MEASUREMENT OF THE ENZYME *N*-ACETYL-β-D-GLUCOSAMINIDASE FOR DETECTION OF SUBCLINICAL MASTITIS IN A RANDOM SAMPLE OF BULK TANK MILK IN PRINCE EDWARD ISLAND

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ABSTRACT

The objective of this study was to compare the fluorometric and the colorimetric methods of *N*-Acetyl-β-D-Glucosaminidase (NAG) measurement in bulk tank milk (BTM) as indicators of subclinical mastitis in dairy cattle. Over a four month period a random pool of 120 BTM samples was selected from the regular collection of 520 fluid milk producing herds on Prince Edward Island (PEI). The levels of the enzyme activity in the BTM determined by fluorometry (NAG(F)) and colorimetry (NAG(C)) were compared with the somatic cell count (SCC), as the most frequently used indicator of subclinical mastitis in dairy cattle.

The fluorometric method was preferred to the colorimetric method in this study, for its simplicity and rapidity. These two methods gave similar results and were positively correlated in BTM, r=0.845. The value of NAG(F) may be predicted by NAG(C) by the following equation: NAG(F)=1.43 + 0.639(NAG(C)).

Observation of an effect of time in frozen storage on NAG activity was not significant for the fluorometric method and was significant for the colorimetric method in this study. Enzyme activity determined by the fluorometric method was less affected by days in storage.

The pattern of the frequency distributions of NAG and SCC in BTM were distinctly different. Linear regression analysis required the log transformation of the SCC data. Linear regression of $\log_e$(SCC) on NAG resulted in the model $\log_e$(SCC)=3.44 + 0.522(NAG(F)). Alternatively, the linear score (LS) somatic cell count model was LS=1.32 + 0.753(NAG(F). Both models were statistically significant r=0.719, ($p<0.001$). The fluorometric method of NAG measurement had a stronger association with SCC than the colorimetric method. A SCC threshold $\geq$200,000 cells/mL as the definition of subclinical mastitis in BTM was applied to the data. This had the effect of separating the data (n=117) into disease positive (D+) and disease negative (D-) groups. The "relative" sensitivity and specificity of NAG(F) was summerized in a receiver operator characteristics curve. The means (SD) for the D- group (n=52) were: NAG(F) 3.2 U/mL (0.621), and SCC 130,000 (42,820). The D+ group (n=65) means (SD) were: NAG(F) 4.4 U/mL (0.882), and SCC 433,400 (195,000).

The range of NAG(F) in the BTM collected in this study was 2-7 U/mL. Threshold value NAG(F)> 3.2 U/mL may be considered an indicator of the onset of subclinical mastitis. Results of this study suggest that the fluorometric method may be applied in a manual test for subclinical mastitis.

DEDICATION

I dedicate this work to my family and friends for their love and support throughout. This alone made it all worthwhile.

" Love your neighbour as yourself." Love does no harm to its neighbor. (Romans 13: 9,10. NIV)

Speak and act as those who are going to be judged by the law that gives freedom, because judgement without mercy will be shown to anyone who has not been merciful. Mercy triumphs over judgement. (James 2:12,13 NIV)

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ABBREVIATIONS

AVC	Atlantic Veterinary College
-β-	Beta
BTM	Bulk tank milk (sample)
BTSCC	Bulk tank somatic cell count
^{o}C	Degrees celsius
CM	Composite milk
D+	Disease positive
D-	Disease negative
DCL	Diagnostic Chemicals Limited
DHI	Dairy Herd Improvement corporation
DIM	Days in milk
ELISA	Enzyme-linked immunosorbent assay
F	Fluorometric method
g	Centrifugal force
HCl	Hydrochloric acid
IQR	Inter-quarter ratio
kg	Kilogram
L	Liter
LS	Linear score somatic cell count
log	Logarithm
μL	Microliter
M	Molarity
MeUNAG	Methylumbelliferyl-N-acetyl-β-D-glucosaminide
mg	Milligram
mM	Millimolar
min	Minute
mL	Milliliter
n	Number of samples
nm	nanometer
NAG	N-Acetyl-β-D-Glucosaminidase
NAG(C)	NAG determined by colorimetry (PNP)
NAG(F)	NAG determined by fluorometry
NAG(TRF)	NAG determined by the treated-fluorometric method
NSERC	Natural Sciences and Engineering Research Council of Canada
p.	Page
%	Percent
PEI	Prince Edward Island
PMN	Polymorphonuclear leukocyte
PNP	Para-nitro-phenol

PNP-NAG	Para-nitro-phenyl-glucosamide
r	Correlation coefficient
ROC	Receiver operator characteristics curve
SCC	Somatic cell count in cells/mL
SD	Standard deviation
sec	Second
SEM	Standard error of the mean
TRF	Treated-fluorometric method
U	Unit of activity
$\geq$	Greater than or equal to
$<$	Less than

1. GENERAL PROBLEM OF MASTITIS

1.1 Introduction

1.1.1 Mastitis reduces milk production and quality

Milk producers are concerned with controlling the cost of production. Improving udder health and fertility are major factors that increase income in the dairy industry (Shook, 1989). Mastitis, a common inflammatory disease of the udder, has direct negative economic impact, due to decreases in milk yield and quality, and increases in farm management costs. Mastitis levels in herds can be monitored by analysis of the cell count in milk. Disease detection aids management by determining a herd's current health status and allows disease trends to be followed over time (Reneau, 1986b). A general summary of loss from mastitis gives estimates from $140 to $300 per cow per year (Gill et al, 1990). The high costs of mastitis are the result of treatment of clinical cases, replacement of diseased animals, decreased milk quality and yield, loss of genetic material due to culling and increased labour (Reneau and Packard, 1991).

Lucey and Rowlands (1984) found that a reduction in 305-day milk yield is greatest when clinical mastitis occurs before peak yield, with higher-producing cows having proportionately higher reduction. Blosser (1979) concluded that reduced milk yield constituted 60 percent of the total cost of mastitis. The costs imposed on producers vary with the frequency, forms, duration and severity of the disease. In addition to reduced milk production, mastitis often forces early culling. Dohoo et al.

(1984b) found that clinical mastitis during the first 150 days of lactation increases by a factor of 80 the odds that a cow would be culled. High producing herds cull more cows for mastitis than herds with lower yield (Fetrow et al, 1988). Rearing heifers to replace cows removed from production adds significant costs that are not always considered in estimates of the losses due to mastitis. As much as 15 percent of total culls in a dairy farm may be attributed to mastitis. This estimate is conservative because cows culled for low production due to subclinical infection or post-clinical disease are not included in this group (Fetrow et al, 1988).

Mastitis control procedures are primarily based on hygiene and prevention of mastitis or at least its limitation to within acceptable levels (Fetrow and Anderson, 1987). Acceptable levels are currently defined by the somatic cell count (SCC) because studies have indicated that a decrease in milk yield is associated with a concurrent increase in cell count (Leslie et al, 1983). This fact may therefore be used to predict production losses that occur as a result of subclinical mastitis in herds. The range of the cell count may be broken down into levels that can then be used to describe the percentage of individual cows within a SCC category. The linear score (LS) transformation of SCC is commonly used in mastitis monitoring and is represented by the equation:

$$LS = [\log_e(SCC)/\log_e(2)] - 13.61.$$

This method of reporting SCC was introduced by Shook (1982) because it showed the strongest association between the SCC test and yield losses. In general, a herd average LS not greater than 3 (mean of 100,000 cells/mL of milk for 80 percent of

the herd) is recommended (Fetrow and Anderson, 1987). One unit increase in the natural logorithm cell count was associated with a reduction in daily milk yield of 0.65 to 1.44 kg (Dohoo et al, 1984a; Raubertas and Shook, 1982). Mastitis monitoring serves as a guide post or indicator of the level of prevention. The costs associated with preventive measures are comparatively low with respect to the cost of the disease and have significant positive impact on farm profitability (Philpot, 1984; Fetrow and Anderson, 1987).

Subclinical or 'carrier state' mastitis, is inapparent. Philpot (1984) states that subclinical mastitis is responsible for 70 percent of economic losses resulting from a reduced milk production potential of herds. In general, subclinical mastitis is 15 to 40 times more prevalent than the clinical form and can be of long duration (Philpot, 1984). Raubertas and Shook (1982) described a loss of 187 kg of milk production per lactation with each doubling of the geometric mean somatic cell count/mL of milk for cows in second lactation or greater.

Milk production loss due to mastitis is estimated by comparing actual milk yield with an expected level of production (Schalm and Noorlander, 1957). Predicted yield losses are related to bulk tank milk somatic cell count (BTSCC) by linear regression that is relative to a "normal" BTSCC of 200,000 cells/mL (Eberhart et al, 1982). Mathematical models for composite samples with confounders for differences in age, days in milk (DIM), herd and herd size demonstrate milk production changes in categories that are stratified by log SCC or LS (Raubertas and Shook, 1982; Jones et al, 1984; Tyler et al, 1989). Losses may be converted to dollar equivalents by

estimating the expected value of the predicted milk yield (using the price of milk and the predicted yield of a percentage of the herd in each LS category) and subtracting the actual yield value (according to the number of cows grouped by age and stage of lactation) (Hueston and Heider, 1986). Total losses can be calculated from this herd profile using SCC or LS to estimate subclinical mastitis (Fetrow and Anderson, 1987).

There are two classifications generally applied to mastitis organisms: Major pathogens (*Streptococci, Staphylococcus aureus*, and coliforms); and Minor pathogens (*coagulase-negative Staphylococci, Micrococci,* and *Corynebacterium bovis*). Major pathogens are associated with visible changes in milk that occur in clinical mastitis. The SCC, is greater, in general, as the percentage of major pathogens isolated in milk rises. In a Virginia study of Dairy Herd Improvement (DHI) tested herds, only 5.9% of milk samples with SCC less than 100,000 cells/mL contained major pathogens associated with mastitis. There was a 12% isolation rate of major pathogens from composite milk samples with a SCC of 100,000-200,000, 17-19% from samples with a SCC of 200,000-400,000 and 23% from milk samples with a SCC over 400,000 (Jones et al, 1984).

Classification of subclinical mastitis by LS was recommended by Shook in 1982 because the direct relationship between production losses and LS assists interpretation (Fetrow et al, 1988). The rise in SCC associated with declining milk yield in cases of subclinical mastitis is usually accompanied by a rising bacterial infection level (Jones et al, 1984). After many attempts to define mastitis using SCC and bacteriology, the SCC alone was found to be a good predictor of pathogen status

of milk and, therefore, of subclinical mastitis in the cow (Reneau, 1986a). Since 1984, DHI analysis uses the LS on composite milk (CM) samples (a combined sample of milk from all four quarters of a cow) to classify individuals with subclinical mastitis (Hueston and Heider, 1986; Fetrow and Anderson, 1987). Generally, 80% of bacteriologically acceptable CM samples have somatic cell counts under 200,000 cells/mL. A LS of 5 in composite milk is interpreted as infected (Reneau, 1986b). Fetrow (1980) suggests that a threshold of 283,000 cells/mL provides a reasonable level for monitoring mastitis control programs in herds of average mastitis prevalence. The strength of the prediction using LS = 5 is better with repeated testing. Similarly, an individual cow with a count less than 100,000 cells/mL is usually not infected (Jones et al, 1984). Cows with LS of 5 are likely infected with a virulent pathogen (Philpot, 1984; Kirk, 1984; Dohoo et al, 1981). Counts between these two extremes (LS 3 to 5) are considered to be developing an infection (Jones and Heald, 1982). Shook (1982) found that the LS of individual cow SCC and the total herd count strongly related to the (predicted) prevalence of pathogenic infections.

