presented in Figure 3.4. The CCC had high values

for both preserved and non-preserved milk samples. The lowest CCC value was observed for non-preserved milk that had been kept frozen for 244 days. This presentation of the data agrees with the BA plot showed in Figure 3.4c, where more outlying points were observed and a linear pattern was present between mean values and the differences. However, the paired t-tests showed very small, but statistically significant, increases in OD values with storage of milk samples 7 or 42 days by freezing or preserving.

3.4.5 Variation among quarters and influence of SCC

The distributions of OD_D by cow are presented in Figure 3.5. The box and whisker plot shows that most of the cows had small variation in OD_D values among the four quarters. The highest variation was observed in cow number 374. In this cow, two quarters were reported to have had *Streptococcus uberis* infection and high CMT score. The variable CMT was re-classified as follow: 0=0; T, 1 and 2 =1 and 3=2 (CMT_3) because there was no significant differences in OD_D between T, 1 and 2 SCC scores.

The relation between OD_D and quarter and the influence of SCC on test results are presented in Table 3.5. The GEE model yielded an estimate of the intra-cow correlation coefficient of 0.76. While there was no difference among quarters on OD_D, a significant positive effect of CMT score and presence of *Streptococcus uberis* on OD was observed.

3.5 Discussion

Expressing raw OD values as a proportion of positive control sera (OD_B) has been commonly used for reporting ELISA test results (known as "percent positivity"). However,

the OD_D normalization method has also been reported in other studies (8; 9) as the OD ratio, but the reason for selection of one method over another has not been reported.

3.5.1 Within plate repeatability

The ELISA had high repeatability within plate. The values of CV obtained were within the standard empirical criteria suggested by Jacobson (10) of less than 20 % for raw values, indicating also acceptable intra-plate repeatability. Although the number of replicates per plate suggested by this author is three or four, in the current study these parameters were estimated by using two samples.

3.5.2 Between plate repeatability

There have not been any published reports of the between plate repeatability of the ELISA test using a crude antigen for *O.ostertagi*. It was found that in the two trials done in the current study OD_D had the highest CCC. In trial 1, it also had the lowest CV and it was within the range accepted as "normal". The logistic transformation used by Ploeger et al. (5) and Poot et al. (6) where the raw values are adjusted to a standard curve is an alternative to adjusting the raw values to the standard controls in each plate. Although this method allows evaluating the fit of the data, it is more time consuming as it requires sequential sample dilutions and thus fewer samples can be tested on each plate. In the current study, less repeatable results were obtained in all of the normalization methods used when the OD values of the control sera were higher than were expected. In these situations, it was necessary to retest the samples.

3.5.3 *Between batches of antigen*

ELISA results depend heavily of the characteristics of the antigen used in the test system. Venkatesan and Wakelin (14) discuss factors which could affect the antigen protein and plastic interactions, with one factor being the nature of the antigen. Work done by Kenny and Dunsmoor (13) showed some of the problems of using a heterogeneous mixture of antigen, such as the crude antigen used in this study. They suggested that such mixtures must contain many antigens and only some of them will be coated in large enough quantity to the plate. In addition, when the antibodies are specific to some of these antigens, the test response will depend on how much of the reacting antigen is coated to the plate. The results of the current study showed that the test results obtained by using different antigen batches and also coated at different times had highly repeatable results when they were normalized by OD_D. Based on Kenny's hypothesis, one possible explanation for this high repeatability found in the current study might be because this antigen presents cross-reaction with other parasites, and therefore these antibodies are less species specific.

3.5.4 *Preserving and freezing trial*

Because milk samples are often preserved when collected for the dairy laboratories, it was important to measure the effect of preserving compounds on ODs. The results of the current study showed a high repeatability between preserved and non-preserved samples, and between fresh and frozen milk samples. These findings agree with that obtained by Sweeney et al. (17) who found no significant effects of adding bronopol on milk ELISA for antibodies against *Mycobacterium paratuberculosis*. In relation to the storage time, there was a high

repeatability among different periods, although it did decrease slightly at 244 days. Although the mean values were significantly different at previous time points, the differences were all small. The CCC and BA plots were a more useful way of evaluating repeatability than simply comparing measures with a paired t-test as they quantify the agreement over the full range of results rather than just comparing the means.

3.5.5 Variation among quarters and influence of SCC

There were only small differences between OD of milk samples taken from the 4 quarters of the udder. The estimated correlation among quarters was 0.73 meaning that milk samples could be taken from any of the quarters, although sampling from infected quarters should be avoided. Similar results have been observed previously (16). In the cited study, the authors showed no difference in IgG1 concentrations in milk relative to location of uninfected quarters. Also, analysis done in the mentioned study indicated no correlation between SCC and IgG1 concentration in milk in uninfected quarters. On the other hand, a positive correlation between SCC and IgG1 was observed in infected quarters. *Staphylococcus aureus* caused the highest increase in IgG1 levels in milk. The results of the present study agree with those reported above. The OD values were positively influenced by an indirect measure of SCC (CMT score) and a positive increment in OD values was associated with the presence of *Streptococcus uberis* (Table 3.5).

3.5.6 Evaluation of performance

As part of a validation process, it is important to evaluate the performance of an assay in terms of repeatability. The methodology used in this study allows, in an objective way, to evaluate the repeatability of the test. In addition to that, it was possible to identify the range of control values in which we could expect good performance of this particular ELISA.

3.6 Conclusions

In conclusion, these results suggest that it is not necessary to use duplicate samples in each plate in routine testing, although this should be done periodically for quality control. In relation to the normalization procedures, OD_B and OD_D tended to give better agreement between plates with OD_D being slightly better. Although high repeatability was found using OD_D, a great variation in OD values was found in the control sera and the normalization was only acceptable if the controls were within an acceptable range. Consequently, when high OD values in the control serum are obtained, it is necessary to repeat the test.

The use of crude mixed antigens produced repeatable results across batches of antigen. However, due to the complex nature of this kind of antigen, it would be recommended to evaluate each new batch of antigen as part of a quality control process.

Our data suggested that the samples could be preserved and frozen up to 8 months without ELISA test results being seriously adversely affected. When the health status of the udder is unknown it is recommended to take composite milk samples to minimize the effect of quarters having high CMT score on OD values.

The ELISA test results expressed by OD_D had in overall high repeatability and the methodology used to evaluate it provides a practical way to select a normalization procedure.

3.7 References

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Table 3.1. Mean concordance correlation coefficient (CCC), range of CCC, mean coefficient of variation (CV) and range of CV from 963 milk and serum samples tested on duplicate plates to evaluate between – plate repeatability.

Method	CCC	Range CCC	CV %	Range CV %
OD_A	0.74	0.41 – 0.95	24	10 – 54
OD_B	0.92	0.80 – 0.97	11	7 – 23
OD_C	0.87	0.52 – 0.97	14	5 – 27
OD_D	0.94	0.86 – 0.98	12	7 – 20

Table 3.2. Mean concordance correlation coefficient (CCC), range of CCC, mean of coefficient of variation (CV %) and range of CV (%) from 6 plates testing 40 bulk tank milk samples.

Method	CCC[a]	Range of CCC[b]	CV %[c]	Range of CV[d]
OD_A	0.86	0.74 – 0.98	15	8 – 25
OD_B	0.95	0.95 – 0.97	8	3 – 15
OD_C	0.89	0.75 – 0.98	14	6 – 24
OD_D	0.96	0.95 – 0.98	12	4 – 39

[a] average CCC of 6 plates, each composed against the set of average values derived from all 6 plates

[b] range of CCC across the 6 plates

[c] average of each sample's CV among all 6 plates

[d] range of each sample CV

Table 3.3. Mean concordance correlation coefficients (CCC), 95% CCC confidence interval (95% CCC C.I.), mean coefficient of variation (CV %) and 95% CV confidence interval (95% CV C.I.) between old and new batches of antigen tested at different days normalized by OD_D.

Test date	CCC	95% CCC C.I.	CV %
December 1999	0.92	0.88 – 0.97	11
January 2000	0.93	0.89 – 0.98	7
Average two test days	0.96	0.93 – 0.98	10

Table 3.4. Concordance correlation coefficients (CCC) and mean differences in ELISA results normalized by OD_D between unpreserved and preserved milk, by storage time.

	Not preserved			Preserved	
Days after sampling	7	42	244	7	42
CCC [a]	0.97	0.98	0.91	0.94	0.94
CCC [b]				0.97	0.93
Mean difference [a]	0.04*	0.02*	-0.02	0.06*	0.06*
Mean difference [b]				0.02*	0.02

[a]compared with fresh non preserved milk

[b]compared with 1 day preserved milk

* significantly different form 0 by a paired t-test ($P \leq 0.05$)

Table 3.5. Coefficients of the generalized estimated equation (GEE) regression model of effects of quarter, CMT score and presence of *Streptococcus uberis* on OD_D test results. (15 cows and 71 observations; intra-cow correlation coefficient = 0.76).

Variable	Coefficient	*P*	Confidence Interval
Intercept	0.473	0.000	0.369 – 0.578
Quarters			
Right Front	Baseline		
Right Hind	-0.030	0.450	-0.106 – 0.047
Left Front	-0.033	0.356	-0.105 – 0.038
Left Hind	-0.056	0.212	-0.145 – 0.032
CMT			
0	Baseline		
1	0.059	0.037	-0.003 – 0.114
2	0.107	0.016	0.020 – 0.194
Streptococcus uberis presence			
Negative	Baseline		
Positive	0.180	0.063	-0.010 – 0.370

Figure 3.1. Agreement between two sets of OD_D observations from duplicate testing of 56 milk samples on different days (CCC = 0.98).

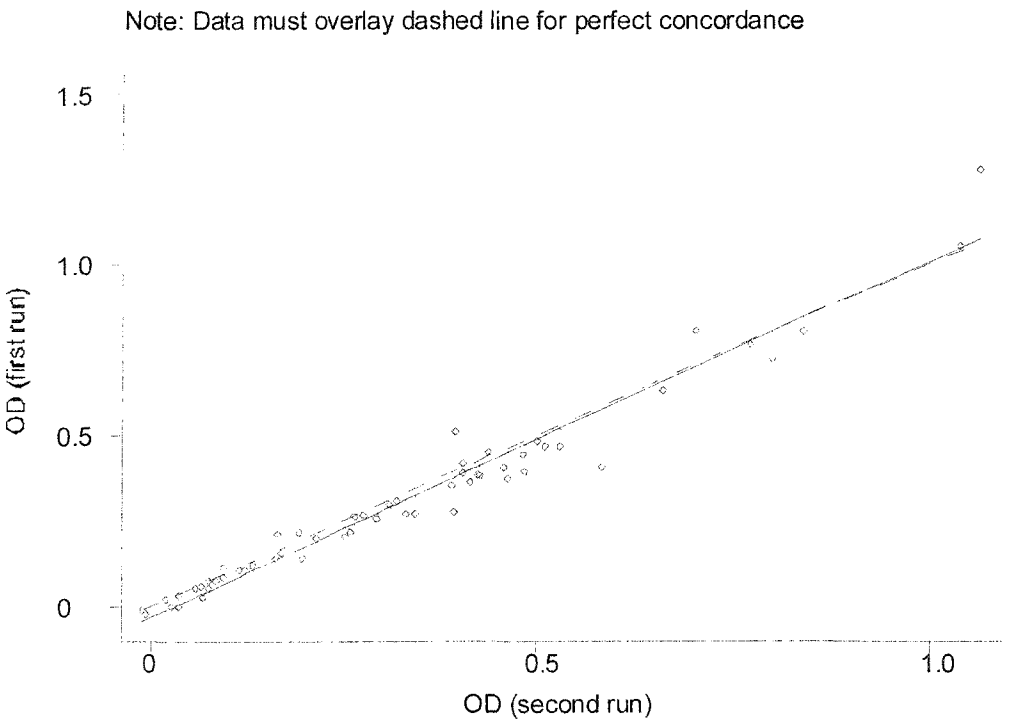


Figure 3.2. Bland-Altman 95% limits of agreement and mean difference of two sets of OD_D observations from duplicate testing of 56 milk samples on different days.

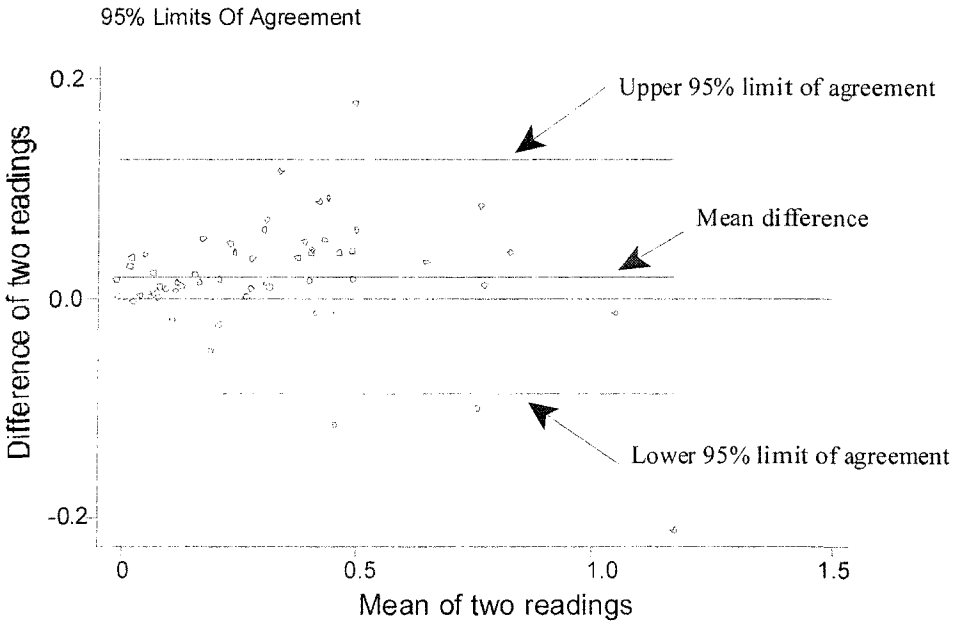


Figure 3.3. Bland and Altman 95% limits of agreement and mean difference of the comparison of new antigen and old antigen. (a) BA plot of December test day. (b) BA plot of January test day. (c) BA plot of average of two test days.

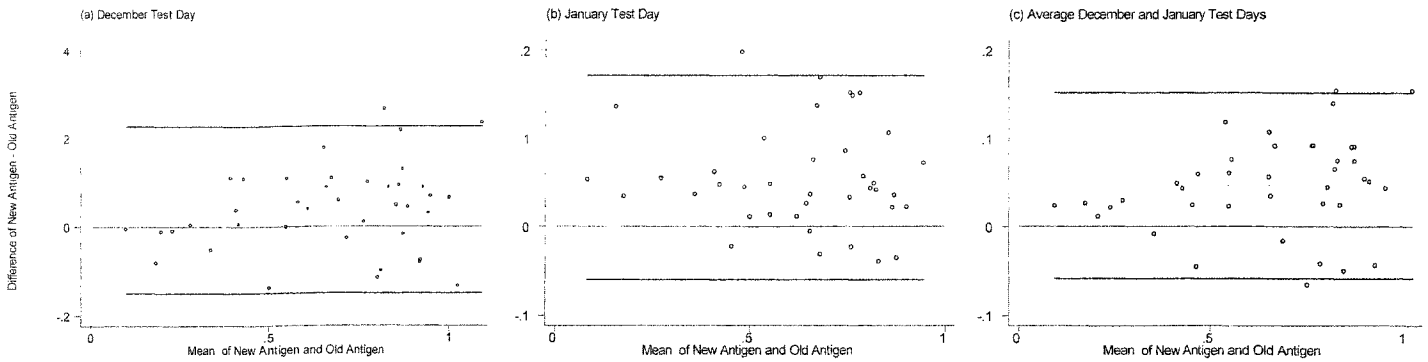


Figure 3.4. Bland and Altman 95% limits of agreement and mean differences of OD_D values comparing storage length and milk preservation with fresh, non preserved milk. (a) BA of 7 days, non-preserved with fresh, non-preserved. (b) BA plot of 42 days, non-preserved and fresh, non-preserved. (c) BA plot of 244 days, non-preserved and fresh, non-preserved. (d) BA plot of fresh, preserved and fresh, non-preserved. (e) BA plot of 7 days, preserved and fresh, non-preserved. (f) BA plot 42 days, preserved and fresh, non- preserved.

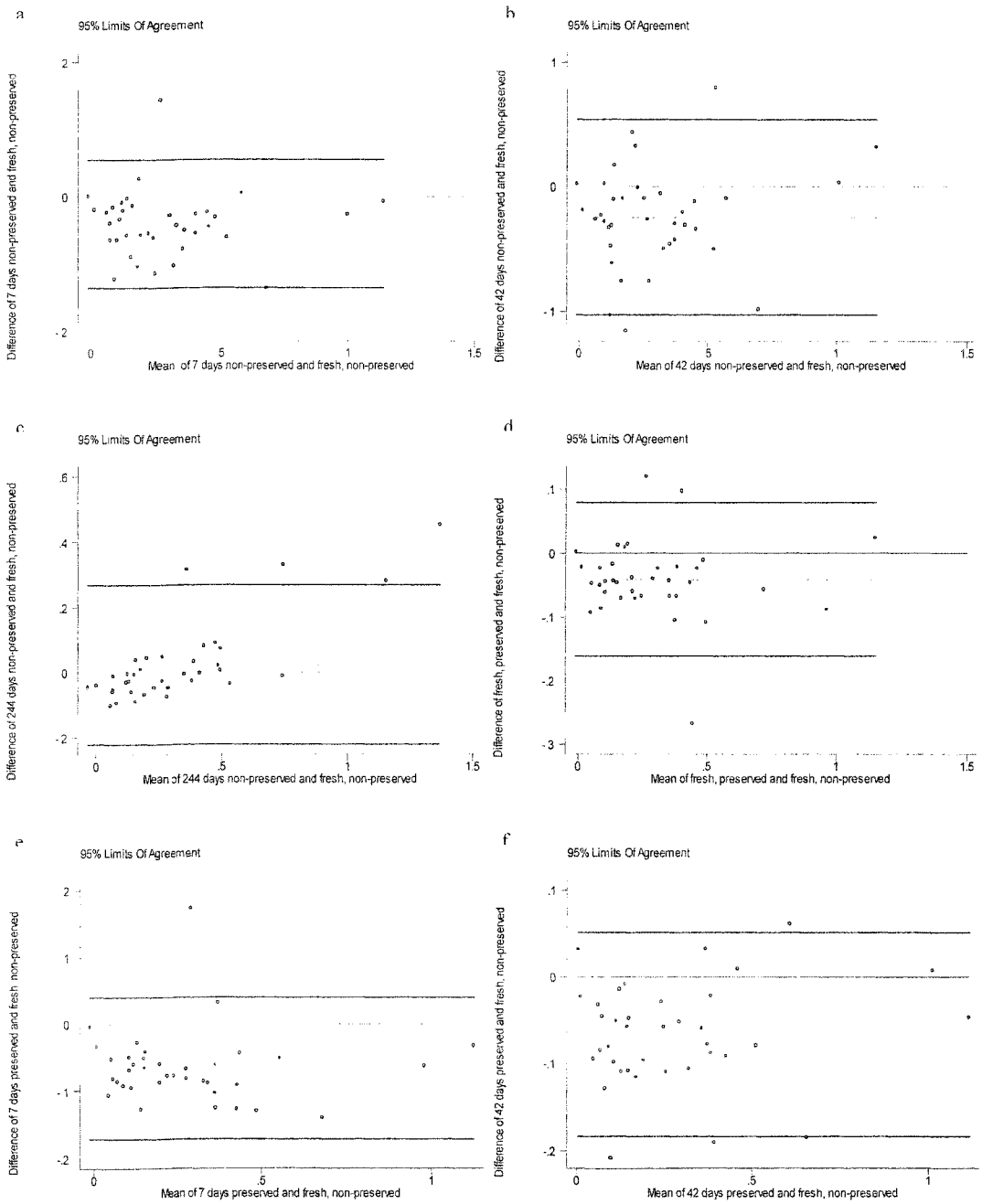
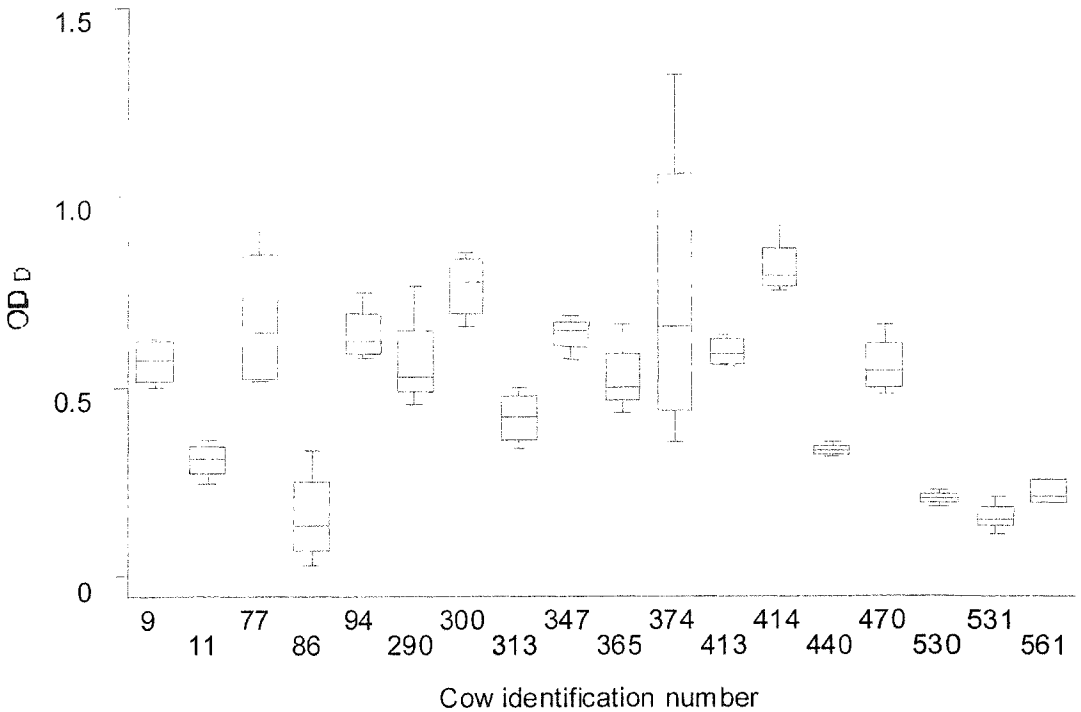


Figure 3.5. Box-and-whisker graph of the quarter OD_D distribution of 18 cows.



4. Milk antibodies against *Ostertagia ostertagi:* Relationships with milk IgG and production parameters in lactating dairy cattle

4.1 Abstract

The present study was carried out to evaluate the relationship between milk optical density ratios (ODRs) from an indirect *Ostertagia ostertagi* ELISA, total milk IgG levels and milk production and then establish a correction factor to adjust ODR. Five hundred and sixty composite milk samples collected from 357 cows on four dairy herds in June and August of 2002 were used in this analysis. The average ODR was 0.34. A positive correlation was found between ODR and IgG values in milk, days in milk, age and log transformed somatic cell counts (SCC). However, ODR was negatively correlated with milk production. The IgG levels and ODR values were constant from 30 to 200 days in milk. However, ODRs increased from 200 days until the end of the lactation. After controlling for age, season, herd and SCC, an increase in milk production of 10 kg/day was associated with a reduction in ODR values of 0.04. The results of the present study suggest that ODR values are not greatly influenced by production factors. ODR follow the same pattern as the IgG variation across lactation and could be adjusted in order to compare ODR values obtained from high producing cows with those obtained from low producing animals.

4.2 Introduction

4.2.1 Parasite immunology

The immune response to parasitism with gastrointestinal nematodes (GIN) is very complex. It is the result of a dynamic relationship between host species, parasite species and the localization of these parasites within the host gut (1). Both cellular and humoral responses are involved in the reaction of the immune system to GIN. Several types of immunoglobulins (Igs) are involved in the humoral response, however, IgG is the main antibody involved in the immune response against GIN (2; 3). Two classes of IgG have been described and have different roles in this immune response. While IgG1 seems to be the main immunoglobulin in serum during either artificial (2) or natural (3) *O.ostertagi* infections, IgG2 levels have been correlated with protection of calves against *Oesophagostomun radiatum* (4). Claerebout and Vercruyesse (1), based in previous work, point out that *Ostertagia*-specific IgG1 antibodies may be an indication of the presence of infection, whereas *Ostertagia*-specific IgG2 response may be correlated with a protective immune response. Moreover, the total IgG levels in serum have been related to acquired immunity to *O.ostertagi* and *Cooperia oncophora* (5). Animals with higher IgG titres in serum had fewer and shorter worms with less ova per female, and more female with reduced vulval flaps (5). However, the level of total IgG response is not only dependent on the acquired immunity, but also on the antigenic stimulation, i.e. the level of exposure to pasture (1). Kloosterman et al. (6) reported that there is a positive relationship between levels of parasite exposure and total IgG. A positive and significant correlation exists between IgG levels as determined by a crude *O.ostertagi* ELISA in adult dairy cattle and the number of parasite larvae on the pasture (7).

4.2.2 Immunoglobulins in ruminants

In cattle as well as in all other mammals, IgG is the major Ig in serum (8). The serum levels of Igs are significantly influenced by the reproductive cycle. After the cessation of lactation, IgG1 is transferred selectively from the blood into colostrum by a receptor mediated mechanism across the gland secretory epithelium (9). Serum IgG1 levels decrease precipitously three or four weeks prior to parturition. This corresponds to the time at which the colostrum-forming gland is selectively accumulating levels of IgG (10). The total Ig levels in milk are very high at parturition but then decline rapidly to approximately 0.7 - 1.0 mg/ml during the first two weeks of lactation. IgG1, however, remains the predominant Ig subclass in these secretions (11; 12). Unlike the situation in either swine or horses, IgA never becomes the major Ig in the mature cow milk. The persistence of IgG1 could reflect the efficiency of the IgG1 transport mechanism during normal lactation, when, although the transport mechanisms is downregulated, the transport of IgG into milk still exceeds the amount of IgA that the udder can produce (8). The predominance of IgG1, even in the milk of mature cows, may also reflect local synthesis within the gland or in nearby mammary lymph nodes (8). Moreover, it has been suggested that the level of milk yield acts as a dilution factor, so at the end of the lactation, higher concentrations of IgG might be expected (13).

