THE EVALUATION OF THE UTILITY OF BULK TANK TESTS FOR SURVEILLENCE OF JOHNE’S DISEASE AND THE EFFECT OF STORAGE TIME AND TEMPERATURE ON JOHNE’S MILK ELISA RESULTS

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CAROLYN INNES

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ABSTRACT

THE EVALUATION OF THE UTILITY OF BULK TANK TESTS FOR SURVEILLANCE AND THE EFFECT OF TIME AND TEMPERATURE ON JOHNE’S ELISA RESULTS

Carolyn Innes
University of Guelph, 2011

The first objective of this study was to evaluate the utility of bulk tank tests to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) antibody in dairy herds for the purpose of Johne’s disease surveillance. Individual cow milk samples were collected by CanWest Dairy Herd Improvement customer service representatives in herds across Ontario, Canada. These samples, along with bronopol preserved bulk tank samples were collected from herds participating in the Ontario Johne’s Education and Management Assistance Program (OJEMAP), a producer funded Johne’s control scheme. Overall, there were 309 farms tested, with herd size from 15 to 986 milking cows. The relative sensitivity and specificity of the bulk tank ELISA test when a positive herd was defined as 1 or more positive cows was 54.7% and 90.6%, respectively. The second objective was to determine the effect of milk storage temperature and duration on the Johne’s milk ELISA test result. When herd level factors were considered in a logistic model, average monthly protein (%) and the percent of positive milk contributed to the bulk tank by milk ELISA positive cows were found to be significantly (p<0.05) associated with the probability of a herd testing positive on the bulk tank Hyper ELISA protocol.
Positive and negative MAP milk samples were stored for varying times and under different temperature conditions. In a mixed linear model, time was found to be significantly (<0.001) associated with the log transformed ELISA optical density. When the results were dichotomized into positive and negative by the cut-off of 0.10 and cross classified, the amount of misclassification was considered biologically negligible.
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Chapter One: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The presence of disease in a herd can be a very costly problem to dairy producers (Miller and Dorn, 1990; Kossaibati and Esslemont, 1997). A high prevalence of disease can have profound effects on milk production, fertility, reproduction and general health and welfare of the animals. The prevention of disease by the implementation of proactive management strategies is important with regard to controlling disease (LeBlanc et al. 2006). Veterinarians and producers alike are proactively testing for production limiting diseases on dairy farms. There have also been many screening and surveillance programs implemented for certain diseases of trade importance (Nuotio et al. 2003; Bitsch et al. 2000). The quality and effectiveness of the diagnostic test applied in a screening or disease-control program is of the highest importance.

Disease detection is a very important aspect of herd management and herd health programs. Detecting diseases in a timely manner can help to reduce transmission within the herd as well as reduce subsequent costs associated with the presence of disease in the herd. Rather than test each individual cow for disease screening, many producers opt to pool samples from several animals, in order to reduce the costs, time and labour. In this chapter, the strategies to detect disease in a dairy herd will be reviewed, with emphasis on Johne’s Disease (JD) and bulk tank milk testing.

1.2 Pooled Samples

Testing for disease can be very costly to producers. The collection, transportation and testing of samples all have time and labour costs associated with them. There can be high costs associated with sampling (fecal, blood, milk etc) and testing each individual cow, especially if
the herd is large and has a low prevalence of disease (van Schaik et al., 2007). Increasingly more producers and veterinarians opt to use pooled samples to keep costs at a minimum (Kalis et al., 2000). There are many advantages to pooling samples from cattle in order to detect the presence of disease in a herd. It is convenient, less expensive and a relatively easy method to sample many herds and individuals at a reduced cost and in a rapid manner. The cost of one individual sample compared to a pooled sample from several individuals will cost less per animal (Kalis et al., 2000). These cost savings can be used to increase testing frequency to monitor infection status and may increase the overall probability of detecting new infections earlier.

There are some limitations to testing schemes based on pooled samples, especially if the scheme involves follow-up of positive pool tests with individual animal sampling and testing. The lag time that occurs between testing a pooled sample and finding an infected individual could pose potential problems. If a pooled sample is confirmed to be positive it takes time to retest all individuals in that pool. An individual who is heavily infected could contaminate the environment for a longer period of time and increase the risk of transmission of the disease agent throughout the entire herd (van Schaik et al., 2003). In addition, sensitivity and specificity estimates for the same test applied to pools and individuals will differ, and can be affected by a number of factors including the effects of dilution of the sample by negative animals and the concentration of antibodies produced by each animal depending on the stage of lactation, parity of the animals infected, and the stage of infection (Christensen & Gardner, 2000).

In the field of veterinary diagnostic medicine, pooled samples are used to diagnose many disease pathogens of dairy cattle. These include bovine leukemia virus (BLV) infection, neospora caninum, bovine viral diarrhoea virus (BVDV) and a range of others among different species of production animals. There are different tests available for use with different types of
pooled samples. Diagnostic tests such as standard bacteriologic culture, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are all routinely used in animal health laboratories. Pooled samples can be utilized to test feces, serum, milk and environmental samples such as soil and/or manure from storage areas, alleyways and pastures. These pooled samples include the collection of milk through a single bulk tank sample or selective pooling of a certain number of cows who may be at a higher risk for the disease. Bulk milk antibody testing has been routinely used to identify herds infected with many agents including BVDV and BLV (Beaudeau et al., 2001; Nuotio, 2003).

Fecal pool culture is commonly used to screen cattle herds for JD (Stabel, 2002). Combining many individual samples of feces into one representative sample is less expensive than testing an individual sample from every cow in the herd. If quick results and individual results are needed for purposes such as culling, this is not an effective option. Culture can take upwards of three months for results to be obtained because some bacteria such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is very slow growing (Whitlock, 1996).

While strategic pooling of feces can be used to identify individuals who are more likely to be affected with the particular disease, the primary purpose of using pooled fecal samples is to screen herds that are thought to be uninfected and thereby to verify the negative herd status. The main concern is whether pooling fecal samples will affect the herd sensitivity. It was found that herd sensitivity of pooled fecal culture to detect MAP (73%) was not significantly different from that of individual fecal culture (64%) (Kalis et al., 2000). Thus, with the equivalent herd sensitivity of pooled culture, it will deliver significant cost savings to the producer and be equally as effective.
In a study to compare pooling versus individual culture, Wells et al. (2003) used 24 dairy herds to determine the sensitivity of each method. Purposive sampling was used in order to select herds with differing prevalence of MAP infection based on the herd history. In their study, fecal samples that had been pooled and contained feces from at least 1 cow with a high or moderate shedding status were identified as MAP positive 94 and 88% of the time, respectively. A strong correlation \( r = 0.96; P < 0.001 \) was observed between prevalence of infection determined by culture of individual and pooled fecal samples. Thus, it was concluded that pooled sample testing programs for JD are sensitive enough to detect infected herds.

Producers can have many differing objectives and goals for testing the whole herd, for example, to determine the status of the herd. The pool size (number of cow samples) that is needed will differ depending on the aims and goals of the producer. Depending on these goals, the number of animals in a pool might differ. Increasing the pool size will lower the costs but decrease the probability of identifying infection, because of the effect of dilution (Whittington et al., 2000; Kalis et al., 2000). Pooled fecal culture among different herd sizes and prevalence was studied by van Schaik et al. (2003). A spreadsheet model was developed to determine the costs associated with testing a whole herd with pooled fecal samples at different herd sizes and 2 different herd prevalence levels (low and high). The model that was developed showed that, in most cases, pooling of fecal samples was more reasonable than sampling individual animals in a herd. The case where it was not efficient to pool samples was with herd sizes of 100 and 250 that had a low prevalence of disease. At this herd size, the probability to detect a MAP infection became poor when samples were pooled. The optimal pool size increased with increasing herd size. In another study, van Schaik et al., 2007, determined the costs of pooled fecal samples versus individual blood or fecal samples to determine infection status of MAP. Pools of 5 and 10
samples were formed by age. The herds were classified by their prevalence into low (≤4%), medium (4-12%) and high (> 12%) herds. Pools with low shedders had a lower sensitivity than pools that included medium or high shedders. There was only a slight, non-significant, difference in the sensitivity of pools of 5 and 10 individual animals. Therefore, pooling could be a suitable alternative to whole herd testing depending on the objective of the farmer, their enthusiasm to pay for the testing and the timeline for test results that is expected. It may take a few months for culture results to be obtained, while an individual ELISA or PCR can be done within 24 hours of receiving a sample. While it may take months for culture results to be completed, there are many control strategies that can be implemented without knowing disease prevalence at the individual level. These strategies include maintaining a high degree of general hygiene and biosecurity measures to limit the transmission of pathogens, as well as the separation of new animals and youngstock. This strategy will benefit the whole herd’s health and disease status and prevent the transmission of many diseases instead of focusing on the individual cow disease status.

Instead of using pooled fecal samples, pooled serum is another option that has been used to detect BVDV in dairy herds. Weinstock et al. (2001) used a reverse transcriptase (RT) PCR assay to evaluate the use of serum pools to detect BVDV. In this study results from the pooled sera were compared to individual results. The agreement between the RT PCR results on pooled serum and the individual virus isolation results was 100% in this study. The RT PCR technique was able to detect BVDV in serum pools of up to 100 samples. Serum samples could also be strategically pooled when attempting to detect BVDV in a herd. A higher number of serum samples can be pooled from older, multiparous cows, which are expected to have a lower prevalence of BVDV, while serum from younger cows expected to have a higher prevalence of disease can be pooled into smaller amounts. This could increase the efficiency of the system and
may decrease the number of animals being tested if strategic pooling is used. Strategic pooling may increase the likelihood of detecting an infected herd by increasing the likelihood of identifying an infected fecal pool through inclusion of more than one positive individual.

### 1.3 Bulk Milk Testing

A program which monitors disease status through individual animal testing could become very expensive as an ongoing program if it includes repeated periodic testing of the entire herd population. An alternative to whole herd testing for surveillance of disease could be based on periodic testing of a bulk tank milk sample as a representation of the whole milking herd. A bulk tank sample used as a combined herd sample is convenient, easy to access and it does not involve any restraint of animals. As a limitation, this kind of sampling method excludes youngstock such as heifers and calves, as well as dry cows who are not lactating at the time of sampling. In order to overcome this issue the addition of spot testing of young animals may be appropriate. The sensitivity and specificity of most tests that are used on an individual animal basis will differ when they are used at the bulk milk level (Christensen & Gardner, 2000). The variability in the milk volume contribution of cows to the overall bulk tank composition will have an effect on the bulk tank result and performance of the test. The milk yield of cows that are infected may influence the probability of a positive bulk tank result, as well as the dilution effect of negative milk with positive milk.

In dairy herds, the level of antibodies to BVDV and *Neospora caninum* in bulk tank milk, measured by an ELISA, has been shown to be a cost effective and valuable tool for herds to assess within-herd prevalence (Beaudeau et al., 2001; Frössling et al., 2006). ELISAs are used on bulk tank milk to identify and screen herds in the control and eradication programs for BVDV (Joly et al. 2005; Lindberg & Alenius 1999). Diseases where infection is often sub-clinical or has
mild clinical signs after a long incubation period make bulk tank testing with an ELISA useful by detecting the presence of antibodies secreted into milk by subclinical animals.