The monthly SCC DHI corporation results are summarized by group based on age and stage of lactation. Complete herd SCC evaluation for mastitis status includes: bulk tank and individual cow (composite milk sample) SCC reports; culture results; and clinical mastitis records (Reneau, 1986a,b). While qu rter sampling for subclinical mastitis is more accurate because ≈80 percent of infected cows are affected in only one quarter (Reneau, 1986a), it is considered too expensive to offer in current monitoring programs.

1.1.2 The herd as a factor in disease detection

The prevalence and incidence of mastitis on a farm are governed by microbial population, dynamics of herd immunity, genetics, environment (Feichmuth, 1975) and management factors (diet, hygiene, teat dip, selective dry cow therapy) (Leslie et al, 1983; Gill et al, 1990; Tolle, 1975; Bodoh et al, 1976; Dohoo and Meek, 1982). Resistance or susceptiblity to mastitis depends in part upon individual genetic makeup. Susceptibility also depends upon age and stage of lactation, anatomical makeup and immunological and cytological status of the mammary gland (Tolle, 1975).

A common SCC threshold for the detection of mastitis in all herds may be unreliable (Sheldrake et al, 1983). Evaluation of SCC data for the purposes of mastitis control is assisted by individual herd interpretation (Lindstrom et al, 1981; McDermott et al, 1982). Relative SCC thresholds that reflect herd prevalence may also be of merit (Reneau, 1986b).

1.1.3 The somatic cell count of the bulk tank milk sample as an indicator of the level of mastitis in the dairy herd.

The BTSCC appears to be the most useful test to date in identifying potential microbial contamination of milk in herds. It also assists in predicting shelf-life of the finished product, estimates yield losses and acts as a monitor for prevalence of mastitis. The ability of one level of testing such as BTSCC, to predict infection levels in another level, quarter infection rate or composite milk SCC, presents an additional challenge for mastitis researchers (Hueston and Heider, 1986; Reneau, 1990; Reneau and Packard, 1991). Bulk milk SCC $\geq$ 200,000 cells/mL was related to the % herd production loss and prevalence of mammary infection in a study by Eberhart et al. (1982). Estimates of up to 6% quarter infection rate for BTSCC 200,000 cells/mL, 16% quarter infection rate and 6.5% of herd production loss for BTSCC 500,000 cells/mL, and 32% quarter infection rate and 17.7% of herd production loss for BTSCC 1,000,000 cells/mL were reported (Eberhart et al, 1982). In a recent study of Ontario herds, Godkin (1989) showed that factors such as herd size or milk volume may influence the ability of bulk tank (BT) culture, as a screening test, to detect some organisms. Herd size, was significant when used in simple regression models to determine the predictive value of BT culture for the prevalence of pathogens. Variation in the BT count for coliforms and *Streptococci agalactiae* had a coefficient of determination of .11 and .33 respectively. This amount of variation in the ability of the bacterial count in the bulk tank milk (BTM) sample to predict the prevalence of cows infected in the herd, was explained by herd size and percent cows positive

for the organism studied (Godkin, 1989).

Bulk tank milk is representative of the herd and its samples are considered an indicator of milk quality. Milk samples are analysed monthly for composition, bacterial content, freezing point and SCC in government laboratories. Bulk tank milk SCC determination is considered to be an economical, reliable and specific test that acts as an indicator of herd level mastitis. The BTSCC test is used to monitor mastitis because of a strong relationship between cellularity in milk and prevalence of the disease (Reichmuth, 1975; Eberhart et al, 1982). Usually, however, only a small proportion of the herd contributes most of the cells (Britt, 1987) and an infected quarter is partly masked by the dilution effect of the healthy ones (Leslie et al, 1983; Kirk, 1984). This relationship may be exacerbated by a compensatory increase in yield by uninfected quarters in some cows (Woolford, 1985). The estimated percentage of quarters infected varies between herds as a result of differences in severity of infection and mean milk yields within herds (Westgarth, 1975). The accuracy of this SCC estimate is improved with successive counts for trends over time, which overcome the cytological count differences within farms because of the variation in herd size (Reichmuth, 1975) and yield differences (Emanuelson and Funke, 1991).

Part of the decrease seen in recent years in SCC may be attributed to the concurrent increase in production. The degree of association between prevalence of mastitis and BTSCC is variable. Emanuelson and Funke (1991) reported an overall mean of herd prevalence of mastitis at 26.7%, corresponding to the overall mean

BTSCC of 204,000 cells/mL. Within this study, the average herd prevalence increased within BTSCC level. The increased production resulted in a dilution that masked the 'true' increase in BTSCC. Both inflammatory and non-pathological factors contributed to this effect. The prevalence factor accounted for 43% of the variation in BTSCC when herd average milk yield was included in the model. For this reason, BTSCC serves as a general monitor for herd management. Higher yielding herds may need to lower the BTSCC goal in order to control subclinical mastitis.

It may be possible to give a more definite diagnosis of mastitis by using more than one screening test at a time on a milk sample (Kitchen et al, 1984b). On a per farm basis, BTSCC or equivalent BT test can serve as a major indicator of the prevalence of mastitis (Emanuelson and Funke, 1991). Bulk tank milk samples can be analysed for antibodies to *Staphylococcus aureus* by an enzyme-linked immunosorbent assay (ELISA). This ELISA screening test can then be used on BTM samples to predict herd prevalence of mastitis (Jones, 1991). Herds with average BTSCC linear scores greater than 3.0 should be followed-up with individual tests to identify infected cows and types of pathogens (Jones, 1991).

The mean SCC for composite milk of a herd approximates that of the BTSCC (Schultz, 1975). Composite milk SCC testing identifies an individual cow's SCC contributions to the herd's milk (Schultz, 1975). The most detailed individual cow data routinely collected is the monthly sampling program carried out by the DHI corporation. Animals under treatment for clinical mastitis recorded as "condition affecting record" (CAR code) (Bartlett et al, 1991) are not included in the mastitis

estimate. This selection bias tends to underestimate the level of mastitis in a herd.

1.2 Methods of Detection of Subclinical Mastitis in Bovine Milk

1.2.1 The somatic cell count

The purpose of cell counting in milk is to determine the amount of pathological secretion in the milk supply (Schmidt Madsen, 1975). The first method available since 1900 to determine the total cell count is the manual microscopic count of milk cells in a dry stained smear. This method is tedious and subject to errors (Heeschen, 1975). Mechanization of the procedure, electonic particle counting, using the Coulter principle permits individual cells to be counted as the only particles of a certain size range after disruption of fat globules. The current method of cell counting by the Fossomtic instrument developed in 1974 represents an advancement to direct cell counting. Fluorescing nucleated cells dyed with Ethidium bromide (dilution 1: 20) emitt a signal detected by a photomultiplier and recorded as a cell count. A standard particle suspension is used to ensure the precision and accuracy of the results. Heeschen (1975) found the Fossomatic instrument to be the superior method of cell counting based on its ease and practical operation, as well as higher rate of sample throughput.

The inflammatory response of the mammary gland to microbial infection can be monitored by compositional changes in bovine milk. Leukocytes constitute the main defense mechanism of the udder once the microbe has entered the teat cistern

(Paape, 1979). The rapid recruitment of inflammatory cells via the blood stream into the gland and subsequently into the milk is usually beneficial to the host and serves to eliminate the offending organism(s) (Jain et al, 1972; Opdebeeck, 1982). This inflammatory process however may also affect the integrity of the cells in the mammary gland. Neutrophils and macrophages are the most numerous phagocytic cells in milk during subclinical mastitis (Azzara and Dimick, 1985); experimentally induced neutropenia results in conversion of chronic staphylococcal mastitis into an acute gangrenous form (Schalm et al, 1976).

The infectious status of the ruminant mammary quarter is the most important factor affecting SCC. The resulting inflammation and cellularity is quantified by the SCC test in quarter, composite and BT milk samples. The recognition of possible confounding factors enables a more accurate determination of mastitis in SCC analysis (breed, age, stage of lactation, herd and season) (Reichmuth, 1975). Since differences in methods, time of collection, storage and handling of the milk sample can alter the SCC, standardization of SCC procedure is essential for correct interpretation (Heeschen, 1975; Leslie et al, 1983; Heald, 1982; Heeschen et al, 1978).

Economic considerations favour detection of subclinical mastitis on a more frequent basis and therefore the development of a rapid, simple, less expensive technique.

1.2.2 The lysosomal enzyme *N*-Acetyl-β-D-Glucosaminidase (NAG) in bovine milk

A milk test that offers additional sample information to the SCC test and increases the reliability of mastitis detection is needed (Miller and Schultz, 1981). Monitoring changes in enzyme levels of body fluids is a well established methodology for diagnosing pathological conditions in humans and animals (Kitchen, 1978). *N*-Acetyl-Glucosaminidase (2-acetamido-2-deoxy-β-D-glucosamide acetamidodeoxyglucohydrolase, E.C.3.2.1.30, NAG) is one of the many hydrolytic glycosidases involved in the catabolism of glycoproteins, glycolipids and glucosaminoglycans (Calvo et al, 1982). It is found in the lysosomes of peripheral blood leukocytes and inflammatory cells as well as cells of the kidney, testes, liver, submaxillary gland and many other tissues (Pugh and Walker, 1960). The first recorded measurement of NAG in milk was made by Mellors (1968) using a colorimetric method. The introduction of NAG as a monitoring tool for mastitis is based on the fact that changes in SCC and NAG in quarter milk samples are closely related (r= 0.86) (Kitchen, 1978; Kitchen and Middleton, 1976; Emanuelsson et al, 1987). The fluorometric assay is superior in its ability to measure an absolute level of the end product and is without the problem of background 'noise' that is encountered in the colorimetric method using p-nitrophenyl-N-acetyl-B-glucosaminide as substrate (Kitchen, 1978). The fluorometric method is more rapid (Kitchen, 1978) and is now used as the standard for NAG determination. The rate of neutrophil recruitment into the udder during the initial phase of mastitis and the rate of

recognition of bacteria by phagocytes determine the disease outcome of the host (Nickerson, 1986). Compared to other tissues, phagocytosis in the mammary gland is less effective because of impaired function of polymorphonuclear leukocytes (PMNs) in milk, a result of the phagocytosis of lipid particles (Paape, 1979; Vecht, 1985). Phagocytic cells release hydrolytic enzymes during phagocytosis or cell death (Slauson and Cooper, 1990). Macrophages (Schnyder and Baggiolini, 1978) and PMNs (Baggiolini and Dewald, 1985) release the NAG enzyme. The rapid release of NAG upon zymosan stimulation as well as with the addition of detergent have been reported in milk (Kaartinen et al, 1988). In clinical enzymology, the release of NAG is linked to phagocytic activation and can be used as a predictor for mastitis (Kaartinen et al, 1988). Timms (1984) found that log transformation of milk sample NAG data was the best predictor of milk yield when both tests are performed on the same day (test-day). Timms (1984) also reported the log transformation of SCC as the best predictor of infectious status based upon positive bacteriological isolation. During inflammation, increases in NAG and phagocytic cells, primarily PMNs, are associated with severity of mastitis, virulence of bacteria (Mattila et al, 1986) and loss of integrity of epithelial cells (Capuco et al, 1986; Mattila et al, 1986).