4.2.3 ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) using a whole worm extract *O.ostertagi* antigen developed by Keus (14), and recently evaluated by Sanchez et al. (15), has shown promising results as a monitoring tool in lactating dairy cattle (16; 17).

One drawback to the validation process of this particular ELISA is the lack of a "gold standard" technique. Counting the abomasal worm burden might be useful when related to the ELISA optical densities results, but the lag-time between the number of parasites and the development of the antibody response, makes this kind of study very difficult to carry out (18). This was demonstrated in two slaughterhouse studies, where there were low and non-significant correlations between parasite loads and ELISA optical densities (19; 20). Different indirect ways to validate this technique have been proposed. The relationship between ELISA optical densities and those management practices that are related to gastrointestinal parasitism have been studied (21; 22). Similarly, the association between ELISA OD and milk production has also been evaluated (21-23) and the level of the parasite in the pasture has shown a positive and significant correlation with the ELISA's OD values (7). Finally, the magnitude of response to anthelmintic treatment has been related to ELISA OD values (16; 17; 24; 25). Collectively, the studies referred to above suggest that the ELISA is a useful measure of parasite burdens in adult cows.

4.2.4 Objective

The objectives of the present study were to determine if the variation in ELISA optical density ratios (ODR) in milk follows a similar pattern to the variation in total IgG levels in milk, and to evaluate the effect of various cow and herd factors on this ODR variation. Additionally, a method of comparing ODR values at different stages of lactation was examined.

4.3 Material and Methods

4.3.1 Animals

Lactating Holstein cows from four dairy herds (two from Prince Edward Island and two from Nova Scotia), participating in a clinical trial of eprinomectin treatment at calving, were selected for this study. These herds allowed the dry cows to graze a pasture during the summer, but lactating cows were confined.

4.3.2 Samples

Composite individual milk samples from the whole herd were collected from the provincial dairy laboratory in June and August of 2002. Only samples from non-treated cows in the clinical trial were used in the analyses.

The milk samples were preserved with bronopol; the lacto-serum was obtained by centrifugation at 16000 x *g* for 4 min. The fat was removed and the supernatant was stored at – 20 °C until tested according to Sanchez et al. (15). The ELISA ODRs were then determined on all samples. A subset of cows, which were between 30 and 400 days in milk, were randomly selected from each herd in June and August of 2002. The milk samples from those cows were tested with a sandwich ELISA to determine total IgG levels.

4.3.3 ELISA techniques

4.3.3.1 Indirect O.ostertagi *ELISA*

An indirect ELISA was performed, using a crude saline-extract of the whole worm *O.ostertagi* as antigen, as described by Sanchez et al. (15). Briefly, 1µg/ml of antigen per well

was coated in a flat-bottom, 96-well polysterene microplate (Dynex, Chantilly, VI, USA). Plates were left at room temperature for one hour, and then frozen at -20 °C until needed. Negative control serum was obtained from pooled samples from 3-month-old helminth-naive calves. A positive control serum was obtained from a hyperimmune calf after repeated artificial infections with L3 of *O.ostertagi*. Control sera were diluted 1:400 and added to test wells in quadruplicate on each plate. Plates were incubated at room temperature for one hour. Anti-bovine IgG conjugated to horseradish peroxidase was then added to each well. Finally, substrate consisting of 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and H_2O_2 was added. Plates were incubated at room temperature for 35 minutes. Absorbance was read at 405/490 nm, using a Spectromax ELISA reader. The optical density (OD) values were expressed as:

$$ODR = \frac{(OD - Nt)}{(Pst - Nt)}$$

where OD is the absorbance value of the sample, and Pt and Nt are the mean absorbance values of the 4 positive and 4 negative controls on each plate, respectively. OD values from the blank cells were subtracted from all cells.

4.3.3.2 Sandwich IgG ELISA

IgG levels in milk were measured using a sandwich ELISA as described by Kummer et al. (26). Briefly, a 96 well polystyrene plate (Dynex, Chantilly, VI, USA) was coated with 100 ul/well of rabbit anti-bovine IgG (H+L) (Cedarlane, Hornby, ON, CA), which was diluted to 1ug/ml in carbonate bicarbonate buffer pH 9.6 and incubated for 18 hours at room

temperature. The plates were washed three times with phosphate buffered saline with Tween 20 (PBS-T20). The plates were blocked with 200 µl/well of 1% gelatin (Difco, Detroit, MI, USA) in PBST, for one hour at room temperature after which they were wrapped in plastic film and frozen at –20°C until needed. Bovine IgG1 standards were included on each plate (Cedarlane). The standards were serially diluted in PBS-T20 plus 0.33% gelatin to give 0.05 to 100 ng/ ml of IgG1 in 100 µl/well. The test milk samples were centrifuged, defatted and diluted to 1/68,276 in PBS-T20/gelatin, dispensed at 100 µl/well and incubated one hour at 37°C. Plates were washed as described and 100 µl/well of horseradish peroxidase labeled rabbit anti-bovine IgG (Cedarlane) diluted 1/1000 in PBS-T20/gelatin was added and the plate incubated for one hour at 37°C. Plates were washed and 100 ul/well of substrate consisting of 0.22 mg/ml of 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt in 0.1M citric acid and 0.2M phosphate with 0.09% of a 30% solution of H_2O_2 was added and the plate incubated for one hour at 37°C. Absorbance was read at 405/490 nm on a Spectramax ELISA reader. The optical densities of the milk samples were recorded and their IgG values, which were extrapolated from the standard curve, were multiplied by the initial dilution factor of 68,276 to calculate the IgG content in mg/ml.

4.3.4 Production data

Daily milk production (kg/cow), days in milk, lactation number, age and somatic cell counts (SCC) were obtained electronically from the Canadian Dairy Herd Management System.

4.3.5 Analysis

4.3.5.1 Descriptive statistics and univariable analyses

Descriptive statistics and Pearson correlation coefficients were computed for ODR, IgG and the production parameters. Scatterplots of ODR and IgG values on stage of lactation and milk yield with a Kernel smooth mean (using a bandwidth of 0.5) were obtained. Samples taken between 30 and 400 days in milk were used for this analysis.

4.3.5.2 Multivariable analyses

Because some of the cows were tested in both June and August, having potentially two observations per animal, independence of the observations was not assumed. A robust estimate of the variance (27) was applied to the multivariable linear regression models. Two sets of models were fit using ODR values as dependent variables. One set consisted of all the records that had ODR readings, while the other used only the observations from which IgG values had also been determined. The following covariates were included in those models: milk yield, days in milk, lactation category (1, 2 and 3 or greater lactation), log transformed somatic cell counts, IgG, test month and herd. All the main effects that were significant at $P \leq 0.05$ were left in the model. When a final model was obtained, a residual analysis was performed to evaluate the assumptions of these statistical procedures. All the analyses were carried out in Version 8 of the statistical package Stata (28).

4.4 Results

A total of 560 milk samples from 358 cows between five and 636 days in milk were collected from the database. A summary of the main production parameters by month is shown in Table 4.1. The ODR average was 0.34, and ranged from –0.08 to 1.12. The ODR distributions by month, and their distribution by herd-month, are presented in Figure 4.1 and Table 4.2, respectively. Overall, the samples taken in August had higher values than those taken in June.

Total IgG concentrations were determined in 279 milk samples taken between 30 and 381 days in milk from 229 cows (145 in June and 134 in August). The IgG concentration average was of 0.28 mg/ml and ranged from 0.07 to 0.91 mg/ml. The variation of total IgG levels and ODR values by days in milk is depicted in Figure 4.2. The IgG levels and ODR values were constant from 30 to 200 days in milk and then they increased up until the end of the lactation. The respective average values at 30-99, 100-199, and 200-300 days in milk were 0.26, 0.26, and 0.33 for IgG concentrations and 0.30, 0.31 and 0.40 for ODR values. The Pearson correlation coefficients among ODR, IgG (mg/ml), days in milk and milk production are presented in Table 4.3. ODR values were moderately positively correlated with IgG, days in milk, age and log SCC. On the other hand, ODR values were negatively correlated with milk production. Similar correlations were observed for IgG values with these variables. The variation of IgG and ODR values by levels of milk production is shown in Figure 4.3. They presented similar patterns with higher values at 15-25 kg of milk production, decreasing at 25-40 kg/day; the lowest values were when milk production was between 40-60 kg/day.

The linear regression model using all the observations with ODR values is shown in Table 4.4. There was a statistically significant difference between herds in ODR values (P = 0.001). Similarly, older animals and test month (August) had higher antibody levels than younger cows and cows tested in June, respectively. Because milk production was not linearly related to ODR (Figure 4.3), a second term (milk square) was added to the model. However, this term was only marginally statistically significant. Thus, a model without this variable was fit to compare the change in the coefficients (Table 4.4). From the latter model, an increase of 13 kg in milk yield (IQR: 25-38) was associated with a reduction of 0.052 in ODR values. The reduced model (i.e. using only those observations with IgG values) had IgG ($\beta = 0.39$, $P < 0.001$) and test month (August vs June, $\beta = 0.05$, $P = 0.019$) as significant predictors of ODR (model not shown). Once adjusted for IgG levels, herd, age, stage of lactation and SCC were all non-significant. Similarly, an increase of 0.14 (IQR: 0.19-0.33) in IgG was associated with an increase of 0.05 in ODR values. The residual analysis of these models did not show outlier or influential observation, and they were normally distributed.

4.5 Discussion and Conclusion

The mean ODR found in this study were lower than values reported in similar studies also carried out in eastern Canada (16; 21; 22). The difference among these studies is presumably related to the sampling period and level of cow exposure to pasture. However, the range of ODR values was similar to those in the present study.

The correlations for ODR and IgG between stage of lactation, milk yield, age and log SCC followed the same patterns. However, IgG values presented a stronger correlation. The

association between IgG levels in milk and age could reflect greater tissue damage in older animals, with an increase leakage of IgG from serum into milk (as is observed with SCC). Caffin et al. (13) did not find any differences in total IgG1 concentrations in milk for the first three lactations but beyond that the IgG1 concentrations increased significantly. On the other hand, Levieux and Ollier (11) found that first lactation cows had significantly lower IgG concentrations in colostrum samples compared with cows in the second or greater lactation. The latter may suggest that physiologically, younger animals might have also lower IgG concentrations in mature milk.

The relationship between total IgG in milk and days in milk observed in Figure 4.2 has been reported previously (13), although less precisely estimated. It has been suggested that this pattern could be explained by a decrease of the selective transport process of IgG in the mammary gland during peak lactation or contrarily by a low level of milk production with constant rate of IgG synthesis at the end of the lactation (13). Caffin et al. (13) also found that IgG1 levels in milk were similar at 30 and 150 days of lactation, but were significantly greater at the end of lactation (270 days in milk). Other factors such as breed, age and udder health have been found to influence the immunoglobulin concentrations in milk (29; 30). On the other hand, Caffin et al. (13) did not find any difference in IgG1 concentrations relative to location of uninfected quarters. However, this relationship changed depending on the pathogens affecting the udder quarters. Quarters infected by *Staphylococcus aureus* had increased levels of IgG1; there was a less pronounced increase in quarters harboring minor pathogens.

It has been suggested that the level of IgG response against GIN appears to be dependent on levels of antigenic stimulation (1). The increased ODR levels in older animals observed in this study might be the result of a higher level of acquired immunity due to repeated parasite-host contact. The correlation coefficients between ODR, milk yield, days in milk and age are similar to those reported by Kloosterman et al. (31). A dilution effect (13) or more efficient and increased IgG transport into the mammary gland (9) have been suggested as possible explanations. The positive correlation of milk titres with age is controversial because older cows produce more milk so lower titres might be expected. However, when controlling for others factors (including milk yield) (Table 4.4), either second or third or greater lactation animals showed higher ODR values than did primiparous cows. Consequently, this association may be the result of a greater parasite exposure in older cows, which is reflected in higher antibody titres (1; 6), or might be related to genetic differences in the ability to respond to GIN (32; 33). The latter is not likely in this study because cows of different ages were evenly distributed among the herds participating in this study, suggesting comparable genetics across age groups.

From the reduced model (only observations with IgG values), and Figures 4.2 and 4.3, it can be observed that IgG levels and ODRs are strongly associated. Chang et al. (34) reported a substantial increase in IgG antibodies in lacteal secretions in animals immunized through an intestinal fistula. Even though the *O.ostertagi* ELISA is measuring a small portion of the total IgG levels, and these originate from parasite-host contact in the gut, it seems to reflect the magnitude of the parasite contact in the digestive tract. The strong association observed between IgG and ODR values might be related to the different capacity of cows to

transfer IgG from serum into milk or it might reflect that cows producing more IgG tend to respond better to the parasite antigens.

The relationship between ODR and days in milk observed in Figure 4.2 seems to disappear when other variables are taken into account (Table 4.4). Among them, the coefficient for milk yield after controlling for season, age, SCC and herd, is –0.004, which may be used as a correction factor. For instance, when comparing ODR from a cow producing 25 kg of milk per day with that from a cow producing 38 kg, a value of -0.052 should be subtracted from the former to create a corrected ODR. In conclusion, the results of the present study suggest that ODR values are not greatly influenced by milk productions. They follow the same pattern of the IgG variation across lactation. However, they might be adjusted in order to compare ODR values obtained from high producing cows with those obtained from low producing animals.

4.6 References

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Table 4.1. Descriptive statistics of production parameters of 328 and 292 cows from four dairy herds sampled in June and August of 2002, respectively *.

	Month				Total	
	June		August			
Variable	Mean	IQR	Mean	IQR	Mean	IQR
Milk yield [a]	33.5	26.6-39.6	31.6	24.2-37.7	32.5	25.3-38.7
DIM[b]	197	124-274	208	126-270	202	124-272
SCC[c]	182.5	24-106	223.5	36-138	203	29-120
Lactation number	2.2	1-3	2.3	1-3	2.3	1-3

[a] kg/cow/day

[b] days in milk

[c] somatic cell counts (1000s)

IQR = Interquartile range (25^{th} and 75^{th} percentiles)

* Milk samples from placebo cows

Table 4.2. Mean and interquartile range (IQR) of *O.ostertagi* ELISA optical density ratios and number of milk samples tested by herd and month.

Herd	June			August		
	Mean	IQR	n	Mean	IQR	n
1	0.37	0.23 – 0.55	69	0.38	0.24 – 0.51	59
2	0.29	0.16 – 0.45	35	0.34	0.16 – 0.44	31
3	0.23	0.11 – 0.31	48	0.37	0.22 – 0.50	78
4	0.27	0.15 – 0.38	123	0.39	0.24 – 0.53	117
Total	0.29	0.16 – 0.41	275	0.38	0.23 – 0.51	285

Table 4.3. Pearson correlation coefficients between *O.ostertagi* ELISA optical density ratios (ODR), total IgG levels (mg/ml), days in milk (DIM), daily milk production (kg/cow/day), age (years) and log transformed somatic cell counts (SCC) (number of observations) (all correlations statistically significant, P <0.01).

	ODR	IgG	DIM	Milk Yield	Age
IgG	0.37	-	-	-	-
DIM	0.16	0.35	-	-	-
Milk yield [a]	-0.18	-0.31	-0.57	-	-
Age [b]	0.13	0.17	0.12	0.13	-
Log SCC [c]	0.12	0.43	0.14	0.19	0.21

Correlations based on approximately 550 samples except for those involving IgG (which were based on approximately 270 samples)

[a] milk yield between 15 and 55 kg/cow/day

[b] age ≤ 8 years

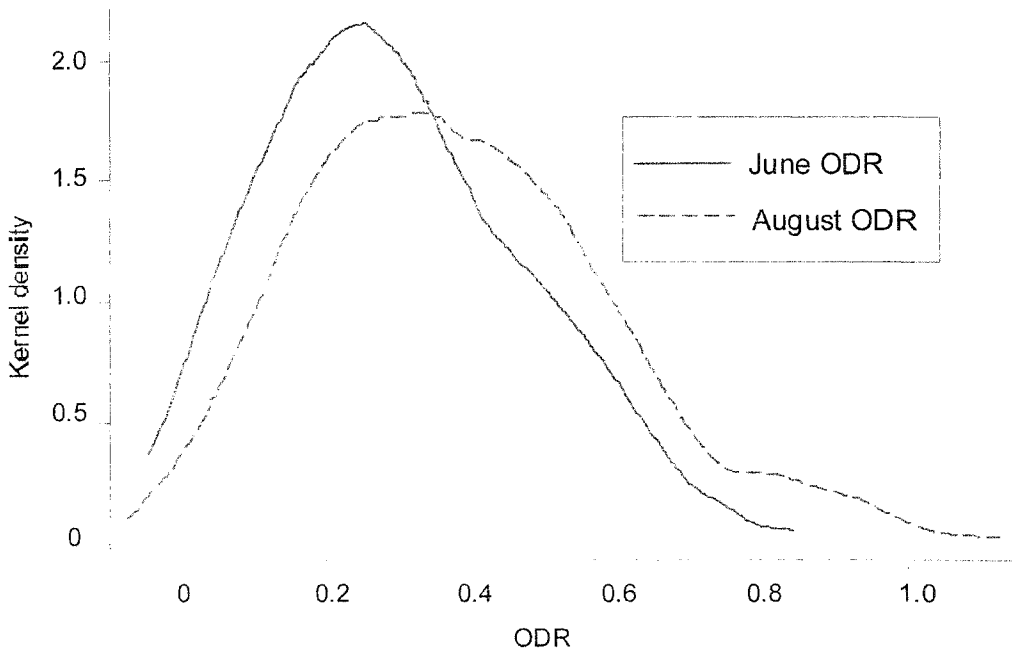
[c] Log SCC : log transformed somatic cell counts

Table 4.4. Fixed effect coefficients and *P*- values for *O.ostertagi* optical density ratios from two linear regression models using the complete dataset (cows=357, tests=558).

	Model 1		Model 2	
Fixed effect	Coefficient	*P*	Coefficient	*P*
Milk yield	-0.013	0.004	-0.004	0.000
Milk yield square	0.0001	0.051	-	-
Log SCC	0.027	0.000	0.027	0.000
Lactation group				
First	Baseline			
Second	0.055	0.001	0.059	0.005
Third or greater	0.051	0.013	0.058	0.005
August (vs. June)	0.077	0.000	0.079	0.000
Herd				
1	Baseline			
2	-0.036	0.267	-0.037	0.253
3	-0.094	0.000	-0.093	0.000
4	-0.020	0.340	-0.015	0.466
Intercept	0.45	0.000	0.31	0.000

Days in milk was not significant and was dropped from the model

Figure 4.1. Kernel smoothed estimates of the distribution of the optical density ratio (ODR) by sampling month.



*only samples from placebo cows

Figure 4.2. Kernel smoothed estimates of the distribution of total IgG levels in milk (n=279) and the *O.ostertagi* ELISA optical density ratio values (n=509) by stage of lactation (observations between 30 and 400 days in milk).

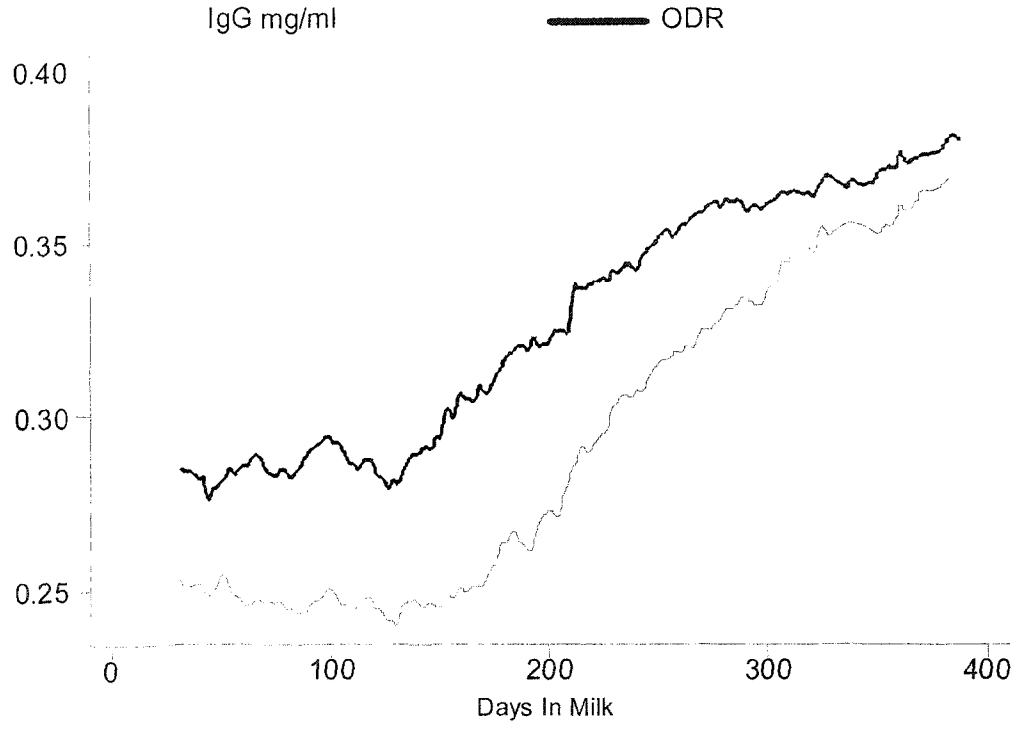
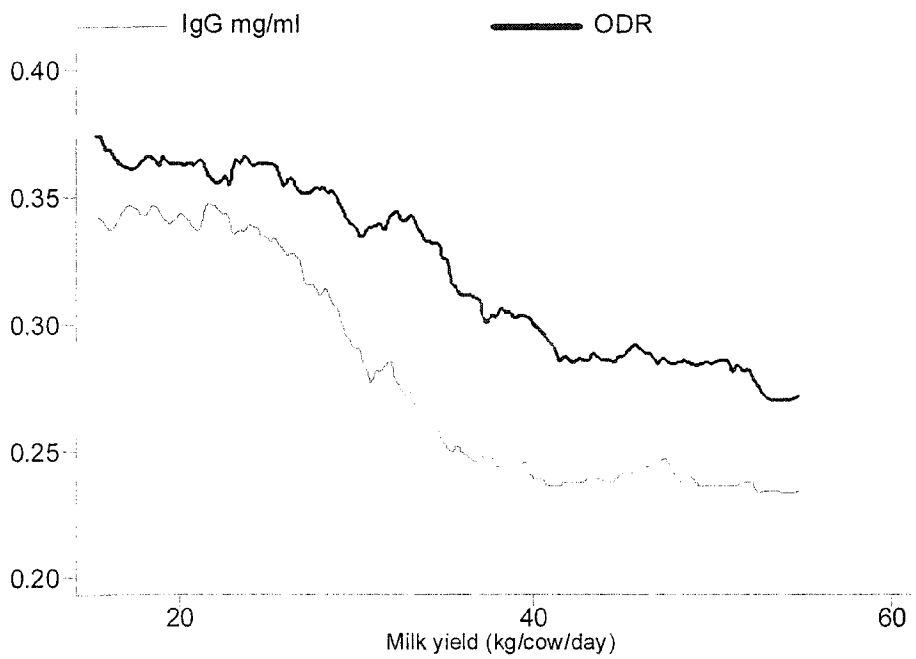


Figure 4.3. Kernel smoothed estimates of the distribution of total IgG levels (n=264) and the *O.ostertagi* ELISA optical density ratio values (n=531) by milk yield (only observation between 15 and 55 kg/cow/day).



5. A bulk tank milk survey of *Ostertagia ostertagi* antibodies in dairy herds in Prince Edward Island and their relationship with herd management factors and milk yield.

5.1 Abstract

The objectives of this study were to quantify the relationship between the levels of antibodies to *Ostertagia ostertagi* in bulk tank milk samples from Prince Edward Island (PEI) dairy farms with milk production and herd management practices potentially related to gastrointestinal nematode infections. Bulk tank milk samples were obtained from 289 to 322 dairy farms during 2000 while production and management data were available from 197 and 200 farms, respectively. Cow exposure to pasture and whole herd anthelmintic treatment were the only significant herd management variables associated with antibody levels in the fall of 2000. An increase in antibody levels from the observed 25th percentile to the 75th percentile (interquantile range) was associated with a drop in milk production of 1.2 kg/cow/day. The results of this study indicate that the *O.ostertagi* antibody ELISA is a potentially useful diagnostic technique to measure parasite exposure in adult dairy cows and that parasite burdens in lactating cattle in PEI have an important impact on milk production.

5.2 Introduction

Gastrointestinal nematode infections occur frequently in young cattle in temperate regions. Although parasite burdens tend to decrease with age, they remain present in lactating dairy cattle. Two recent slaughterhouse studies carried out in Belgium and The Netherlands in adult dairy cows have shown that more than 90% of the cows examined were infected and that some of them harbored up to 99,000 parasites (1; 2). In these studies, *Ostertagia ostertagi* was the most prevalent parasite and between 15% and 20% of the animals had total parasite burdens greater than 10,000 worms. In adult cattle, the effect of these parasites has been assessed by evaluating the milk production response after anthelmintic treatment. A review of more than 80 anthelmintic field trials using different study designs and treatment protocols suggested that after anthelmintic treatment, a median increase in milk production of 0.63 kg/d could be expected (3). In addition, a recent clinical trial carried out in pastured dairy herds in 2 provinces of Canada, in which cows received either placebo or eprinomectin pour-on (Ivomec® Eprinex®, Merial Canada Inc., 500 Boul. Morgan, Suite 1 Baie d'Urfe, Montreal, QC H9X 3V1) at calving showed an average increase in milk production of 0.94 kg/cow/d during the first 6 mo of lactation (4). In spite of the evidence that gastrointestinal nematode infection has an adverse effect on milk yield, there is considerable variability between farms in terms of milk response after anthelmintic treatment. In relation to this, Vercruysse and Claerebout (5) discussed the need to identify a parameter that can be used to identify animals or herds with a level of parasite infection that would justify anthelmintic treatment. A partial budgeting analysis of internal parasite control on dairy farms in Michigan reported a benefit

of $US 15 per head assuming that all animals with parasite burdens were correctly diagnosed and that they responded positively to the treatment (6).

An indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *Ostertagia ostertagi* developed in The Netherlands (7) has been evaluated for monitoring gastrointestinal parasites in dairy cattle (8). It has a moderate correlation with fecal egg counts (FECs) when herd average optical density (OD) values are compared with herd average FECs. However, FECs in adult animals are not well correlated with parasite burdens (3). Consequently, evaluation of the ELISA requires that OD values be compared with some other indirect estimators of parasite infection (e.g. factors that increase or reduce the risk of gastrointestinal parasitism, production measures). In two studies using bulk tank milk samples, a significant positive relationship has been found between ELISA OD values and levels of exposure to pasture (housing system: confinement, yard, paddock or pasture) (9), and a negative relationship between ELISA OD values and anthelmintic treatment (10).

Finally, the relationship between ELISA OD values and production measures has been evaluated. Guitian et al. (9) found that an increase in bulk tank milk OD values from 0.53 to 0.83 (the interquartile range of all observed values) was associated with a reduction in milk production of 1.25 kg/cow/d in dairy herds in Nova Scotia. In addition, Hovingh (10) found that a significant reduction in the fall milk production was associated with high levels of antibody to *O.ostertagi* in bulk tank milk samples in dairy herds in Prince Edward Island (PEI). The use of OD values to predict the milk production response after anthelmintic treatment has also been investigated. Ploeger et al. (11), using serum samples, and Sanchez et al. (unpublished observations) using milk samples, have found statistically significant

associations in which high OD values cows had greater response to treatment. Similarly, Kloosterman et al. (12) reported a trend toward a higher milk yield response from herds with high levels of antibody in bulk tank milk samples, but it was not statistically significant.

The objectives of this study were 1) to quantify the relationship between antibody levels determined using an *O.ostertagi* indirect ELISA on bulk tank milk samples and herd management practices related to gastrointestinal nematode infection, and 2) to evaluate the association between antibody levels and measures of milk production.

5.3 Materials and methods

5.3.1 Study design and study population

A cross-sectional study, in which levels of antibody in bulk-tank milk, herd management practices, and milk yield measures were determined, was conducted between January 2000 and December 2000. The study population consisted of all dairy herds in PEI.

5.3.2 Sample collection and laboratory methods

A complete set of bulk tank milk samples submitted to the PEI provincial milk quality laboratory in each of January, May, September and October 2000 were used in this study. The samples were kept frozen (-20°C) until they were tested and milk *O.ostertagi* IgG levels were determined in an indirect ELISA. Crude adult antigen extracts were coated in 96 well microplates (pH 9.6) in a concentration of 1ug/ml. Positive and negative control sera were diluted 1:140 in phosphate buffered saline in quadruplicate on each plate. Anti-bovine IgG coupled to horseradish peroxidase was used as conjugate. The substrate used was in ABTS

([2,2'-azino-bis-(3-ethyl-benzth-iazoline-sulfonic acid]) diluted in citrate buffer (0.1 M), sodium phosphate buffer (0.2 M) and 0.09 % of H_2O_2. Optical density (OD) was measured at 405/490 nm and was expressed as optical density ratio (ODR) values calculated according to the following formula:

$$ODR = \frac{OD - Neg}{Pos - Neg}$$

where OD is the sample absorbance, and Pos and Neg are the mean absorbance values of the four positive and four negative control samples on the ELISA plate, respectively. Although this ELISA cross reacts with other helminths (mainly *Cooperia* spp.), this is not a serious problem when used for monitoring parasite burdens where an overall estimation of the effect of the gastrointestinal parasitism is desired. In addition, good reproducibility values of this ELISA have been determined (7; Sanchez et al., unpublished observations).

5.3.3 Farm management practices

During September 2000, a one-page closed-response questionnaire (Appendix A) was mailed to all registered dairy producers asking for information on factors that are hypothesized to be associated with exposure to gastrointestinal parasites. Thus, data on housing systems, pasture management, and anthelmintic treatment programs for heifers (nulliparous cows) and milking cows were obtained (definition of all management practice variables are presented in Table 5.1).

5.3.4 *Milk production data*

Individual cow milk yield data from January 2000 to December 2000 were extracted from the Canadian Dairy Herd Management System (CDHMS) database for all study herds. From these data, herd average values of individual cows' milk production (kg/cow/d) were computed for annual milk production (January – December 2000), fall milk production (October – December 2000), and seasonal decline (average of October -December as a proportion of average of May - July). Herd averages for annual and fall days in milk (DIM), lactation number, and log somatic cell counts (SCC) were also computed.

5.3.5 *Data analyses*

Means, standard deviations, and ranges of bulk tank ODR values and milk yield were obtained. The variation in bulk tank ODR values was evaluated using a mixed linear regression model that was fit with the restricted generalized iterative least-square (RIGLS) algorithm in the statistical package MLwiN (13). The contribution of herd and test month to the total variance was obtained from a random intercept model containing only the intercept (null model).

The fall ODR value (average of September and October ODR values) was the only ELISA measure used in the following models. The association between the fall ODR value and herd management practices, obtained from the questionnaire, and between milk production and the fall ODR values were evaluated using a backwards-stepwise regression with elimination of non-significant effects ($P > 0.05$). All the main effects that were

significant at $P \leq 0.05$ were left in the model and two-way interactions of these variables were evaluated. Once the final model was selected, the potential confounding effect of the eliminated variables was assessed by evaluating the change in the coefficients of the remaining variables in the model that resulted from removal of the potential confounders. Pearson correlation coefficients were used to check for collinearity among explanatory variables. Analyses of the residuals and influential observations were performed on all these models. All of these analyses were carried using Stata Ver.7 (Stata Statistical Software, Release 7.0, Stata Corporation, College Station, Texas, USA).

For the model evaluating the associations between fall ODR values (dependent variable) and management practices, cow and heifer housing variables were recategorized with confinement and exercise yard combined into a single category.

Two sets of models were fit using each of the 3 milk production measures (herd average annual milk production, herd average fall milk production, and seasonal decline in milk production) as the dependent variables. One set of models included fall ODR values, DIM, parity, and SCC as the predictors and was based on data from all herds in the province. The second set also included a variable for pasture exposure, which was dichotomized as nonpastured (confinement, yard and paddock) and pastured herds, and anthelmintic treatment protocols. This second set was limited to those herds for which a response to the questionnaire had been received.

5.4 Results

5.4.1 Descriptive statistics

The number of farms sampled during the study period ranged from 289 to 322 per month (mean=313) with 333 herds contributing to the fall ODR values. In total, 1239 bulk tank milk samples were tested for antibodies against *O.ostertagi.* The mean ODR was 0.54 with a standard deviation of 0.26 and the ODR values ranged from 0.03 to 1.90. The distribution of ODR values by month is depicted in Figure 5.1. The proportional contribution of herd and test month to the total variance of ELISA ODR obtained from the mixed linear model containing only the intercept was 0.64 and 0.36, respectively.

Out of the 313 surveys mailed, 200 farms (64 %) returned the questionnaire. Milk production data were obtained for 197 of these 313 herds, but only 191 of these had fall ODR values. The mean fall ODR value for the responding and the nonresponding farms were 0.55 and 0.66, respectively. The fall ODR and those measures obtained from the milk production database are presented in Table 5.2.

5.4.2 Association between herd management factors and ELISA results

The pairwise correlation coefficients of the explanatory variables used in these models showed a moderate correlation coefficient (r = 0.69) between whole herd treatment and lactating cow treatment, so it was decided to include the former variable. Apart from that, the highest pairwise correlation observed was 0.33, which suggested that no serious multicollinearity problem was expected.

The regression coefficients, 95% confidence intervals and *P*-values from the final model of factors affecting fall ODR values are presented in Table 5.3. Cow exposure to pasture and whole herd treatment were the only significant variables associated with fall ODR values. The lower the level of exposure to pasture, the lower was the fall bulk tank antibody level. Whole herd deworming of milking cows, significantly reduced the fall ODR values. A model that was restricted to pastured herds only, showed similar results, with the only significant variable associated with fall ODR values being whole herd treatment ($\beta = -.21$, $P < 0.001$, $R^2 = 0.14$). No heteroscedasticity was observed in the residual analysis from either model and only one outlying observation was present in each model. However, this observation did not have a large influence on the coefficients, so it was left in the model.

5.4.3 Association between milk production and ELISA results

The descriptive statistics of the three measures of milk production by cow housing system are shown in Table 5.4. The Pearson correlation coefficients between fall ODR values and annual milk production, fall milk production, and seasonal decline were -0.38, -0.44, and -0.29, respectively. Annual and fall milk production were both significantly negatively associated with the fall ODR values. A unit of increase in fall ODR values was associated with a reduction of 5.82 kg/d ($P < 0.001$) and 6.29 kg/d ($P < 0.001$) based on the annual milk production and fall milk production, respectively. A similar association was found when seasonal decline was the outcome variable; a unit increase in fall ODR values was associated with a reduction of 9% in this parameter.

Cow exposure to pasture was the only significant management variable when herd management practices were included in the previous models. The associations between milk production measures and fall ODR values are presented in Table 5.5. After controlling for pasture exposure, a unit of increase in fall ODR values was associated with a reduction of 3.42 kg/d ($P = 0.041$) based on annual milk production and a reduction of 2.89 kg/d based on fall milk production ($P = 0.096$) (Table 5.5). However, these models were based on a smaller number of observations (n = 120 and n = 118) than were the models without pasture exposure (n = 189 and n = 186). For seasonal decline, no significant effect of cow exposure to pasture was observed. However, the association between fall ODR values and seasonal decline was similar to the value reported above. The interaction between cow exposure to pasture and fall ODR values could be not evaluated in these models due to the small number of non-pastured herds.

5.5 Discussion

The overall mean ODR values were higher than that found in a longitudinal study of lactating dairy cows from 4 provinces of Canada between September 1999 to October 2000 (Sanchez et al., unpublished observations). In that study, only 38 dairy herds were sampled monthly throughout the year and they had a mean bulk tank ODR value of 0.36 with a range from 0.03 to 1.03.

Little variation in ODR values could be observed between sampling dates (Figure 5.1), and it is difficult to describe a seasonal pattern in this study, because samples were not collected during the summer months, when a rise in ODR values might be expected. The

proportion of the variance in fall ODR values explained by herd and test day was similar to the values observed in a longitudinal study of gastrointestinal parasitism in lactating dairy cattle (Sanchez et al., unpublished observations). This agrees with results reported by Dohoo et al. (8), who suggested that ELISA OD might be a more stable indicator than fecal egg counts of gastrointestinal parasitism at farm level. Kloosterman et al. (14) have also reported that milk samples were as efficient as serum samples to discriminate between herd levels of infection. Finally, Berghen et al. (15) have suggested that antibody levels against *O.ostertagi* are the most valuable parameter for estimating the variation in levels of parasite exposure among herds.

The fall ODR value of 0.60 was similar to that of 0.58 obtained by Hovingh (10) from 74 dairy herds in PEI in October 1994 and lower than 0.69 reported by Guitian et al. (9) from 402 dairy herds in Nova Scotia during the late summer (July-September) of 1998. However, the previous two studies reported "raw" OD values rather than ODR values that makes these results less comparable. On the other hand, the higher OD values observed in the Nova Scotia study could be attributable to higher levels of parasite exposure during the summer compared with the fall.

In contrast with the study done in Nova Scotia (9), we found only cow housing system and whole herd treatment significantly associated with fall ODR values. This model had an R^2 of 0.16, meaning that after controlling for cow exposure to pasture and whole herd treatment, there was a large amount of the variation in the fall ODR values that had not been explained by factors in this study. In the Nova Scotia study, heifer housing system and spring anthelmintic treatment of the heifers were also significantly associated with ELISA OD.

Hovingh (10) also reported a significant negative association of ELISA OD values with anthelmintic treatment of mature cows.

Although the effect of pasture grazing system (continuous versus rotational) on fall ODR values did not have a significant effect, conflicting results are to be found in the literature related to this factor. Stromberg et al. (16) summarized the results of several parasitological studies, that evaluated the effect of rotational versus continuous system on parasite burdens; while some of them found a higher parasite load in rotational systems, the others did not find such a difference for either egg or worm counts. Gasbarre et al. (17), using a survey questionnaire of management practices in the northeast USA, reported that a rotational program and other uses of pasture did not influence the farmer's perception of the importance of parasites in his herd. These authors concluded that "given the complexity of the parasite biology plus all the factors that regulate the egg output and larval survival on pastures, there will be no simple answer to the question of whether rotational grazing system increases or decreases parasite transmission".

The negative relationship between antibody levels and milk production observed in this study is in agreement with that reported in other studies (9; 10; 14). Contrary to results reported by Guitian et al. (9) the fall ODR values were also significantly associated with seasonal decline in milk production, as was reported by Hovingh (10). Sanchez et al. (unpublished observations) have also found that high ODR (> 0.5) late lactation cows did have a higher milk response after anthelmintic treatment at calving compared with low ODR late lactation cows.

Higher levels of exposure to pasture have been found to be related with lower levels of milk production (9). Similarly, Leslie et al. (18) using a conjoint analysis survey of expert opinion determined that confinement housing systems, anthelmintic treatment of replacement heifer and lactating cow were predictors of increasing milk production, while rotational grazing systems on pasture where manure was spread were expected to decrease milk production. Consequently, a coefficient of –3 kg/d (an intermediate value taken from models in which pasture exposure had been controlled; Table 5.5) would probably be a better estimate of the association between fall ODR values and milk production. With a fall ODR interquartile range from 0.38 to 0.78 and a coefficient of approximately –3 kg/d, a herd at the 75th percentile would be expected to produce 1.2 kg/cow/day (-3 x (0.78-0.38)) less milk than a herd at the 25th percentile. However, since exposure to pasture was relatively crudely estimated in the current study, the confounding effect on milk production may not have been totally controlled and the effect of ODR may still be biased upwards.

In conclusion, a high proportion of the variation in fall ODR values was explained by herd (as opposed to within herd variation between test dates). The ELISA test results (ODR values) were associated with factors that biologically would increase or reduce the risk of gastrointestinal parasitism. However, it is still necessary to identify other factors that would explain the large amount of unexplained variation. Moreover, the consistently observed negative association between bulk tank milk ODR values and milk production, plus some observations that high ODR value cows did perform better in terms of milk yield after anthelmintic treatment, provides evidence that high ODR value cows and/or herds are

suffering parasite-induced losses in milk production (Sanchez, et al., unpublished observations). Collectively this information supports the potential value of this ELISA as a diagnostic test to measure parasite burdens. However, further research is needed to establish a threshold value for bulk tank milk ODR values, at which treatment is warranted economically.

5.6 References

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Table 5.1. Description and proportions of the herd management variables obtained from a survey of dairy herds on Prince Edward Island (September-October 2000) along with mean fall ELISA optical density ratios (ODRs).

Variable Description	N	Yes		No	
		%	Mean fall ODR	%	Mean fall ODR
Heifers pasture/paddock (vs. confinement/yard)	178	85	0.57	15	0.46
Heifers graze on pastures also grazed by dry cows	141	50	0.55	50	0.59
Heifers graze on pastures also grazed by milking cows	160	14	0.66	86	0.56
Heifers dewormed in the fall 1999	188	56	0.54	44	0.59
Heifers dewormed in the spring 2000	188	33	0.53	67	0.58
Heifers given a sustained-release bolus in the summer 2000	188	8	0.50	92	0.56
Heifers dewormed in the fall 2000	188	4	0.66	96	0.55
Milking cows pasture/paddock (vs. confinement/yard)	185	97	0.56	3	0.31
Milking cows dewormed with oral product in the last 12 months	195	4	0.73	96	0.55
Milking cows treated with pour-on or injectable treatment at drying off in the past 12 months	195	8	0.68	92	0.55
Cows treated with pour-on or injectable treatment at calving in the past 12 months	195	10	0.48	90	0.57
Anthelmintic treatment in milking cows in the last 12 months	195	45	0.51	55	0.60
Whole herd treated with pour-on or injectable treatment in the past 12 months	195	29	0.43	71	0.61

Variable Description	N	Yes		No	
		%	Mean fall ODR	%	Mean fall ODR
Pastures are managed using some form of controlled access grazing (rotation or strip) vs continuous access	180	71	0.58	29	0.58
Manure mechanically spread on pastures used for grazing	183	41	0.62	59	0.55
Pastures dragged or harrowed	182	31	0.59	69	0.56
Pastures clipped	183	78	0.57	22	0.60

Table 5.2. Descriptive statistics of ELISA optical density ratios (ODRs) and milk production data in dairy herds on Prince Edward Island. Data from 333 herds for which the fall ODR values were obtained and from 197 herds for which milk yield data were available.

Variable Description	Mean	SD	Range
Average of 2 months (September - October) values of ODR (fall ODR)	0.60	0.27	0.05, 1.70
Average milk production (kg/cow/day) from January 2000 to December 2000	26.80	4.13	11.7, 36.34
Average milk production (kg/cow/day) from October 2000 to December 2000	25.03	4.74	13.2, 35.1
Fall milk production divided by spring milk production.	0.88	0.12	0.59, 1.26
Herd average natural log somatic cell counts from January-December 2000	5.41	0.39	4.39, 6.42
Herd average natural log somatic cell counts from October-December 2000	5.39	0.46	4.11, 6.63
Herd average days-in-milk from January to December 2000	183	23	140, 269
Herd average days-in-milk from October to December 2000	194	28	128, 274
Herd average lactation number from January to December 2000	2.85	0.51	1.33, 4.95
Herd average lactation number from October to December 2000	2.89	0.53	1.39, 5.21

Table 5.3. Regression coefficients, 95% confidence interval (CI), and *P*-values for a multiple regression model predicting fall ELISA test results. Data from 184 dairy herds on Prince Edward Island. The dependent variable was the average optical density readings from bulk tank samples collected in September and October 2000. ($R^2 = 0.16$).

Variable	β	95% CI	*P*
Intercept	0.64	[0.59, 0.68]	0.00
Cow housing [a]			
Pasture	Baseline		
Paddock	-0.16	[-0.29, -0.03]	0.02
Confinement / yard	-0.26	[-0.46, -0.06]	0.01
Whole herd treatment			
No	Baseline		
Yes	-0.19	[-0.27, -0.11]	0.00

[a] overall significance of cow housing categories based on Wald test was P=0.003

Table 5.4. Descriptive statistics of 3 measures of milk production (annual, fall, and seasonal decline) for different levels of milking cow housing. Data from dairy herds on Prince Edward Island for which both herd management data and milk production data were available.

Housing system	N	Mean	SD
Annual milk production (Milk_annual)			
confinement/yard/paddock	15	29.20	4.10
pasture	105	26.59	4.22
Fall milk production (Milk_fall)			
confinement/yard/paddock	15	28.61	4.18
pasture	103	24.74	4.71
Seasonal decline in milk production (Milk_decline)			
confinement/yard/paddock	15	0.95	0.11
pasture	102	0.87	0.11

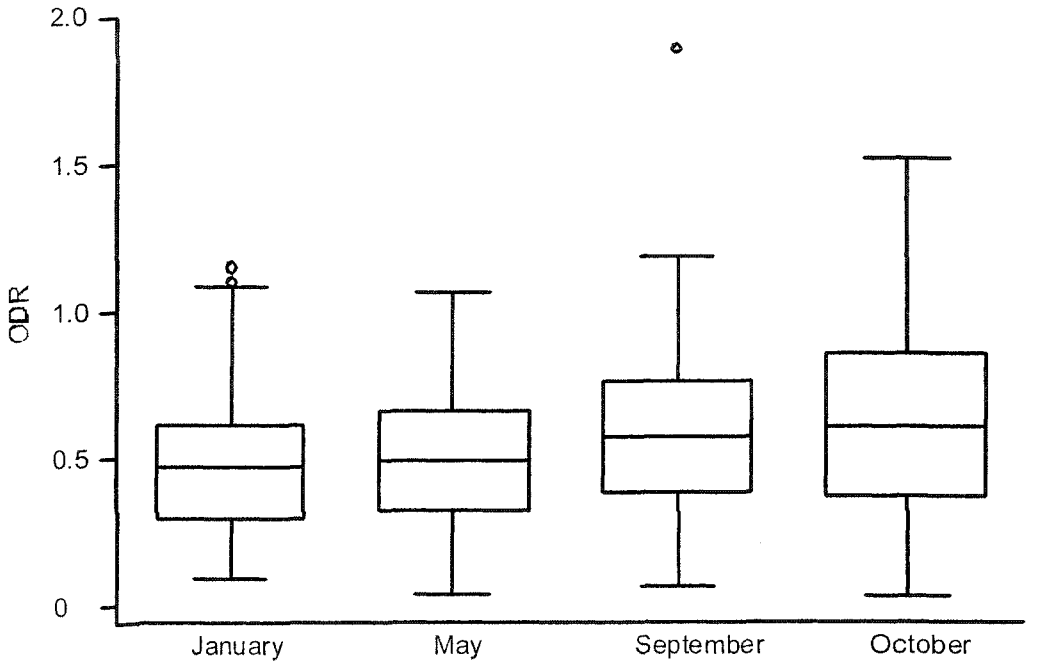
Table 5.5. Regression coefficients, 95% confidence interval, and *P*-values of the associations between milk production (annual milk production, fall milk production) and levels of antibody to *Ostertagia ostertagi* in bulk tank milk after controlling for cow exposure to pasture, average days in milk, average parity number, and average log-SCC [a].

Variable	β	95% CI	*P*
Annual milk production model (n=120, $R^2 = 0.17$)			
ODR [b]	-3.42	[-6.68, -0.15]	0.04
Fall milk production model (n=118, $R^2 = 0.35$)			
ODR [b]	-2.89	[-6.30, 0.52]	0.09

[a] coefficients for controlled factors omitted

[b] optical density ratios from ELISA applied to September and October bulk tank milk samples

Figure 5.1. Box-and-whisker plot of bulk-tank milk optical density ratio (ODR) by test month from dairy farms on Prince Edward Island during January-December 2000.



6. A longitudinal study of gastrointestinal parasites in Canadian dairy farms: the value of an indirect *Ostertagia ostertagi* ELISA as a monitoring tool.

6.1 Abstract

The study evaluated a crude *Ostertagia ostertagi* antigen ELISA for monitoring gastrointestinal parasites in lactating dairy cattle. A longitudinal study of gastrointestinal parasites in lactating dairy cows was carried out in 38 herds in four provinces of Canada (Prince Edward Island, Quebec, Ontario and Saskatchewan) from September 1999 to October 2000. Bulk-tank milk, cow milk, serum and fecal samples were collected monthly or quarterly from all these farms. Information on herd management factors was collected by a standard questionnaire and individual cow production data were obtained from an electronic database. The overall mean optical density ratio (ODR) was 0.30 and ranged from –0.05 to 1.55. Although a clear seasonal pattern was not observed, the ODR values tended to decrease during the housing period and start increasing in the spring before the cows went out to pasture. The second and third or greater lactation cows had significantly higher ODR values compared with first lactation animals. The individual cow ODR had a very low correlation with individual squared root fecal egg counts but showed a reasonably high correlation when herd averages values were computed ($r = 0.73$). A moderate correlation ($r \approx 0.50$) between the bulk-tank and herd average ODR was observed. Milk yield was negatively associated with individual cow milk ODR and a quadratic effect on ODR was observed for days in milk. Twenty-eight of the herds participated in a clinical trial of eprinomectin (Ivomec® Eprinex®) treatment at calving. The cow level ODR values determined late in the previous lactation had

a marginally significant effect (P = 0.07) on treatment response, suggesting that high OD cows responded better to the anthelmintic treatment. However, because of the small sample size available in this model, more research is needed to better understand this relationship. In conclusion, the indirect ELISA using an *O.ostertagi* crude antigen appears useful as a technique for monitoring gastrointestinal parasite burdens in adult dairy cows and holds promise as a potential predictor of response to anthelmintic treatment.

6.2 Introduction

Gastrointestinal nematode infections are one of the most important production limiting diseases of ruminant livestock. Their effect on milk production has broadly been studied with equivocal results from multiple clinical trials of anthelmintic treatment being found in the literature. Gross et al. (1) reviewed more than 80 field trials that were based on different study designs, treatment strategies and products. They reported an overall median increase in milk production after anthelmintic treatment of 0.63 kg/day. Recently a randomized clinical trial performed in 28 Canadian herds reported an increase in daily milk production of 0.94 kg during the first 6 months of lactation after the use of an eprinomectin pour-on solution (IVOMEC® EPRINEX®, Merial Inc.) administered at calving (2).

An important question with regard to anthelmintic treatment is how to identify animals or herds that will benefit from treatment (3). This appears to be most important in adult animals where subclinical parasitism is the primary form of the disease. Traditional diagnostic methods, such as fecal egg counts (FEC), do not perform well in these animals and therefore it is difficult to identify those animals or herds requiring treatment. Another important point needing to be addressed when evaluating the usefulness of anthelmintic products is the necessity of defining the threshold for treatment (3). These authors mentioned three possible thresholds; among them the "economic" threshold is intended to measure the effects of the sub-clinical parasitism and to associate these parasite levels with production parameters, such as milk production.

6.2.1 *Diagnostics Techniques*

Identifying the thresholds for treatment, and therefore, the animals that could have a positive treatment response, depend on the possibility of having reliable diagnostic techniques for gastrointestinal parasitism. Two of the most promising diagnostic methods to be used for this purpose are pepsinogen levels and an immunological assessment (ELISA) of antibody titres (4). Immunological tests currently appear to be the most encouraging tool for monitoring gastrointestinal parasite burdens in adult animals because pepsinogen levels may overestimate the adult parasite burden in adult animals due to a hypersensitivity type reaction (5).

An ELISA using a crude adult *O. ostertagi* antigen has been available during the last 20 years (6). However, the lack of standardization between plates and laboratories and the difficult to obtain high quality antigen have been reported as the main drawbacks of this technique. On the other hand, a recent validation study of this technique has shown a high repeatability between batches of antigen (7), when using test results adjusted according to values from positives and negative controls.