1.3.1 Bovine Viral Diarrhea Virus

Bovine Viral Diarrhoea Virus (BVDV) infection has been detected using bulk tank milk samples. Infection with BVDV can often be subclinical, mild or have non-specific signs and failure to identify infected animals in a herd can lead to the production of a persistently infected (PI) calf through transplacental infection (Baker, 1995). Bulk milk testing is a useful way to detect new infections in herds that were previously uninfected, by detecting changes in ELISA result between sequential samples (Lindberg and Alenius, 1999). The existence of a few lactating cows with high antibody titres to BVDV within a herd of sero-negative cows will positively increase a bulk milk test (Niskanen, 1993). A study which looked at the relationship between BVDV persistent infections and the incidence of disease on dairy farms found that the test used on bulk tank milk was a useful method to detect PI animals in dairy herds (Kozasa et al., 2005). In this study, a RT PCR was used. Sixteen out of 265 dairy herds in this study were identified as BVDV positive. Of these 16 herds, six were identified as having a history of BVDV infection. In this study, using bulk tank samples, this test was confirmed to detect one PI animal in a herd of 150 cows. In another study (Renshaw et al., 2000), a RT PCR was compared to a virus isolation technique on bulk tank milk samples to detect the presence of BVDV infection. One hundred and forty-four samples from 97 farms were tested using these two techniques. On a farm by farm basis, the correlation between the results of the two methods was very high at 95.9%. These studies support the argument that the RT PCR is a useful tool for the detection of BVDV in bulk tank milk samples.
The ELISAs applied to bulk tank milk are also used to identify and monitor herds for BVDV (Niskanen et al., 1991; Lindberg and Alenius, 1999; Joly et al., 2005). The accuracy of bulk tank milk antibody ELISA in detecting likely infected herds was evaluated by Thobokwe and Heuer (2004) using a random sample of 724 dairy herds. The bulk tank samples were organized into quartiles by their ELISA result expressed as a percent inhibition. In each of the quartiles a random sample of 20 herds were selected for blood sampling. In order to determine the sensitivity and specificity of the bulk tank test, the herd status was assessed based on the number of sero-positive animals and cross classified with the apparent herd status of the bulk tank ELISA (at varying cut-offs). A receiver-operator characteristic (ROC) analysis showed that the optimal cut-off for bulk tank samples was associated with 81% sensitivity and 91% specificity for likely herd infection with BVDV.

In another similar study by Beaudeau et al (2001), two strategies were used to evaluate the relationship between the within-herd prevalence of positive animals and the percentage inhibition of the bulk milk. In the second strategy two cut-off values (35% and 60%) for the ELISA test were chosen based on a ROC analysis. This allowed the categorization of the bulk milk results into three levels. The researchers concluded that herds with a bulk tank inhibition value of 35% or less would be expected to be BVDV free. Using tests on a bulk tank level and varying the cut-offs and taking herd size into account is needed to evaluate the outcome of the test and come to a conclusion on the interpretation with regards to the within herd prevalence.

1.3.3 Q Fever

Q fever, caused by *Coxiella burnetii*, is a zoonotic disease that affects ruminants. Cattle, sheep, and goats are considered the main sources of infection for humans. Clinical signs of infection are abortion in sheep and goats and reproductive failure in cattle (Berri et al., 2001).
The use of bulk tank milk to screen for this disease was recently studied (Guatteo et al., 2007). The within herd prevalence of *Coxiella burnetii* shedding cows was compared to the bulk tank milk result using a real-time PCR. Bulk tank milk samples were collected along with individual milk samples. It was found that the PCR on a bulk milk sample interpreted in a qualitative way (positive or negative) was poor at assessing the within herd prevalence and detecting the proportion of high shedding cows. Estimated titres were also recorded for the bulk tank results. The titres performed well in estimating the within herd prevalence, and the proportion of high shedding cows.

1.3.4 Neosporosis

*Neospora caninum* is a parasite that causes neosporosis in dairy herds. This disease is a cause of sporadic abortion in cattle all over the world (Dubey and Lindsay 1996). It can be detected using bulk tank milk. The presence of specific antibodies indicates the presence of this parasite in cattle. The detection of *Neospora caninum* antibody in bulk tank milk was evaluated using an immunostimulating complex (iscom) ELISA test (Frössling et al., 2006). Individual blood samples were taken and compared to a milk sample from the farm’s bulk tank. This study confirmed that the milk composition affects the performance of the bulk tank test. Increasing volumes of milk from negative cows significantly decreased the probability of a positive outcome at two different cut-offs of the test. This study concluded that a high cut-off can be successfully applied to rule-in infection, and for screening purposes a lower cut-off can be used to detect neosporosis in a herd.

Similarly, another comparative study to evaluate the use of three ELISA tests to detect *Neospora caninum* was conducted on 162 Dutch dairy herds (Bartels et al., 2005). Blood samples from all lactating cows were collected in these herds, along with a bulk tank milk
sample on the same day. In this study, one in-house ELISA and two commercial ELISAs were evaluated on three different test days. The seroprevalence value of 15% was considered the positive cut-off value. Both commercial ELISAs performed satisfactorily at detecting a within herd seroprevalence of 15% or higher. However, the in-house ELISA in this study lacked specificity and there was a significant difference in the results for test days two and three between the in house ELISA and the two commercial ELISAs. In contrast, a study by Chanlun et al. (2002) conducted in Thailand on a smaller scale, with only 11 herds, found no direct association between bulk milk results and herd seroprevalence. Lactation stage and milk yield may have influenced these results, especially in smaller herds with fewer cows contributing to the bulk tank milk yield. The use of repeated sampling eventually including heifers and non-lactating cows is a method to increase the accuracy of classifying a herd as positive or negative by over time once they enter the milking herd.

1.4 Detection of MAP and MAP Antibody in Bulk Tank Milk

The potential for animal to human transmission of important zoonotic diseases is the major reason that some animal disease eradication and control programs have been established. Known diseases such as brucellosis, Q fever and tuberculosis have been nearly completely controlled through pasteurization and also eradication programs implemented in the developed world. A number of foodborne pathogens can be present in milk and also pose a hazard to human health if they are consumed. These pathogens can also be detected using bulk tank samples (Jayarao & Henning, 2001). There are also diseases that may be zoonotic in nature, but where clear evidence is still lacking. Johne’s Disease in cattle is one such disease which is caused by MAP, and has been speculated to be associated with Crohn’s disease in humans (Behr and Kapur, 2008; Skovgaard, 2007).
Johne’s disease in dairy cattle is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This bacterium persists in the intestinal mucosa of the animal and causes inflammation. The disease is characterized by intermittent bouts of diarrhea, weight loss and a decrease in milk production (Tiwari et al., 2009). Major monetary losses are associated with the disease. These losses are due to the lower milk production, decreased fertility, loss of income due to early culling and the reduced slaughter value of the carcasses of infected cows (Benedictus et al., 1987). Johne’s disease spreads slowly and young infected cows do not usually show clinical signs, which are chronic, profuse diarrhea and weight loss (Baumgartner & Khol, 2006). In the clinical stage, affected cows become lethargic and may be too weak to rise (Manning and Collins, 2001). These clinical stages are not usually seen since the cows are often culled for low production before the signs appear. In a Canadian study, it was estimated based on an average herd size of 50 cows with 7% seroprevalence, that Johne’s disease would cost an average producer $2472 per year (Chi et al, 2002).

The diagnosis of Johne’s disease is difficult because most infected animals on a farm are subclinically infected. It may take years before signs begin to show in an animal that was infected within hours of birth. Furthermore, the delayed humoral response to the MAP organism makes it difficult to detect MAP antibodies (Tiwari et al., 2007). In clinically affected cows diagnosis becomes easier because they will be actively shedding more bacteria in their feces as well as producing a large amount of antibodies against the bacteria compared to subclinically infected cows. It has been suggested that the prolonged preclinical phase of the infection coupled with the poor sensitivity of diagnostic tests, results in the likely exposure of the rest of the herd to these subclinical cows (Whitlock 1996).
An ELISA assay is a quick and low cost test that measures the amount of antibody produced against MAP and can be performed on serum or milk, however it is less sensitive and specific than fecal culture for identifying infected animals (Whitlock, 2000). The ELISA method was first used to detect MAP antibodies in serum in the late 1980’s (Jorgensen and Jensen, 1978), and its application to the testing of bulk tank samples has the potential to be very helpful to the dairy industry because many herds can be screened for Johne’s disease in a short period of time. Bulk tank samples are easily collected and would not be an inconvenience if implemented as part of a surveillance program.

There are many different ELISA tests available to detect MAP infection in cattle. One of these is the Pourquier ELISA kit (Institut Pourquier). A study by van Weering et al., (2007) validated the diagnostic performance of this test in bulk tank milk samples. Three hundred and eighty-three bulk tank milk samples were collected along with individual serum samples to determine the relationship between the herd seroprevalence and the bulk tank result. There was a fair correlation between the two parameters (r=0.70). At a cut-off of 12.5% S/P (sample to positive ratio) and a herd prevalence of ≥3%, the sensitivity and specificity were 85% and 96%, respectively. While at lower prevalence levels (≤2% seroprevalence) the performance of the ELISA was poor.

A similar study by Duthie et al. (2005) (Biobest Laboratories) looked at the same ELISA (Insitiut Pourquier) for the investigation of Johne’s disease in bulk tank milk. In this study 155 cows were sampled by taking matched serum and milk samples. Bulk milk samples were taken from 26 dairy herds, eight of these were known to be infected with Johne’s disease. They found a 95% agreement between the matched serum and milk sample results. There was a significant difference between the bulk milk results from positive and negative herds. The bulk milk test
was found to have a sensitivity of 67% and a specificity of 88%. More research is needed on bulk tank milk tests and the corresponding herd prevalence because small variations in the cut-off value could affect the estimated prevalence in a herd, and could make it unreliable to detect the presence of MAP (Neillen, 2000).

ELISA and PCR methods specifically designed to detect MAP at the bulk milk level were evaluated by Wilson et al. (2010). Both tests were performed on each bulk tank sample. The sensitivity for detection of MAP was calculated by dividing the total number of positive bulk tank results by the total number of bulk tank samples tested from the MAP positive herds. Using this method, the sensitivity for a single bulk tank test interpreted in parallel (ELISA or PCR) was 57%. Of the 952 tests performed on the 476 bulk tank samples, both the ELISA and PCR results on the same sample agreed 726 times. The agreement between the two bulk tank tests was 76% and between only the MAP positive herds, it was a little lower at 53%. The lower agreement in the MAP positive herds could be attributed to the test target of different components (DNA and antibody). The delay in the humoral response to MAP (Stabel, 2007) could have contributed to the discordant results when the ELISA was compared to the PCR.

The PCR test for MAP targets the insertion sequence IS900 (Collins et al. 1986), which is MAP specific. MAP can be differentiated from other similar isolates of *Mycobacteria* due to the presence of multiple copies (+20) (Olsen, 2002) of this specific insertion element. This element has become the standard marker for MAP. This technique was evaluated on bulk tank milk samples (Pillai & B M Jayarao, 2002). A total of 211 animals from 5 herds with a known history of Johne’s disease had composite samples collected and a total of 20 bulk tank samples (4 from each herd) were collected and evaluated by PCR and milk culture. In the bulk tank samples, 10 out of the 20 (50%) were positive by PCR while only 1 of 20 were positive by milk culture.
(5%). These results indicate that the use of PCR to detect MAP in bulk milk could be implemented to estimate prevalence of MAP in a population of dairy herds.

Detection of MAP in bulk tank milk was evaluated by using 2 PCR assays which detect different parts of the MAP genome (F57 and IS900) and a milk culture method in a study by Slana et al., (2009). Two hundred and twenty herds were sampled. MAP was not detected by culture in any samples. Sixty-three bulk tank samples were positive by the PCR. Forty nine were positive by the IS900 PCR assay only and 14 by both of the PCRs. These results show that there is increased sensitivity of the PCR method compared to standard cultures.