The use of NAG enzyme levels as a quarter milk sample screening test to select cows for SCC and bacterial culture can reduce the total number of samples assessed by these more costly methods (Ball and Greer, 1991). The response of NAG as an indicator of cellular damage differs with the pathogen type (Timms, 1984; Mattila et al, 1986). The NAG enzyme has been identified as a species-specific

enzyme for *Streptococcus* isolated from cows with mastitis (Schaufuss et al, 1986).

According to Timms (1984) NAG enzyme is relatively stable in raw milk and is unaffected by freezing based on evaluation of internal milk standards. Therefore, sample handling is believed to be less critical than for SCC (Mattila et al, 1986). Retained NAG activity in frozen milk can be useful when testing is not readily possible, although there are small differences between the fresh and frozen milk NAG measurements (Sandholm and Mattila, 1986). Another advantage is that NAG in milk is less responsive than SCC to non-infectious sources of variation such as environmental stess and returns to normal ranges within 24 to 36 hours after stimulation has ceased (Wilson et al, 1991). In addition to being a simple and inexpensive test, NAG may not require a sterile milk sample (Kitchen, 1976) and may be a useful cost saving tool when applied as a screening test for quarter milk samples in problem herds (Ball and Greer, 1991). Other advantages of NAG enzyme test are related to rate of sample throughput, ease of handling, and cost. The fluorometric method of detecting NAG (Leaback and Walker, 1961; Kitchen et al, 1978) has been adapted for a rapid 96-well microplate determination (Mattila, 1985; Linko-Lopponen and Makinen, 1985). This technology combined with computerized analysis of NAG inter-quarter ratios (IQRs) determines the level of inflammation. In quarter milk sampling the IQR is used to eliminate variation due to confounding factors, such as age and stage of lactation (Sandholm and Mattila, 1986). The rationale for the application of IQR variation in the analysis of inflammatory markers in milk is to identify the portion of variation from pathological sources from that related to normal

physiological sources. Non-pathological factors are assumed to affect all four quarters equally (Knudsen and Jensen, 1990). An IQR is calculated by dividing quarter NAG measurements per cow by the lowest NAG value of the individual. The quarter with the lowest NAG serves as a baseline reference for the individual cow. The difference in NAG measurement between the lowest and the highest quarter can also be used to identify mastitic glands (Fox et al, 1985).

1.2.3 Factors affecting NAG measurement in milk

Many types of cells contribute to the NAG activity in milk, including fibroblasts, leukocytes and mammary epithelial cells. During inflammation, leukocytes actively produce and secrete NAG as part of phagocytosis and release it upon cell lysis (Capuco et al, 1986; Kaartinen et al, 1988). Lysed phagocytes, epithelial cells and fibroblasts may contribute the enzyme, NAG, but do not contribute to the SCC and can result in discrepancies between NAG and SCC test results. Other mammary cells, resident macrophages and bovine blood plasma are also believed to be a source of NAG (Kitchen et al, 1978; Kitchen et al, 1980). Serum-derived sources of NAG in milk increase during inflammation and pregnancy. It is well established that serum and urinary NAG increase with the progression of pregnancy in humans (Woollen et al, 1961; Lombardo et al, 1984), during lactation in the milk of the pregnant guinea pig (Roets et al, 1989), and during pregnancy and lactation in murine mammary tissue (Van Hekken and Eigel, 1986). Lombardo et al, (1984) has suggested that

NAG increases throughout pregnancy can be used to predict its regular course in women. This information has not yet been applied to NAG levels in the dairy cow who is in progressive stages of pregnacy during much of her lactation. Instead, the pregnancy and lactational effect is described in part as the 'stage of lactation effect' on milk NAG levels. The IQR provides a means to control this source of variation in NAG analysis by comparing quarters within the same cow (Miller and Paape, 1988). However, an interaction between NAG due to inflammation and NAG as a result of the gestational/lactational effects makes the interpretation of a disease effect more difficult in milk testing (Timms and Schultz, 1985; Mattila and Sandholm, 1985).

Two major forms of the enzyme, A and B, a third form, the human P-isoenzyme for pregnancy; two intermediate forms, I_1 and I_2; and a serum form, S have been identified (Pierce et al, 1978). The A and B forms have been isolated from the bovine mammary gland (Kitchen and Masters, 1985). Douglas (1981) found that increases in human serum albumin and IgG increase the secretion of the lysosomal enzyme, hexosaminidase, from placental villi in a dose and time-dependent manner. It is suggested that endocytosis may be a trigger mechanism for secretion of the lysosomal iso-enzyme, hexosaminidase (Hex A) during pregnancy. Secretion of a lysosomal enzyme can be correlated with experimental phagocytosis of microspheres by PMNs and can be measured by the liberation of fluorescent substrate 4-methylumbelliferone (Suzuki et al, 1986).

Many mastitis-causing bacteria may themselves be additional sources of NAG (Maddocks and Greenan, 1975; Robison, 1984; Slifkin and Gil, 1983; Watts, 1988;

Mathews et al, 1991; Mattila et al, 1986). Whether the identity of a pathogen may be suggested by the rate of change in NAG measurement in milk, has not been determined. Milk NAG activity fluctuates in mild subclinical mastitis and tends to increase and stabilize in more severe subclinical cases, reflecting bacterial pathogenicity (Mattila et al, 1986). In conclusion, NAG in milk has been shown to vary with the severity of mastitis, the virulence of bacteria, the stage of lactation and the host defence ability (Nagahata et al, 1987a). The NAG test has also been shown to be affected by age and pregnancy in other species.

Standardization of sampling and processing methods is needed for accurate NAG determination because differences exist between frozen and fresh milk samples and between fractions of milk (Kaartinen et al, 1988). While skimmed milk fraction NAG level is not affected by the addition of detergent to the sample, the more cellular fractions (cream and sediment) are altered by the addition of detergent (Kaartinen et al, 1988). Temperature of storage affects NAG release from cells; storage at 4°C stabilizes the distribution between fractions (Kaartinen et al, 1988). Release of NAG from within the cellular compartments in milk is enhanced by the addition of 2 percent Triton X-100, a freeze-thaw cycle, or stimulation of cells by opsonized zymosan or endotoxin (Kaartinen et al, 1988).

1.3 The Objective of This Study

The primary objective of this study was to compare the fluorometric and colorimetric methods of NAG measurement as indicators of subclinical mastitis in the bulk tank milk sample. In order to meet this prime objective, the purposes of this study were 1) to determine the correlation between the fluorometric and colorimetric methods of NAG measurement in milk; 2) to determine the range of NAG activity present in bulk tank milk; 3) to relate NAG to SCC in a mathematical model inorder to interpret the NAG test with SCC as the standard for detection of subclinical mastitis. This work was designed as a preliminary step in the development of a manual NAG test for milk.

2. COMPARISON OF COLORIMETRIC AND FLUOROMETRIC METHODS OF NAG MEASUREMENT IN BULK TANK MILK

2.1 Introduction

The activity of the lysosomal enzyme, *N*-Acetyl-β-D-Glucosaminidase (NAG; EC 1.2.3.30) can be determined by the fluorometric method using 4-methylumbelliferyl *N*-acetyl-β-D-glucosamide (4MeUNAG) (Leaback and Walker, 1961; Dance et al, 1969; Kitchen, 1976) and by the colorimetric assay method using p-nitrophenyl-*N*-acetyl-β-D-glucosamide (PNP-NAG) as substrates (Leaback, 1963; Mellors, 1968; Horak et al, 1981). Comparison to NAG determined by the fluorometric method (the standard) is used to determine the acceptability of new colorimetric methodology (Noto et al, 1983; Yuen et al, 1984).

Determinations of NAG activity in urine have been used to detect renal disturbances in patients with diabetes, hypertension, rejection of renal transplants, and nephrotoxicity. Optimal conditions for the assay in urine have been described (Tucker et al, 1975; Thomas, 1969; Maruhn, 1976). The automated fluorometric assay for urine was achieved more than a decade ago (Tucker et al, 1975). A manual colorimetric method for the quantification of NAG in urine exists as a test kit with instructions for automation (Price, 1979). More recently, a manual assay has been developed for use in clinics on a portable miniphotometer in which the freeze-dried colorimetric substrate turns red in NAG containing urine (Yuen et al, 1984). The elimination of a sample blank permits the direct reading of urinary NAG without the

need to account for unwanted background 'noise' of the sample. Many substrates for the colorimetric assay have been developed for use in urine (Makise et al, 1988; Noto et al, 1983; Yuen et al, 1982).

The first determination of NAG in bovine milk by the colorimetric method was reported by Mellors (1968) in Guelph, Ontario. In the last decade, the fluorometric NAG measurement in milk has been used as a diagnostic test for bovine mastitis (Kitchen, 1978; Mattila, 1985). This method is sensitive in its ability to accurately measure levels of enzyme activity and rapid as it offers the advantage of being applied to raw (untreated) milk samples (Kitchen, 1981).

The objective of this study was to compare the fluorometric and colorimetric NAG assay methods for the detection of subclinical mastitis as preliminary work on the development of a manual NAG test for milk. To meet this objective, the purpose of this study was to determine the correlation between the fluorometric and colorimetric methods of measurement of levels of NAG enzyme activity in a randomly selected pool of BTM. In addition, an observation was made of an effect related to the time the sample was frozen at -70°C on the NAG level in milk.

2.2 Materials and Methods

2.2.1 Sample collection and SCC

Over a four month period in the fall of 1988 a pool of 120 milk samples was randomly selected from the routine collection of BTM at the Prince Edward Island

(PEI) Provincial Dairy Laboratory, Charlottetown, PEI. This pool of BTM samples represents ≈ 20% of the 520 fluid milk producing herds on PEI. Two samples were discarded as a result of a labelling and recording error. In Chapter 3 an additional sample was identified as an unusual result with respect to SCC and subsequently removed from the data.

The BTSCC was determined at the PEI Dairy Laboratory using standardardized methodology on the Fossomatic cell counter (Foss Electric, Hillerod, Denmark). Before processing, the milk samples were warmed to 40°C and mixed as per the SCC procedure. The BTM samples were held overnight at 4°C and transported the following morning on ice to the University of PEI for further processing and NAG determinations (Figure 1). The milk samples were warmed to room temperature and vortexed lightly for 5 sec initially and between aliquots. Multiple aliquots (15-20) of 1.3 mL were made from each BTM sample and stored at -70°C in 1.5 mL polyethylene microtubes.