6.2.2 *Assessment of the ELISA*

Fecal egg counts (FEC) have been mostly widely used for the diagnosis of gastrointestinal parasitism in cattle. In first grazing season animals, the correlation between FEC and parasite burdens depends on the initial parasite infection level in the pasture(4). In adult dairy cows, FECs have a low correlation with parasite burdens; thus, they are considered to be a poor indicator of gastrointestinal nematode infection level (1). Data from two recent

European studies of culled dairy cows also suggest that the FECs are not recommended for adult cows (8; 9). The authors of these studies found that 90 % of the cows harbored gastrointestinal parasites, while FEC only revealed 14 % and 30 % of positive cows.

Despite its inadequacies, FEC remains a standard diagnostic technique of gastrointestinal parasitism. As a result the ELISA technique has been evaluated by comparing the optical density (OD) values with either direct indicators of gastrointestinal parasitism such as FEC (10; 11), as well as indirect estimators such as those management practices that increase the risk of parasitism (12). Also, correlations between serum and individual milk samples and bulk tank milk samples have been done to assess the ELISA (11; 13). Factors such as age, stage of lactation and seasonality have been evaluated and reported in different studies to influence ELISA optical density values (8; 9; 11; 13)

Finally, several studies have been carried out using a crude adult *O. ostertagi* antigen to evaluate if the ELISA can predict the response in milk production after anthelmintic treatment. Ploeger et al. (14), reported a positive correlation between serum antibody titres and milk response. However, a similar association could not be determined in a subsequent study (15). Moreover, Kloosterman et al. (16) reported a higher herd average milk response to treatment in high bulk tank milk OD herds, but the difference between the groups lacked statistical significance.

The general objective of the current study was to evaluate a crude *O. ostertagi* antigen ELISA for monitoring gastrointestinal parasites in lactating dairy cattle. The specific objectives were: to evaluate correlations among FEC and OD values; to identify factors influencing the ELISA test results such as milk yield, stage of lactation, parity, seasonality

and herd management practices; and to evaluate the ability of this ELISA to predict the milk production response to anthelmintic treatment.

6.3 Materials and methods

6.3.1 Study population

A longitudinal study was carried out in 38 dairy farms in four provinces of Canada from September 1999 to October 2000. These farms were distributed as follows; 14 farms in Prince Edward Island; 14 farms in southern Quebec; 5 in southern Ontario and 5 from Saskatchewan. Three criteria for herd selection were used: the milking cows had to have some degree of exposure to pasture during the grazing season (mid May to mid November), no use of broad spectrum endectocides in adult animals in the previous 6 months, and the farms had to be enrolled on a milk production recording program. In September 1999, 4 primiparous and 4 second or greater lactation Holstein cows were randomly selected on each farm. The selected cows were identified with a plastic leg-band. Milk and fecal samples were collected from those cows during the study period according to following schedule:

- Cow milk and fecal samples were collected monthly from PEI herds;
- Cow milk and fecal samples, monthly up to April 2000 and bimonthly up to October 2000 from Quebec herds;
- Cow milk and fecal samples were collected monthly and quarterly, respectively from Ontario and Saskatchewan farms.
- Bulk tank milk samples were collected at monthly intervals from all the participating herds.

In addition, in order to have an estimation of the level of parasitism in each farm at the beginning of the study, 5 heifers, the 5 most recently calved primiparous and the 5 most recently calved second or greater lactation cows were sampled at the initial visit. At this time blood samples were also collected from all the study animals. The animals from PEI and Quebec farms were also involved in a randomized clinical trial where they received either eprinomectin pour-on (Ivomec® Eprinex®) or a placebo solution at calving (2). Information on pasture management and other management factors was collected by a standard questionnaire administered to all participating farms (Appendix B).

6.3.2 Techniques

Fecal samples were taken rectally from each cow. Egg counts per 5 gram of feces (FEC) were determined using the modified Wisconsin technique (17).

The sera were obtained by centrifuging the clotted blood samples at 850 x g for 10 min. Milk samples were centrifuged at 16000 x g for 4 min. The fat was skimmed off and the supernatant was removed from any solid precipitate. These supernatants were frozen (-20 °C) in plastic tubes. All samples were processed according to this standard procedure until they were tested. An indirect microtitre enzyme-linked immunosorbent assay (ELISA) using a crude saline extract of an adult *Ostertagia ostertagi* preparation as the antigen (6) was performed according to Sanchez et al. (7) to determine IgG antibody levels towards this parasite. Briefly, the crude adult extract was coated in 96-well microplate (pH 9.6) in a concentration of 1 ug/ml. Negative and positive control serum were obtained from pooled samples from 3 month old helminth-naïve calves and from a hyperimmune calf, respectively.

Anti-bovine IgG coupled to horseradish peroxidase was used as conjugate. The substrate used was in ABTS ([2,2'-azino-bis-(3-ethyl-benzth-iazoline-sulfonic acid]) diluted in citrate buffer (0.1 M), sodium phosphate buffer (0.2 M) and 0.09 % of H_2O_2. The ELISA results were expressed as optical density ratios (ODR) calculated according to the following formula:

$$ODR = \frac{OD - Nt}{Pt - Nt}$$

Where OD is the absorbance value of the sample at 405/492nm, Pt and Nt are the mean absorbance values of the four positive and four negative control samples, respectively.

6.3.3 Production data

Individual daily milk yields, days in milk, calving date and lactation number were obtained from the Canadian Dairy Herd Management System (CDHMS) database through electronic data transfer.

6.3.4 Analysis

6.3.4.1 Descriptive statistics and unconditional associations

Unless otherwise indicated, all statistical analyses were performed in Stata version 7 (18). The mean, standard deviation and range of the ODR and FEC values were calculated. Because the FEC values were highly right skewed, they were square root transformed in order to reduce the impact of the few high values. Unless specifically indicated otherwise, all ODR analyses were carried out using data from "untreated" cows. For herds on the clinical trial, these data came from samples collected before the use of the eprinomectin treatment. All data

from placebo cows that participated in the clinical trial and all the cows from the 2 other Canadian provinces were also included. Four bulk tank milk ODR categories were created according to the approximate quartiles of all bulk tank milk ODR values throughout the year. Then, based on each individual herd's monthly bulk tank ODR value, the correspondent group category was assigned to each herd for that month. The distribution of the cow milk ODR values within the 4 bulk tank ODR categories was then plotted. Scatterplots with lowess smoothed average lines were generated to evaluate the effect of stage of lactation on ODR. Lowess smoothed curves of the mean ODR by province and parity were generated to evaluate the effect of season on ODR. Correlations between FEC (squared root transformed) and ODR values were obtained at various levels of aggregation from individual cow-test day values up to annual herd average values.

The proportion of variance in cow ODR values and square root transformed FEC at each of the three levels of the hierarchy (test day, cow, herd) was determined by fitting a multilevel random intercept model containing only the intercept (null model) using the restricted generalized iterative least-square (RIGLS) algorithm in MLwiN (19). A similar two level (test day, herd) model was used to determine the components of variance in the bulk tank milk ODR.

6.3.4.2 Multivariable analysis

6.3.4.2.1 Multilevel model – factors affecting ODR

These models included data from all clinical trial and non-clinical trial herds. A stepwise backward elimination procedure using generalized estimated equation (GEE)

algorithm was carried out to identify those herd management variables that were associated with cow milk ODR values. Briefly, these management factors were related to housing systems, pasture management and anthelmintic treatments used for heifers, dry cows and milking cows. A more detailed description of these variables are described in another study using the same questionnaire (20). Individual test day observations were assumed to cluster within cows. Only those variables statistically significant at $P \leq 0.15$ were kept and used in the following model.

Because of the hierarchical structure of the data, multilevel models were used to evaluate associations between cow milk ODRs and factors such as daily milk production, days in milk, seasonality, parity, province, and those significant herd management factors from the stepwise regression model. A multilevel model that was an extension of the previously described null model was used to account for the clustering of observations within the database (herd, cows and repeated test measures per cow). Prior to fitting the model, ODR values were log transformed. An evaluation of the residuals and influential observations was performed to check for the assumptions of the model. The model assumed a compound symmetry correlation structure for the repeated measures, and this was checked by evaluating the correlations among level one residuals across sampling times.

6.3.4.2.2 Multilevel model – effect of treatment on ODR

For the following model and the model under the sub-heading "*multilevel model – effect of ODR on milk production*", only cows from PEI and Quebec involved in the clinical trial of eprinomectin (2) were included in the analyses and the ODR values were kept

untransformed.

The same approach as described above was used to fit this model, using only observations recorded from calving until the end of the study, and only derived from herds on the clinical trial (i.e. treated with eprinomectin or placebo at calving). An interaction term between treatment and days in milk was used to evaluate the effect of treatment on cow milk ODR over time.

6.3.4.2.3 Multilevel model – effect of ODR on milk production

In this model, test-day milk production (in kgs.) was the dependent variable. ODR values derived from milk samples collected within a window of 120 days before calving up until the day of calving were selected for use in this model. If an animal had more than one ODR measurement in this time period, the average value was used for the calculations. These pre-calving (late lactation) ODR values were then categorized as high if the average optical density was greater than, or equal to 0.5 and low if it was below 0.5.

A random effects regression model of the overall effect of the pre-calving ODR and anthelmintic treatment on individual test day milk production for the first six tests after calving/ treatment was built using the same structure and procedure (SAS, Proc Mixed) (21) as previously reported (2). The data used were a subset of those from the full analysis of the clinical trial results. Briefly, the cow was identified as a clustering variable and a first order auto-regressive (AR1) correlation structure for the within-cow correlation was used. Herd was included as a random effect and lactation group (first, second and third or greater), calving season (fall 1999, winter 2000, spring 2000 and summer 2000) and test month (from

September 1999 to October 2000) and days in milk were included in the model as fixed effects. The effect of days in milk on test day yield was included using the Wilmink's function (days in milk + (days in milk)$^{-0.05}$) (22). All these variables were forced into the model because they were significant for the full dataset model. Before the interaction between the pre-calving ODR and treatment were evaluated, a model with only the main effects was fit to estimate if the overall effect of treatment for the subset of data was similar to that obtained from the full dataset.

6.4 Results

6.4.1 Descriptive statistics and unconditional associations

6.4.1.1 Individual cow samples

The overall mean, standard deviation and range of serum, individual cow milk and bulk tank milk samples tested in this study are presented in Table 6.1. The four quartile-based categories of bulk tank milk ODR were: 0-0.3; 0.3-0.5; 0.5-0.7 and 0.7-1.0. The distribution of individual cow milk samples within each bulk tank category is presented in Figure 6.1. There were groups of cows in the lowest bulk tank category (0.00 – 0.30) that had high ODR values, and on the opposite side there were cows with low ODR values in the highest bulk tank category (0.71 – 1.00). Figure 6.2 and Figure 6.3 present the lowess-smoothed estimates as well as the individual data points of the variation of cow milk ODRs by stage of lactation for all untreated cows (Figure 6.2) and by treatment group (Figure 6.3).

There was a quadratic effect of days in milk on untreated cow ODRs with the lowest values between 100 and 200 days in lactation (Figure 6.2). Comparing treated and untreated

cows, the ODRs declined until up approximately 60 days after calving for both groups, but the decline was more pronounced in the treated group (Figure 6.3). ODRs then increased up until day 200, with the data becoming sparse after that.

Figure 6.4 and Figure 6.5 present the lowess-smoothed estimates of the cow milk ODR variation by month for each province and lactation group, respectively. PEI and Quebec tended to have higher ODR values than the other provinces but there were limited data for Saskatchewan and Ontario. The seasonal patterns were not consistent across the provinces. All lactation groups tended to have higher ODR values by summer 2000 (Figure 6.5). A small peak in ODR was observed in all 3 age groups in February 2000 with the effect being most noticeable in the oldest cows.

Pearson correlation coefficients (r) and their 95% confidence interval (CI) between cow milk ODR values and square root FEC at different levels of aggregation are shown in Table 6.2. When both parameters were averaged for a herd over the full year, the correlation was moderately high ($r = 0.73$). The correlation coefficient between individual cow milk and cow serum sample ODR values was 0.53 (95 % CI=[0.46, 0.58]). The correlation coefficients by stage of lactation were: 0.34 ($n = 38$), 0.65 ($n = 133$) and 0.54 ($n = 104$) for the first 30 days, between 30 and 150 days and between 150 and 270 days, respectively.

When no fixed effects were included in the multilevel model of individual cow ODR values (null model), the proportions of the cow milk ODR variance explained by herd, cow and test day were 0.24, 0.21 and 0.55, respectively. For squared root FEC, the variance explained by the same factors were 0.10, 0.42 and 0.48, respectively.

6.4.1.2 Bulk tank milk samples

The distribution of bulk tank milk ELISA ODR values between herds in each province for the whole study period is presented in Figure 6.6. Considerable variation, not only between herds and provinces, but also within a herd (between test days) was evident. The proportions of the bulk tank ODR variance explained by herd and test day obtained from the null multilevel model were 0.63 and 0.37, respectively. The Pearson correlation coefficients between bulk tank milk ODR and cow milk ODR values and between bulk tank ODR values and squared root FEC are shown in Table 6.3. The bulk tank - cow milk coefficients were higher than the bulk tank-FEC coefficients for both average test day and average annual correlations. However, there was only a small difference in those coefficients within each comparison group.

6.4.2 Multivariable methods

6.4.2.1 Multilevel model – factors affecting ODR

The herd management variables that were significant from the stepwise backward elimination GEE model and initially included in the random intercept model of log ODR were: heifers on pasture, lactating cow on pasture, spread of manure on pasture, continuous grazing of dry cows, if pasture was cut for hay, if pasture was clipped and if pasture was dragged (model details not shown). However, none of these variables were significant ($P > 0.05$) in the random intercept model and were consequently removed.

The final variables included in the random intercept model and their coefficients, standard errors and p-values are shown in Table 6.4. Lower daily milk yields were associated

with higher ODR values, while second and third or older parity cows had higher ODR values compared with first parity cows. ODR values were higher in the winter, spring and summer than during the fall. Overall, province was statistically significant. As was previously observed in Figure 6.2, days in milk had a significant quadratic effect on ODR values. The analysis of the level-one residuals (test day) showed no pattern of correlations declining over time, which supported the assumption that a constant correlation among observations could be used. The residuals were approximately normally distributed and no outlying observations or influential values were observed.

6.4.2.2 Multilevel model – effect of treatment on ODR

There was a small and marginally significant effect of treatment on ODR values ($\beta = -0.06$, $P = 0.09$). The cows treated with eprinomectin group had lower ODR than the placebo cows. However the interaction between treatment and time, evaluated in this model, was not significant ($P > 0.1$) (model details not shown).

6.4.2.3 Multilevel model – effect of ODR on milk production response to treatment

One hundred and twenty three cows (123) had pre-calving ODR values recorded and were included in the model. Ignoring the pre-calving ODR, the cows treated with eprinomectin produced 1.26 kg/day more milk over the first 6 months of lactation compared to the placebo cows. This estimate was close to the overall estimate of 0.94 kg/day derived from the full dataset (Nødtvedt, et al., 2002b). The coefficients, standard errors and p-values of the model including the interaction between treatment and the pre-calving ODR are

presented in Table 6.5. When the interaction of treatment and pre-calving ODR was evaluated, the effect of treatment depended on ODR group (P=0.07). High ODR (n = 59) cows had an increase of 2.87 kg/day (95% confidence interval =[0.49, 5.26]) following treatment while there was no apparent effect in low ODR (n = 64) cows (0.11 kg/day, 95% confidence interval =[-2.30, 2.08]).

6.5 Discussion

The mean ODR serum values from our study were similar but generally slightly lower than those reported by Borgsteede et al. (9) and Agneessens et al. (8). The correlations between serum and milk ODR values (r = 0.53) was similar to the values of 0.45 and 0.47 reported previously (11; 13). The low to moderate correlation found during different intervals of the lactation period may be a consequence of milk IgG in ruminants being primarily produced locally in the mammary gland (23) as opposed to being derived from serum antibodies. It also appeared that milk ODR values were inversely proportional to the level of milk production and this dilution effect may reduce the correlation between serum and milk levels. The lowest correlation was observed early in lactation, a period where the active transport of IgG into the mammary gland might still occur (23).

6.5.1 Seasonal pattern

When provincial ODR values were plotted against month (Figure 6.4), a seasonal pattern could be observed but it was not as distinct as those observed in the two previously cited studies (9). The difference observed among provinces could be explained by different

weather conditions or management practices (especially level of exposure to pasture) and the pattern was similar to those seen in FECs collected at the same time (24). Although none of the management practices were significantly associated with ODR, the study had little power to detect such effects because it was based on only 38 herds. Three studies carried out in eastern Canada have reported a significant association between level of pasture exposure, pasture management and anthelmintic treatment strategies with ELISA test results (12; 20; 25). From the multilevel model (Table 6.4) it appeared that there were significant differences among provinces. A more obvious seasonal pattern was observed when parity group was graphed against time (Figure 6.5). The seasonal pattern observed follows the expected larval intake, with ODR values decreasing during the housing period and increasing when the cows go out to pasture. There was some evidence that ODR may have started to rise before cows were put on pasture. A similar pattern was observed by Nodtvedt et al. (24) in FEC. The peak in ODRs observed during February may have been a response to emerging hypobiotic larvae. These findings have been also observed in an experimental infection study, where non-treated cows had a progressive increase in antibody levels by the end of the winter compared with treated and non-artificially infected cows (26). Three studies of worm burdens in adult cows carried out in Europe have shown that the resumption of the arrested larvae occurs during this period of the year (8; 9; 27). Similarly, Armour and Duncan (28) pointed out that in Canada and northern United States, the proportion of arrested larvae is higher during the fall and early winter with a resumption of the development by the following spring.

6.5.2 ODR related to age

The second and third or greater lactation cows had significantly higher ODR values compared with first lactation animals. A similar age effect has been previously reported (11; 13). Two possible explanations have been discussed by these authors. It was suggested that older animals have a higher general level of immunity, or that older animals may have a greater capability to transmit worm antibodies from serum to milk due to some difference in udder physiology.

6.5.3 FEC – ODR relationship

The Pearson correlation coefficient between individual cow ODR values and square root FEC were lower than those previously reported (11). However, similar values were observed when average values either at test day, or at herd level were computed. Gasbarre et al. (10), in a study of influence of host genetics factors on antibody levels in first year calves reported no correlation between serum OD values and log transformed FEC after controlling by sex, age of the calf, age of the dam and sire. The authors concluded that FEC and antibody response are under genetic control, but are not influenced by the same genes. Another possible explanation is that in adult cows, the FEC technique is an unreliable indicator of subclinical parasite burdens.

While most of the variation in cow milk ODR and square root FEC was observed between test days, the former had a higher proportion of variance explained by herd. Similar results were observed by Dohoo et al. (11) who reported a higher intra-herd correlation coefficient from OD values (indirect crude antigen *Cooperia* spp. ELISA) than the one

obtained from square root FEC.

6.5.4 Relationship between ODR and milk production

The influence of milk yield on ELISA test results revealed the same relationship found in a previous study (13), suggesting that milk production is negatively associated with milk optical density values. Days in milk had a quadratic effect on cow milk ODR. Both a linear relationship and quadratic relationship have been observed in previous studies (11; 13).

6.5.5 Effect of ODR on milk production response to treatment

For the production model, the interaction between the treatment and the cow pre-calving ODR value was marginally significant ($P = 0.07$), but given the sample size available for this analysis a P-value of 0.10 was accepted as the cut-off value. Even though different study design and statistical methodologies were used in this study compared with those studies described by Ploeger et al. (14; 15) and Koosterman et al. (16) similar results were found. In 1989, Ploeger et al. (14) described a statistically significant positive correlation between individual serum antibody levels and response in milk production. However in Ploeger et al. (15) the same trend was present but the association was not statistically significant. The main difference pointed out by the authors was that in the second study the antibody levels were significantly lower compared with the first study. On the other hand, in the Kloosterman's study (16), herds were selected with high and low bulk-tank antibody levels and milk production response to treatment was measured. While a higher response was observed in the high antibody level herds, the difference between the two herd groups was not

statistically significant. Overall, the current results agree with those from these studies. There was a positive response in high antibody level cows and no effect in the low antibody level cows. One other possible explanation for the lack of significance in previous studies is the choice of statistical analyses used. It has been shown that for diseases that have an impact on milk production in a small part of the lactation period, a test-day model (as was used in this study), is better able to measure the effect of the disease on daily milk production (29) than a model based on lactation total milk production. The same principle would apply if the impact of parasitism varied much across the lactation.

6.5.6 Bulk Tank ODR

The high variation in bulk tank ODR values between herds presented in Figure 6.6 agrees with the observation that 63% of the total variance was explained by herd in the multilevel null model. This also was discussed by Kloosterman et al. (13), who suggested that the bulk tank OD values were better able to discriminate antibodies levels between herds compared with either individual serum or milk values.

Considering the bulk-tank ODR value as an overall herd estimate of gastrointestinal parasite burdens, it can be seen from Figure 6.1 that there were some cows with high individual test values in all bulk-tank groups. Results from a slaughter house study in which worm counts were high in 15% of the animals examined (8) supports this observation of large between cow variability in parasite burdens.

The correlations between bulk-tank milk ODR and average cow milk ODR ($r = 0.46 - 0.6$) also were similar to the values of 0.49 and 0.64 reported in the two previously cited

studies. The relatively low correlation between the bulk-tank and herd average ODR values suggests that any monitoring program may want to include both bulk-tank and individual cow samples

6.6 Conclusions

The ODR appears useful as a measure of gastrointestinal parasite burdens in dairy cows. The ODR correlates moderately well with FEC if both are summarized over a herd and over a year. The bulk tank ODR was able to discriminate antibody levels between herds, and this is presumably related to different infection levels. However, within herds with different bulk-tank ODR levels there was considerable variation in individual cow ODR values. ODR values increased from late spring through the summer, and were higher in older cows than younger ones. There was a quadratic relationship between ODR and "days in milk". ODR values were negatively associated with level of milk production. The effect of treatment on ODR requires further investigation. Finally, the antibody levels in late lactation animals detected by this ELISA appeared to predict the response in milk production to anthelmintic treatment. However, because of the small herd sample size available in this study, more research has to be done in order to better understand this relationship.

6.7 References

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Table 6.1. Mean, standard deviation and range of all ELISA test values (ODR) from serum, individual cow milk and bulk tank milk samples from 38 dairy herds from 4 provinces of Canada (September 1999 to October 2000).

	Serum	Individual cow milk	Bulk tank milk
N	718	2781	378
Mean	0.589	0.297	0.363
Standard deviation	0.336	0.251	0.198
Range	-0.033 – 1.764	-0.051 – 1.558	0.033 – 1.030

Table 6.2. Pearson correlation coefficients (r) and 95% confidence interval (95% C.I.) between cow milk ODR values and squared root FEC from "untreated" cows in 38 herds from 4 provinces of Canada (September 1999 to October 2000).

Level	n	r	95% C.I.
Cow / test day	2108	0.16	[0.12, 0.20]
Average cow / year	418	0.17	[0.08, 0.26]
Average herd / test day	328	0.39	[0.29, 0.48]
Average herd / year	38	0.73	[0.53, 0.85]

Table 6.3. Pearson correlation coefficients (r) and 95% confidence interval (95% C.I.) between bulk tank milk ODR and "untreated" cow milk ODR values and bulk tank milk ODR values and square root transformed FEC (38 herds from 4 provinces of Canada).

	n	r	95% C.I.
Bulk Tank OD - Cow OD (herd / test day)	355	0.46	[0.38, 0.54]
Bulk Tank OD - Cow OD (herd / year)	38	0.56	[0.29, 0.75]
Bulk Tank OD – epg5 (herd / test day)	286	0.31	[0.20, 0.41]
Bulk Tank OD – epg5 (herd / year)	38	0.34	[0.02, 0.60]

Table 6.4. Multilevel linear model of the cow milk log-transformed ODR from untreated cows in four provinces of Canada from September 1999 to October 2000 (37 dairy herds, 439 cows and 1663 test measurements).

Fixed effects			
Variable	β	Standard error	P
Intercept	-0.869	0.145	0.000
Province			0.004
PEI	Baseline		
Quebec	-0.157	0.143	
Ontario	-0.673	0.192	
Saskatchewan	-0.381	0.217	
Lactation group			0.000
First	Baseline		
Second	0.121	0.055	
Third or greater	0.391	0.061	
Season			0.000
Fall 1999	Baseline		
Winter 2000	0.169	0.037	
Spring 2000	0.369	0.042	
Summer 2000	0.310	0.048	
Daily milk production	-0.019	0.002	0.000
Days in milk	-0.003	0.000	0.000

Fixed effects			
Variable	β	Standard error	P
Days in milk squared	0.00001	0.000	0.000

Random effects	Variance	Standard error
Herd	0.115	0.033
Cow	0.171	0.020
Test	0.276	0.011

Table 6.5. Mixed model of association between treatment and pre-calving ODR on test-day milk production using an AR1 correlation pattern adjusted for parity group, calving season, stage of lactation and month of the test [a]. Data were a subset (27 dairy herds, 123 cows and 676 test-day measurements) from a larger clinical trial of eprinomectin in Canada.

Variable	β	Standard error	P
Intercept	169.09	13.19	0.000
Treatment with eprinomectin	-0.11	1.11	0.919
Pre-calving High ODR	-1.83	1.35	0.177
Eprinomectin – High ODR	2.99	1.66	0.073

[a] Coefficients from adjustment variables not reported for sake of clarity.

Figure 6.1. Distribution of individual cow milk ODR values from untreated cows by bulk tank milk ODR category. Individual cow ODR values were assigned to 1 of the 4 categories based upon the corresponding bulk tank ODR value for the herd in the month the sample was collected. The distribution shows the range of values in individual cow ODRs to be expected when the bulk tank ODR is very low, low, moderate or high.