One factor to consider when using PCR is that it does not differentiate between live and dead cells (Stabel, 2000). Fecal contamination of the milk could be a possible source of the MAP, and therefore could be more easily detected in bulk milk compared to a non-contaminated bulk milk sample (Giese & Ahrens, 2000).

A bulk milk ELISA and PCR testing strategy was compared to environmental fecal culture on 515 US dairy farms. The ELISA had a higher sensitivity (51.8%) and an equal specificity (93.9%) when compared to the sensitivity of the PCR (39.8%) (Biswa et al., 2008). The ELISA optical density increased in farms that had a higher number of positive environmental culture sites. This indicates that there were increased levels of antibodies to MAP in the bulk tank. Therefore, periodic bulk tank testing could detect changes in levels of infection over time and could potentially be a useful herd monitoring tool.

Testing of dairy herds for MAP should be utilized in the dairy industry to help control Johne’s disease. In Denmark, a successful control program was launched in 2006 which uses a testing strategy that uses a milk ELISA on the whole lactating herd four times a year (Neilsen,
Using a repeated-testing strategy, such as collecting monthly samples, on bulk-tank milk could also be a practical and convenient way to monitor for the presence of MAP in herds. More studies on the use of bulk tank milk tests are needed. When evaluating a diagnostic test it is essential to consider its future use in the field. There need to be studies to evaluate bulk milk ELISAs and/or PCR assays as screening and monitoring tools to detect above average within-herd MAP infection. For those herds that will test positive, a subsequent protocol for future MAP testing should be developed.

1.5 Environmental Sampling

Some control programs have used pooled fecal cultures to screen herds for MAP infection status. For small herds with a low prevalence and large herds with a high prevalence, pools of 5 to 10 samples/pool have proved to be cost effective (Kalies et al., 2000; van Schaik et al., 2003; Wells et al., 2003). But, this process has not been suitable for medium to large dairy herds with a lower prevalence of MAP (Wells et al., 2003). Pooling feces requires collection of individual cow samples and can differ in sensitivity based on the stage of disease and the shedding level of the infected cows.

Instead of sampling the cows directly, whether it is feces or milk, the environment is another source of MAP that can be collected in a screening program. The environment of dairy cows is a place that can harbour MAP and can be a source of infection. This can include places such as the soil, manure from slurry storage or gutters, water troughs, and high traffic areas such as alleyways and holding pens. It does not replicate outside of the host, but it can survive for over a year in the environment (Whittington et al., 2004). Studies have been performed to assess the extent of MAP in the environment on and surrounding dairy farms. The organism has been found in various locations in the environment including calving pens and weaned calf pens.
(Berghaus et al., 2006). Some studies have described long term MAP survival under a range of conditions in vitro (Jorgenson, 1977). These results suggest that the organism can survive in conditions similar to those found on dairy farms.

Environmental sampling can reduce the labour of collection because there is no need to handle the animals to obtain the samples. This method can be beneficial to large dairies, who may want to avoid the cost of testing a large number of individual cows. Berghaus et al., (2006) evaluated this method for detecting MAP on large dairies in California. There were three locations used for sample collection and they were the lagoon water, sick/fresh pens and an alleyway where cows exited the milking parlour. They found that lagoon water was most likely to yield a positive result. In this study, 65-74% of infected farms who were previously identified by individual sampling were also identified positive by environmental sampling.

In a similar study that involved Minnesota operations, Raizman et al., (2004) found that cow alleyways and manure storage areas yielded the greatest proportion of positive results. This study involved the sampling of both free-stall and tie stall types of housing. The researchers concluded that targeted sampling of cow alleyways and manure storage areas could be an alternative strategy to determining herd infection status and herd level fecal prevalence, rather than initial individual cow testing. A study that assessed the relationship between the culture status of MAP in the farm environment and herd infection status collected 5 environmental samples per farm. Samples were collected from common alleyways, manure pits, manures spreaders, lagoons, floors of holding pens, and parlor exits (Lombard et al., 2006). In this study individual samples were taken to determine if herds were infected based on the criteria of one or more positive animals in the herd. It was shown that environmental sampling could be effectively implemented as an initial test to determine whether a herd is positive or negative. In
contrast, a more recent study showed that the sensitivity of environmental sampling is low and should be used with caution when determining herd infection status (Smith et al., 2011 in press). In this study the relative sensitivity of a single environmental sampling was 40%, although the presence of MAP in the environment of a farm or pen was found to be associated with fecal shedding in the cows. Targeted environmental sampling is a method in which certain high traffic areas of the barn or manure storage are sampled to increase the chances of detecting MAP (Pillars et al., 2009). These areas can include high traffic areas such as alleyways and near water troughs. This method is good for determining herd status (Pillars et al., 2009) and not to determine herd prevalence or the individual status for culling decisions. It is valuable for an initial screening program to flag potential herds that are positive and then continuing to monitor these herds in the future. Culture of environmental samples was more cost effective than individual animal sampling, but does not include any information on which animals are likely to be excreting MAP (Tavornpanich et al., 2008).

1.6  Storage and Handling of MAP samples

Diagnostic samples for MAP identification are generally collected, shipped, and stored under various conditions that may influence the viability of MAP (Richards, 1981; Richards and Cohen, 1977). Research papers may not report how the samples were transported or when they were processed and this could affect the subsequent test results. There can be unforeseen laboratory delays in processing samples. Samples can be stored at different temperatures for unknown or differing time periods. Some reports do not state how the samples were transported or when they were processed Some may also report delays in processing; therefore the evidence is inconclusive regarding whether storage temperature or duration has an effect on the ability to detect MAP in samples.
To our knowledge, the effects of sample handling on milk antibody levels to MAP have not been published in the literature. Data reported on serum antibodies (Alinovi et al, 2009) indicate the values of some ELISA tests are dependent on storage conditions. Storage for one week was found to cause discrepancies in MAP ELISA scores, compared to samples processed within 24 hours of collection. During this time, a sample may change status, resulting in a misdiagnosis of the animal. So, if there is an adverse affect on the way samples are handled and their subsequent ELISA score, a low shedding cow’s status could change from positive to negative.

Khare et al., (2008) looked at different handling and storage scenarios on fecal samples and the recovery of MAP. This study indicated that the storage of fecal samples at -20°C had adverse effects on the viability of MAP. Although all samples were positive by real-time PCR, the higher cycle threshold value for samples stored at 4°C for 48 hours and then -20°C for 1 week indicated the loss of viable bacteria. This handling technique could often be used by veterinarians and researchers when they are sampling small populations or individual cases of MAP infection. Short term storage of fecal samples at 4°C and longer term storage at -70°C appeared to have no adverse effects on MAP viability in this study.

1.7 Rationale for Thesis Project

Diagnostic tests that are used to diagnose Johne’s disease in dairy cows have been well researched and documented in the literature (Hendrick et al., 2005; Lombard et al., 2006; Whitlock et al., 2000). The tests that are currently used such as ELISAs and PCR detection methods have low sensitivities. Sensitivities for ELISAs will vary with the state of infection of the animal and fecal shedding status. They can range from 15%-87% (Whitlock, 2000). The sensitivity of PCR on milk has been estimated at 41.3% (Gao et al., 2009). The strategy of using
bulk tank milk samples as a means of surveillance for Johne’s disease is not commonly used in the dairy industry in Ontario. The utility of the testing on bulk milk has been studied with conflicting results (van Weering et al., 2007; Wilson et al., 2010). The concern is how well these tests perform when compared to herd level prevalence and whether they can indicate the level of infection of the herd. The conditions and length of time milk samples can be stored for subsequent testing has not been studied thoroughly. Alinovi et al., (2009) looked at the processing of serum for MAP detection. Samples were evaluated by time to processing (one day, one week) and storage temperature (4 °C, -20 °C). Sample storage for one week at -20 °C resulted in significantly lower Johne’s ELISA scores, regardless of the handling method, compared to samples stored at 4 °C for one week. The method of sample preparation, as well as transportation temperature, and medium term storage temperature affect Johne’s ELISA results. The variability in time to testing and time of collection can be large in some research projects. This may cause results to differ quite considerably depending on the location of the labs, shipping time and the amount of samples the labs may have to process. There is presently not any published literature on the effect of storage duration and temperature on milk samples and Johne’s ELISA result. In an unpublished pilot study that included a small sample size of 104 milk samples, there were no statistically significant differences between testing on days 1, 7 and 10 and between storage conditions which included freezing (-14°C), room temperature (22°C), incubated (32°C) and refrigeration (5°C) (Burns and Kelton, personal communication).

1.8 Objectives of Thesis Project

The overall objective of this thesis is to evaluate the utility of bulk tank tests to detect the presence of Johne’s disease in a dairy herd for the purpose of surveillance.

The specific objectives of this thesis project were:
1. To determine the effect of storage conditions and length of storage on the Johne’s milk ELISA result in individual milk samples.

2. To determine the utility of bulk tank tests in a surveillance program for dairy herds.

3. Determine whether herd level and cow production parameters influence the outcome of a bulk tank milk result.
REFERENCES


Chapter Two: THE EFFECT OF STORAGE CONDITIONS AND STORAGE DURATION ON MILK ELISA RESULTS FOR JOHNE’S DISEASE

2.1 INTRODUCTION

The diagnosis of Johne’s Disease (JD) in dairy cattle is difficult because of its extremely long latent period and hence the predominance of subclinically affected cattle in most herds (Baumartner and Khol, 2006). It may take 2-10 years before clinical signs begin to show in the animal. The delay in the humoral response makes it difficult to detect Mycobacterium avium spp. paratuberculosis (MAP) antibodies early in disease development (Lepper et al., 1989). Therefore, this disease is difficult to diagnose, and coupled with the fact that it is also untreatable, the control and prevention of transmission is very important on dairy farms with JD.

In Ontario, in order to help manage this disease, the Ontario Johne’s Disease Education and Management Assistance Program (OJEMAP) was launched in January 2010 (Godkin, 2011). This program is designed to help educate producers about the disease and assist them with management strategies to help reduce the disease in their herds. As part of this program, producers are encouraged to test for JD within their herds using available milk and serum ELISA tests. All of the milk samples collected and tested through this program are sent to the CanWest DHI laboratory in Guelph, Ontario. Given that this laboratory services all dairy herds across the province of Ontario, some milk samples will travel a considerable distance and will be subjected to a variety of environmental conditions prior to arriving at the laboratory for testing. These variable transport and storage conditions, including the time between sample collection and testing, could have an effect on the Johne’s ELISA results.

In a review of Johne’s related publications there is no published evidence about how storage temperature and duration effect the detection of antibodies to MAP in milk samples.
Based on one published study, serum ELISA results were significantly lower after storage for one week at -20°C compared to samples stored at 4°C for one week (Alinovi et al., 2009). This study found discrepancies in MAP ELISA scores between samples stored for one week and samples that were processed within 24 hours of collection. The variability in the time from collection to processing can be large in some cases, depending on the location of the laboratory relative to the farm, shipping route and time, and the volume of samples that the laboratory may have to process relative to their capacity. In a small unpublished pilot study that included 104 cow milk samples from one herd (4 positive, 3 suspect and 97 negative on initial testing with a Johne’s milk ELISA test), there were no statistically significant differences between results when testing was repeated on days 1, 7 and 10 after collection and among storage conditions which included incubation at 32.2°C, refrigeration at 5°C, freezing at -14°C, and room temperature at 22°C (Burns and Kelton, personal communication). Unfortunately, the small number of positive samples limited the power of this pilot study.

The aim of this study was to evaluate the effect of storage time and storage conditions on the Johne’s milk ELISA results.