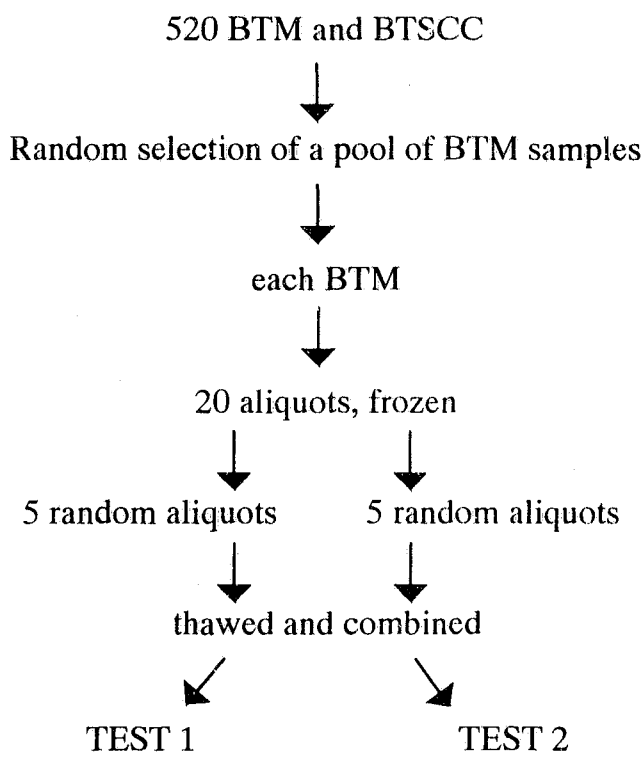
Standards for the fluorometric and colorimetric NAG assays were prepared by mixing soluble NAG enzyme (Jackbean, Sigma Chemical Co., St.Louis, MO, USA) at concentrations of 0, 2.5, 5.0, 7.5, 10.0 U/mL in milk from a normal cow. The purpose of preparing NAG standards in the presence of normal milk components rather than buffer was to compensate for the unknown effect of milk on the assay. The criterion used to ensure the quality of the base milk was a SCC of 34-40,000 cells/mL. The base milk was collected during the study period and stored at -70°C in 30 mL vials until test day. Uniform base milk ensured consistency in the

preparation of the standards which were made up daily and accompanied each run. The validity of the data depends on the assumption that the NAG standards within the runs were sufficient for quality control for the determination of test sample NAG activity levels.

On test day, randomly chosen groups of 5 aliquots per BTM were thawed at room temperature and combined. Each BTM sample and standard was performed in duplicate and accompanied by a sample blank. The resultant NAG measurement was the mean of the duplicates after subtraction of the sample and reagent blanks. The conversion to NAG activity in U/mL was interpolated from the standard curve of the run (Appendix A).

The preparation of the milk and the application of the colorimetric NAG (NAG(C)) assay was performed on the same day and on the same test sample following the fluorometric NAG (NAG(F)) determination. A flow diagram of the experimental design is shown in Figure 1.

Figure 1. Flow Chart



2.2.2 NAG Determinations

Fluorometric Method

The lysosomal enzyme activity, NAG, was determined fluorometrically by the method of Kitchen et al. (1980) using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MeUNAG) (Sigma Chemical Co., St. Louis, MO.) as substrate in citrate buffer (2.25 mM in 0.25 M citrate buffer, pH 4.60). This method was developed from the original assay by Leaback and Walker (1961) and modified by Kitchen (1976). The fluorometric method measured the generation of 4-methylumbelliferone (4MeU) as the fluorescent end-product of hydrolysis. The NAG activity was reported as the mean of duplicate measurements and determined to be proportional to the absorbance of the liberated end-product, after correction for the absorbance of the sample blank. The reading was compared to the standard curve of the run that accompanied the unknown test samples.

In the fluorometric procedure the thawed milk samples (100 μL) were mixed with 400 μL of substrate, 4MeUNAG, at pH 4.60. After 15 min incubation in a waterbath at 37°C, the reaction was stopped by raising the pH with the addition of 1.5 mL of 0.2 M glycine buffer, pH 10.7 (Appendix B). A reagent blank (RB) was included in each run. The samples' NAG measurements were read without delay. The fluorometric intensity of 4MeU was measured on a Perkin Elmer 204 fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT, USA) at excitation and emission wavelengths of 355 nm and 460 nm respectively. The NAG measurement

was determined as the activity units (U/mL) predicted by interpolation using the standard curve of the run (Appendix A).

Colorimetric Method

On test day, the test BTM subsamples were thawed at room temperature, combined and NAG(F) determined using the raw/thawed milk sample. The same combined BTM sample was then prepared for colorimetry by the addition of 50 μL of chloroform per mL of raw/thawed milk and 35 μL of 1 N HCl to lower the pH to 4.50. The mixture was centrifuged at g = 16,000 for 8 min in a microcentrifuge to clarify the milk sample. The supernatant was decanted into new test tubes and used as the new test material for the colorimetric assay (Appendix E).

The NAG(C) method using the reagent, 6.2 mM para-nitrophenyl-glucosaminide (PNP-NAG) (Sigma Chemical Co., St. Louis, MO., USA) in 0.25 M citrate buffer, pH 4.50 as substrate was mixed with the sample supernatant (Kitchen, 1976). In the colorimetric procedure 0.75 mL of the prepared test material was mixed with an equal volume of substrate reagent (6.2 mM PNP-G in 250 mM citrate buffer, pH 4.50). After 15 min incubation in a waterbath at 37°C, the reaction was stopped by the addition of 0.5 mL of 2 M glycine buffer, pH 11.3. The samples and blanks were centrifuged for 5 min at g = 16,000 in the microcentrifuge and the supernatant decanted into new test tubes. The absorbance of PNP generated from the reaction was read on a Hewlett Packard Diode Array Spectrophotometer (Model 8452) with an absorbance wavelength set at 405 nm. The resultant colored PNP of

the unknown test samples, corrected for the sample blank, were read against the NAG standards described in Appendix A to determine the NAG content. The result represented the mean of duplicates minus the sample and reagent blanks.

Treated-Fluorometric Method

The fluorometric assay method was applied to the supernatant of the prepared milk sample to observe the biochemical effect of altering the raw (thawed) milk on NAG enzyme levels. The altered fluorometric test was called the 'treated-fluorometric' (TRF) method, in this study.

The experiment consisted of paired testing of the original BTM sample's NAG measurement using the fluorometric, treated-fluorometric and colorimetric assays. All three methods were performed on the same day and on the same reconstituted (Test 1) BTM sample that had been frozen and thawed once. For the purposes of evaluation of the colorimetric (C) assay, the fluorometric (F) method served as the standard for NAG measurement in milk.

2.2.3 Observation of an effect of the difference of days in frozen storage (DDF) on NAG methods of measurement in bulk tank milk.

The series of 3 NAG assay methods were repeated on 70 of the original pool, test 2 (see Figure 1). The second BTM subsample was made by combining a group of 5 aliquots for use as a duplicate pair. The entire pool of 118 BTM samples was used for the comparison of paired samples determined by fluorometry and for the NAG(F) and NAG(C) comparison. A pool of 70 BTM was used to examine the

difference between paired samples within the TRF and C methods. The difference in days in frozen (DDF) storage (-70°C) ranged from 10 to 18 days and was recorded as the factor DDF. The second NAG measurement (NAG-2) was subtracted from the first NAG measurement (NAG-1) to quantitate the difference between paired subsamples, delta NAG, for each method. Regression analysis was used to evaluate the effect of DDF on the change in NAG levels determined by the fluorometric method :

delta NAG(F) = (NAG(F-1) minus NAG(F-2))

for each BTM sample pair. The observation of an effect of DDF on NAF(C) and NAG(TRF) were assessed in a similar manner.

2.2.4 Statistical analysis

Statistical analysis consisted of linear regression, correlation, analysis of variance and descriptive statistics computed using the Minitab statistical package (version 7.1).

Analysis of variance (ANOVA) was used to test the effect of different methods of analysis on NAG measurement in milk. The difference between the means of NAG measurement between the methods were isolated by the Student Newman Kuels (SNK) test (Glantz, 1987).

The fluorometric, colorimetric and treated-fluorometric methods were repeated as test 2 of the pool of samples (n=70). A difference between pairs for

each method (delta NAG) was observed by subtracting test 1 NAG level from the level in the second subset, test 2. The paired t test was used to determine the significance of delta NAG for each method. Regression analysis was used to determine the effect that frozen storage as a factor had on the difference between pairs in NAG measurements for the F, C and TRF methods.

2.3 Results

2.3.1 Comparison of NAG methods

The comparison of the test 1 NAG activity levels determined by the colorimetric (C) and fluorometric (F) methods, is expressed by the regression equation:

$$NAG(F) = 1.43 + 0.639(NAG(C)) \qquad (1)$$

($F = 284.61, (p<0.001), n=118$). Conversely, the regression of NAG(C) on NAG(F) yielded the equation:

$$NAG(C) = -0.484 + 1.11(NAG(F)). \qquad (2)$$

The NAG(F) and NAG(C) are highly and positively correlated ($r = 0.845$). The comparison of the data for NAG(F) and NAG(C), shown as a scatter plot in Figure 2, illustrates the small differences in the levels of NAG enzyme activity levels between methods.

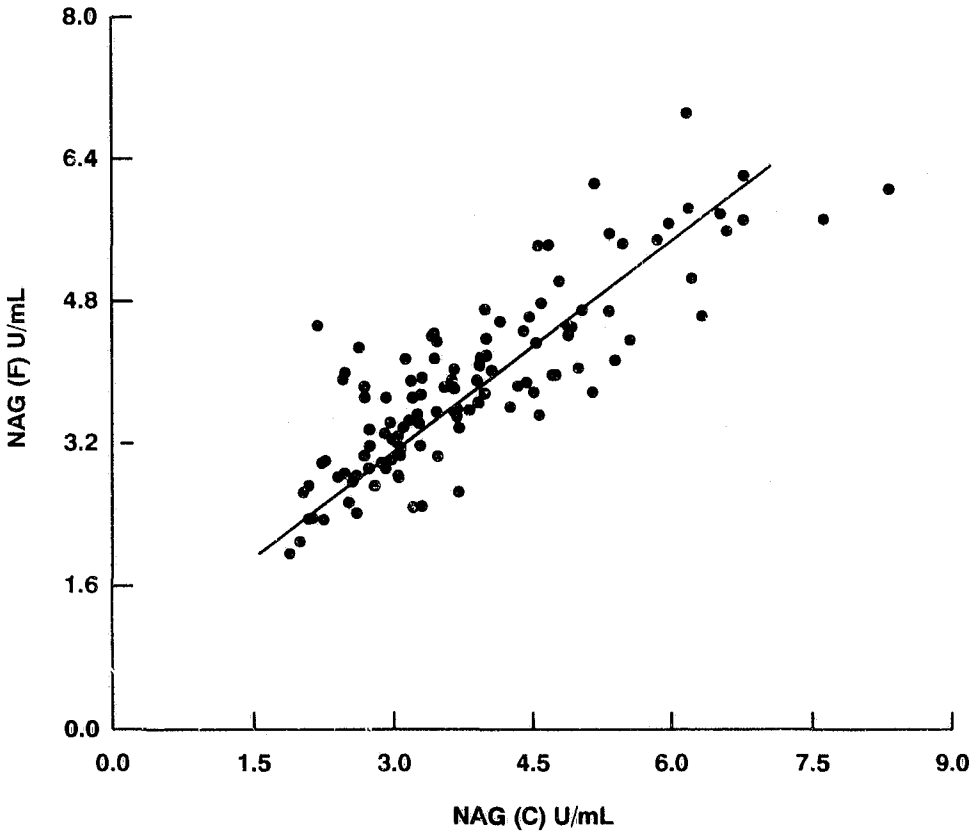


Figure 2. Scatter plot of relationship between the colorimetric NAG and fluorometric NAG measurements in paired samples of a random pool of bulk tank milk (n = 118).