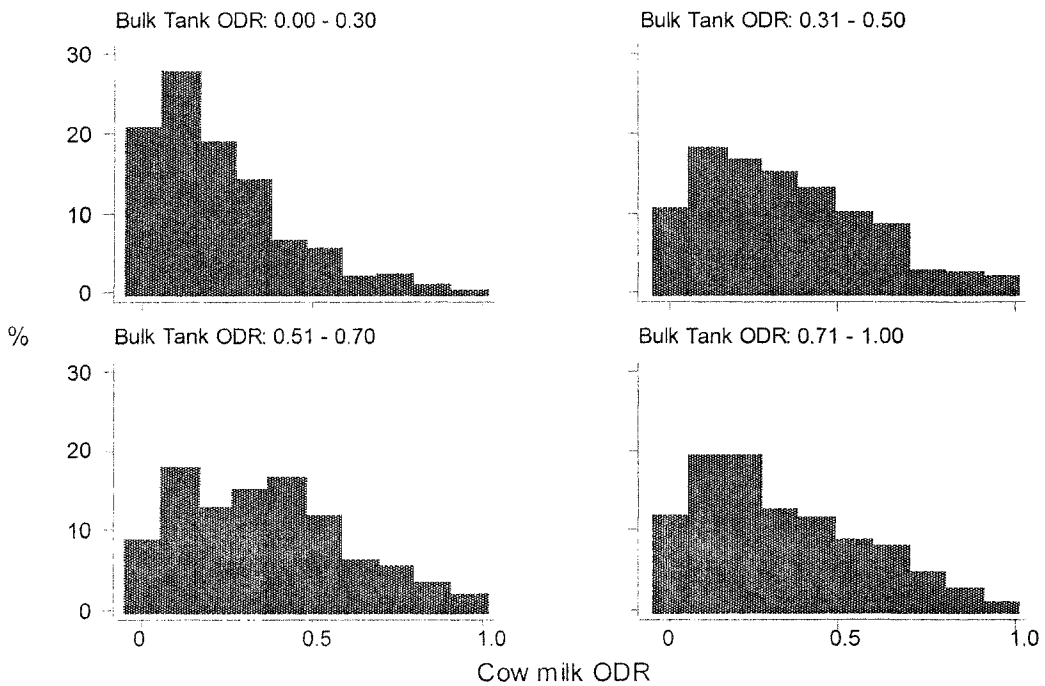


Figure 6.2. Graph of the cow optical density ratios by days in milk and their lowess smooth estimate for untreated cows (37 dairy herds and 1675 observations).

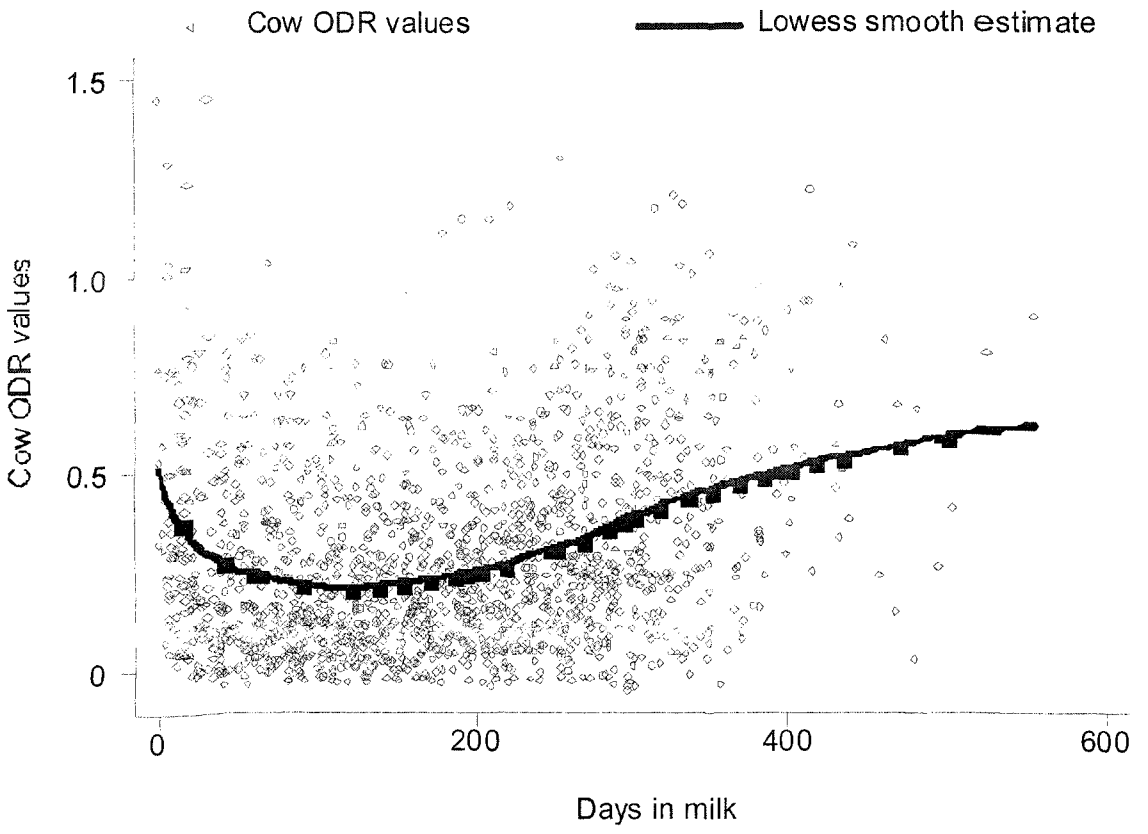


Figure 6.3. Graph of the cow optical density ratios (ODR) for all cows by stage of lactation (days related to calving) and their lowess smooth estimate by treatment group (28 dairy herds and 1252 observations).

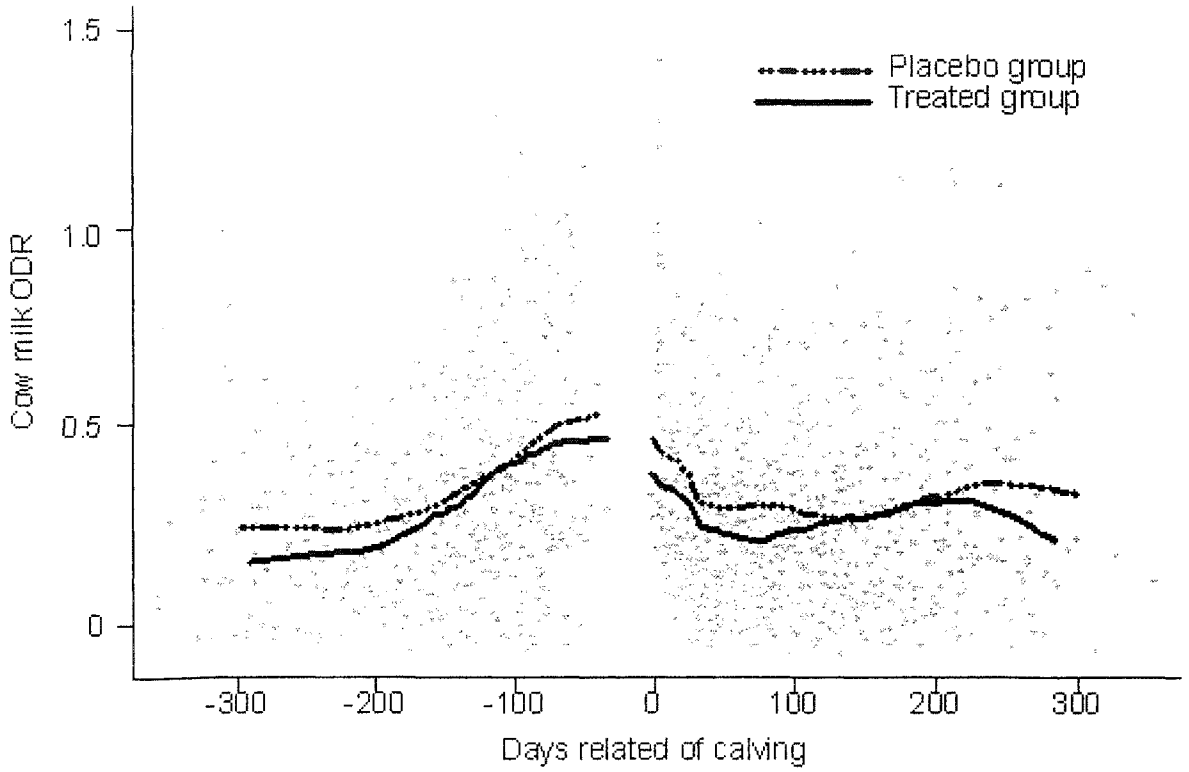


Figure 6.4. Graph of the lowess smooth of cow milk optical density ratios by test month and by province. Data from "untreated" cows in 37 Canadian dairy herds (September 1999 - October 2000).

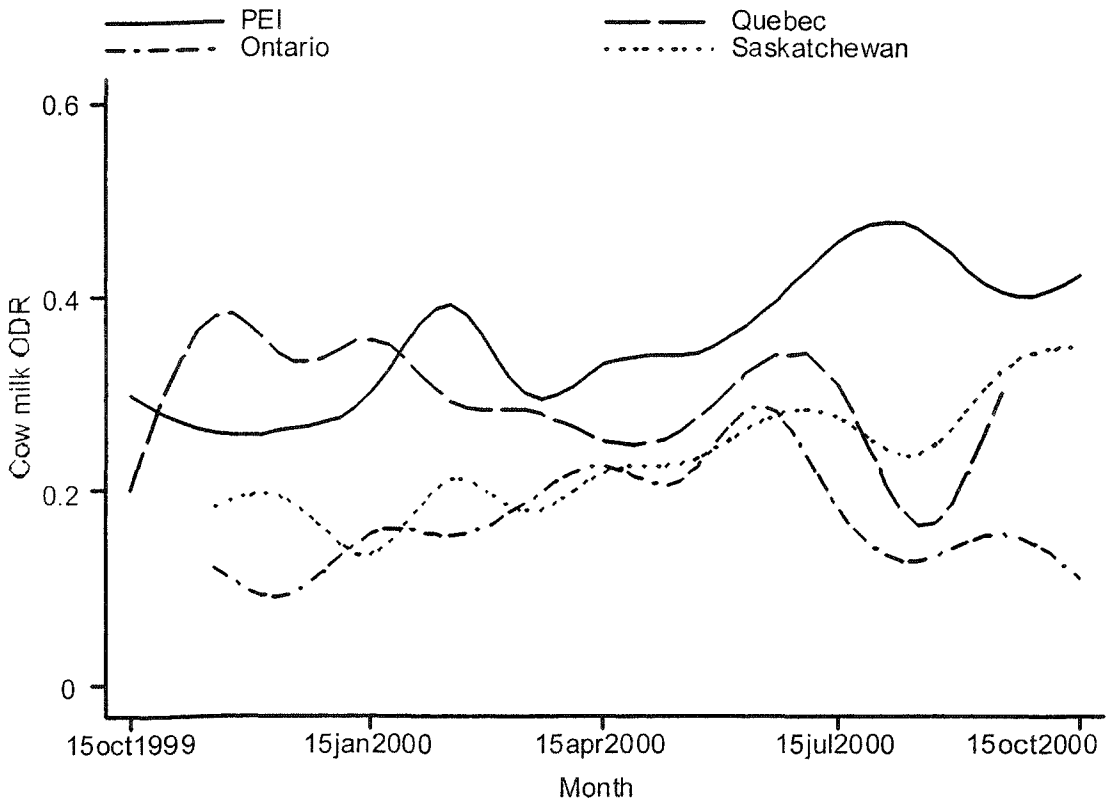


Figure 6.5. Graph of the lowess smooth cow milk optical density ratios by test month and by lactation group. Data from "untreated" cows in 37 Canadian dairy herds (September 1999 - September 2000).

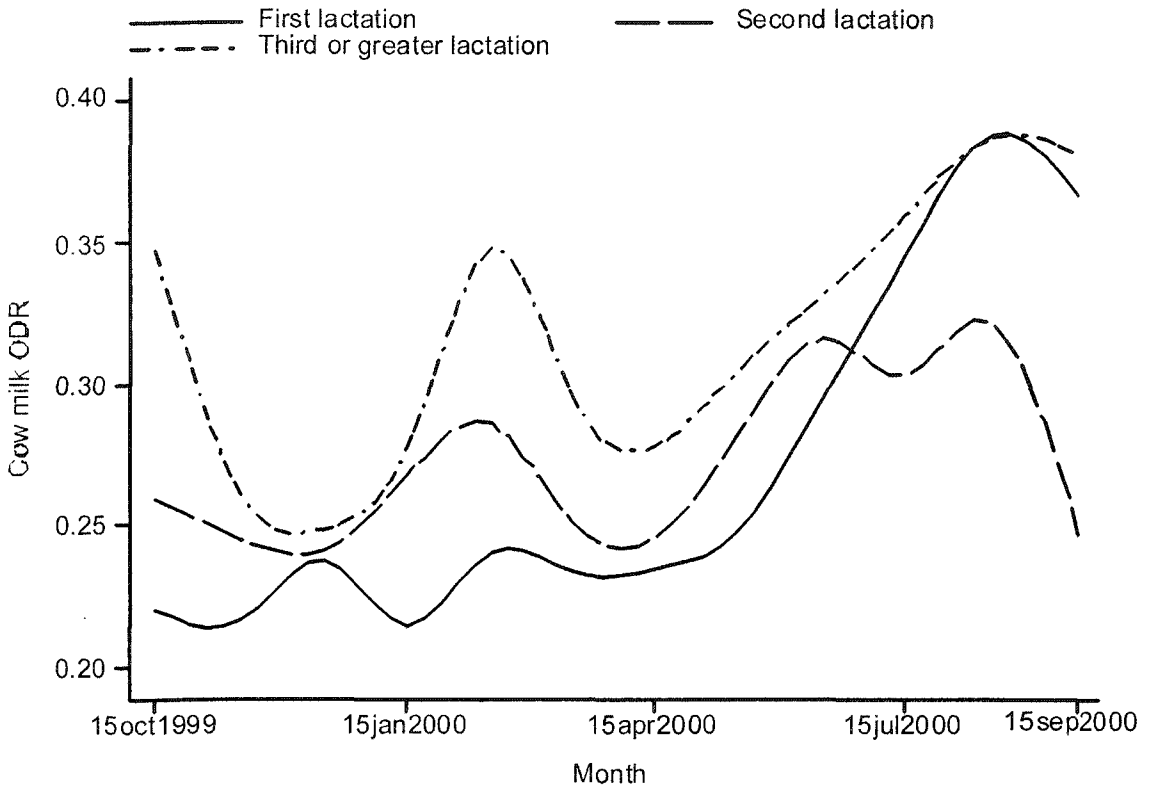
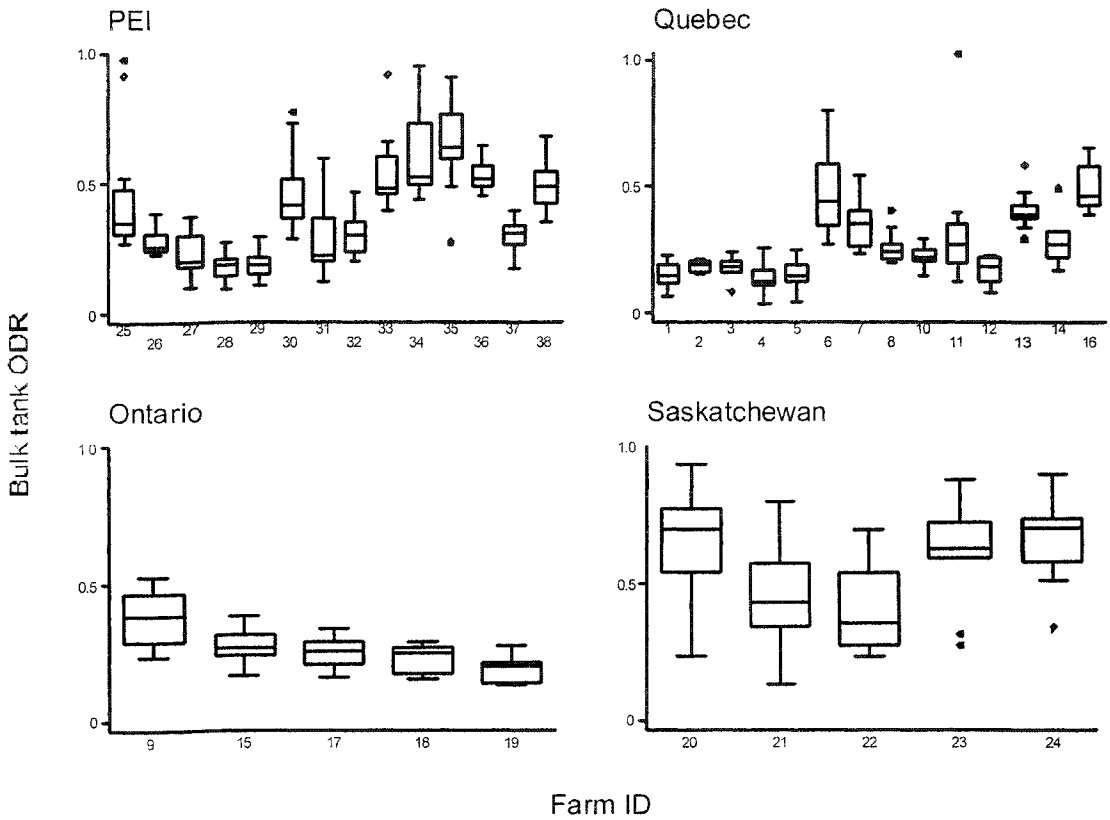


Figure 6.6. Box-and-whisker plots of bulk tank milk ELISA ODR values from September 1999 to October 2000 stratified by herd and province. Data from "untreated" cows in 38 Canadian dairy herds.



7. The effect of eprinomectin treatment at calving on reproduction parameters in adult dairy cows in Canada

7.1 Abstract

A clinical trial was carried out in two provinces of Canada to investigate the effect of treatment with eprinomectin at calving on production parameters in adult dairy cattle in 1999-2000. One of the objectives of this study was to evaluate the impact of treatment on reproductive performance as measured by: calving- to-conception interval, calving-to-first service interval and number of services per conception. The ability of an indirect ELISA using a crude adult *Ostertagia ostertagi* antigen to predict response to treatment also was evaluated. All lactating cows in 20 dairy herds were randomly allocated to receive either eprinomectin pour-on or placebo at calving. Information on reproductive parameters was obtained from computerized cow records. Survival models were used to evaluate the effect of treatment on the two intervals and a Poisson model was used to evaluate the number of services to conception. A total of 549 cows were included in these analyses. A marginally significant treatment effect on calving-to-conception interval was observed (hazard ratio = 1.24, P = 0.06) but not on calving-to-first service interval. A significant reduction in the number of breedings to conception for treated animals was also observed with a longer effect in cows with short interval to first service. Milk samples from a subset of 109 late lactation cows were tested for antibodies against *Ostertagia ostertagi*. The ELISA optical density (ODR) values obtained between 120 days before calving and drying off were categorized as high ODR (>=0.5) and low ODR (< 0.5). Among untreated animals, the hazard of conception was lower

(hazard ratio=0.38, 95% CI=[0.19,0.75]) for high ODR cows compared to low ODR cows suggesting that higher parasite burdens had an adverse effect on reproductive performance. Treated high ODR cows had a hazard of conception equivalent to the hazard for all cows in the low ODR group indicating that treatment prevented the negative effect associated with these higher parasite burdens.

7.2 Introduction

One of the main challenges when deciding on a parasite-treatment strategy in a herd is to determine whether application of an anthelmintic will produce an economic gain that will pay for the treatment (1). Gross et al. (2) reviewed more than 80 clinical trials using different anthelmintics, and concluded that there was an overall gain in milk production of 0.63 kg/d following anti-parasite treatment. Newer anthelmintics of the macrocyclic lactone family - such as eprinomectin - have no milk withdrawal following use. This leads to the possibility of treating dairy cattle at any stage of lactation. Evaluation of the milk production data for the clinical trial described in this paper showed an average increase in production of 0.94 kg./day for the first 6 months of lactation for the treated cows (3) suggesting that in eastern Canada internal parasites play an important role in dairy cattle productivity. Three studies have shown *O.ostertagi* and *Cooperia* spp. as the most prevalent bovine gastrointestinal nematodes in Canada (4-6) The pattern of pasture larval contamination in eastern Canada is similar to that observed in other regions in North America with increased contamination starting when the animals are turned out on pasture, and a peak has been observed by the end of the grazing season (5).

Little work has been published on the effect of anthelmintic treatment on reproductive performance in dairy cattle. A clinical trial in dairy cows in Australia detected an increase in milk production of 74 l over the first 100 days in milk (7). Those researchers used a single dose of ivermectin injectable during the dry period, and also found that treated animals had on average a 4.8-days-shorter calving-to-conception interval than the controls. Climatic conditions and dairy-herd management are expected to differ greatly between Australia and

North America, and the herds in the Australian study had seasonal calving. Also, the calving-to-conception interval in the Australian study was analysed using ANOVA rather than a survival model (which is better able to handle data from animals that did not conceive or were censored). A New Zealand study (8) reported improved reproductive performance in first lactation animals treated with eprinomectin at calving. However, when all cows were included in the analysis, the effect was no longer statistically significant. Hawkins (9) pointed out that a beneficial anthelmintic treatment effect on reproductive performance has been seen (inconsistently) in beef cattle. A study performed in Georgia, USA found higher number of pregnant cows (98% vs. 75%, $P = 0.12$) and calved cows (90% vs. 68%, $P = 0.03$) for beef cattle treated with fenbendazole (10). Another study of fenbendazole (in dual-purpose cattle in Gambia) showed an improvement in annual calving risk (52% vs 44%, $P < 0.001$) in the treated group of animals (11).

The standard diagnostic test for gastrointestinal parasitism is the fecal egg count, but in adult animals it shows poor correlation with either worm burden present or production response to anthelmintic treatment. A microtitre ELISA test has shown promise as a diagnostic tool for herd level monitoring of gastrointestinal parasite levels in dairy cows (12). Previous work done by Ploeger et al. (13) and Kloosterman et al. (14) have used ELISA results to predict milk production response following anthelmintic treatment.

The time from calving to first service or conception are commonly used measures of reproductive performance (15) and time-to-event data are commonly analysed using survival analysis (particularly, the Cox proportional-hazards model). The number of times an animal is bred before conception is an alternative parameter that can be used to compare reproductive

performance between two groups. For count data, Poisson regression is commonly used but it assumes that the mean and the variance for the outcome variable are similar and this assumption must be evaluated (16). Because animals in the same herd are exposed to similar management and breeding practices, independence of observations cannot be assumed. This clustering of cows at the herd level can be dealt with either by including a random error term for herd in the Cox proportional hazards and Poisson regression models, or by adjusting the standard errors of the coefficients using a robust variance estimate algorithm.

The objective of this study was to use multivariable models (the Cox proportional hazards model and Poisson regression model) to investigate whether use of eprinomectin pour-on solution at calving would affect calving- to-conception interval, calving-to-first service interval or number of services per conception in dairy cows that had some level of pasture exposure. A secondary objective was to test whether a crude *Ostertatgia ostertagi* antigen ELISA test used in milk samples taken 120 to 60 days pre-calving could predict which cows would respond positively to eprinomectin treatment in terms of reproductive performance.

7.3 Materials and methods

7.3.1 Study animals

Holstein cows from 28 herds in two different regions of Canada were included in a clinical trial of eprinomectin treatment at calving (3). Of these herds, 20 (6 in Prince Edward Island (PEI) and 14 in Quebec) kept computerized records of cow reproductive performance, and were included in this study. The selected herds were a convenience sample based on

expected compliance from the producer and proximity to the Farm Service Units at the Veterinary Colleges of the University of Prince Edward Island and the University of Montreal, respectively. The herd-selection criteria for the trial included farms where the adult cattle met some of their nutritional requirements from pasture (or they had been exposed to a small grassed paddock for exercise), participation in the Canadian Dairy Herd Monitoring System (CDHMS) for recording milk production data, and a herd history of no use of broad-spectrum endectocides in lactating animals in the 6 months before the onset of the study. All cows due to calve within 12 months of the start of the trial in PEI and within 6 months in Quebec, were eligible for inclusion in the study. All the herds included had cows calving throughout the year.

7.3.2 Treatment protocol

The study was a double-blind randomized clinical trial, with anthelmintic and placebo being delivered in indistinguishable bottles labelled with a unique number and letter. As they calved, cows were randomly allocated (using computer generated random numbers) to treatment with eprinomectin pour-on solution or placebo within blocks of 10 cows, to ensure that both treatment groups would have a balanced distribution of animals calving through the duration of the study. Application was done according to label use for IVOMEC® EPRINEX®. The weight of the animal was estimated using a weight tape provided by the researchers, and the pour-on solution applied at 1 ml/10 kg (500 μg eprinomectin per kg body weight) by the producer on (or close to) the day of calving. Treatment date and dose applied were recorded by the person performing the treatment.

7.3.3 Reproduction records

Information on calving date and lactation number was obtained from the CDHMS database in Montreal. First-service date, number of breedings and conception date were obtained from computerized records kept at the Farm Service Ambulatory Units at the two study sites. The number of cows from any individual herd that were included in the analyses was limited to 80 cows to avoid a few (n=3) large herds having excessive influence of the parameters estimated in the regression models.

7.3.4 ELISA optical density

A subset of eight cows (4 first lactation and 4 second or greater lactation) in each herd was randomly (computer generated random numbers) selected from a farm-list of first lactation and second or greater lactation cows at the time of the first farm visit. Selected cows were identified using a plastic leg band. Milk samples from the monitored cows were collected monthly or bimonthly from September 1999 to October 2000. Only samples obtained within a window of 120 days before calving up until the dry-off day (approximately 60 days before calving) were used for analyses in this study. All samples were shipped to PEI and stored at -20 °C until processing. An indirect microtitre ELISA (using crude adult *Ostertagia ostertagi* antigen) as first described by Keus et al. (1981) was used to determine the level of antibodies towards the parasite in individual-cow milk samples(17). Optical-density values were adjusted based on the reading from the positive and negative control samples on each plate and results were expressed as an optical density ratio (ODR) (18) .

If an animal had more than one ODR measurement in the time period before calving (-120 days up to calving), the average value was used for the calculations. The results were classified as "high" if the average ODR was greater than or equal to the mean ODR (i.e. ≥0.5) and "low " if it was below 0.5.