2.2 MATERIALS AND METHODS

2.2.1 Sample Size and Sample Collection

The sample size was calculated based on data from the pilot study (Burns and Kelton, personal communication). Using an effect size of 0.10 (optical density), which was defined as the smallest difference in the mean value of the outcome variable between positive and negative samples, and a two-sided hypothesis test ($\alpha = 0.05$, Power = 0.80), it was determined that 80 positive samples with matched negative controls would be required.
Positive and negative milk samples from cows in herds testing for Johne’s disease through the CanWest DHI laboratory were saved and subsequently used to determine the effect of time and temperature on Johne’s ELISA score. All samples in this study were preserved using a 1.5 mg tablet of bronopol (2-bromo-2-nitro-propane-1,3-diol) because of collection protocols by DHI. Samples were first tested on arrival at the laboratory as per normal operational routine. All test positive samples were flagged for inclusion in the study. The negative controls were chosen by taking the next negative sample in the testing sequence within the same herd as the positive sample. All samples were aliquoted into 14 sub-samples and then stored according to the study protocol. Each aliquot contained 1.5 ml of milk and was used once for a specific time and treatment test to avoid repeated freezing and thawing of the same sample.

2.2.2 Sample Storage Temperature and Duration

The temperature conditions at which the milk samples were stored at were the following: ambient or room temperature (22°C), refrigerated (4°C) and frozen (-20°C). The refrigerated and frozen milk samples were tested using the ELISA assay on days 4, 7, 10, 14 and 28. The samples that were stored at ambient temperature were tested on days 4, 7, 10 and 14. All samples collected and aliquoted in the same day were stored in plastic bags. All testing dates for every sample were recorded on a calendar in the lab. A schematic of the study design is shown in Figure 2.1.

2.2.3 ELISA Assay

The ELISA kit that was used on all of the milk samples in this study was the Parachek® (Prionics USA Inc., La Vista, NE) Johne’s milk ELISA kit. This kit is used routinely by the CanWest DHI laboratory as a service offered to their dairy producer clients. The assay was performed according to the manufacturer’s instructions. The ELISA scores were reported as
corrected optical density (ODₐ) readings. Readings below 0.07 are considered negative for MAP, those that are within the range of 0.07-0.10 are considered to be suspect for MAP, and those above 0.10 are considered positive for MAP. Samples and testing dates were recorded and tracked on data sheets in the lab and entered into the database.

2.2.4 Statistical Analysis

Data were entered in Microsoft Excel (Microsoft, USA) and exported into STATA 10.1 (StataCorp, College Station, Texas) for variable screening and statistical modeling. All variables were screened for abnormal or missing values. Negative corrected ODₐ are common due to the variation in the negative controls when the ELISA is carried out. The negative range comes from subtracting the average of the negative controls from an already very low optical density. From the study design (Figure 2.1) it is evident that the testing scheme with respect to days after original test, does not follow a random pattern. Therefore, a mixed model approach was used to analyse these data. The statistical analysis was based on a linear mixed model with fixed effects for condition and sample status (positive or negative on the original test), and random intercept for sample (cow) and a random slope for time. The outcome (ODₐ) was transformed to normalize residuals (Dohoo et al., 2003); 0.1 units were added to each score to have positive values for all outcomes and then a log transformation was applied. An unstructured covariance pattern was used for the random effect matrices. To determine if there was any misclassification between the different test days, the results of Day 1 and days 14 and 28 were cross classified into two by two tables. To see how well the results agreed between test days, concordance correlation coefficients (CCCs) were determined. CCC evaluates the agreement between two series of continuous measurements, where values close to 1 indicate very good agreement while values approaching zero reflect very poor agreement (Dohoo et al., 2003). The CCC is computed as the
product of a measure of accuracy (the bias correction factor, BCF), and a measure of precision (the Pearson correlation coefficient, PCC) \((CC = BCF \times PCC)\) (Lin, 1989). The BCF measures how close a regression line through the data points falls to a line of perfect agreement (45° line), while the PCC measures how closely the data points are clustered around the regression line. The Bland and Altman method of assessing agreement was also used. It plots the mean of the paired measurements (x-axis) against their difference (y-axis). The 95% limits of agreement were computed as the mean difference plus or minus 1.96 times the standard deviation of the difference (Steichan and Cox, 1998; Bland and Altman, 1995).

2.3 RESULTS

Overall, there were 186 milk samples originally collected. Eight of these were discarded because the positive sample did not test positive when re-tested the next day (as part of routine laboratory procedure all positive samples are re-tested before reporting the results to the herd owner). Of the remaining 178 samples, there were 89 negative samples, 79 positive samples and 10 suspect samples. Of the positive samples, eight of them initially tested high (greater than 1.0). The 10 suspect samples were treated as negative for purposes of this study. Therefore, for the purposes of data analysis, there were 99 negative samples and 79 positive samples. From each original sample 14 aliquots were created, therefore in total there were 2670 aliquots from 178 samples that were tested across conditions at different times with the Johne’s milk ELISA. Descriptive means of the ELISA results across conditions and storage lengths are presented in Tables 2.1 and 2.2. The mean result across time and storage temperature is presented in graphical form in Figure 2.2. In this graph, the positive samples show more variation across time than the negative samples. The results of the mixed linear model are presented in Table 2.3. Storage condition was determined to not be significant in the final model. Time was modelled as
a continuous variable and was significant (p<0.05) in the final model. The predicted values for the back transformed ELISA results are shown in Figure 2.3. In order to determine if there was a significant risk of sample misclassification over time, the results were dichotomised as positive or negative and cross-classified among the samples that were refrigerated (the most common storage condition for short term storage of milk samples prior to routine testing). The cross classified results for day 1 and days 14 and 28 are presented in Tables 2.4, 2.5 and 2.6 respectively. When comparing results of individual samples and storage time, it was found that two samples changed from positive to negative based on ELISA score and a cut-point of 0.1 after two weeks of refrigerated storage. Five samples that were in the suspect range of values retested positive after a month of refrigerated storage. The CCCs were determined between day 1 and days 4, 7, 10 and 28 for the negative (n=99) and positive (n=79) groups of samples, for a total of ten combinations. Figures 2.4 and 2.6 display the CCC plots where the results of the 5 testing days are compared to day 1. The range of CCC values was 0.734-0.918. Figures 2.5 and 2.7 show the BA plots for each of the test days. The mean limits of agreement for the positive samples were -0.485-0.4046 with a mean difference of -0.04. The mean limits of agreement for the negative samples was -0.048-0.0422, with a mean difference of -0.0022.

2.4 DISCUSSION

In this study the storage temperature of the milk samples did not significantly affect the outcome of the ELISA. Interestingly, data reported in other studies (Holten-Andersen et al., 2003; Doherty et al., 2005) suggest that the values of some ELISAs are significantly affected and dependent on storage conditions, although these ELISAs were not detecting antibodies produced against MAP. In our study, storage for four days (Table 2.4) was found to be sufficient to cause some discrepancies in reported ELISA scores compared to the milk samples processed within 24
hours of the laboratory receiving them. In a field setting, four days from sample collection to delivery to laboratory and final processing may occur. Longer storage times might occur in some situations, and within study protocols for some research projects. Many research projects utilize milk samples acquired from DHI which could be subjected to a variety of handling and processing procedures, which may generate delays between sample acquisition and testing.

The potential impact of storage time on the Johne’s milk ELISA, makes the interpretation of results of studies utilizing stored milk samples problematic. Storage length significantly increased ELISA scores in milk samples in this study. However, while statistically significant, the degree of change in test results may not be biologically significant from a practical standpoint. From a realistic perspective, the potential misclassification of a sample is important, not whether over time there is variation in the ELISA result. Since this study was conducted in the same laboratory there is no effect of laboratory to laboratory variation. Plate to plate variability between the sample days could be present with the milk ELISA results, however given that positive and negative control samples are run with each plate, and test results are adjusted accordingly, the impact should be minimal.

The repeatability of serum ELISA results on individual samples has been evaluated (Collins et al, 1993; Sweeney et al, 1995). Although the overall repeatability for serum MAP ELISAs has been found to be acceptable, samples just above or just below the cut-off point, when retested, may yield the opposite dichotomous results (Collins et al, 1993; Sweeney et al, 1995). The construction of the 2x2 tables (Tables 2.4 - 2.6) and the kappa statistic shows a strong agreement between sample status on Day 1 and day 4, 14 and 28. The samples that were misclassified over time hover around the cut-off in this study. These samples will become the problematic ones when it comes time to interpret the ELISA result if a delay has occurred.
between sample collection and processing. The maximum number of samples that were misclassified in the refrigerated treatment group across all testing days was eight. This represents 4% (8/178) of the total samples. This small percentage of misclassified samples is negligible from a practical standpoint.

As with any test measuring a biological parameter there will be variability and samples close to the cut-off may change classification over time. In another study (Vanderstichel et al., 2010), a significant difference was found between milk samples tested for antibodies to Ostertagia ostertagi subjected to a variety of stressors, but the estimated difference between normally handled samples and ones subjected to the most extreme stressor represented less than 5% of the range of ELISA results, and was thus considered biologically insignificant.

The importance of these study results is that the test status of cows may be misclassified as a result of storage duration. For example, some laboratories process samples immediately, while others store samples until their next scheduled test date. Also, studies may collect milk over time from numerous cows and store all of the milk samples until the end of the study period, then process the samples all at once. It is possible that differing results might be detected if samples are processed immediately after collection. The proportion of samples that have ELISA results within and around the suspect range may be large enough to have an impact on the outcome of the study results if processing is delayed. Overall, although the outcome of the test is influenced by the storage length in the mixed linear model, the classification differences between positive and negative samples were minimal.
Figure 2.1. Flow chart describing the study design.
Table 2.1. Descriptive summary (Mean)(SD)(95% CI) of Johne’s ELISA results (ODc) of milk samples tested on days 0, 4, 7, 10, 14, 28.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Positive Mean(SD) (95% CI)</th>
<th>Negative Mean(SD) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (initial test)</td>
<td>0.50(0.45) (0.45-0.57)</td>
<td>-0.026(0.042) (-0.03(-0.02))</td>
</tr>
<tr>
<td>4</td>
<td>0.53(0.48) (0.46-0.59)</td>
<td>-0.031(0.04) (-0.04(-0.03))</td>
</tr>
<tr>
<td>7</td>
<td>0.50(0.42) (0.44-0.55)</td>
<td>-0.025(0.037) (-0.03(-0.02))</td>
</tr>
<tr>
<td>10</td>
<td>0.56(0.54) (0.49-0.63)</td>
<td>-0.022(0.039) (-0.03(-0.02))</td>
</tr>
<tr>
<td>14</td>
<td>0.52(0.50) (0.46-0.59)</td>
<td>-0.022(0.041) (-0.03(-0.02))</td>
</tr>
<tr>
<td>28</td>
<td>0.57(0.52) (0.48-0.65)</td>
<td>-0.013(0.041) (-0.02(-0.01))</td>
</tr>
</tbody>
</table>
Table 2.2. Descriptive summary (Mean)(SD)(95% CI) of Johne’s ELISA results (OD<sub>c</sub>) by different storage temperatures.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(SD)</td>
<td>Mean(SD)</td>
</tr>
<tr>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Ambient</td>
<td>0.52(0.47)</td>
<td>-0.024(0.04)</td>
</tr>
<tr>
<td></td>
<td>(0.47-0.57)</td>
<td>(-0.03-(-0.02))</td>
</tr>
<tr>
<td>Refrigerated</td>
<td>0.54(0.49)</td>
<td>-0.024(0.04)</td>
</tr>
<tr>
<td></td>
<td>(0.49-0.58)</td>
<td>(-0.03-(-0.02))</td>
</tr>
<tr>
<td>Frozen</td>
<td>0.53(0.49)</td>
<td>-0.023(0.041)</td>
</tr>
<tr>
<td></td>
<td>(0.48 -0.57)</td>
<td>(-0.03-(-0.02))</td>
</tr>
</tbody>
</table>
Figure 2.2. Graphical representation of the mean of the Johne’s milk ELISA result (OD<sub>c</sub>) over time and three different storage conditions among the samples initially tested as positive (black line) or negative (grey line).
Table 2.3. Summary of variables significant in the final model for the log transformed ELISA results.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.094 (0.025)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Time</td>
<td>0.002 (0.001)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Sample Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Positive</td>
<td>0.647(0.033)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Condition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Refrigerated</td>
<td>-0.003 (0.004)</td>
<td>0.394</td>
</tr>
<tr>
<td>Frozen</td>
<td>-0.004 (0.004)</td>
<td>0.269</td>
</tr>
<tr>
<td><strong>Random Effects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>3.13e-05</td>
<td>3.94e-06</td>
</tr>
<tr>
<td>Sample</td>
<td>0.071</td>
<td>1.85e-03</td>
</tr>
</tbody>
</table>
Figure 2.3. Predicted values of back transformed ELISA results (OD$_c$) for positive and negative Johne’s milk samples when condition is held constant (refrigerated).
Table 2.4. Cross-classification of Johne’s ELISA results from the initial test results of 178 milk samples and results after refrigerated storage for four days.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>75</td>
<td>4</td>
<td>79</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>99</td>
<td>178</td>
</tr>
</tbody>
</table>