Similarly, the observed relationship of the NAG(F) and NAG(TRF) measurement for a subset of 70 samples is expressed by the equation;

$$NAG(F) = 0.981 + 0.61(NAG(TRF)) \qquad (3)$$

($F = 173.47$, $p < 0.001$) and can also be described by the equation:

$$NAG(TRF) = 0.222 + 1.18(NAG(F)) \qquad (4)$$

with $r = 0.847$, ($n=70$). The linear regression analysis of NAG(TRF) and NAG(C) is expressed by the equation:

$$NAG(TRF) = 1.05 + 0.928(NAG(C)) \qquad (5)$$

($F = 559.27$, $p< 0.001$) and $r= 0.944$, ($n=70$). The high r-value proves that the prepared milk NAG methods are highly positively correlated.

The summary of the comparison of NAG results (means and SEM) for the F,C and TRF methods in Table I shows small differences between the colorimetric and fluorometric methods and a larger difference in NAG measurement in the altered method, the treated-fluorometric group. The difference is also shown in Figure 3. The frequency distribution of NAG activity determined by the colorimetric, fluorometric and treated-fluorometric protocols depicting the differences between methods of pairs of BTM samples is shown in Figure 4.

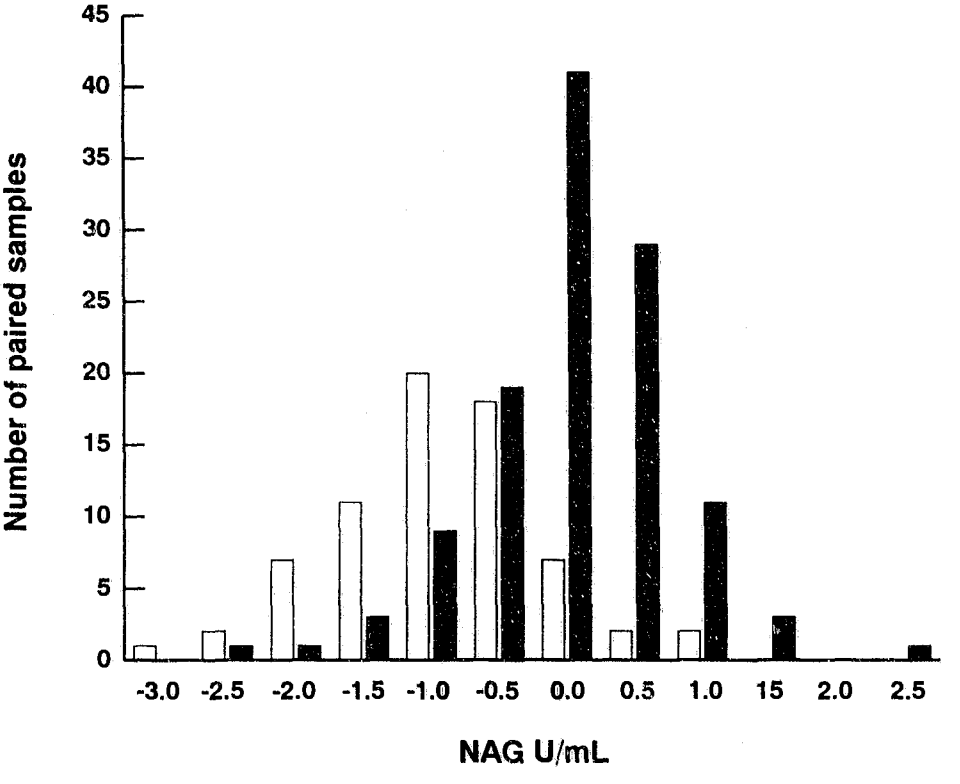


Figure 3. Differences in NAG U/mL between fluorometric NAG measurement and treated-fluorometric methods (solid bar) (n=70), and fluorometric NAG and colorimetric NAG in paired bulk tank milk samples (open bar) (n=118).

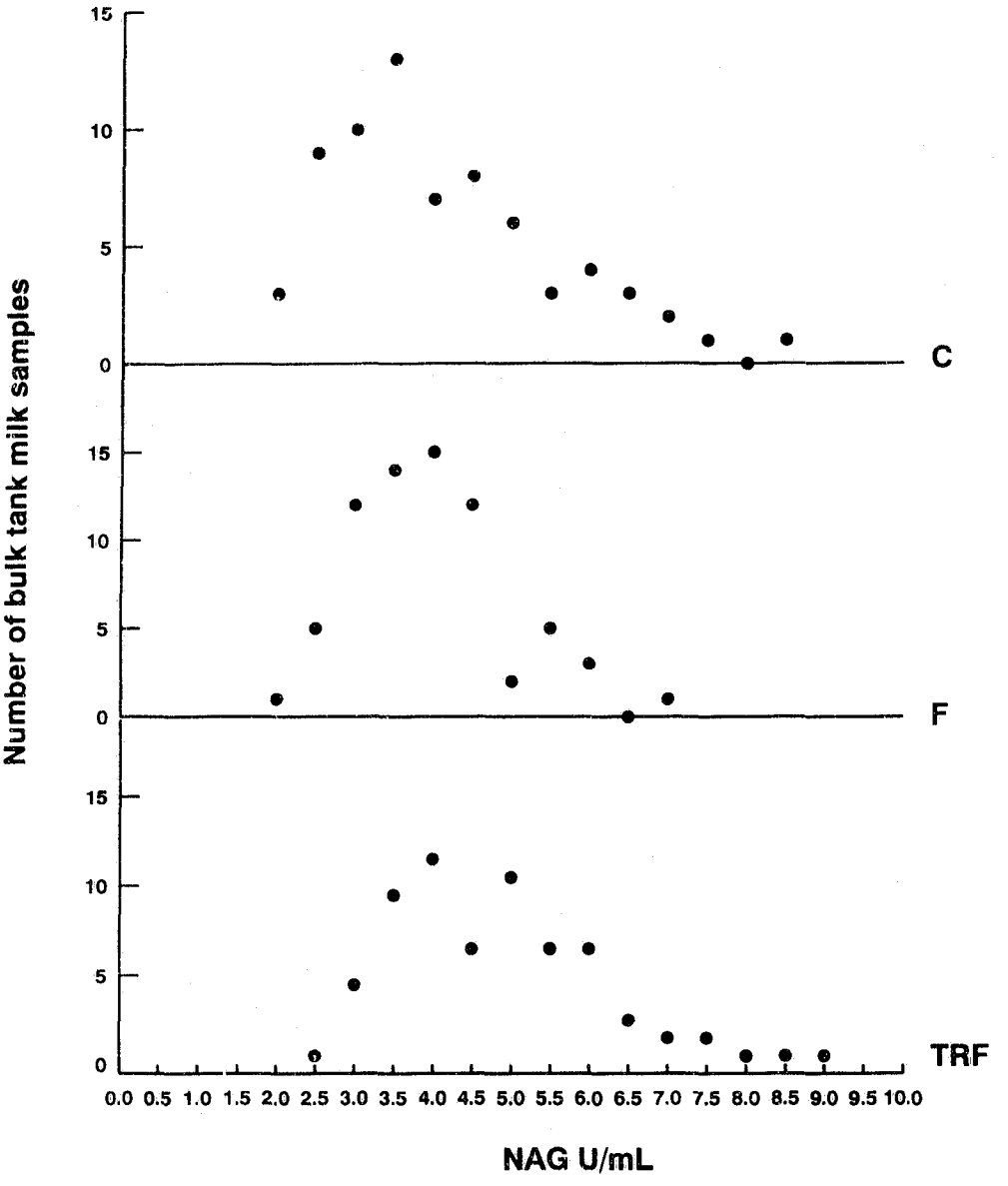


Figure 4. Frequency distribution of NAG activity determined by the colorimetric (C), fluorometric (F) and treated-fluorometric (TRF) methods in paired bulk tank miik samples (n = 70).

Table I. Methods of NAG Measurement: Mean and SEM

Method	n	Mean U/mL	SEM
Fluorometric (F)	70	3.9641	0.168
Treated-F (TRF)	70	4.8842	0.119 *
Colorimetric (C)	70	4.1319	0.165

* significant difference ($p < 0.05$) from F and C Methods

Analysis of variance (ANOVA) was used to test the effect of NAG(F), NAG(C) and NAG(TRF) treatments on NAG measurement for a subset of 70 BTM samples. The global hypothesis of "no effect" yielded a significant statistical relationship with $F = 71.23$ ($p<0.001$), ($n=70$). The comparison of the means of the treatments in Table I was made to determine if the fluorometrically determined NAG was statistically different from the colorimetric measurement.

The differences between the means for NAG activity levels between the F, C and TRF methods were isolated by the Student Newman Kuels (SNK) test (Glantz, 1987). The differences between the NAG(TRF) and NAG(F) gave a $q= 16.207$. The SNK test for differences between means for NAG(TRF) and NAG(C) was $q= 12.966$. Both were significantly different at $\alpha = 0.05$, ($n=70$) (Table I).

The differences between means for the fluorometric and colorimetric NAG determinations were, however, not statistically significant with a SNK test with $q = 2.897$ at $\alpha = 0.05$ (Table II). There is no significant statistical difference in NAG(F) and NAG(C) measurements. Correlation coefficient for NAG(F) and NAG(C), test 1 ($n=118$) of BTM pool of samples is expressed as $r= 0.845$. The linear models for NAG conversion between methods are shown in Table II.

TABLE II. Summary of Methods: linear regressions, p and r values.

(1) NAG(F) = 1.43 + 0.639(NAG(C))	($p < 0.001$), $r = 0.845$
(3) NAG(F) = 0.981 + 0.611(NAG(TRF))	($p < 0.001$), $r = 0.847$
(5) NAG(TRF) = 1.05 + 0.928(NAG(C))	($p < 0.001$), $r = 0.944$

(1) (n=118); (3) and (5) (n=70).

2.3.2 Observation of an effect of the difference in days in frozen storage as a factor in paired BTM sample NAG levels.

An assessment of the effect that the difference in days held in frozen storage, as a factor, may have had on the difference in NAG activity between paired samples was made. In order to do this, it was first necessary to determine if NAG by the first fluorometric determination (NAG(F-1)) was significantly different from NAG by the second determination (NAG(F-2)), that is, delta NAG(F). Delta NAG(F) computed for paired samples was indeed statistically significant ($p<0.05$, paired t-test, n=70). The mean and standard error of the mean (SEM) in NAG activity determined by fluorometry for test 1 and test 2 are 3.964 (0.119) and 3.858 (0.136), respectively.