7.3.5 Data analysis

Although the degree of clustering of reproduction performance within dairy herds is relatively small (19), independence of observations was not assumed and a robust estimate of variance to adjust for clustering (20) was applied in all the statistical models. This produces valid standard errors even if the assumption of independence of observations within groups is not valid. Independent variables tested in all models were treatment with eprinomectin, daily milk production over the first 3 months of lactation, province, calving season, and parity. The study period was split into four calving seasons: Fall (October to December 1999), Winter (January to March 2000), Spring (April to June 2000) and Summer (July to September 2000). Parity was classified as 1^{st}, 2^{nd} or 3^{rd} lactation and older. The significance of categorical variables and two-way interactions was tested using a likelihood-ratio test and variables were kept in the model if $P \leq 0.1$ with parameters having P-values between 0.05 and 0.10 being reported as marginally significant. Treatment with eprinomectin was forced to remain in all models. Cows that were not bred at all during the study period were excluded from all analyses. Four separate Cox models were fitted. Follow-up time for all cows was set to 180 days, after which animals that had not yet conceived were considered censored. Cows with calving-to-conception intervals < 40 days and calving-to-first service interval < 40 days were

excluded from the analysis because they were assumed to be recording errors. The four models were: overall effect of treatment on time from calving-to-first service, overall effect of treatment on time from calving-to-conception, effect of treatment on calving-to-conception stratified by late lactation ODR, effect of treatment on calving-to-conception comparing placebo-treated, high ODR cows to all others. Testing of the proportional-hazards assumption and an evaluation of residuals were done for each model. The goodness-of-fit of the model was evaluated by incorporating nine design variables (based on the ranked values of the deciles of the estimated risk) into the fitted proportional hazards model. A partial likelihood ratio test was then applied to compare the two models with a non-significant result suggesting that the model fit the data (21). Each analysis was complemented by plotting the quantiles of the cumulative observed versus the cumulative estimated expected number of events (Arjas plot). If the model was correct, the points approximately followed a 45° degree line beginning at the origin. A Poisson model was applied to compare the number of services required for conception in each treatment group. Only cows that conceived were considered in this analysis. In addition to the independent variables mentioned above, days to first service (separate linear effects from days 40 to 90 and 91 to 180) and an interaction effect with treatment were also included in this model. The goodness-of-fit of this model was assessed by comparing the sum of the squared deviance residuals to its expected chi-squared value.

All analyses were carried out using the software package Stata version 7 (22).

7.4 Results

Data from a total of 549 animals were analyzed (307 in PEI and 242 in Quebec), out of which 271 were treated with eprinomectin pour-on and 278 were controls. Those cows calved between September of 1999 and September of 2000 and between November 1999 and June 2000 for PEI and Quebec, respectively. The distribution of calving cows by calving season and province is shown in Figure 7.1 (80 % of the cows calved between November 1999 and May 2000). The mean number of animals treated per farm was 44 (range was 5 to 80). At the end of the follow-up time of 180 days, 391 (71.2%) of the cows were pregnant. The average time at risk until conception for the treated animals was 117 days compared to 126 days for the controls. Average time-at-risk until first service was 81days for both groups. The mean number of services was 1.68 (n = 180) and 1.93 (n = 168) for the eprinomectin-treated and placebo-treated groups, respectively

Late lactation milk samples were obtained from 56 eprinomectin-treated and 53 placebo-treated cows. The average time at risk until conception for this subgroup of animals was 117 days for the treated animals and 131 for the controls. The ODRs ranged from 0 to 1.5 (mean = 0.49, median = 0.47; Figure 7.2). The number of cows in the "high" and "low" ODR category was 49 and 60, respectively.

7.4.1 Survival analyses

The survival curves (calving-to-conception interval) for treated and control animals are depicted in Figure 7.3. The treated animals seemed to conceive slightly earlier. Table 7.1 summarizes the coefficients, robust standard errors, *P*-values and hazard ratios (HR) from the

Cox model based on data from all 549 cows. Eprinomectin-treated animals had a marginally significantly higher hazard of conception (HR = 1.23, $P = 0.06$). Lactation category was overall (i.e. considering all categories together) significant ($P = 0.02$ from the likelihood ratio test). Calving-to-conception intervals were shorter in second lactation animals compared to other age groups. Milk production, calving season, province and two-way interactions were not significant. Both the likelihood ratio test for the goodness-of-fit of the data ($P = 0.66$) and the Arjas plot suggested that the model fits the data reasonable well. For the calving-to-first service interval model, treatment with eprinomectin was not significant ($P = 0.12$, data not shown).

The survival curves for the 109 treated and control animals by ODR group are presented in Figure 7.4. Overall, animals with an ELISA ODR greater than 0.5 that did not receive the anthelmintic treatment had the longest calving-to-conception intervals. Table 7.2 presents the coefficients for treatment, ODR group and the interaction between the two terms from a Cox proportional hazards model of the calving-to-conception interval in this subgroup of 109 animals. Milk production, province, parity and calving season were not significant. The main effect of ODR was significant ($P < 0.01$) as was the treatment-ODR interaction term ($P = 0.05$). The Arjas plot of the observed versus expected number of events suggested a good fit of the model. Table 7.3 shows the calculated HRs with 95% confidence intervals for combinations of treatment and ODR groups (compared to placebo-low ODR cows), based on the coefficients from the model in Table 7.2. For all the Cox proportional hazard models, the basic assumptions of proportional hazards and independence between outcome and censoring

were met; residual diagnostics and checks for influential data points did not reveal any major shortcomings.

7.4.2 Number of breedings

A kernel-smoothed mean plot of the log of the number of services per conception (by treatment group) versus days to first service is shown in Figure 7.5. The eprinomectin-treated cows tended to have a lower number of services than the placebo cows but the difference was only evident in cows first bred at < 90 days. Based on these findings two linear splines with a "knot point" at 90 days were created and included in the Poisson regression model, along with an interaction term between the first spline and treatment.

Treatment group and days to first service (40 - 90 days) as well as the interaction between these two variables were significantly associated with the number of services required per conception in the group of pregnant cows during the study period. Contrarily, parity, calving season, province, milk production and days to first service (91 - 180 days) were not significant. The effect of eprinomectin depended on the interval from calving to first service (Table 7.4). An eprinomectin-treated cow that was first bred at 72 days post calving required 11 % fewer services per conception than a placebo cow, whereas an eprinomectin-treated cow first bred at 50 days required 25 % fewer services per conception than a placebo cow. However, the beneficial effect of eprinomectin had disappeared if the cow received its first breeding after 90 days. The goodness-of-fit test was not significant, suggesting that the model fit the data reasonably well.

7.5 Discussion

Overall, a marginally statistically significant improvement in calving-to-conception interval was observed for the treated animals included in the model based on 549 cows. However, no treatment effect was found for calving-to-first service interval. Although, therapeutic concentrations of this drug have been observed for up to 14 days after treatment (23) the apparent beneficial effect was observed at breedings from 40 - 180 days after treatment. Two possible reasons may explain this prolonged treatment effect. Firstly, the elimination of parasites around calving might improve the energy balance during the postpartum period and consequently, improve the cow reproductive performance. Metabolic disorders during the peri-partum period have been associated with higher incidence of reproductive disorders (24-27). The other reason might be related to a low larvae exposure during the study period given that approximately 80 % of the animals calved during the non-grazing season.

Contrary to the results reported from New Zealand by McPherson et al. (8), the treatment effect in the present study appeared to be the same across all parity groups. The improvement in calving-to-conception interval and lack of effect on calving-to-first service agree with those findings reported from Australia by Walsh et al. (7). Although a seasonal pattern of the gastrointestinal parasites has been described for one of the regions where the current study was performed (5), the treatment effect did not appear to depend on calving season. However, the study would have had limited power to detect treatment by season interactions. The weather conditions and dairy management practices found in New Zealand and Australia differ from those found in the present study. Under those conditions, cows are

kept on pasture all year round and calved in a limited season. However, similar responses to anthelmintic treatment were observed, indicating that even a minimal exposure to parasites (i.e. shorter grazing season in Canada) is enough to impair the reproductive performance in lactating cows.

When untreated animals with high levels of antibodies against *O.ostertagi* (i.e. high ODR) were compared to the low untreated ODR group, the hazard of conception was lower (Table 7.3 and Figure 7.4) suggesting that higher parasite burdens did impair reproductive performance. However, high ODR animals that received the treatment had a hazard ratio that was comparable to the two low ODR groups. The hazard ratio comparing the two low ODR groups plus the eprinomectin-treated high ODR group, combined, to the placebo cows with high ODR, was 2.44 (P=0.02) (model details not presented). These two models suggest that a cow with either high ODR and receiving anthelmintic treatment or low ODR in late lactation was approximately 2.0 - 2.5 times more likely to conceive at any given time during the study period compared with non-treated high ODR cows.

Similar associations have been reported between ELISA values and milk production response to treatment (13; 14). Finally, a significant interaction between ODR groups and treatment response of milk production was also observed for the cows involved in the present clinical trial (Sanchez et al., unpublished observations). High late lactation ODR cows that were treated had a much higher milk production response compared to late lactation low ODR cows. While, the ability of the ELISA to discriminate between groups of animals that might benefit from anthelmintic treatment is promising it warrants further evaluation given the small sample size in this study.

An increase in number of services per conception has been reported in cows that had experienced reproductive disorders during the early postpartum period (28; 29). The more pronounced beneficial effect of eprinomectin observed in cow first bred early in lactation may be related to an improved energy metabolism and a reduction of incidence of early postpartum diseases (27). This beneficial effect appeared to have disappeared if first breeding was delayed until after 90 days.

7.6 Conclusion

Overall, treatment with eprinomectin pour-on at calving had an effect on both calving-to-conception interval and number of services per conception in a group of 549 adult dairy cows that had been exposed to pasture. Contrarily, no effect on calving-to first service was observed. Animals with high milk antibody levels (based on a crude antigen *O.ostertagi* ELISA) to gastrointestinal nematodes at the end of the previous lactation had a significantly reduced hazard of conceiving in the following lactation. This negative effect of parasites was eliminated by eprinomectin treatment in these cows.

7.7 References

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Table 7.1. Cox proportional-hazard model of calving-to-conception interval in 271 eprinomectin-treated and 278 control Holstein cows in a clinical trial of anthelmintic treatment at calving in Canada (1999 - 2000)[a].

Variable	Level	β	Robust S.E. [b]	*P*	HR	95 % C.I. HR
Eprinomectin	no	0.00	-	-	1.00	-
	yes	0.20	0.13	0.06	1.23	0.99, 1.52
Lactation [c]	1st	0.00	-	-	1.00	-
	2nd	0.41	0.20	0.00	1.51	1.16, 1.97
	3rd and older	0.17	0.19	0.29	1.18	0.86, 1.61

[a] milk production, calving season, province and all 2-way interactions were not significant and dropped from the model.

[b] standard errors adjusted for clustering (herd effect)

[c]overall significance of lactation categories based on likelihood ratio test was P=0.02

Table 7.2. Cox proportional-hazard model of calving-to-conception interval for 109 Holstein cows which had late lactation milk antibody (*O.ostertagi*) levels determined in a clinical trial of eprinomectin in Canada (1999 - 2000) [a].

Variable	Level	β	Robust S.E. [b]	*P*	HR	95% C.I. HR
Eprinomectin	no	0.00	-	-	1.00	-
	yes	-0.16	0.32	0.62	0.85	0.45, 1.59
ODR group [c]	low	0.00	-	-	1.00	-
	high	-0.97	0.35	0.00	0.38	0.19, 0.75
Eprinomectin*ODR		1.07	0.56	0.05	2.92	0.98, 8.68

[a] milk production, calving season, lactation group and province were not significant and dropped from the model.

[b] standard errors adjusted for clustering (herd effect)

[c] *O.ostertagi* ELISA optical density in late lactation

Table 7.3. Hazard ratio and 95% confidence interval for contrast terms derived from a Cox proportional-hazard model of calving to conception interval by treatment and ODR group (Table 7.2).

Treatment and ODR group	N	HR	95% C.I. HR
Placebo, low ODR	28	1.00	-
Placebo, high ODR	25	0.38	0.19, 0.75
Eprinomectin, low ODR	32	0.85	0.46, 1.59
Eprinomectin, high ODR	24	0.94	0.46, 1.92

Table 7.4. Coefficients, robust standard errors and *P*-values from a Poisson model of the number of breedings in 391 dairy cows in a clinical trial of eprinomectin in Canada (1999 - 2000) [a].

Variable	β	Robust S.E. [b]	*P*	Count Ratio
Treated with eprinomectin	-0.11	0.04	0.01	0.89
Days to first service (40-90 days) [c]	-0.01	0.00	0.00	0.99
Eprinomectin * days to first service (40-90 days)	0.008	0.005	0.09	1.10
Intercept	0.63	0.04	0.00	-

[a] calving season, lactation group, province, milk production and days to first service (91-180 days) were not significant and dropped from the model.

[b] standard errors adjusted for clustering (herd effect)

[c] spline of days to first service from 40 to 90 days centered at 72 days

Figure 7.1. Frequency distribution of calving cows by calving season and province. Data from 549 Holstein cows in a clinical trial in Canada (1999 - 2000).

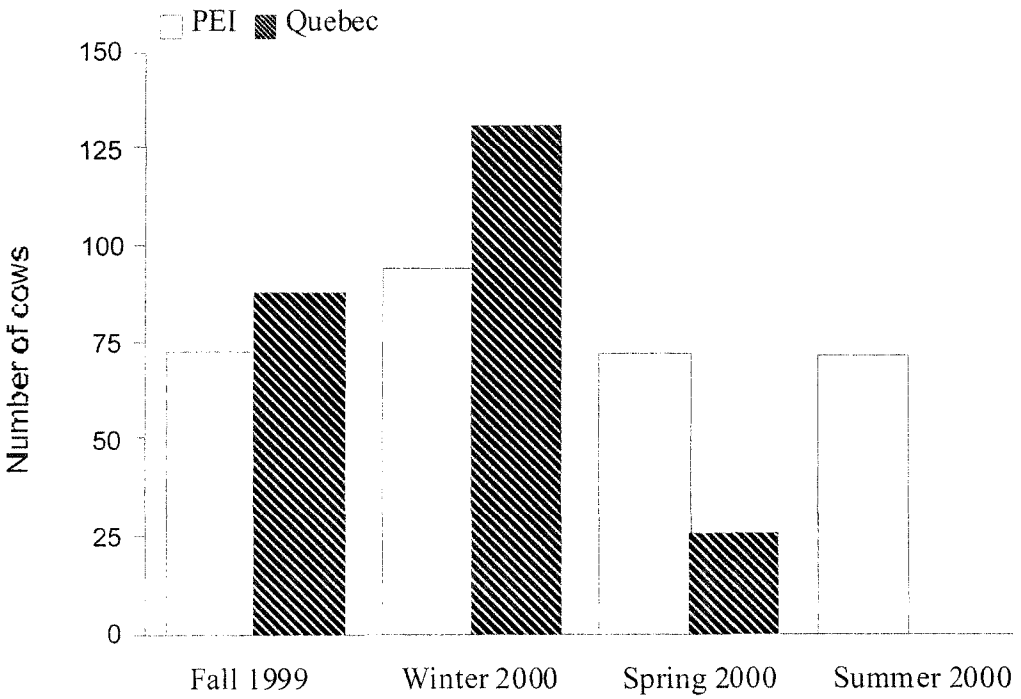


Figure 7.2. Frequency distribution of *O.ostertagi* optical-density ratios (ODR) from milk ELISAs from 109 late lactation (or dry) Holstein cows in a clinical trial of eprinomectin in Canada (1999 - 2000).

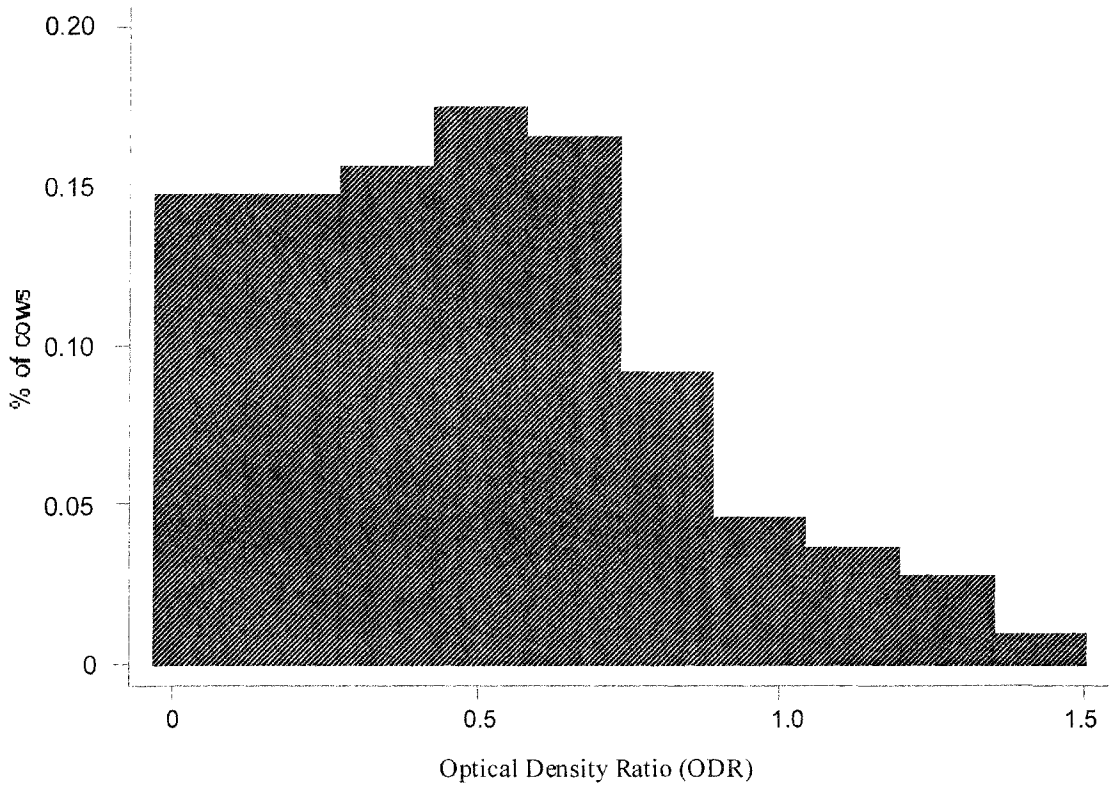


Figure 7.3. Survival curves for calving to conception interval, 549 Holstein cows treated with eprinomectin or placebo at calving (Canada, 1999 - 2000).

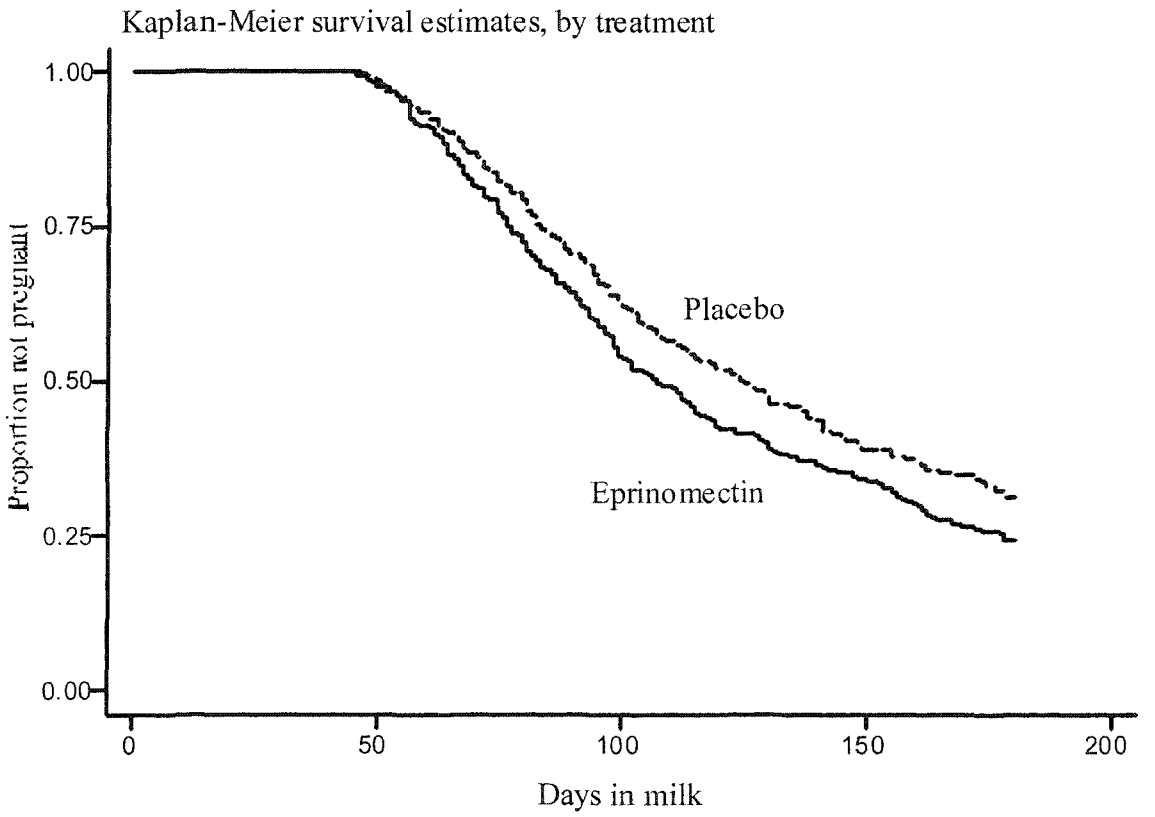


Figure 7.4. Survival curves for calving-to-conception interval by treatment and ELISA optical-density (ODR) group (a measure of parasite antibody levels; "high" = ODR ≥0.5, "low" = ODR < 0.5). Data from 109 Holstein cows from a clinical trial of eprinomectin in Canada (1999 - 2000).

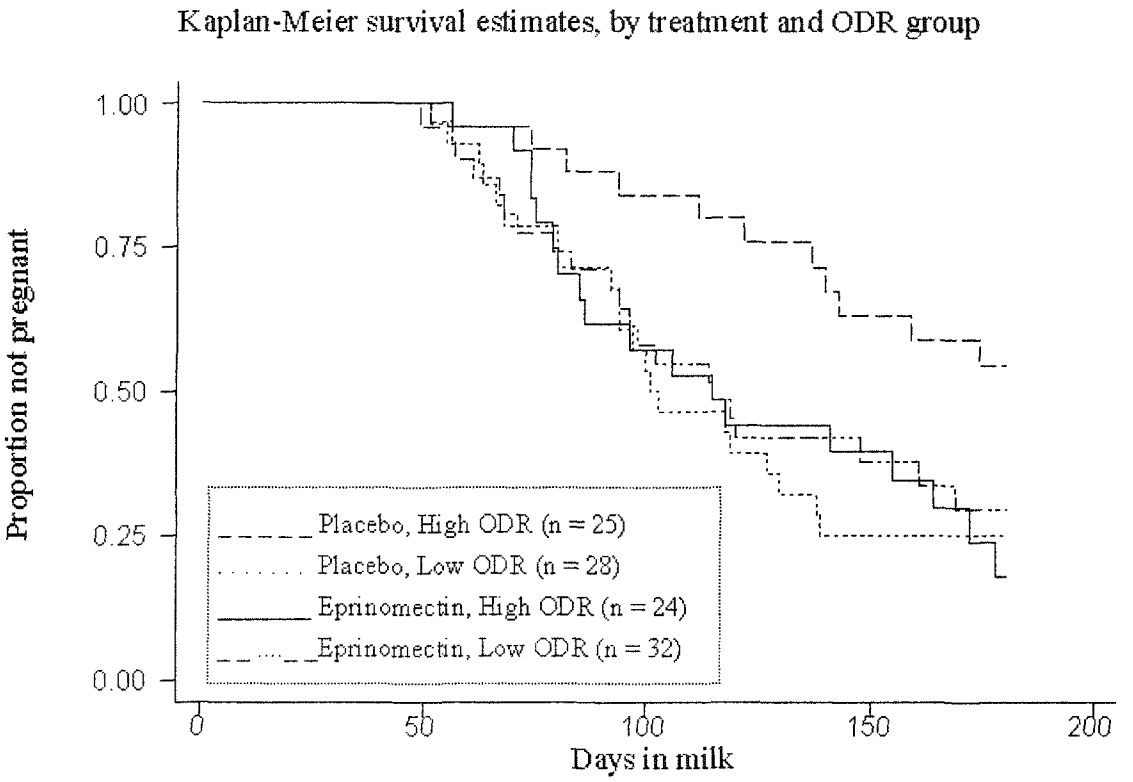
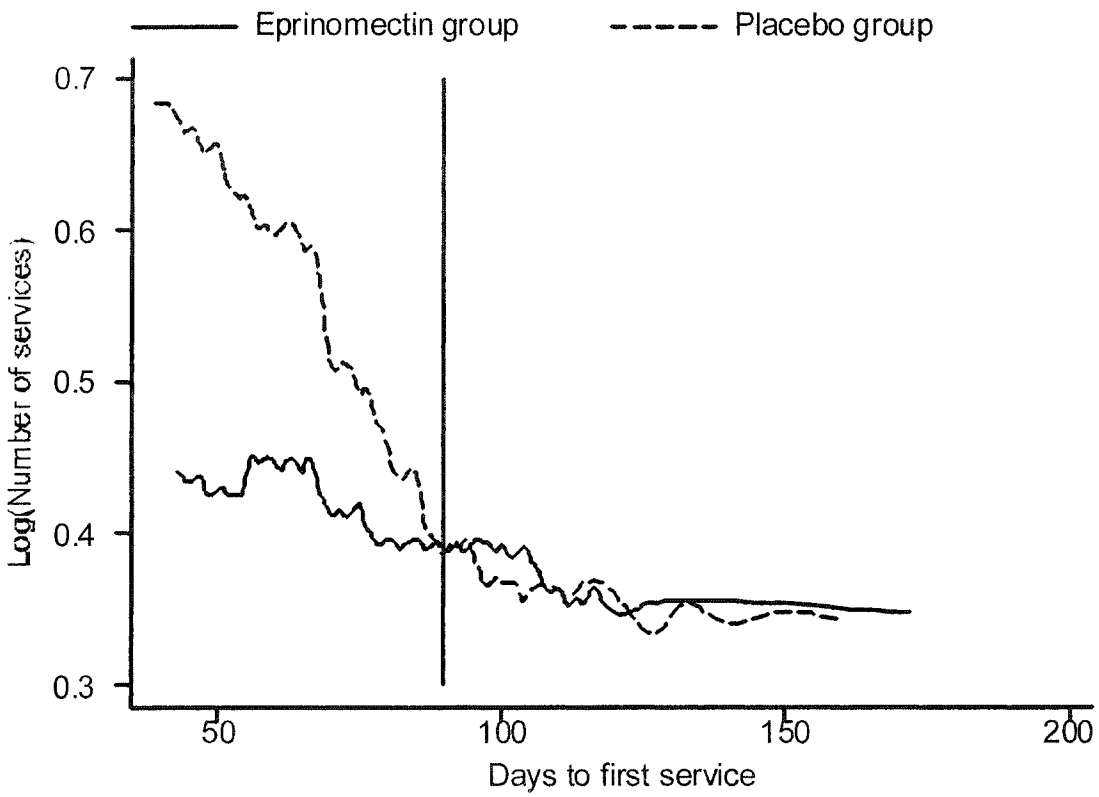


Figure 7.5. Kernel smoothed estimates of the natural log of the number of services by treatment group from 40 to 180 days to first service. Data from 391 Holstein cows from a clinical trial of eprinomectin (Canada, 1999 - 2000).