κ = 0.910, (exact McNemar test, P=1.000)
Table 2.5. Cross-classification of Johne’s ELISA results from the initial test results of 178 milk samples and results after refrigerated storage for 14 days.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>75</td>
<td>4</td>
<td>79</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>101</td>
<td>178</td>
</tr>
</tbody>
</table>

κ=0.930, (exact McNemar test, P = 0.688)
Table 2.6. Cross-classification of Johne’s ELISA results from the initial test results of 178 milk samples and results after refrigerated storage for 28 days.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>76</td>
<td>3</td>
<td>79</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>94</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>97</td>
<td>178</td>
</tr>
</tbody>
</table>

κ=0.910, (exact McNemar test, P = 0.726)
Figure 2.4. Concordance correlation coefficient plots of 79 positive Johne’s ELISA milk samples tested on days 4, 7, 10, 14, 28 of refrigerated storage.
Figure 2.5. Bland-Altman plots of 79 positive Johne’s ELISA milk samples tested on days 4, 7, 10, 14 and 28 of refrigerated storage.
Figure 2.6. Concordance correlation coefficient plots of 99 negative Johne’s ELISA milk samples tested on days 4, 7, 10, 14, 28 of refrigerated storage.
Figure 2.7 Bland-Altman plots of 99 negative Johne’s ELISA milk samples tested on days 4, 7, 10, 14 and 28 of refrigerated storage.
REFERENCES


Chapter Three: THE EVALUATION OF THE UTILITY OF THREE BULK TANK TESTS FOR HERD LEVEL JOHNE’S DISEASE SURVEILLANCE.

3.1 INTRODUCTION

The adaptation of Enzyme Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) technology for bulk tank milk samples for the purpose of testing whole dairy herds for *Mycobacterium avium* ssp. *paratuberculosis* (MAP) could be very cost effective. Bulk tank samples are a convenient sampling method and would decrease the labour associated with collecting individual cow samples. If these tests could be reliably applied to bulk tank samples, periodic bulk tank testing could be incorporated into a provincial surveillance program for Johne’s Disease (JD). There are many publications which address the testing of individual cow milk samples by ELISA assays (Stabel et al., 2002; Nielsen et al., 2002; Hendrick et al., 2005; Lombard et al., 2006) and PCR assays (Giese and Ahrens, 2000; Pillai and Jayarao, 2002; Pinedo et al., 2008). However, very few studies address the application of these diagnostic tests for bulk milk analysis for the presence of MAP in dairy herds (Nielsen et al., 2000; Slana et al., 2005; van Weering et al., 2007; Wilson et al, 2010). The objective of this study was to evaluate the accuracy and utility of available ELISA and PCR tests for Johne’s Disease applied to bulk tank milk samples from Ontario dairy farms.

3.2 MATERIAL AND METHODS

3.2.1 Animals and Herds

Individual cow milk samples (n=20,618) were collected by CanWest Dairy Herd Improvement (DHI) customer service representatives from 309 dairy herds across different regions of Ontario, on each herd’s particular DHI test day. These samples were collected from the herds participating in the Ontario Johne’s Education and Management Assistance Program
(OJEMAP) which was implemented in January 2010 (www.johnes.ca). Each herd was asked to participate in the present study by allowing the collection of a supplementary bulk tank milk sample on the next pick up after their DHI test day.

3.2.2 Sample and Data Collection

The sampling of herds took place from March 2010 to December 2010. The DHI customer service representatives were given bulk tank test packets that were used to collect, with permission from the dairy producer, a sample from the testing herd’s bulk tank. The packets included two 50 ml vials, each containing a tablet of preservative, along with an instruction and permission form for the producer to sign. The DHI customer service representatives were instructed to leave the packet for the milk transporter to use on the next pick up day. The milk transporter took a sample from the farm’s bulk tank, following their routine bulk tank testing protocol. The samples were preserved with one tablet (1.5 mg) of bronopol (2-bromo-2-nitropropane-1,3-diol) and inverted to ensure even mixing of the sample with the preservative. The bulk tank samples were transported by courier with the routinely collected regulatory and payment bulk tank samples to the Dairy Laboratory Division of the University of Guelph. The study bulk tank samples were sorted, separated and refrigerated by the lab technicians employed by the University of Guelph. Samples were picked up twice weekly and transported to the CanWest DHI Laboratory in Guelph for analysis.

3.2.3 Diagnostic Tests

In order to detect the presence of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in the DHI individual cow milk samples the Paracheck® milk ELISA (Prionics USA Inc., La Vista, NE) was used. This test can be conducted on fresh or preserved samples. The test was carried out according to the manufacturer’s instructions. The tests that were used
on the bulk tank samples were the IDEXX Milk ELISA (IDEXX Laboratories, Westbrook, Me.), a “Hyper” ELISA method based on the IDEXX test kit components but with a modified protocol as described by Wilson et al. (2010) and the Mpara-teQ Milk Assay (AntelBio® Lansing, MI).

For the Parachek® ELISA, results were expressed as percentage S/P (sample to positive), as described by Institut Pourquier, calculated as:

\[
\% \text{ S/P} = 100 \times \frac{\text{Sample} \times [A(450)] - \text{NC}}{\text{PC}_x - \text{NC}}.
\]

For bulk tank samples a S/P ratio of \( \geq 30\% \) was considered positive for MAP, a ratio of 20-30\% was considered to be suspect for MAP and \(<20\% \) was considered negative. The Hyper ELISA results were expressed as a corrected optical density value. A positive sample had an optical density of 0.10 or higher. The PCR was carried out as per the manufacturer’s instructions.

### 3.2.4 Statistical Analysis

Results of the diagnostic tests on the individual cows and bulk tanks were stored in Microsoft Excel (Microsoft, USA), and exported into STATA 10.1 (Stata Corp, College Station, Texas) for statistical analyses. Values of P <0.05 were considered significant. The level of agreement between results for herd level status and bulk tank milk samples were determined by calculating the kappa statistic. The McNemar test was used to compare paired proportions of positive results for the three tests. The exact McNemar test was used as well because in some cases the 2x2 tables were highly unbalanced. For the ELISA and PCR used to analyze bulk tank milk samples, relative sensitivities were calculated by comparison with herd status as determined by ELISA results from individual cows.
3.3 RESULTS AND DISCUSSION

Overall there were 313 herds enrolled in the study. Of these 313 herds, four herds were excluded due to missing herd results or sample labelling errors. There were 309 dairy herds included in the final dataset with completed bulk tank milk and individual cow milk test results. A summary of farms by the number of cows testing positive is presented in Table 3.1. In the 309 herds tested, there were 20,618 lactating cattle that had individual milk ELISA results. Of these 20,618 cows, there were 136 (0.67%) that tested positive and 37 tested as suspect. Of the 136 that were positive 16 were high positives (0.08%) with an OD ≥1.0. Overall, 75 of the 309 (24.3%) participating herds had at least 1 cow test positive and 30 of the 309 (9.70%) herds had 2 or more cows testing positive.

The agreement between Hyper ELISA bulk tank milk results and herd status where at least one positive cow defined a positive herd was only moderate (κ = 0.48; Table 3.2), and the P value for the McNemar test indicated that proportions of positive results for bulk tank milk and herd status were not significantly different. In contrast, the level of agreement between results for Hyper ELISA bulk milk samples and herd status results when two or more cows testing positive was considered a positive herd was only fair (κ = 0.32; Table 3.3), and the P value for the McNemar test indicated that proportions of positive results for bulk milk and herd status were significantly different (Table 3.2). The Hyper ELISA underestimated the proportion of positive herds. This suggests that the results for the Hyper ELISA used for bulk milk samples agreed more closely with herd status results when one or more positive cows identified a positive herd than did the herd status results when two or more positive cows were used to define a positive herd.
The quantitative real-time PCR (Q-PCR) applied to bulk tank sample identified only three positive herds among those that had at least one cow test positive on the milk ELISA (4.0%) (Table 3.4). When the criteria for a positive herd was two or more cows testing positive, the number testing positive dropped to two of 30 (6.0%) (Table 3.5). The exact McNemar test, applied to test whether the two tests (Q-PCR and herd status) were classifying similar proportions of the herds positive had significant P values indicating significant differences between the proportions testing positive for bulk milk Q-PCR and herd status (for both criteria).

The IDEXX ELISA without any modification to the manufacturer’s protocol and applied to bulk tank samples did not identify any MAP positive herds.

Herd status based on cow ELISA results was used to evaluate the relative sensitivity and specificity of the Hyper ELISA and PCR. Using one or more positive cows to identify a positive herd as the “gold standard”, the relative sensitivity for the Hyper ELISA was not very good, at 54.5% and the relative specificity was 90.60%. When the criteria increased to 2 or more cows, the relative sensitivity and specificity were only slightly better at 63.3% and 84.2%. The milk ELISA has a low sensitivity for identifying individual cows infected with MAP (Hendrick et al., 2005), so it will underestimate the prevalence of infection at the cow and herd level.

The potential advantage of the modified Hyper ELISA protocol is that it more sensitive than other regular ELISAs used for individual milk samples. The relative sensitivity of the Hyper ELISA used on bulk milk samples in the present study was similar to values reported previously for other bulk tank tests (Biswas et al., 2008; Wilson et al., 2010).

This present study provided some information on the function and interpretation of the modified (Hyper) and unmodified ELISA and Q-PCR used for bulk milk samples. Because an
ELISA is more convenient and relatively cheaper (~$8/milk sample) than mycobacterial culture (~$15/fecal sample), it may be useful in monitoring dairy herds for MAP. The proportion of positive Hyper ELISA bulk milk samples was not significantly different from the proportion of herds with one or more positive cow results, suggesting that the Hyper ELISA protocol used to test bulk milk samples may be a potentially useful and convenient method of estimating herd level prevalence of MAP in dairy herds, but would not be sufficient to screen or monitor individual herds over time.