TABLE III. The difference in mean values of NAG activity by method between pairs of BTM samples.

	Delta Mean U/mL	SEM
Fluorometric	0.1060	0.05
Colorimetric	0.002	0.104
Treated-Fluo.	0.082	0.10

(n=70)

To assess the contributing effect that time in frozen storage may have made to the difference between paired NAG(F) values (delta NAG), the factor, difference in days of frozen (DDF) storage, was used in linear regression analysis. The linear regression equation may be expressed as:

$$\text{delta NAG(F)} = -0.137 + 0.014(\text{DDF}) \qquad (6)$$

with an associated $F = 2.96$, ($p < 0.090$), $r = 0.205$, ($n=70$). A portion of the difference between pairs of samples of BTM could be accounted for by the factor DDF as used in this study. Although the result was not significant at $\alpha = 0.05$ for the fluorometric NAG method, it suggests that DDF may explain a minor part of the differences in fluorometrically determined NAG activity between paired samples. The factor DDF was not statistically significant at $\alpha = 0.05$ in NAG(F) BTM.

The frequency distribution of the difference in paired samples revealed small differences within the BTM subsamples. This is one of the fundamental problems when dealing with a biologically complex secretory matrix such as milk. The

histogram of the residual differences between paired samples and DDF determined by regression analysis revealed small effects of the additional factor DDF. The paired BTM subsamples used to evaluate DDF had small differences in NAG activity. It was observed that the days in frozen storage made a small but ambiguious contribution to this difference between pairs.

In order to determine if the observable effect of DDF was repeated uniformly between methods; delta NAG(C), the difference in paired colorimetric determinations, and DDF was also examined. The linear regression equation can be described by the equation:

$$\text{delta NAG(C)} = 0.956 - 0.056(\text{DDF}) \qquad (7)$$

with an associated $F = 12.59$ ($p<0.001$) and $r = 0.392$, ($n=70$).

Similarly, for the NAG(TRF) group and DDF the regression equation was:

$$\text{delta NAG(TRF)} = 0.859 - 0.046(\text{DDF}) \qquad (8)$$

with an associated $F= 8.57$ ($p<0.005$) and $r= 0.335$, ($n=70$). Equations (7) and (8) represent the statistically significant effect of DDF on the treated milk NAG measurements. The observation was made that a small change in NAG activity was associated with time in frozen storage for the colorimetric and TRF groups. Although small, the effect was statistically significant and indicated that days in frozen storage (DDF) had an observed effect on milk NAG determined by the colorimetric method that was relatively larger than for the fluorometric method as performed in this study.

2.4 Discussion

There was no significant difference in the colorimetric and fluorometric methods of NAG measurement in BTM and the two methods were highly positively correlated, in this study. Small differences were noted between paired subsamples of BTM in NAG level determinations. Some of this effect was attributed to the difference in the number of days frozen. The observed effect of DDF could be accounted for as a confounding variable for NAG determination in frozen samples and it may be of greater import in colorimetry. A better approach to this problem would be to conduct a study on the effect of storage over time using multiple regression analysis.

The fluorometric method was applied to the prepared samples supernatants used in the colorimetric procedure, in order to make the observation of an effect of the biochemical alteration on NAG measurement. Live and frozen cells in milk are capable of releasing more NAG upon stimulation or disruption of membranes. Since phagocytes can die upon phagocytosis and release their cellular constituents this contribution is present in the soluble milk fraction when cells have disintegrated. Cells may be further disintegrated and release NAG as a result of freezing and thawing. Furthermore, the preparation of the milk sample for colorimetry may enhance the activation of the enzyme by lowering the milk pH to within the optimum range for NAG. This may have the effect of preincubating the enzyme at its optimum pH thus enhancing its activity during the assay. The differences between

methods attributable to the biochemical alteration (clarification) of the milk sample may in part represent the "latency" of the enzyme (Holtzman, 1989), the increase of the activity that occurs as a result of optimizing conditions such as inducing a conformational change in the enzyme or inducing modifiers of enzyme activity (Harper, 1975). Loss of activity due to freezing may be the result of the denaturation of the protein over time. Some of the difference may relate to substrate, equipment, handling (preincubation) and unknown effects.

Cells are unevenly distributed in milk (Mellors, 1968). This was demonstrated by the difference between paired NAG(F) subsamples of the random pool of BTM. The data demonstrate a range of effects of the chemical treatment of milk and the factor DDF on NAG levels. The speculation is that this may reflect unknown differences in cellular NAG iso-enzymes. The release of NAG is affected by storage, freezing, and cell stimulants (Kaartinen et al, 1988). The freeze/thaw cycle may have effects on the membrane bound, free or isoenzyme components of the total NAG measurent in milk. Some of the difference between paired samples may be due to the uneven distribution of cells that occurs in the emulsion (Mellors, 1968; Kaartinen et al, 1988) and therefore of the cell associated NAG of the BTM aliquots. The cellular and free portions of NAG within the paired samples may be affected differently by the freeze/thaw cycle and/or the difference may represent an altered NAG activity (cytosolic, lysosomal or membrane bound induced activity). The random selection of aliquots to create paired samples was an attempt to overcome this effect. The differences between the fluorometric and colorimetric methods may

in part be due to the rupturing of membrane bonds by HCl and chloroform used in the colorimetric method. The increased handling and the chemical preparation (chloroform and HCl) of the sample in the TRF procedure may have ruptured more cell membranes and released amounts of intracellular and/or membrane bound NAG, altered iso-enzymes, induced modifiers or decreased interference resulting in an increase in the total NAG activity level in the test samples. The procedure may enhance the activity of an iso-enzyme thus raising the activity of a portion of the total NAG. The unwanted background 'noise' in fluorometric measurement was reduced in the TRF blank. An observable positive shift of NAG activity resulted from the reduced blank values of the prepared test material. The treated-fluorometric group had an increase in NAG relative to the paired fluorometric group. The rest of the shift was unexplained but may be due to interference from other enzymes or substances in the milk.

A portion of the differences between the colorimetric, fluorometric and treated-fluorometric methods was observed to be attributed to the number of days in frozen storage which had an effect on NAG measurement in BTM. The effect of DDF was observed to be inconsistent between methods with respect to the change in the amount of NAG activity recovered from the paired BTM samples. Various isoenzymes of NAG may be differently affected by time in frozen storage and by treatment of the milk.

In conclusion, the fluorometric and colorimetric methods of NAG determination are strongly correlated in frozen samples of bulk tank milk and can be

used with equal confidence. An effect of storage time on total NAG activity level measured by the fluorometric method was observed to be minimal and not statistically significant within the limited range of days used in this study. The treated milk showed more of an effect on NAG that was statistically significant but this may only be a reflection of the small delta mean. To study an effect of time in storage, as a factor, the change in NAG level must be determined over a wider range of days and applied in a model determined by multiple regression analysis. In an assessment of the fluorometric and colorimetric NAG methods with respect to work-time and handling qualities, the fluorometric form of the test was favored for its simplicity and ease, both of which resulted in reduced work-time.

3. N-Acetyl-B-D-Glucosaminidase (NAG) AS AN INDICATOR OF SUBCLINICAL MASTITIS IN BULK TANK MILK

3.1 Introduction

The function of the glycosidic enzyme, **NAG**, and its isoenzymes remain unknown. Determination of the total NAG activity in body fluids appears useful in the subclinical detection of several pathological conditions of humans (Powell et al, 1983) and animals. Human kidney, lung and liver lysosomes are high in NAG activity (Powell et al, 1983). The presence of NAG in urine is a reliable predictor of early renal disease (Wellwood et al, 1976). Serum and urinary NAG can also be useful in the detection of the early onset of diabetes (Whiting et al, 1979).

The NAG test is at least as sensitive as the somatic cell count (SCC) in detecting subclinical mastitis (Kitchen et al, 1978; Mattila, 1985; Emanuelsson et al, 1987). Kitchen (1981) suggested that milk NAG values change as a function of the normal physiology and pathology of the mammary gland, reflecting tissue damage (Kitchen et al, 1980; Kitchen et al, 1978), increases in cell counts, and increases in serum NAG sources (Nagahata et al, 1987b). The amount of activity considered to be derived from somatic cells in the milk varied with the type of sample and the mastitis status of the mammary gland quarter (Kitchen et al, 1978). Milk NAG levels are also positively correlated with *in vitro* bacterial replication rates (Mattila and Sandholm, 1986). The NAG test in bovine milk can be applied in quarter, composite and bulk tank milk samples.

The potential use of NAG as a monitoring tool for mastitis is based upon the fact that it accurately predicts production losses at the level of the mammary quarter (Fox et al, 1985). It is a simple test for milk that can be adapted to a high capacity microtitre plate (EFLAB NAGase system) (Mattila, 1985) for rapid analysis at room temperature or 37°C (Linko-Lopponen and Makinen, 1985). Future use of computer analysis linked to the microtitre plate technology will assist monitoring for mastitis detection. Cost benefit analysis will determine its utility as a practical tool for subclinical mastitis detection in the future.

A rapid method for NAG determination in milk may permit improved monitoring of subclinical mastitis in the bovine. Given the economic impact of this disease, monitoring of clinical and subclinical mastitis may be assisted by the more rapid microplate laboratory technology and a manual NAG test for on farm testing of individual quarter milk samples. At present, the California mastitis test (CMT), an indirect cell count and pH test, is the only semi-quantitative manual test for quarter milk. Milk pH has been shown to decrease during inflammation caused by mastitis as well as during involution of the gland (Marschke and Kitchen, 1985). Over the last decade NAG determination in milk has been used as a laboratory method for the detection of mammary gland inflammation in research. It is presently being adapted for field use in the hope that it will detect subclinical disease at a level more sensitive than, or below that of the CMT.

Bulk tank milk was used in this study because it is the industrial milk sample which is farthest removed from individual cow sources of confounding variation. Bulk

tank milk NAG measurement may be confounded by herd factors such as herd size, age and stage of lactation and prevalence of disease within the herd. Other issues such as risk factors have historically been addressed by epidemiological studies (Schukken et al, 1990).

The primary purpose to this study was to determine the preferred method of NAG measurement (colorimetric or fluorometric) in detecting subclinical mastitis in milk based on the relationship to the somatic cell count. Bulk tank milk (BTM) is monitored by monthly SCC analysis for evidence of mastitis in herds and as a measure of milk quality in PEI, Canada. A randomly selected pool of BTM was used as the reference population. In this comparison, the ranges of NAG levels in BTM were determined for incorporation into a manual format of the test. The relationships of NAG(F) and NAG(C) to SCC were expressed in mathematical models using linear regression analysis. The strength of the correlation coefficients (r-value) were used to compare the fluorometric and colorimetric determinations of NAG with the corresponding SCC of the BTM samples.