8. The use of an indirect *Ostertagia ostertagi* ELISA to predict milk production response after anthelmintic treatment in non-pastured dairy herds.

8.1 Abstract

This chapter presents the preliminary results of a longitudinal study carried out between 2002 and 2003, which evaluated the use of an indirect *Ostertagia ostertagi* ELISA in confined and semi-confined dairy herds to predict milk production response after anthelmintic treatment.

Holstein cows from 30 dairy farms from PEI, central Nova Scotia and southern Ontario participating in a clinical trial of anthelmintic treatment at calving were used in this study. The cows were randomly treated with either eprinomectin pour-on endectocide or a placebo solution around calving. Milk samples were obtained from cows between 200 and 700 days in milk and were tested for antibodies to GIN using the indirect ELISA. Production records were obtained from a computerized database of dairy herd improvement data. Pre-calving ODRS showed a seasonal pattern, they were higher in the summer and fall and lower during the winter months. Older animals had higher pre-calving ODR values compared with younger cows. Similarly, cows from semi-confined herds had higher parasite milk antibody levels compared with cows from confined herds. The anthelmintic treatment did not affect the milk production response in the study animals. In addition, the interaction effect between treatment and pre-calving ODR on milk production response after anthelmintic treatment was not significant. These preliminary results showed that the indirect *O.ostertagi* ELISA was related to those known factors related to parasitism levels in herds with little expose to

pasture. However, the factors did not predict the milk production response after anthelmintic treatment.

8.2 Introduction

The effect of gastrointestinal nematodes (GIN) on milk production in adult dairy cattle has been of increased interest recently. The nematodes, *Ostertagia ostertagi* and several species of *Cooperia*, are the main gastrointestinal parasites in cattle in temperate regions (1). Young stock are more susceptible to GIN, but adult animals can harbor a significant number of GIN, comparable to that found in first grazing season animals (2; 3).

A recent literature review has reported that a median milk production increase of 0.63 kg/cow/day may be expected after anthelmintic treatment (4). Similarly, the meta-analysis described in Chapter 2 has demonstrated that an increase of 0.35 kg/cow/day might be obtained after anthelmintic treatment. However, the treatment response varied greatly between studies (-2.17 to +3.16 kg/cow/day), which suggests that the anthelmintic treatment had an effect in some, but not all, cows/farms.

Cattle under grazing production systems are considered at higher risk of suffering the negative impact of GIN than cattle from non-pastured herds. However, based on results from an indirect *O.ostertagi* ELISA, cows from herds with little exposure to pasture might also be prone to production losses due to GIN (5); the putative effect on production has not been well documented. Most of the field trials to measure the impact of gastrointestinal parasitism on milk production have been carried out in herds with grazing cows.

Vercruysse and Claerebout (6) pointed out that the negative impact of GIN is the result of a balance between the development of protective immunity and the characteristic of the production system. They recommended identifying the level of parasitism that justifies anthelmintic treatment. However, the lack of a reliable diagnostic technique, especially in adult animals, has made it difficult to identify this threshold value.

The indirect ELISA used in this project has been related to management factors that are related to GIN (7-10). Moreover, a negative correlation between test results and milk production has also been reported (8; 9). Finally, this test was useful in predicting milk production and reproductive performance response after anthelmintic treatment in cows exposed to pasture (5).

The objective of this study was to use the indirect *O.ostertagi* ELISA in confined and semi-confined dairy herds to evaluate the ability of this test to predict milk production response after anthelmintic treatment.

8.3 Material and Methods

8.3.1 Test animals

Holstein cows from 30 dairy farms in Prince Edward Island (PEI) (n = 5), central Nova Scotia (n = 9) and southern Ontario (n = 16) participating in a double blind randomized clinical trial of anthelmintic treatment around calving were used in this study. The lactating cows from these farms either had no access to pasture (confined) or they had access only to a small paddock for exercise (semi-confined) during the summer of 2002.

8.3.2 Treatment protocol

All cows due to calve within the next 12 months of the start of the trial (end of February 2002) were eligible for inclusion in the study. The study was a double blind randomized clinical trial, with anthelmintic and placebo being delivered in one-cow treatment bottles identified with a unique number. Each bottle contained 72.5 ml of either eprinomectin pour-on endectocide or placebo solution. As each cow calved, they were randomly assigned to

receive one bottle. The bottles were packed in box of 36 units and they were kept at the farm. Cow name, treatment date, calving date, and bottle number were recorded by the person performing the treatment.

8.3.3 Milk samples

Composite individual cow milk samples preserved with bronopol immediately after sampling were obtained from the provincial dairy laboratory in each of the participating provinces after routine testing of fat, protein, SCC and milk urea nitrogen. The samples were frozen at –20 °C and sent to the Atlantic Veterinary College where they were thawed and centrifuged at 16,000 x *g* for 4 minutes. Finally the fat fraction was removed and the skim milk was stored at –20°C until tested for parasite antibodies.

8.3.4 O.ostertagi *ELISA*

An indirect *O.ostertagi* ELISA was performed on all these samples as described in Sanchez et al., (11; Chapter 3).

8.3.5 Production data

Individual daily milk yield, days in milk, calving date, lactation number and somatic cell counts were obtained from the Canadian Dairy Herd Management System (CDHMS) database through electronic data transfer.

8.3.6 Statistical analysis

8.3.6.1 Descriptive statistics

A flow-chart of the dataset structure used in this study is presented in Figure 8.1. The number of treated cows, the distribution of cows by province, herds and calving season, as well as the distribution of pre-calving optical density ratios (ODR) by province and herds, were computed using the software package Stata version 8 (12).

8.3.6.2 Multivariable analysis

8.3.6.2.1 Multilevel model – factors affecting ELISA ODR

The influence of several factors on ODR was evaluated using a multilevel random intercept model in MLwiN (13). This model included all the individual ELISA values obtained from cows between 150 and 22 days before calving (n = 909) (Figure 8.1). The following predictors were evaluated in this model: lactation group (first, second, and third or greater lactation); test month (month of the milk test); calving season (Winter-02 :Jan-Feb-Mar/02; Spring-02:Apr-May-Jun/03; Summer-02: Jul-Aug-Sep/02; Fall-02:Oct-Nov-Dec/02 and Winter-03:Jan-Feb/03); housing (confined and semi-confined); region (PEI, Nova Scotia and Ontario); days in milk, and log transformed SCC.

8.3.6.2.2 Mixed model – relationship between ODR and treatment response

The effect of treatment and pre-calving ODR on daily milk yield was evaluated using the PROC MIXED command in SAS version 8.1 (14). In addition to these predictors, the model also included the independent variables used in the previous model. Only the first six milk tests after calving were used to fit this model. Cow was identified as the clustering

variable and a first-order autoregressive (AR1) correlation structure was used to account for the repeated measures between milk tests. The effect of days in milk on daily milk yield was included using the Wilmink's function (15).

The linear relationship between pre-calving ODR and milk production was evaluated by dividing the pre-calving ODR values into three groups based on the 25^{th}, 50^{th} and 75^{th} quartiles. If a trend was identified, ODR was used as a continuous variable in the model.

The predictability of pre-calving ODR on daily milk yield response was investigated by including an interaction term between pre-calving ODR and treatment in the mixed model. Pre-calving ODR was centered to the mean value (0.28) to reduce the collinearity between the main effect and the interaction term.

Plots of residuals and predicted values were performed to evaluate heteroscedasticity, and it was evaluated whether the residuals had a normal distribution as assumed in these models.

8.4 Results

8.4.1 Test animals

A total of 2805 cows calved between February 2002 and March 2003. At the end of the study period, 82 % of the calved cows were enrolled in the clinical trial and 88% (n = 2089) of the enrolled cows were treated between three weeks before and one week after calving (Table 8.1). The number of cows treated by province is shown in Table 8.1. The calving distribution by province and season is presented in Table 2. Most of the cows (79 %) calved between April and December of 2002 (Table 8.2). Similarly, 83% of the cows with pre-calving ODR and production records calved during the same period of time (Table 8.5).

8.4.2 Milk samples and ELISA

A total of 3736 milk samples from 1759 late lactation cows (mean: 288 DIM, SD: 53.4) were collected from March to November of 2002. Out of the 1759 cows, 1057 were enrolled in the clinical trial. Pre-calving ODR from 909 cows enrolled in the clinical trial (1555 samples) were obtained between 150 and 22 days before calving. The distribution of the pre-calving ODR values as well as their distribution by herd is shown in Figure 8.2 and Figure 8.3, respectively. The mean pre-calving ODR of these cows was 0.28 and the median and inter-quartile range, by province is described in Table 8.3. The data from the 909 cows were merged with the production records resulting in complete data for 824 cows (Figure 8.1). The distribution of treated and placebo cows by province and calving season are presented in Table 8.4 and 8.5, respectively. Table 8.6 summarizes the number of animals with both pre-calving ODR and production records, by herd. These cows had a mean pre-calving ODR of 0.28 (median = 0.22, IQR = 0.11 – 0.41).

8.4.3 Multivariable analysis

8.4.3.1 Multilevel model – factors affecting ODR

A total of 909 cows (1555 samples) (Figure 8.1) were used for this analysis. The average number of ELISA values was 1.7 per cow and ranged from 1 to 4. The results of this model are presented in Table 8.7. Fourteen of the 30 herds used in this study were confined herds. The others 16 herds were classified as semi-confined. Confined herds had lower pre-calving ODR values compared with semi-confined farms. The pre-calving ODR showed a seasonal pattern: they were higher during the summer and fall and lower during the winter months. Second or greater lactation cows had higher pre-calving ODR values compared with

first lactation animals. After all the significant predictors were included in the model, 80% of the pre-calving ODR variation was at herd and cow level.

8.4.3.2 Mixed model – effect of treatment and pre-calving ODR on milk production response

The results of this model are shown in Table 8.8. The treatment effect was not significantly associated with daily milk production, but the pre-calving ODR was negatively associated with daily milk production. Province was the only predictor not associated with milk production. The rest of the predictors presented the expected association with milk yield (eg. cows from confined herds produced 2.14 kg/day more than cows from semi-confined herds). To evaluate if the milk production treatment response depended on the GIN antibody levels, an interaction term between treatment and pre-calving ODR was added to this model. The coefficients, SE and *P*-values obtained from this model are presented in Table 8.9. These results suggested that the treatment effect did not depend on GIN antibody levels.

8.5 Discussion

The median pre-calving ODR obtained from the 909 cows that had a late lactation reading (Figure 8.1) was smaller than that observed previously in similar group of animals (16). However, these results are difficult to compare because most of the samples from the present study were taken during the summer and fall, while in the cited study most of the late lactation samples were from the late fall and winter months. This might suggest a larger difference in pre-calving ODR values between these two studies, which may be a result of the greater exposure to parasites in the former study.

8.5.1 Factors affecting ODR

The association between ODR and housing was similar to previous studies (8; 9). Confined herds had lower milk ODR, which may also reflect the degree of parasite exposure. The seasonal trend is related to the epidemiology of the GIN in the central-east of Canada (17). The summer and fall presented the highest ODR values which probably reflects an increased availability of parasite larvae on the pasture for the herds where cows have access to grass.

Herds with lactating cows totally confined had lower ODR than herds where the lactating group was allowed to use a yard or small paddock for exercise. This finding agrees with similar studies carried out using bulk tank milk samples (8; 9). These studies reported that totally confined herds had lower ODR compared with herds where cows grazed pasture during the summer. Similarly, Cadwell et al. (10) reported that cows exposed to pasture, as well as pastures used by heifers or pasture with incomplete rotation were associated with higher bulk tank *O.ostertagi* titers. Also, Eysker et al. (18) found that optical density values from a crude *O.ostertagi* ELISA had a moderate and significant correlation ($r = 0.53$, $P < 0.01$) with pasture larvae contamination levels.

A seasonal pattern was observed in pre-calving ODR as previously reported by Sanchez et al. (5). Winter pre-calving ODR had the lowest values, while pre-calving ODR during the summer and fall showed higher values compared with the spring. This pattern agrees with epidemiology of GIN parasitism in Canada (17).

The relationship of ODR with age has been observed in others studies (5; 19). Animals in their second or greater lactation had significantly higher ODR values compared with first

lactation animals. Kloosterman et al. (20) found similar associations and suggested that the ability to transmit antibodies from serum to milk might be related to genetic differences between animals. On the other hand, other research indicated that total IgG1 levels in milk were similar during the first three lactations with a significant increment beyond the third lactation (21).

Most of the variance in pre-calving ODR was explained by cow level factors (40 %) (Table 8.7), followed by herd factors (35 %) and test measurements (25 %). This pattern was different from that reported by Sanchez et al. (5) where most of the variance (49 %) was explained at the test measurement level. This difference might be related to a more homogeneous parasite exposure between herds in that study, (e.g. all the herds allowed cows to graze pasture during the summer and fall). However, in the present study, 14 out of the 30 herds were totally confined with no exposure to pasture, which might explain the larger variation at herd level. This agrees with the pattern observed in Figure 8.3, which shows some herds with high pre-calving ODR and some herds with a large variation between cows.

8.5.2 Relationship between pre-calving ODR and milk production

The negative association between ODR and milk production has been observed in previous studies (5; 8; 9). Based on the inter-quartile range (0.11 – 0.41) and the coefficient from this model (-4.07) (Table 8.8), an increase in pre-calving ODR from 0.11 to 0.41 was associated with a reduction in milk production of 1.22 kg/cow/day during the following lactation. Similar figures have been reported in studies carried out in Nova Scotia (8), Prince Edward Island (9) and Virginia dairy herds (22) using bulk tank milk samples. However, the milk production response after anthelmintic treatment did not depend on the level of

antibodies measured by the *O.ostertagi* ELISA (the interaction term between treatment and pre-calving ODR in Table 8.9 was not significant). However, with only 824 cows used in the current analysis and the generally low level of gastrointestinal parasitism, the study may have had insufficient power to detect an interaction between pre-calving ODR and treatment effect.

8.6 Conclusions

The data used in this chapter are only based on preliminary production records; the complete dataset will be obtained in September 2003. These preliminary results showed that the indirect *O.ostertagi* ELISA were related to those known factors related to parasitism levels in the herd. An important amount of the variance in pre-calving ODR was explained by herd and cow factors. In addition, the pre-calving ODR were related to lower levels of milk production. However, pre-calving ODR did not predict the milk production response after anthelmintic treatment.

At the time of this analysis, only production records from animals that calved before February 2003 were used and production data were only available up to the end of February 2003. Because of that, some of the cows had less than 6 milk production tests. Moreover, cows that had pre-calving ODR, and calved after the end of the clinical trial, will be included in the final analysis.

One issue that could not be fully explored at time of writing this chapter was related to the association between herd and pre-calving ODR. There seems to be a latent variable that greatly influenced the association between pre-calving ODR and milk production (e.g. when herd was included as either fixed or random effects, pre-calving ODR was positively but not

significantly associated with milk production). For this reason, all the production models did not include herd. This will be fully addressed in the final analysis.

8.7 References

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Table 8.1. Number of cows enrolled in the clinical trial between February 2002 and February 2003 by province (cows treated between three weeks before calving and one week after calving).

Province	Treatment group		Total
	Eprinomectin	Placebo	
PEI	167 (49.6 %)	170 (50.4 %)	337 (16.1 %)
Nova Scotia	376 (49.7 %)	381 (50.3 %)	757 (36.2 %)
Ontario	500 (50.3 %)	495 (49.7 %)	995 (47.7 %)
Total	1043 (49.9 %)	1046 (50.1 %)	2089 (100.0 %)

Table 8.2. The number of cows calving in a clinical trial of eprinomectin pour-on solution, by season and province.

Province	Season					Total
	February/ March-02	April/ June - 02	July / September-02	October/ December-02	January/ March -03	
PEI	45	69	91	94	38	337
Nova Scotia	82	178	194	224	79	757
Ontario	48	240	292	270	145	995
Total	175	487	577	588	262	2089

Table 8.3. Median and inter-quartile range pre-calving optical density ratios (ODR) from milk samples obtained from cows between 150 and 22 days before (approximately last 100 days of lactation), by province.

Province	Median ODR	IQR	Number of cows	Number of samples
PEI	0.31	0.18 – 0.47	143	223
Nova Scotia	0.29	0.13 – 0.49	314	583
Ontario	0.17	0.07 – 0.30	452	749
Total	0.22	0.10 – 0.41	909	1555

IQR = Interquartile range (25^{th} and 75^{th} percentiles)

Table 8.4. The number of cows by treatment group and province from the subset of animals (n = 824) that had both pre-calving ODR and production records.

Province	Treatment group		Total
	Eprinomectin	Placebo	
PEI	64 (50.0 %)	64 (50.0 %)	128 (15.5 %)
Nova Scotia	157 (51.1 %)	150 (48.9 %)	307 (37.3 %)
Ontario	197 (50.6 %)	192 (49.4 %)	389 (47.2 %)
Total	418 (50.7 %)	406 (49.3 %)	824 (100.0 %)

Table 8.5. The number of cows calving in a clinical trial of eprinomectin pour-on solution, by season and province from the subset of cows (n = 824) that had both pre-calving ODR and production records.

Province	Calving Season					Total
	February/ March-02	April/ June - 02	July / September-02	October/ December-02	January/ March -03	
PEI	0	5	52	50	21	128
Nova Scotia	0	33	114	116	44	307
Ontario	0	2	157	159	71	389
Total	0	40	323	325	136	824

Table 8.6. Mean, minimum and maximum number of cows enrolled in the clinical trial between February 2002 and March 2003. Subset of cows that had both pre-calving ODR and production records.

Province	Mean	Minimum	Maximum	Number of herds
Prince Edward Island	29	15	40	5
Nova Scotia	44	12	70	9
Ontario	41	1	77	16

Table 8.7. Coefficients, standard errors and P values from the multilevel linear model of the pre-calving ODR. Milk samples collected from three provinces of Canada from March 2002 to November 2002 (30 herds, 909 cows and 1555 test measurements).

Variable	ß	SE	*P*
Fixed Effects			
Intercept	0.29	0.04	< 0.001
Housing			
Semi-confined	Baseline		
Confined	-0.20	0.05	<0.001
Season			<0.001
Spring	Baseline		
Winter	-0.05	0.02	
Summer	0.09	0.01	
Fall	0.12	0.01	
Lactation group			<0.001
First lactation	Baseline		
Second lactation	0.10	0.01	
Third or greater lactation	0.15	0.01	
Random Effects	Variance	Standard Error	
Herd	0.018	0.005	
Cow	0.020	0.001	
Test	0.013	0.001	

Province was not significant ($P = 0.48$)

Table 8.8. Mixed model of the association between treatment and pre-calving ODR on test-day milk production using an autoregressive (AR1) correlation structure (30 herds, 824 cows and 2901 test measurements).

Variable	ß	SE	*P*
Intercept	198.2	5.70	<0.0001
Anthelmintic treatment			
Placebo	Baseline		
Eprinomectin	-0.21	0.47	0.657
Pre-calving ODR	-4.07	1.13	0.0003
Housing			
Semi-confined	Baseline		
Confined	2.00	0.50	<0.0001
Lactation group			
Second lactation	Baseline		
Third or greater lactation	3.00	0.50	<0.0001
Days in milk	-0.16	0.01	<0.0001
Days in milk - Wilmink	-171.5	6.63	<0.0001
Log SCC	-1.15	0.10	<0.0001

Test month ($P < 0.0001$) not shown for sake of simplicity. Province and calving season were not significant ($P > 0.10$) and dropped from the model.

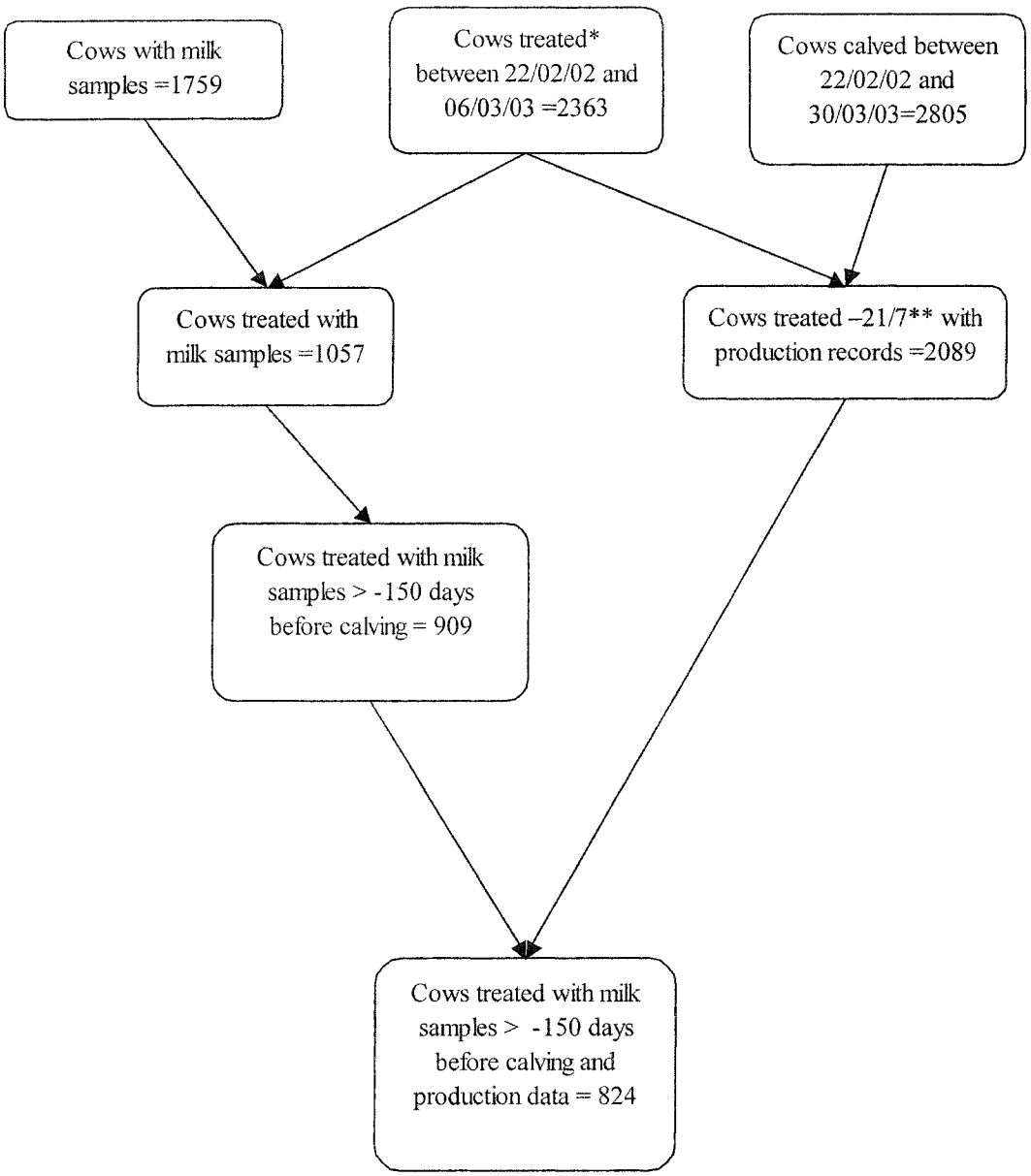
Table 8.9. Mixed model of the association between treatment and pre-calving ODR on test-day milk production using an autoregressive (AR1) correlation structure adjusted for parity group, test month, stage of lactation, calving season, housing type. Data are from 30 herds, 824 cows and 2901 test measurements [a].

Variable	ß	SE	*P*
Intercept	197.1	5.69	< 0.0001
Treatment with eprinomectin	-0.23	0.47	0.621
Pre-calving ODR[b]	-3.26	1.46	0.026
Eprinomectin – Pre-calving ODR	-1.76	2.03	0.386

[a] Coefficients from adjustment variables not reported for sake of simplicity.

[b] Pre-calving ODR = ODR > -150 days before calving centered to the mean value (0.28)

Figure 8.1. Flow-chart diagram of the dataset structure used in this study.



* treated: cows treated with both anthelmintic and placebo drugs

** -21/7 = Cows treated between 21 before and 7 days after the calving date

Figure 8.2. Distribution of the pre-calving optical density ratios (ODR) from milk samples obtained from cows that had production records, between 150 and 22 days before calving during March 2002 -November 2002 (n = 909).