The use of a PCR for testing bulk tank milk samples has been demonstrated to be useful for estimating the prevalence of infection with MAP in some studies (Corti and Stephan, 2002; Grant et al., 2002; Stabel et al., 2002). Grant et al. (2002) performed an immuno-magnetic PCR on 244 bulk tank milk samples and detected a herd level prevalence of 7.8%. In another study, Corti and Stephan (2002) tested bulk-tank milk samples from Switzerland and found that 19.7% of the 1,384 milk samples were IS900 PCR positive. Similarly, it was shown that MAP could be detected directly from bulk-tank milk by an IS900 PCR (Pillai and Jayarao, 2002). Our inability to detect MAP from the bulk milk of most MAP positive herds using the PCR was surprising, because the Q-PCR did detect MAP in a previous study on bulk tank milk (Wilson et al, 2010). There could be many reasons why it did not detect MAP in many of the positive herds in this study. The previous study was not based on individual cow results; instead their definition of a positive herd was having at least one positive bulk tank result using an ELISA and Q-PCR. Therefore, the specificity was assumed to be 100% as false positive reactions are fairly uncommon (Lombard et al., 2006). The cow level prevalence in these US herds could have been much higher than 136/20,433 (0.67%) found in our current study. Thus having a higher cow-level prevalence of the disease would increase the number of herds which were positive by the
PCR. As part of a survey by the USDA National Animal Health Monitoring System (NAHMS) conducted in 1996, it was estimated that the percentage of dairy cows in the United States with Johne’s disease is 2.4 to 3.0%. Also, the herd level prevalence of MAP was higher in the Utah study (39%) compared to what was discovered in this study (24%). Also, a dilution of milk occurs after collection from individual animals when combined with milk from the rest of the herd in the bulk tank (Christensen and Gardner, 2000). These factors can make detection of MAP fairly difficult in bulk tank milk.

Some sub-clinically infected animals may not shed MAP in milk, and shedding can be low and intermittent (Corti and Stephan, 2002). It is a possibility that on DHI test day the sub-clinically infected animals may not have shed MAP in their milk and therefore the DNA would not be present in bulk tank milk to detect.

Based on the current study, the PCR is insufficient to detect MAP in bulk tank milk samples. The Hyper ELISA protocol may be a tool that can be implemented to estimate the proportion of positive herds, but it is not a good test for monitoring individual herds.
Table 3.1. Summary of the bulk tank (PCR and Hyper ELISA) results of herds with milk ELISA positive cows by herd size and the number of milk ELISA positive cows in each herd.

<table>
<thead>
<tr>
<th>Positive cows (#)</th>
<th>Herd Size*</th>
<th>Number of herds</th>
<th>BT** Positive</th>
<th>PCR Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>45</td>
<td>22/45</td>
<td>1/45</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>13</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>18</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>14</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>11/16</td>
<td>1/16</td>
</tr>
<tr>
<td>2</td>
<td>Small</td>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>7</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3/7</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>75</td>
<td>41</td>
<td>3</td>
</tr>
</tbody>
</table>

*Small: ≤50 Medium: 51-100 Large: >100

** Bulk Tank Hyper ELISA
Table 3.2. Cross-classification of results for a Hyper ELISA on bulk tank (BT) milk samples from 309 herds and herd level status, positive herd status defined as one or more positive cows in a herd, identified by individual milk ELISA results.

<table>
<thead>
<tr>
<th>Herd Status</th>
<th>BT ELISA</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>41</td>
<td>22</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>34</td>
<td>212</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>234</td>
<td>309</td>
<td></td>
</tr>
</tbody>
</table>

*At least one cow milk ELISA positive.
Relative sensitivity 54.6% (95% CI, 43.5% to 65.4%)
Relative specificity 90.60% (95% CI, 86.18% to 93.71%)
κ= 0.4787. P-value for McNemar test = .1088
Table 3.3. Cross-classification of results for a Hyper ELISA on bulk tank (BT) milk samples from 309 herds and herd level status, positive herd status defined as two or more positive cows in a herd, identified by individual milk ELISA results.

<table>
<thead>
<tr>
<th>Herd Status</th>
<th>BT ELISA</th>
<th>Positive*</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>19</td>
<td>44</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>235</td>
<td></td>
<td>246</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>279</td>
<td></td>
<td>309</td>
</tr>
</tbody>
</table>

*At least two cows milk ELISA positive
Relative sensitivity 63.3% (95% CI, 45.5% to 78.1%)
Relative specificity 84.23% (95% CI, 79.49% to 88.04%)
κ=0.3190. P value for McNemar test <0.001
Table 3.4. Cross-classification of results of a Q-PCR in bulk tank (BT) milk samples from 309 herds and herd level status, positive herd status defined as one or more positive cows in a herd, identified by individual milk ELISA results.

<table>
<thead>
<tr>
<th>Herd Status</th>
<th>BT Q-PCR</th>
<th>Positive*</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>72</td>
<td>233</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>234</td>
<td>309</td>
<td></td>
</tr>
</tbody>
</table>

*At least one cow milk ELISA positive.
Relative sensitivity 4.00% (95% CI, 1.37% to 11.11%)
Relative specificity 99.57% (95% CI, 97.62 to 99.92%)
κ = 0.0527. P value for Exact McNemar test <0.001
Table 3.5. Cross-classification of results of a Q-PCR in bulk tank (BT) milk samples from 309 herds and herd level status, positive herd status defined as two or more positive cows in a herd, identified by individual milk ELISA results.

<table>
<thead>
<tr>
<th>Herd Status</th>
<th>BT Q-PCR</th>
<th>Positive*</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>277</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>279</td>
<td>309</td>
<td></td>
</tr>
</tbody>
</table>

*At least two cows milk ELISA positive.
Relative sensitivity 6.67% (95% CI, 1.85% to 21.32%)
Relative specificity 99.28% (95% CI, 97.42% to 99.80%)
κ=0.0970. P value for Exact McNemar test <0.001
REFERENCES


Chapter Four: THE EVALUATION OF A HYPER ELISA PROTOCOL FOR THE DETECTION OF ANTIBODIES TO MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN BULK TANK MILK SAMPLES

4.1 INTRODUCTION

Johne’s disease is a chronic digestive tract disease that affects wild and domestic ruminants. Affected animals may exhibit no clinical signs, or may show milk yield loss, weight loss and intermittent or chronic diarrhea. *Mycobacterium avium ssp. paratuberculosis* (MAP) is the bacteria which is responsible for this disease (Stabel, 1998). Johne’s disease presents a challenge for the veterinarian and the producer, in its identification and its management. The disease can cause serious financial losses for dairy farmers because of loss of milk and early culling of infected animals (Hasonova and Pavlik, 2006; Clark et al., 2008).

The most likely and frequent transmission route to animals is fecal–oral, where infected animals shed MAP in their feces and milk. This causes feed, water and different components of the environment to be contaminated with MAP. Most sensitive to infection with MAP are young animals that are infected through contaminated colostrum or milk, dirty teats and equipment (Giese and Ahrens, 2000; Slana et al., 2008). Most infected animals do not show any clinical signs for a long period of time after they are infected.

The most commonly used diagnostic tests are fecal culture and ELISA assays which detect the presence of antibodies against MAP (Klausen et al., 2003). Direct detection of MAP in feces, milk or blood by quantitative real-time PCR (qPCR) has also been described in several studies (Rodriguez-Lazaro et al., 2005; Pillai et al., 2002; Alinovi et al., 2009; Pinedo et al., 2008). Commercially available ELISA assays designed to quantify antibodies to MAP have a low sensitivity (Collins 2005; Sweeney, 1995). Despite this limitation, the test is inexpensive, easily performed and gives a quick result so that it could be a useful tool in the surveillance of
Johne’s disease (Caldow, 2004). Although this test sensitivity is low, it increases as disease moves to the clinical stage (Collins, 2002). The ELISA can be of value in identifying cows that are likely to be shedding the bacteria and at risk of transmitting the disease to the rest of the herd.

Bulk milk antibody testing is routinely being applied to measure antibodies to several infectious agents such as bovine viral diarrhea virus (BVDV) and bovine viral leucosis (BLV) (Pritchard 2001; Mars and van Mannen 2005; Nuotio, 2003). Analysis of bulk milk has been used to estimate the prevalence of viral diseases in dairy cattle herds (Paton et al., 1998), and has been used in control programs for BVDV (Lindberg and Alenius, 1999). There are many advantages in using pooled samples, and bulk milk testing could be an economical and convenient method to sample a large number of herds. The bulk milk test could be a tool suited for initial screening of a herd, or for screening populations. By repeated bulk milk sampling, bovine infections such as bovine leucosis, bovine viral diarrhea and infectious bovine rhinotracheitis have been successfully controlled (Lindberg and Alenius, 1999; Nylin et al., 2000; Nuotio et al., 2003).

The milk ELISA is currently being used in a Johne’s disease control program in Ontario called the Ontario Johne’s Education and Management Assistance Program (OJEMAP) to identify cows likely to be infected with MAP and high shedding cows that are at risk for transmitting MAP, as well as estimating the herd prevalence in Ontario. Further research focusing on herd-level factors could increase the efficacy of test program performance by determining the herd characteristics that may influence the bulk tank test result.
The objective of this study was to investigate the relationship between herd-level factors associated with the outcome of a Johne’s Disease ELISA test applied to bulk tank milk samples in a subset of Ontario dairy herds.

4.2 MATERIAL AND METHODS

4.2.1 Sample and Data Collection

Bulk tank and individual cow milk samples were collected as per the protocol described in detail in Chapter Three. In brief, on the producers test day for OJEMAP, a bulk tank sample was taken on the following pick up day. Written permission of dairy producers was required to sample bulk-tank milk for MAP testing. Permission forms were distributed by DHI customer service representatives. Bulk tank samples in this study were the same herds and samples as were included in Chapter Three, so the methods of sample collection were the same. The herd level data were extracted from the DHI database and matched to each farm by the month their sampling was carried out.

4.2.3 Diagnostic Tests

In order to detect the presence of *Mycobacterium avium* subsp. *paratuberculosis* in the individual cow milk samples the Parachek® Milk ELISA (Prionics USA Inc., La Vista, NE) was used. This test can be conducted on fresh or preserved samples. The test was carried out according to the manufacturer’s instructions. The tests that were carried out on the bulk tank samples were the same as described in Chapter Three and included the IDEXX Milk ELISA, the AntelBio *Mpara*-teQ Milk Assay and a “Hyper” ELISA method carried out with the IDEXX test kit ingredients. For this paper, only the Hyper ELISA bulk tank results were analysed since the other two tests performed poorly.
4.2.2 Statistical Analysis

Data were entered into Microsoft Excel (Microsoft, USA) and exported into Stata 10.1 (StataCorp. College Station, Texas) for variable screening and modeling. Variables were screened for abnormal or missing values and checked against original data sources. To quantify the impact of other herd level factors on the probability of a herd testing positive on a bulk tank test, logistic regression analyses were conducted with the dichotomous outcome being a positive or negative Hyper ELISA bulk tank result. Collinearity of predictor variables was assessed by calculating Pearson correlation coefficients. If predictor variables were highly correlated (r>0.75), the predictor that was considered to be more informative was retained. All variables hypothesized to be related to the outcome of interest were screened for unconditional associations with the outcome variable in a univariable analysis. Variables that were significant at the 20% (p<=0.20) level of significance were included in a multivariable model. The variable of percent milk contributed to the bulk tank by positive cows was calculated by summing the milk yield on test day from each milk ELISA positive cow and dividing it by the total test day milk yield to come up with a percentage of milk that reflects the bulk tank. Average days in milk, parity and breed were assessed as potential confounders. Breed was categorized as Holstein or other. If a herd was comprised of 70% or more Holstein it was coded as a Holstein herd. If there was more than 30% of other breeds (Jersey, Brown Swiss, Milking shorthorn, Guernsey, Ayrshire etc) comprising the herd it was coded as other. Linearity of continuous predictors with the outcome were assessed visually using lowess smoothers. To address non-linearity, quadratic terms were assessed using a p-value cut-off of ≤ 0.05. If a quadratic term was significant and adequately described the relationship between the predictor and the outcome (identified by the lowess smoother), then it was included in the model. If this did not work the variable was
categorized. The predictor of percent positive milk was categorized into three groups (0-2%, 2-4%, >4%). Main effects variables in the model were checked for interaction by inclusion of two-way interaction terms between variables significant in the univariable analysis. All hypothesized two-way interactions were tested in a multivariable model. Statistically significant interaction terms (p<0.05) were considered to be evidence of interaction, and were retained in the final model. Main effects variables were considered significant and included in the final model if p<0.05. Confounding was assessed throughout model development, following stepwise removal of non-significant (>0.05) variables in the multivariable model. Variables were retained in the model regardless of their p-value, if their removal from the model resulted in a change of more than 20% in a model coefficient. Pearson residuals were calculated for each covariate pattern and residuals were explored for outliers and unusual patterns and associations. The overall fit of the model was assessed with the Hosmer–Lemeshow test, with the data divided into g = 10 groups (Hosmer and Lemeshow, 2000).