3.2 Materials and Methods

3.2.1 Collection of milk samples and SCC

The BTSCC was determined at the PEI Dairy Laboratory using standardized methodology on the Fossomatic electronic cell counter. The cells in the milk sample were dyed with a dilute solution of ethidium bromide, dried and the fluorescent

emission from the stained nuclei of the cells was converted to a cell count or SCC. The milk samples used for the SCC determination were then stored overnight and transported to the Atlantic Veterinary College (AVC) the following morning, processed and NAG levels determined as reported in Chapter 2. One additional BTM sample was removed from the data as an unusual finding (outlier) and not included in this section describing the NAG test relative to SCC (n=117).

3.2.2 Statistical analysis

Statistical analysis consisted of linear regression of $\log_e$(SCC) on NAG and descriptive statistics using the Minitab statistical package (version 7.1).

3.3 Results

3.3.1 Range of NAG in a random sample of BTM

The range of NAG in BTM was determined from the frequency distribution of a randomly selected pool of BTM in PEI shown in Figure 5. The range varied from 2-7 U/mL NAG(F) within a BTSCC range of 100,000-1,000,000 cells/mL shown in Figure 6.

3.3.2 Comparison of NAG with $\log_e$(SCC)

The linear regression model for fluorometrically determined NAG (NAG(F)) and SCC test required the $\log_e$ transformation of the BTSCC data, based on the evaluation of residuals. The untransformed SCC data was seriously skewed where as the $\log_e$(SCC) was normally distributed. The non-logged SCC and NAG(F) data had an R^2 = 52.3% (r=0.723) with non-normal residuals and therefore did not meet this requirement for regression. The relationship of NAG(F) and untransformed SCC is demonstrated as a scatter plot of the data in Figure 7.

The $\log_e$ transformation of SCC represents the best fit of the data with NAG(F) to the regression line. The model is justified by the normal distribution of the residuals. The correlation of NAG and $\log_e$(SCC) is R^2= 51.7%.

The relationship between NAG(F) and BTSCC can be described by the linear regression model:

$$\log_e(SCC) = 3.44 + 0.522(NAG(F)) \qquad (9)$$

(r=0.719, p<0.001). The linear score somatic cell count (LS), a form of the log transformation of SCC, expressed by the equation:

$$LS = [\log_e(SCC)/\log_e(2)] - 13.61,$$

was applied to the SCC data. The linear regression model with LS predicted by NAG(F) from the BTM data is represented by the equation:

$$LS= 1.32 + 0.753(NAG(F)) \qquad (10)$$

(r = 0.719, p<0.001). The scatter plot of the log transformed LS cell count and

NAG(F) is shown in Figure 8. The residuals as a frequency distribution generated by the linear model for equation (10) are shown in Figure 9.

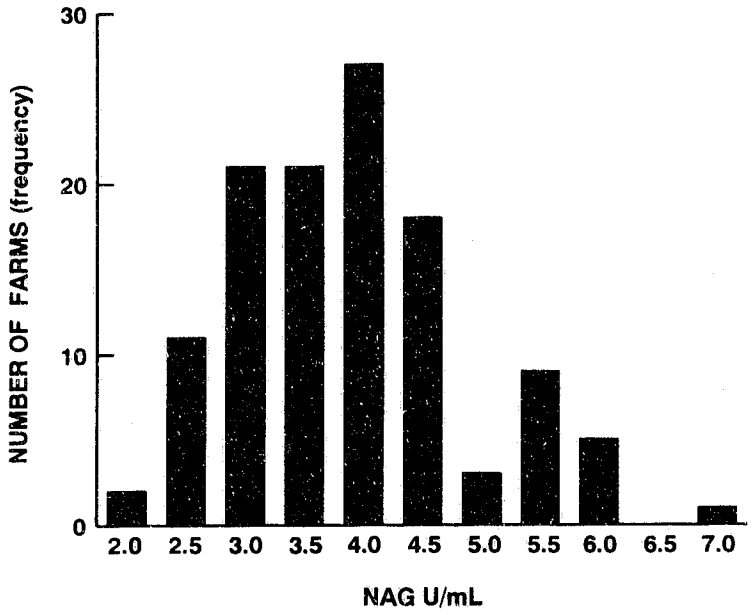


Figure 5. Frequency distribution of NAG (F) activity in bulk tank milk (n = 118).

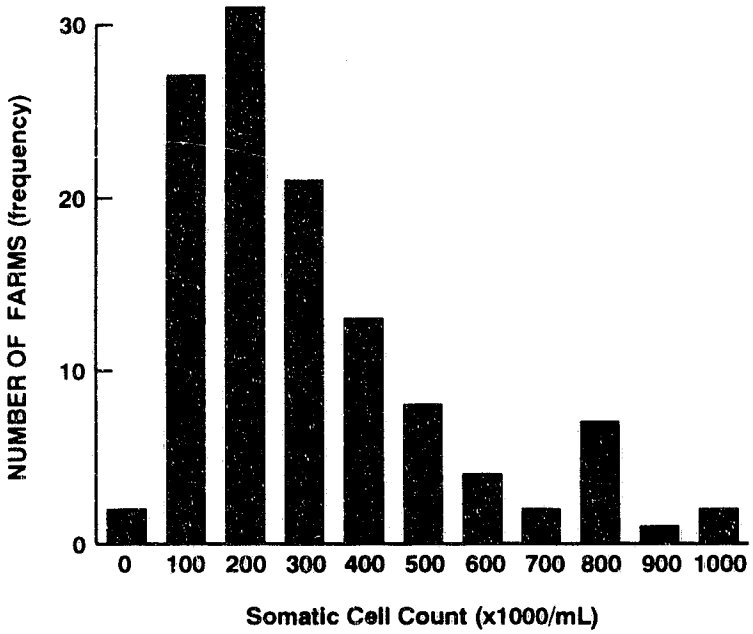


Figure 6. Frequency distribution of total cell count in bulk tank milk (n = 118).

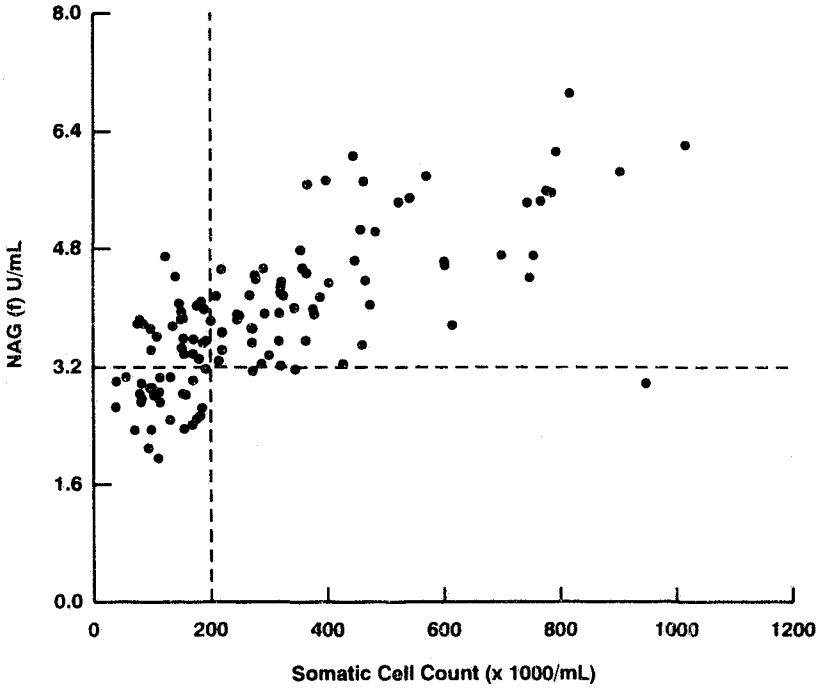


Figure 7. Scatter plot of fluorometric NAG activity and total cell count in a random pool of bulk tank milk (n=118). Each point represent one farm or sample. Normal ranges are indicated by inside lines.

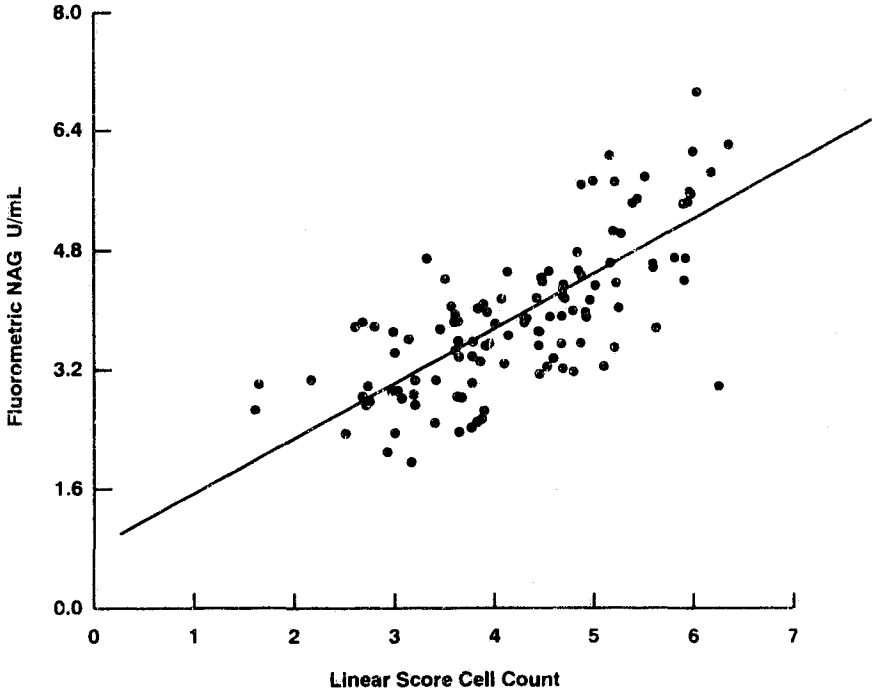


Figure 8. Scatter plot of fluorometric NAG activity and linear score cell count in bulk tank milk. The line represents a least squares regression of the data using the transformed cell count.