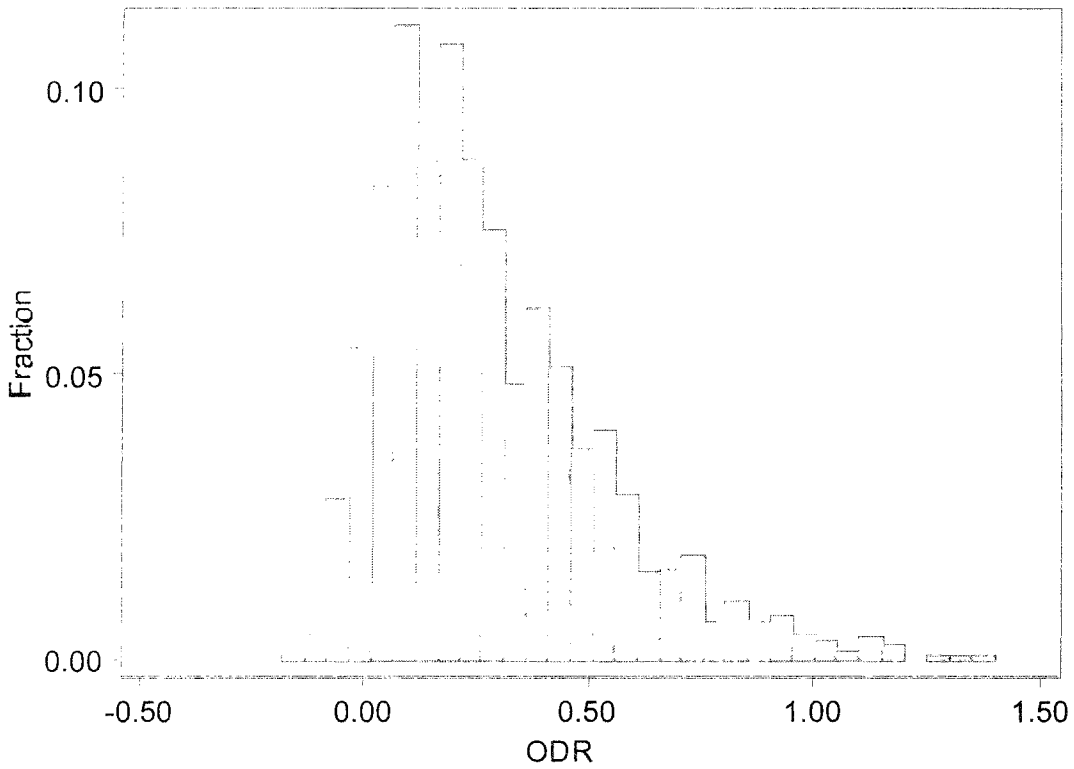
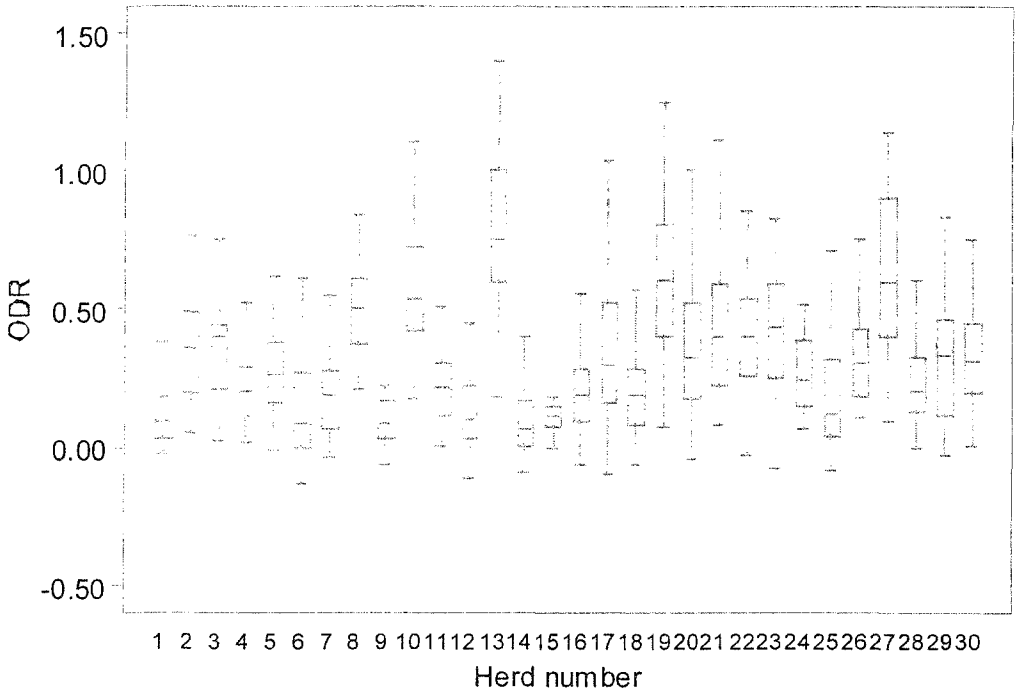


Figure 8.3. Box-plot graph of pre-calving milk optical density ratios (ODR) from cows between 150 and 22 days before calving collected during March 2002 - November 2002 that had production records, by herd (n = 909).



Herd: 1/16 = Ontario, 17/25 = Nova Scotia, 26/30 = Prince Edward Island

9. Summary

9.1 Introduction

The research described in this thesis indicates that the indirect *Ostertagia ostertagi* ELISA was useful in understanding the epidemiology of GINs in adult dairy cows. The impact of these parasites was evaluated by summarizing the published literature of their impact on milk production (chapter 2). A clinical trial was carried out to evaluate the effect of treatment (deworming) in lactating pastured dairy cattle in eastern Canada. The effect of treatment on milk production of the mentioned clinical trial was presented in a separate thesis (Nødtvedt, 2001)[1]. The effect on reproductive performance is presented in Chapter 7. The next steps involved the evaluation of the performance of this ELISA using milk samples (chapters 3 and 4). Later, two epidemiological studies evaluated the use of this ELISA to monitor gastrointestinal parasites in adult cows and find out which factors influence test results (chapters 5 and 6). Because the ELISA is designed to be part of a herd health program as a tool for monitoring parasite burdens and making treatment decisions, two additional analyses were performed to investigate if this ELISA could predict future production performance under different housing management systems (chapters 6, 7 and 8). Finally, suggestions for future research are made.

9.2 Effect of GIN on production performance

The meta-analysis of the published literature suggested that an increase of 0.35 kg/cow/day in milk production might be expected after anthelmintic treatment. Although this was a significant positive response, a large between-study variation was observed (-

[1] Nødtvedt, ACW. Parasites in lactating dairy cattle: epidemiology and response to treatment [MSc thesis]. Charlottetown, Prince Edward Island: University of Prince Edward Island, 2001.

2.17 to +3.16). This variation may be the result of either study design effects, or some underlying risk of GINs not measured in those studies. In relation to these design or risk issues, the results of this meta-analysis showed the presence of publication bias or small study effect, mainly related to low quality studies. Moreover, study design variables such as drug used, time of treatment, outcome measure recorded, age and geographic location were associated with the magnitude of the treatment response. In addition, results from the clinical trial in chapter 7 showed that anthelmintic treatment improved reproductive performance. Treated cows had a shorter calving-to-conception interval. The hazard of conception for this group of animals was 24 % higher compared with the placebo group (HR = 1.24, $P = 0.06$). However, no effect on the calving-to-first service interval was observed. Moreover, the effect of treatment on number of breedings per conception depended on the interval from calving to first service. A treated cow that was first bred at 72 days post-calving required 11% fewer services per conception than a placebo cow.

9.3 Performance of the indirect crude adult *O.ostertagi* antigen ELISA

Several factors were evaluated to determine the operational performance of this ELISA. Firstly, all those factors related to antigen preparation, sampling method, handling, preserving of the samples and udder health of individual cows were evaluated. Secondly, other factors that may influence test results such total IgG levels, stage of lactation and milk production were also quantified. As part of this process, a normalization method that maximized the within and between plate repeatability was also investigated. This test showed a high within plate repeatability, which suggests that it is not necessary to use duplicates samples per plate. Computing normalized values as

optical density ratio (ODR)=(OD – Nt)/(Pt – Nt), gave the most repeatable results. However, when the optical density values were higher than 1.2 and 0.3 for the positive and negative controls, respectively, it was necessary to repeat the test. Batches of antigens did not affect the between plate repeatability. Similarly, the use of preservative and storage of the samples up to eight months did not affect test results. On the other hand, ODR values were affected by SCC score, which indicated that the use of composite milk samples should be used when the udder health status is unknown.

The ELISA test results in milk samples followed a similar pattern to total IgG levels in milk. They were constant between 30 and 200 days of lactation and then they increased until the end of the lactation. However, when ODR values were adjusted for milk production, this relationship disappeared. Milk production might be used to correct the ODR. For instance, when comparing ODR from a cow producing 25 kg/day with that from a cow producing 38 kg/day a value of –0.05 should be subtracted from the former to create a corrected ODR.

Overall, the results from these studies suggested that milk samples, either from individual cows or bulk tank samples, might be used to monitor parasite burdens in dairy farms.

9.4 Use of the *O.ostertagi* ELISA to monitor GIN in dairy farms

A bulk-tank cross-sectional and a longitudinal study were carried out to evaluate the relationship between ELISA results, management practices and milk yield. Bulk-tank milk samples from approximately 300 dairy farms in PEI were collected in the fall of 2000 for ELISA testing. Cow exposure to pasture and whole herd anthelmintic treatment

was associated with parasite antibody levels. An increase in antibody levels from 0.38 to 0.78 was associated with a reduction in milk production of 1.2 kg/cow/day.

Thirty-eight dairy farms participated in the longitudinal study. Bulk tank milk, cow milk, serum and fecal samples were collected monthly or quarterly from all these farms. A moderate correlation between serum and milk ODR values was obtained. A seasonal ODR pattern followed the expected parasite larval intake from the pasture, with ODR values decreasing during the housing period and increasing in the spring when the cows were exposed to pasture. Second and third or greater lactation cows presented higher ODR values compared to first lactation animals. Different ODR patterns were observed between geographical regions in Canada. Dairy herds in Prince Edward Island and Quebec tended to have higher ELISA values (Table 6.4), and a marked variation between herd and month was also observed (Figure 6.6). In Saskatchewan, although cow ELISA values were smaller than those from Maritimes herds, the bulk tank ELISA values were similar to those from PEI herds (Figure 6.6). Contrarily, Ontario dairy herds showed the lowest values and had the smallest variability, suggesting a lower parasite exposure. Similarly, a negative association between ODR and milk production was found in this study. A unit of increase in the log-ODR was associated with a decrease in milk production of 0.019 kg/cow/day. A high proportion of the bulk tank ODR variation was explained by herd level factors (63%), suggesting the bulk tank ODR values were able to discriminate parasite levels. However, high cow ODR levels were observed in low bulk tank ODR herds (Figure 6.1), which may indicate that any monitoring program should include both bulk tank and individual cow milk samples.

9.5 Ability of the *O.ostertagi* ELISA to predict production response

The identification of threshold values that determine animals or herds suffering the detrimental production effect of the GINs is one of the main objectives of any parasite control program. Therefore, the indirect ELISA was evaluated in two longitudinal studies where cows had different levels of pasture exposure. The first study looked at the predictability of this indirect ELISA on milk production and reproductive performance response to anthelmintic treatment in pastured dairy herds. The lactating cows received anthelmintic treatment at calving, and pre-calving ODR were determined using the indirect ELISA. Twenty-eight dairy herds participated in this clinical trial and included 123 cows with pre-calving ODR. Because of the small sample size, the pre-calving ODR were categorized, based on their median value, as high ($\geq$ 0.5) or low ($<$ 0.5). The pre-calving ODR had a significantly effect on treatment response, suggesting that high pre-calving ODR cows responded better to the anthelmintic treatment. When evaluating the reproductive performance of the animals that had pre-calving ODR, it was observed that treated cows with high pre-calving ODR had a hazard of conception equivalent to the hazard for all the cows in the low pre-calving ODR group. Among the untreated group, the high pre-calving ODR had a much lower hazard of conception compared with the low pre-calving ODR group, suggesting that higher parasite burdens had an adverse effect on reproductive performance. In summary, these preliminary findings indicated the potential use of the ELISA to predict future productive performance and may also suggest that a cut-off value of 0.5 could be used to decide which animals warrant treatment.

In order to validate these results, a larger study was performed, but in confined and semi-confined herds and only preliminary analyses were presented in this thesis. Pre-

calving ODR from 824 cows were used to evaluate the treatment response and showed a negative relationship with milk production. Based on this preliminary analysis, the treatment response did not depend on the pre-calving antibody level. This lack of interaction might be related to a low exposure to GINs in these production systems (pre-calving ODR were half of the value of those found in the pastured herds). However, the pre-calving ODR were significantly negatively associated and milk production, which contradict this hypothesis. This relationship will be fully explored in the final analysis, once all of the follow-up production data are available.

9.6 Un-answered questions and future research

The present research was part of a larger research program, where the main aim is to establish a commercial diagnostic kit that can be used as part of a herd health program to monitor parasite burdens in adult dairy cattle. To accomplish this goal, several objectives were reached as result of the work described in this thesis, however there are still other steps that need to be taken.

First, other aspects related to the operational characteristics of the ELISA should be investigated. The reproducibility of the test will be an important issue. Results between laboratories in either the same or different geographical locations should be compared. The performance of other antigens should be also analyzed. Moreover, purified antigens that are easier to standardize and to produce in large quantities will be an asset for commercial purposes. In relation to that, the performance of those antigens under different microplate coating processes should be also evaluated.

Finally, despite the fact that a cow level cut-off value was identified in the first clinical trial, it will be important to identify the functional form of this relationship (on a continuous scale) in order to measure more thoroughly all possible control options. More important will be the determination of a herd level cut-off value by using either bulk-tank samples or any other herd measure of antibody levels, for instance, the proportion of cows with ODR greater than a pre-defined threshold.

Appendix A - Parasite survey questionnaire (Chapter 5)

Dear Dairy Producer:

Veterinarians at the Atlantic Veterinary College are evaluating a new test for measuring parasite (worm) burdens in dairy herds using bulk tank milk samples. All dairy herds in PEI will have bulk tank milk samples tested in September and October 2000.

To assist in the evaluation of the test, we are collecting some basic data about each dairy herd. Would you please take a few minutes to fill in this very brief survey and return it in the self addressed, postage paid envelope.

All information will be used only for research purposes and will be kept strictly confidential.

If you would like the results of your bulk tank milk tests returned to your veterinarian, so you can discuss the results with them, please check the box below and return the completed survey.

☐ Yes, please return the test results to:
Name of veterinarian:

_____

Ian Dohoo
Professor - Epidemiology
Health Management
Atlantic Veterinary College

Murray Mylet
Manager
PEI Milk Marketing Board

Javier Sanchez
Graduate Student
AVC

Heifers (Breeding Age or Pregnant)

1. In the Summer of 2000, heifers were (check one):
   a. totally confined (in the barn) 24 hrs./day. ☐
   b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐
   c. given access to a small field for the purpose of exercise (not primarily for grazing). ☐
   d. spent some time grazing and met some of their nutritional requirements from pasture. ☐

1

2. If heifers have access to pasture for grazing (not just for exercise), do they graze on pastures that have also been grazed by dry cows this year? Yes ☐ No ☐

3. Which of the following treatments were used for worm control in heifers? (Check all that apply)
   a. pour on or injectable deworming in Fall 1999 ☐
   b. pour on or injectable deworming in Spring or Summer 2000 ☐
   c. Ivomec sustained release bolus in Summer 2000 ☐
   d. pour on or injectable deworming in Fall 2000 (before October 1st) ☐
   e. no treatments between September 1999 and Fall 2000 ☐

Milking cows

4. In the Summer of 2000, milking cows were (check one):
   a. totally confined (in the barn) 24 hrs./day. ☐
   b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day ☐
   c. given access to a small field for the purpose of exercise (not primarily for grazing) ☐
   d. spent some time grazing and met some of their nutritional requirements form pasture. ☐

5. If milking cows have access to pasture for grazing (not just for exercise), do they graze on pastures that have also been grazed by heifers this year? Yes ☐ No ☐

6. Which of the following worm control treatments have been used in milking cows in the past 12 months? (Check all that apply)
   a. no treatment ☐
   b. oral dewormers (in feed or by mouth) ☐
   c. pour on or injectable treatment at dry off ☐
   d. pour on or injectable treatment at calving ☐
   e. pour on or injectable treatment of whole herd ☐

Pasture Management (Pastures for milking cows)

7. How were the pastures used by milking cows managed this year? (Check one)
   a. continuous grazing (continuous access for pasture season). ☐
   b. controlled access grazing (rotational or strip grazing). ☐

8. Was any cattle manure mechanically spread on pastures used for grazing by milking cows this year? Yes ☐ No ☐

9. Were these pastures dragged or harrowed this year? ☐ ☐

10. Were these pastures clipped this year? ☐ ☐

Appendix B - Parasite survey questionnaire (Chapter 6)

Owner Lastname: _____ DHI-Herd #_____

A. Herd Size

A1. Average number of lactating cows

A2. Average number of dry cows _____

A3. Average number of heifers (12 mo. - 1st calving) _____

A4. Average number of calves (< 12 mo.) _____

A5. Breed(s) _____

B. Heifers (Breeding Age or Pregnant)

B1. In the Summer of 1999, heifers were (check one):

a. totally confined (in the barn) 24 hrs./day. ☐

b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐

c. given access to a small field for the purpose of exercise (not primarily for grazing). ☐

d. spent some time grazing and met some of their nutritional requirements from pasture. ☐

B2. If heifers were on pasture, which of the following have also grazed on the same pasture(s)

-during the summer of 1999?

a. Calves ☐

b. Dry cows ☐

c. Lactating cows ☐

-during the summer of 1998?

d. Calves ☐

e. Heifers ☐

f. Dry cows ☐

g. Lactating cows ☐

Pasture Management for pastures used by heifers (summer 1999)

If heifers were on pasture during the summer of 1999 complete the following questions.

B3. Date animals first turned out on to pasture in 1999(MM/DD) _____

B4. Total pasture area used for grazing heifers. _____

B5. Number of fields paddocks _____

B6. How long have these pastures been used for grazing since last reseeding :(Check one)

a. < 1 year ☐
b. 2-5 years ☐
c. > 5 years ☐

B7. How were these pastures managed last summer? (Check one).

		Yes	No
a.	continuous grazing (continuous access for pasture season).	☐	☐
b.	controlled access grazing (rotational or strip grazing).	☐	☐

B8. If controlled access grazing is used, how frequently are cattle moved:

a. < 7 days ☐
b. 7 - 14 d. ☐
c. > 14days ☐

B9. Has any cattle manure been mechanically spread on these pastures this year? ☐ ☐

B10. Have these pastures been dragged or harrowed this year? ☐ ☐

B11. Have these pastures been clipped this year? ☐ ☐

B12. Was a cut of hay or silage taken off any of these pastures before they were used for grazing? ☐ ☐

B13. If Yes, how many acres were cut? _____

C. Milking cows

C1. In the Summer of 1998, milking cows were (check one):

a. totally confined (in the barn) 24 hrs./day. ☐
b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐
c. given access to a small field for the purpose of exercise (not primarily for grazing). ☐
d. spent some time grazing and met some of their nutritional requirements form pasture. ☐

C 2. If milking cows were on pastures, which of the following have also grazed on the same pasture(s)

-during the summer of 1999?

a. Calves ☐
b. Dry cows ☐
c. Lactating cows ☐

-during the summer of 1998?

d. Calves ☐
e. Heifers ☐
f. Dry cows ☐
g. Lactating cows ☐

Pasture Management for pastures used by milking cows (summer 1999)

If milking cows were on pasture during the summer of 1999 complete questions:

C3. Date animals first turned out on to pasture in 1999(MM DD) _____

C4. Total pasture area used for grazing milking cows. _____

C5. Number of fields paddocks _____

C6. How long have these been pastures been used for grazing since last reseeding :(Check one)

a. < 1 year ☐
b. 2-5 years ☐
c. > 5 years ☐

		Yes	No
C7.	How were these pastures managed last summer? (Check one).		
a.	continuous grazing (continuous access for pasture season).	☐	☐
b.	controlled access grazing (rotational or strip grazing).	☐	☐

C8. If controlled access grazing is used, how frequently are cattle moved:

a. < 7 days ☐
b. 7 - 14 d ☐
c. > 14days ☐

C9.	Has any cattle manure been mechanically spread on these pastures this year?.	☐	☐
C10.	Have these pastures been dragged or harrowed this year?	☐	☐
C11.	Have these pastures been clipped this year?	☐	☐
C12.	Was a cut of hay or silage taken off any of these pastures before they were used for grazing?	☐	☐

C13. If Yes, how many acres were cut? _____

D. Dry cows

D1. In the Summer of 1998, dry cows were (check one):

a. totally confined (in the barn) 24 hrs./day. ☐
b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐
c. given access to a small field for the purpose of exercise (not primarily for grazing). ☐
d. spent some time grazing and met some of their nutritional requirements form pasture. ☐

D2. If dry cows were on pastures, which of the following have also grazed on the same pasture(s)

-during the summer of 1999?

a. Calves ☐
b. Heifers ☐
c. Lactating cows ☐

-during the summer of 1998?

d. Calves ☐
e. Heifers ☐
f. Dry cows ☐
g. Lactating cows ☐

Pasture management for pastures used by dry cows (summer 1999).

If dry cows were on pasture during the summer of 1999 complete questions

D3. Date animals first turned out on to pasture in 1999(MM-DD) _____

D4. Total pasture area used for grazing dry cows. _____

D5. Number of fields / paddocks. _____

D6. How long have these been pastures been used for grazing since last reseeding? (Check one)

a. < 1 year ☐
b. 2-5 years ☐
c. > 5 years ☐

		Yes	No
D7.	How were these pastures managed last summer? (Check one).		
a.	continuous grazing (continuous access for pasture season).	☐	☐
b.	controlled access grazing (rotational or strip grazing).	☐	☐

D8 If controlled access grazing is used, how frequently are cattle moved?

a. < 7 days ☐
b. 7 - 14 d ☐
c. > 14days ☐

D9. Has any cattle manure been mechanically spread on these pastures grazing this year?. ☐ ☐

D10. Have these pastures been dragged or harrowed this year? ☐ ☐

D11. Have these pastures been clipped this year? ☐ ☐

D12. Was a cut of hay or silage taken off any of these pastures before they were used for grazing? ☐ ☐

D13. If Yes, how many acres were cut? _____

E. Treatments

E1 Which of the following treatments have been used for worm control in cows, heifers and calves over the past year? (Check all that apply).

		Cows	Heifers (>12 mo - 1st calving)	Calves (< 12 mo)
E2	-Pour on or injectable deworming in Fall 1998			
	b. Ivomec pour on	☐	☐	☐
	c. Ivomec injectable	☐	☐	☐
	d. Dectomax pour on	☐	☐	☐
	e. Dectomax injectable	☐	☐	☐
	f. Cydectin pour on	☐	☐	☐
	g. Levasol Tramisol pour on	☐	☐	☐
	h. Ripercol pour on	☐	☐	☐
	i. Other (specify) _____	☐	☐	☐
E3	-Pour on or injectable deworming in Spring or Summer 1999			
	a. Ivomec pour on	☐	☐	☐
	b. Ivomec injectable	☐	☐	☐
	c. Dectomax pour on	☐	☐	☐
	d. Dectomax injectable	☐	☐	☐
	e. Cydectin pour on	☐	☐	☐
	f. Levasol Tramisol pour on	☐	☐	☐
	g. Ripercol pour on	☐	☐	☐
	h. Other(specify) _____	☐	☐	☐
E4	-Sustained release bolus in Summer 1999			
	a. Ivomec bolus	☐	☐	☐
	b. Pamteel flex bolus	☐	☐	☐
	c. Other(specify) _____	☐	☐	☐

		Cows	Heifers (12 mo - 1st calving)	Calves (< 12 mo)
15.	-Oral deworming any time in Fall-winter 1998			
	a. Bannimth II 20 % Premix	☐	☐	☐
	b. Exhelm E Pellets	☐	☐	☐
	c. Safe-Guard Premix 20%	☐	☐	☐
	d. Other-specify)_____	☐	☐	☐
	_____			
16.	-Oral deworming Spring-summer 1999			
	a. Bannimth II 20 % Premix	☐	☐	☐
	b. Exhelm E Pellets	☐	☐	☐
	c. Safe-Guard Premix 20%	☐	☐	☐
	d. Other (specify)_____	☐	☐	☐
17.	No treatments in last 12 months	☐	☐	☐

		Yes	No
18.	In the last 12 months, have you seen signs of tail head mange (lumpy skin, itchy areas, crusty lesions, open scores) at the tail head or top of udder on any cows?.	☐	☐
19.	If yes,		
	a. What % of milking cows were affected ?	_____	
	b. Did you treat these cows?	☐	☐
	c. What type of treatments did you use?	_____	
	d. When did you apply these treatments?	_____	
	e. Do you feel these treatments were effective?	☐	☐

Appendix C - List of published chapters, submitted for publication or in preparation

Chapter 2	J.Sanchez, I.Dohoo, J. Carrier, L. DesCôteaux. A meta-analysis of the milk production response after anthelmintic treatment in adult dairy cattle. Preventive Veterinary Medicine, submitted for publication.
Chapter 3	J. Sanchez, I. Dohoo, F.Markham , K. Leslie, G. Conboy. Evaluation of the repeatability of a crude adult indirect *Ostertagia ostertagi* ELISA and methods of expressing test results. Veterinary Parasitology,109 (2002) 75-90.
Chapter 4	J. Sanchez, F. Markham, I. Dohoo, J. Sheppard, G. Keefe, K. Leslie. Milk antibodies against *Ostertagia ostertagi:* Relationships with milk IgG and production parameters in lactating dairy cattle. Veterinary Parasitology, submitted for publication.
Chapter 5	J. Sanchez, I. Dohoo. A bulk tank milk survey of *Ostertagia ostertagi* antibodies in dairy herds in Prince Edward Island and their relationship with herd management factors and milk yield. Canadian Veterinary Journal, 43 (2002) 454-459.
Chapter 6	J. Sanchez, I. Dohoo, A. Nødtvedt, G. Keefe, F. Markham, K. Leslie, L. DesCôteaux, J. Campbell. A longitudinal study of gastrointestinal parasites in Canadian dairy farms: the value of an indirect *Ostertagia ostertagi* ELISA as a monitoring tool. Veterinary Parasitology, 107 (2002) 209-226.
Chapter 7	J. Sanchez, A. Nødtvedt, I. Dohoo, L. DesCôteaux. The effect of eprinomectin treatment at calving on reproduction parameters in adult dairy cows in Canada. Preventive Veterinary Medicine, 56 (2002) 165-177.
Chapter 8	J. Sanchez, I. Dohoo, K. Leslie, G. Keefe, F. Markham. The use of an indirect *Ostertagia ostertagi* ELISA to predict milk production response after anthelmintic treatment in non-pastured dairy herds. This paper is being prepared for submission to Veterinary Parasitology.