A Receiver Operating Characteristic (ROC) curve was created and the area under the curve (AUC) was estimated to quantify overall test performance. The optimum cut-off value for the Hyper ELISA was investigated and was determined by means of the ROC, a plot of sensitivity (y-axis) by 1 – specificity (x-axis) (Dohoo et al. 2009).

4.3 RESULTS

4.3.1 Herds and Cows

A total of 309 bulk milk tank samples were collected. Overall, there were 305 herds tested which had production records accessible from CanWest Dairy Herd Improvement. There were 20,443 individual animals tested from the 309 herds. One large herd (900+ lactating cows) was removed from analyses because it did not reflect the average herd size (~70 cows) that is
common in Ontario and hence was considered an outlier. The mean of the 304 herds included in this analysis was 64 cows (range 15-381). Graphical representation of the distribution of herd sizes is shown in Figure 4.1. Within-herd prevalence and corresponding bulk milk OD’s are presented in Figure 4.2. The correlation of these measures is 0.60. The majority of the 304 herds had no test positive cows (233/304) and of the positive herds the majority of them only had one test positive cow (45/74). Of the 20,443 cows tested 16 tested high, 120 were positive and 36 suspect ELISA scores. Descriptive statistics of the herd level variables between positive and negative Hyper ELISA herds are presented in Table 4.1.

When bulk milk samples together with individual milk samples from all the lactating cows from 304 dairy herds were analysed for presence of MAP antibodies, the bulk milk ODₖ results were found to range between -0.07 and 1.36, and the prevalence in the herds varied between 0 and 11.49%.

4.3.2 Logistic Regression

Predictor variables collected in the study are summarized according to the Hyper ELISA bulk tank result and presented in Table 4.1. Variables significant in the univariable analysis are presented in Table 4.2 along with their p-values. The variables that were significant in the univariable analysis were average percent protein for the test month (%) (PMP), average test day milk yield per cow (kg) (TDM), presence of a high titre (HT) cow (yes/no), the categorized variable of percent of positive milk contributed to bulk tank on test day (PPM), average milk value (AVM), average yearly somatic cell count (YRSCC), average days in milk (DIM) and percent of cows in first parity (PFP). The final variables included in the multivariable model were PMP, PPM, and breed was forced into the model since it acted as a confounder. Despite testing all possible interactions between variables that were significant in the univariable
analysis, the final multivariable model included only two main effect variables. The p-values and odds ratios are displayed in Table 4.3 for the model. The Hosmer-Lemeshow goodness of fit test for this model was not significant (p-value = 0.6910) suggesting good model fit. The variables significant in the final multivariable model were PMP and PPM (Table 4.3). Herds with higher PMP values are more likely to be positive on the Hyper ELISA test and herds with increasing PPM were more likely to be positive on the Hyper ELISA compared to herds with low PPM.

4.3.2 ROC Analysis

The sensitivity and specificity of the Hyper ELISA bulk tank test was analysed at different cut-offs and values for sensitivity, specificity and overall diagnostic potential as indicated by the area under the curve (AUC) are presented in Table 4.4 for different herd status criteria based the number of individual cows testing positive (1, 2+ or 3+). Test characteristics are presented at cut-off values of 0.8, 0.1, 0.15 and 0.2. The largest AUC (0.8072) value is found when the herd criteria is greater than two cows positive on the milk ELISA. Point estimates of sensitivity and specificity are shown in Figure 4.3 for the herd criteria of one or more cows being milk ELISA positive.

4.4 DISCUSSION

In this study, 304 herds were used to analyse the relationship between herd level factors based on individual and bulk tank milk and bulk milk Hyper ELISA optical densities. The individual test results were translated into a herd-level result, which enabled comparison of the bulk milk test result to the total of individual milk ELISA results. The contribution of milk from the cows with higher milk antibody levels to the bulk tank was expected to influence the outcome of the bulk milk test. The model confirms that the milk tank composition affects the outcome of the bulk milk test in particular, the milk yield from cows that are positive on the milk
ELISA and the protein level of the bulk tank. In this study, herds that had a one percent increase in bulk protein levels were approximately 10.02 times more likely to have a positive Hyper ELISA bulk tank test result. Cross-reactivity of antibodies with the ELISA test is one possibility that can explain this association. The problem of mutual cross-reactivity between antibodies (false positive reactions), which occurs when animals encounter atypical mycobacteria, remains to be solved (Nielsen, 2002). The ELISA targets antibodies in the bulk tank, which can only come from cow milk and are the result of a variable and delayed humoral response to MAP infection (Stabel, 2007). The bulk tank is milk contributed by all cows in the herd with differing antibody levels to different pathogens. Consequently, there could be the presence of cross-reacting antibodies in the bulk tank from non-infected cows in the herd.

The categorized variable of the percent of milk contributed to the bulk tank by milk ELISA positive cows were found to be statistically significant in the final logistic model (Table 4.3). This association comes as no surprise because the milk ELISA cows will contribute milk with a higher level of MAP antibodies compared to a herd with no test positive cows. This association has been documented with other ELISA tests (Frössling et al, 2006).

Depending on the producer or veterinarian’s purpose for testing, results could be followed up by repeated bulk milk testing, or individual milk sampling. The herd status based on individual milk testing cannot be considered a gold standard, regardless of what herd cut-off is applied because the individual milk ELISAs have low sensitivities (Hendrick et al, 2005). However, using two test positive cows as a herd standard, as indicated by the AUC in the ROC analysis gives the best diagnostic potential for the Hyper ELISA test in this study.
It has been shown that bulk tank milk can be used to identify antibodies to infectious agents such as viruses (Elvander et al., 1995; Armstrong and Mathew, 2001), bacteria (Vanzini et al., 2001; Veling et al., 2001) and parasites (Kloosterman et al., 1993; Frössling et al., 2006). Although many examples exist for bulk milk testing for other diseases, only a small amount has been published for MAP (Nielsen et al., 2000; van Weering et al., 2007; Wilson et al., 2010). Nielsen et al. (2000) concluded that the technical performance of an ELISA performed on bulk tank samples was not adequate to provide a tool for surveillance. Wilson et al. (2010) used the same Hyper ELISA protocol for bulk milk testing to determine herd level prevalence and determine the sensitivity of a bulk tank test in a true positive herd. However, in Wilson et al., herd level factors were not collected or analysed and the definition of a positive herd was one which had at least one of four positive bulk tank milk results on either an ELISA or PCR designed for bulk tank milk. They concluded that the utilization of bulk milk sample testing is a practical way to screen dairy herds for presence of MAP. In our study, the level of MAP antibodies in bulk tank milk moderately correlated (r=.60) with the prevalence of antibody positive cows in the herds, however it was lower compared to that in the study of van Weering et al (2007).

As shown in Table 4.4, higher cut-off for the Hyper ELISA that corresponds with a higher specificity of 97.39% bulk milk samples is higher than the cut-off of .10 defined by the manufacturer. By increasing the number of cows positive to define the herd infection status increased diagnostic potential somewhat as shown by the AUC.

Bulk-tank milk testing for diseases is always affected by disease prevalence, intermittent shedding of pathogens and the possible increased dilution of antibodies if uninfected cows produce more average milk than infected cows. Analysis of bulk tank milk samples is a relatively
inexpensive approach for detection and prevalence estimation of different pathogens in dairy herds. MAP ELISA applied to these bulk tank milk samples can provide data on herd exposure and MAP prevalence estimations. Milk sampling is much more convenient than fecal or blood sample testing for any disease of dairy cattle. Bulk milk is easy to collect and it covers most of the herd population. However, a bulk milk sample excludes non-lactating individuals and cows that do not contribute to the bulk milk because they are diseased or treated. It is a simpler method than individual serum sampling and is also harmless to the cows. Furthermore, the producers themselves can collect and submit samples to the laboratory for testing.

These results are consistent with previous studies that report a lower correlation between MAP antibodies in the bulk milk and prevalence of antibody positive cows (Nielson et al, 2000). A probable reason for this could be that not only the proportion of infected cows but also their antibody levels and milk yield influence the bulk milk result. This is likely a reflection of the Ontario dairy herd population which consists of smaller herds that have few cows contributing to the bulk tank milk as well as larger herds with a lower prevalence of infection.
Figure 4.1. Graphical representation of the range of herd sizes comprising the study (milking cows only).
Figure 4.2. Bulk milk test results in relation to within herd prevalence of MAP as measured by a Hyper ELISA (R=0.60) (Negative Hyper ELISA ODs plotted as zero).
Table 4.1. Descriptive summary (Mean)(SD)(95% CI) of predictor variables between Hyper ELISA positive and negative herds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>POS</th>
<th>NEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of milking cows tested</td>
<td>61.96(37.92)</td>
<td>64.54(47.79)</td>
</tr>
<tr>
<td></td>
<td>(51.83-68.79)</td>
<td>(58.41-70.93)</td>
</tr>
<tr>
<td>Average age at calving</td>
<td>1450.18(172.77)</td>
<td>1474.59(171.03)</td>
</tr>
<tr>
<td></td>
<td>(1406.22-1469.08)</td>
<td>(1454.03-1497.82)</td>
</tr>
<tr>
<td>Average test day milk yield per cow</td>
<td>22.5(4.42)</td>
<td>23.37(4.59)</td>
</tr>
<tr>
<td></td>
<td>(21.41-23.59)</td>
<td>(22.76-23.93)</td>
</tr>
<tr>
<td>Average test day protein yield per cow</td>
<td>.73(.14)</td>
<td>.75(.14)</td>
</tr>
<tr>
<td></td>
<td>(0.69-0.76)</td>
<td>(0.72-0.76)</td>
</tr>
<tr>
<td>Total test day milk yield</td>
<td>1755.54(1150.63)</td>
<td>2035.69(2458.08)</td>
</tr>
<tr>
<td></td>
<td>(1470.28-2040.81)</td>
<td>(1715.29-2112.05)</td>
</tr>
<tr>
<td>Total test day protein yield</td>
<td>56.60(37.06)</td>
<td>64.93(79.95)</td>
</tr>
<tr>
<td></td>
<td>(47.41-65.78)</td>
<td>(54.62-67.12)</td>
</tr>
<tr>
<td>Average test day SCC linear score</td>
<td>3.01(0.64)</td>
<td>2.90(0.60)</td>
</tr>
<tr>
<td></td>
<td>(2.84-3.16)</td>
<td>(2.82-2.98)</td>
</tr>
<tr>
<td>Average days in milk</td>
<td>187.16(38.48)</td>
<td>179.87(32.32)</td>
</tr>
<tr>
<td></td>
<td>(176.51-194.91)</td>
<td>(176.22-184.66)</td>
</tr>
<tr>
<td>Percent of herd in first parity</td>
<td>36.87(10.88)</td>
<td>34.94(8.59)</td>
</tr>
<tr>
<td></td>
<td>(34.06-39.54)</td>
<td>(33.81-36.03)</td>
</tr>
<tr>
<td>Average Monthly Bulk Protein</td>
<td>3.38(0.21)</td>
<td>3.32(0.19)</td>
</tr>
<tr>
<td></td>
<td>(3.33-3.44)</td>
<td>(3.29-3.34)</td>
</tr>
<tr>
<td>Monthly Average Bulk Tank SCC</td>
<td>265.30(106.03)</td>
<td>257.28(103.51)</td>
</tr>
<tr>
<td></td>
<td>(238.31-293.48)</td>
<td>(244.71-271.62)</td>
</tr>
<tr>
<td>Average Milk Value</td>
<td>5582.50(914.57)</td>
<td>5799.28(953.92)</td>
</tr>
<tr>
<td></td>
<td>(5348.57-5829.09)</td>
<td>(5683.35-5928.85)</td>
</tr>
<tr>
<td>Average Yearly SCC</td>
<td>280.85(113.98)</td>
<td>260.86(99.67)</td>
</tr>
<tr>
<td></td>
<td>(250.87-311.29)</td>
<td>(247.71-271.62)</td>
</tr>
</tbody>
</table>
Table 4.2. Summary of predictor variables and their significance in a univariable model for the probability of a herd being positive on a Hyper ELISA bulk tank milk test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average monthly BT protein (%)</td>
<td>4.62 (1.173-18.186)</td>
<td>0.029</td>
</tr>
<tr>
<td>Average test day milk yield per cow (kg)</td>
<td>0.960 (.903-1.020)</td>
<td>0.188</td>
</tr>
<tr>
<td><strong>Percent milk yield from positive cows (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2%</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2-4%</td>
<td>7.479(2.963-18.880)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;4%</td>
<td>25.243(5.263-121.070)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>High Cow Present</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>11.540 (2.965-44.910)</td>
<td>0.013</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Days in Milk</td>
<td>1.005(0.998-1.013)</td>
<td>0.135</td>
</tr>
<tr>
<td>Percent of herd in First Parity</td>
<td>1.022(0.0156)</td>
<td>0.137</td>
</tr>
<tr>
<td>Average Milk Value</td>
<td>1.000(0.999-1.001)</td>
<td>0.115</td>
</tr>
<tr>
<td>Average Yearly SCC</td>
<td>1.535(0.891-2.646)</td>
<td>0.123</td>
</tr>
</tbody>
</table>
Table 4.3. Summary of predictor variables significant in the final multivariable logistic regression model for the probability of being positive on a Hyper ELISA bulk tank milk test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percent milk yield from positive cows (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2%</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2-4%</td>
<td>7.23 (2.763-18.945)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;4%</td>
<td>28.34 (5.611-143.136)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Average Monthly Bulk Protein (%)</strong></td>
<td>10.02 (1.393-72.029)</td>
<td>0.022</td>
</tr>
<tr>
<td><strong>Breed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holstein</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Other</td>
<td>0.38 (0.094-1.589)</td>
<td>0.188</td>
</tr>
</tbody>
</table>
Table 4.4. Sensitivity and specificity of the Hyper ELISA for bulk milk samples (n=304) at four different cut-offs and three different herd criteria for positive herd status (between brackets the number of herds fulfilling the criterion)

<table>
<thead>
<tr>
<th>Herd Criteria**</th>
<th>Cut-off= 0.08</th>
<th>Cut-off =0.10</th>
<th>Cut-off= 0.15</th>
<th>Cut-off= 0.20</th>
<th>AUC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Se(%)</td>
<td>Sp(%)</td>
<td>Se(%)</td>
<td>Sp(%)</td>
<td>Se(%)</td>
</tr>
<tr>
<td>1+(74)</td>
<td>63.51</td>
<td>83.04</td>
<td>55.41</td>
<td>90.00</td>
<td>40.54</td>
</tr>
<tr>
<td>2+(29)</td>
<td>72.41</td>
<td>76.36</td>
<td>65.52</td>
<td>83.64</td>
<td>48.28</td>
</tr>
<tr>
<td>3+(13)</td>
<td>69.23</td>
<td>73.54</td>
<td>61.54</td>
<td>80.76</td>
<td>46.15</td>
</tr>
</tbody>
</table>

*Per herd criterion the AUC is given as an indicator of diagnostic potential of the test
**Herd Criteria for positive herd
1+ = one or more cows positive on milk ELISA
2+ = two or more cows positive on milk ELISA
3+= three or more cows positive on milk ELISA
Figure 4.3. Point estimates of sensitivity (Se) and specificity (Sp) of a Hyper ELISA, at various cut-off levels, when used to detect antibodies to MAP in bulk milk from Ontario dairy herds (n=304)
REFERENCES


Collins, M.T. 2002. Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using likelihood ratios. Clinical and Diagnostic Laboratory Immunology. 9: 1367-1371


Chapter Five: GENERAL SUMMARY AND CONCLUSIONS

5.1 CONCLUSIONS

The strategy of using bulk tank milk samples as a means of surveillance for Johne’s disease is not common in the Canadian dairy industry. The utility of bulk milk testing has been studied with conflicting results (van Weering et al., 2007; Wilson et al., 2010). The concern is how well these tests perform when compared to herd level prevalence estimates based on individual cow testing, and hence whether they accurately reflect the level of infection in the herd. The conditions and length of time milk samples can be stored for subsequent testing has not been studied thoroughly. Alinovi et al. (2009) looked at the processing of serum for MAP detection. Sample storage for one week at -20 °C resulted in significantly lower Johne’s ELISA scores, regardless of the handling method, compared to samples stored at 4 °C for one week. There is presently not any published literature on the effect of storage duration and temperature on milk samples and Johne’s ELISA result. In an unpublished pilot study, that included a small sample size of 104 milk samples, there were no statistically significant differences between testing on days 1, 7 and 10 and between storage conditions which included freezing, room temperature, incubated and refrigeration (Burns and Kelton, personal communication).

The goal of this thesis was to determine the utility of bulk tank tests for detecting the presence of Johne’s disease in a dairy herd and the effect of storage duration and temperature on milk ELISA results. This project was divided into two distinct research components, one was the bulk tank study and the other, the time and temperature study.

The first research project component comprised a trial that ran from March 2010 to December 2010. The primary objective of this study was to determine the feasibility of testing bulk tank samples as a surveillance method for Johne’s disease. This study utilized the individual
cow milk ELISA results from 309 herds participating in the OJEMAP, as well as the testing of bulk tank milk samples from these participating farms, using both a modified milk ELISA (Hyper ELISA) and a proprietary PCR test. This study found the relative sensitivity and specificity of the bulk tank Hyper ELISA test, when a positive herd was defined as 1 or more positive cows, was 54.7% and 90.6%, respectively. When two or more positive cows defined a positive herd, the relative sensitivity increased to 63.3% while the specificity decreased to 84.2%. The relative sensitivity of the Hyper ELISA used on bulk milk samples in the present study was similar to values reported previously for other bulk tank tests (Biswas et al., 2008; Wilson et al., 2010). The PCR test did not perform as well as expected and only identified three herds with cows that were ELISA positive for Johne’s disease. To further investigate the influence of herd level factors on the bulk tank ELISA test result, a logistic regression model was developed with the outcome being the probability of a herd testing positive on the Hyper ELISA protocol. After controlling for breed, herds with a higher protein level had higher odds of testing positive for MAP on the Hyper ELISA than herds with a lower level of protein. Also, the proportion of positive milk (milk volume contributed to the bulk tank by ELISA positive cows) was associated with a higher probability of a herd testing positive. The correlation of the bulk tank result to within herd prevalence was 0.60 and was lower than previous reports (van Weering et al, 2007). The AUC was also evaluated at different cut-offs of the Hyper ELISA and at different levels of herd criteria. The highest AUC was given at a herd criterion of two or more milk ELISA positive cows in the herd.

The second component of this project was to evaluate the effect of time and temperature on the milk ELISA test result. This component of the study took place from June 2010-December 2010 and involved a total of 178 positive and negative Johne’s milk samples collected
over this period through DHI laboratory. Concordance correlation coefficients were calculated for each day compared to the initial result from day one. These values showed moderate correlation with values ranging from 0.734-0.918. The final mixed linear model indicated that time was significantly associated with the log transformed ELISA result. When results were dichotomized and compared to the initial result the maximum number of samples that were misclassified were in the refrigerated treatment group, and the total number misclassified across all testing days was eight. This represents 4% (8/178) of the total samples. It was concluded that this small percentage of misclassified samples is negligible from a practical standpoint.

5.2 THESIS PROJECT LIMITATIONS

There were several factors in the design and execution of this research project that may limit the overall findings of this study. The milk ELISA that is performed on individual cows is known to have a low sensitivity (Hendrick et al, 2005). The use of this method as the definition of the gold standard could underestimate the prevalence of cow level infection used as indicators of herd status, and therefore could underestimate herd level infection. On the other hand, if the goal is to identify herds with milk ELISA positive cows, rather than Johne’s infected cows, then this misclassification is less important. Hence the utility of the bulk tank test must be viewed in context of the intentions of testing.

Study herds were selected without prior knowledge of the Johne’s disease status of each herd. Although bulk tank testing did not identify all positive herds, the importance of bulk tank testing for herd level surveillance should not be overlooked. If herds had been purposively selected based on previous Johne’s results rather than a convenience sample based on voluntary involvement results might have been different.
In the second component of the research project only one sample was tested for each day and in each temperature group. In doing this, there was no way to evaluate the possibility of plate to plate and sample to sample variability. In future studies, running multiples of the same milk sample each day would be more helpful in determining the effect of this variability on the ELISA results.

5.3 FUTURE RESEARCH

Although this body of work has confirmed the use of the Hyper ELISA protocol in a field setting, it has led to several questions that should be further investigated. In this study, a relationship between the probability of a positive Hyper ELISA result and average monthly bulk protein levels was observed. No previous study has reported an association between bulk tank results and bulk tank protein levels. Based on the nature of the association, future research is required to determine factors influencing the strength of this association. In further investigations of the test performance of this bulk tank milk test, follow up testing could be done on herds that test positive on the bulk tank test but have negative individual cow results to see if indeed there may be a possibility of cross reactions within the bulk tank milk.

The use of bulk milk in this study was not adequate to provide a tool for herd surveillance. The ease of collection of this milk makes it desirable to find a method that is satisfactory for this purpose. Future research should aim to determine a method to pool individual milk samples coming into the DHI laboratory for monthly analysis. Optimum pool sizes for herd size should be determined. This proposed method could be a viable option for surveillance in the future, instead of the bulk milk fraction.
5.4 IMPLICATIONS

Based on these results, the Hyper ELISA protocol proves to be a satisfactory test to screen herds in order to estimate the infection level in a population of dairy herds. The sensitivity was poor and did not meet the standards that a good diagnostic test should have in order to monitor individual herds. These findings show that a more sensitive bulk tank test is needed if surveillance by bulk tank is to be performed. Sampling handling of Johne’s milk can have an adverse effect on the classification of the ELISA result. Transporting and storing milk samples should be consistent throughout the research study period. But this difference was deemed negligible because misclassification only happened when results were near the cut-off value of the ELISA. This variation is expected in any test around the respective cut-off which classifies results as either positive or negative for the presence of antibodies.
REFERENCES