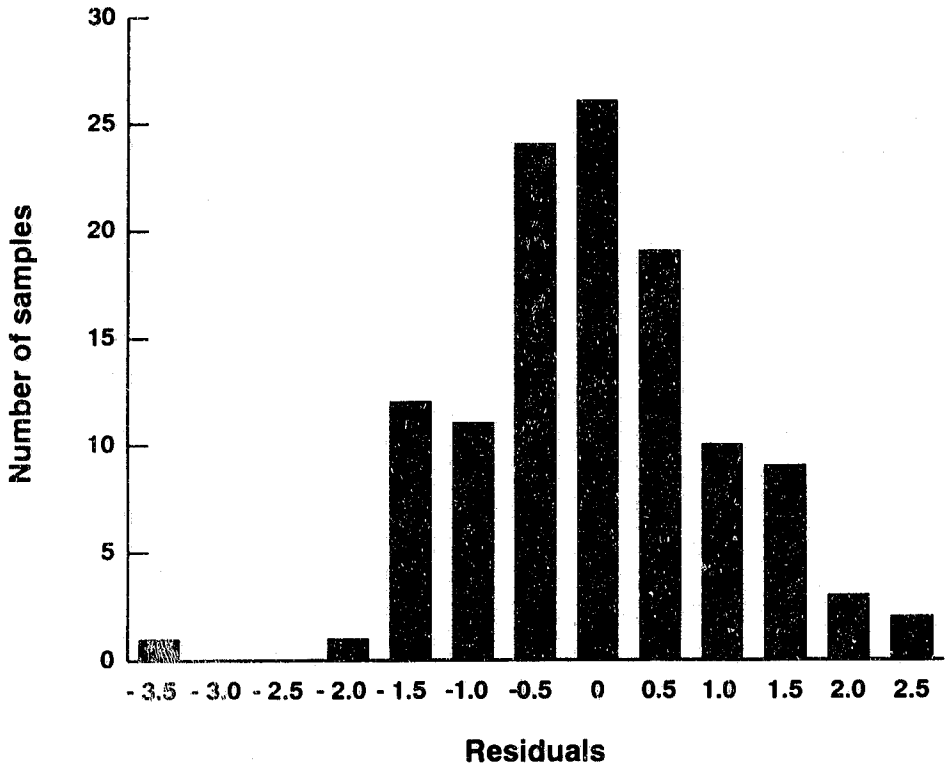


Figure 9. Frequency distribution of residuals from the linear regression of linear score cell count and fluorometric NAG activity in bulk tank milk (n=118).

The $\log_e$(SCC) by NAG(C) regression is represented by the equation:

$$\log_e(SCC) = 4.30 + 0.304(NAG(C)) \qquad (11)$$

($r = 0.553$, $p<0.001$). The colorimetric measurement of NAG and LS using SCC in BTM is expressed by the equation:

$$LS = 2.56 + 0.439(NAG(C)) \qquad (12)$$

($r = 0.553$, $p<0.001$).

The $\log_e$(SCC) or the LS may be used with equal confidence in the mathematical model. The means and standard deviations (SD) of the randomly selected pool of BTM for LS, NAG(F) and NAG(C) are presented in Table IV. The linear models derived from the least squares regression of the data with their associated F and p values are summarized in Table V. The correlation coefficient between colorimetrically determined NAG and $\log_e$(SCC) or LS is $r = 0.553$ and between the fluorometric NAG measurement and $\log_e$(SCC) or LS is $r = 0.719$, ($n=118$).

TABLE IV. MEANS AND STANDARD DEVIATIONS (SD) OF NAG AND LINEAR SCORE IN BULK TANK MILK

	n	Mean U/mL	SD
NAG[1]	118	3.887	0.996
NAG[2]	118	3.838	1.314
LS	118	4.25	1.042

[1] = fluorometric method (Test 1)
[2] = colorimetric method (Test 1)
LS = Linear Score (transformed cell count)

TABLE V. MODELS OF THE RELATIONSHIP BETWEEN SCC AND NAG[1,2]: SUMMARY OF REGRESSION EQUATIONS, F AND P VALUES.

(1) $\log_e(SCC) = 3.44 + 0.522(NAG(F))$	$F = 124.41$ ($p<0.001$)
(2) $LS = 1.32 + 0.753(NAG(F))$	$F = 124.41$ ($p<0.001$)
(3) $\log_e(SCC) = 4.30 + 0.304(NAG(C))$	$F = 51.21$ ($p<0.001$)
(4) $LS = 2.56 + 0.439(NAG(C))$	$F = 51.21$ ($p<0.001$)

[1] = fluorometric method (Test 1)
[2] = colorimetric method (Test 1)
(n=118).

3.3.3 Description of NAG based on the SCC

In order to place NAG values within the context of detection of subclinical mastitis, SCC was used as the standard. In the clinical setting the cut-off point between normal and abnormal is an arbitrary decision (Fletcher et al, 1988). One method used to show the relationship between sensitivity (Se) and specificity (Sp) for a test is to construct a curve relating the two (Fletcher et al, 1988). A definition of disease, BTSCC $\geq$ 200,000 cells/mL, was applied to the BTM samples as shown in Table VI. The data were divided into disease positive (D+) and disease negative (D-) on the basis of this SCC threshold. The data displayed in Figure 7 also demonstrates that this threshold, illustrated by the vertical dashed line, has the effect of dividing the data set into D+ and D- groups. By moving the NAG threshold (horizontal dashed line in Figure 7), the data may represent the "relative" sensitivity (Se) and 1 minus specificity (1-Sp) for a series of cut-off values of NAG(F) as shown in Table VII. Within these fixed categories of D+ and D-, the newly represented data in Table VII is used to generate a receiver operator characteristics (ROC) curve shown in Figure 10. Tests that are good at discriminating are tight in the upper left corner of the curve (Fletcher et al, 1988). This curve describes the accuracy of a new test over a range of NAG values relative to the SCC test. The ROC curve assists in the evaluation of the NAG test relative to the standard, SCC. The picture created is generally thought easier to follow for clinicians.

Table VI. DESCRIPTION OF NAG BASED ON DISEASE = SCC≥200,000 cells/mL IN BULK TANK MILK.

	Disease Negative			Disease Positive		
Variable	n	Mean	SEM	n	Mean	SEM
NAG(F) U/mL	52	3.21	0.086	65	4.42	0.111
LS	52	3.29	0.078	65	5.01	0.076

(n=117)

TABLE VII. TRADE-OFF BETWEEN SENSITIVITY AND SPECIFICITY USING SCC≥200,000 cells/mL AS THE DEFINITION OF SUBCLINICAL MASTITIS IN BULK TANK MILK.

Cut-off Value [a] NAG[1] U/mL	D+	D-	Sensitivity	1-Specificity
Test positive at			%	%
2.0	65	1	100.0	98.0
2.5	65	7	100.0	86.54
3.0	65	22	100.0	57.69
3.5	59	37	90.76	28.85
4.0	40	47	61.54	9.61
4.5	24	51	36.92	1.92
5.0	16	52	24.62	0.00
5.5	12	52	18.6	0.00
6.0	3	52	4.62	0.00

[a] P(disease); values ≥ the cut-off value are test positive (D+); those < the test value are disease negative (D-).

[1] fluorometric NAG assay.

(n=117)

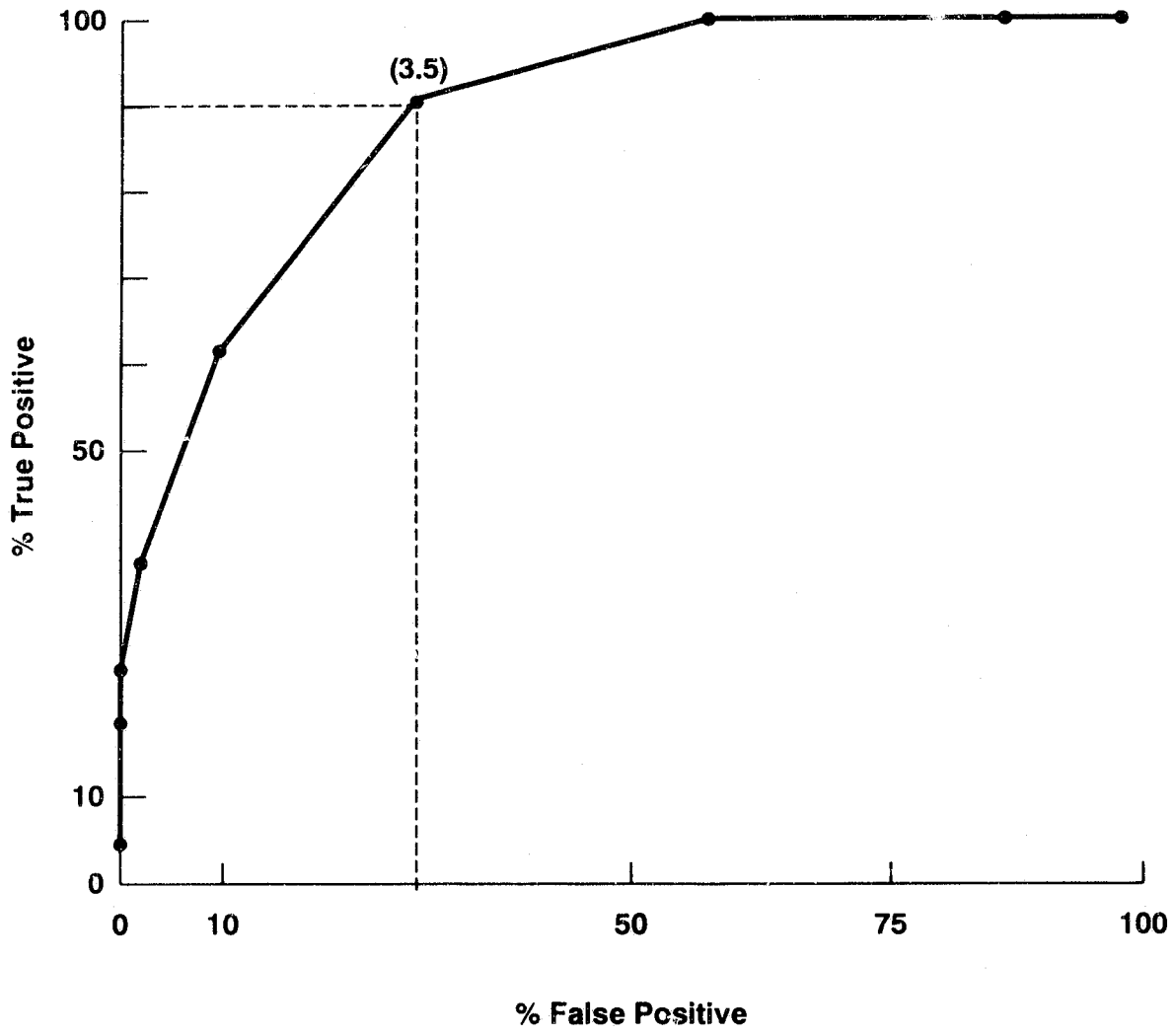


Figure 10. Receiver operating characteristics (ROC) curve for the cut-off values from the top right-hand corner for fluorometric NAG U/mL at 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 in bulk tank milk.

3.4 Discussion

It is well established that the NAG enzyme measurement in milk is of diagnostic value in the detection of sub-clinical mastitis. The definition of D+ was set at BTSCC ≥200,000 cells/mL. Prevalence of subclinical mastitis in a herd may be estimated by the BTSCC and a BTSCC of 200,000 cell/mL has 6% of quarters infected according to Eberhart et al. (1982). Linear regressions of NAG on BTSCC were calculated.

The coefficients of correlation of NAG and SCC imply that the NAG and cellularity changes in milk are related. The NAG(F) test is a reasonable substitute for the somatic cell count in milk illustrated by ROC curve. A range of $\log_e$(SCC) 3.2 - 8.2 per mL in the random pool of BTM used in this study corresponds to a range of NAG activity measured by fluorometry of 2-7 U/mL. This is the range of NAG(F) units suggested by this study for incorporation into a spot test for the BTM sample. The ranges of NAG for quarter and composite milk sample testing need to be similarly assessed. Visual comparison of quarter milk NAGs for the individual cow can assist interpretation as a means of visually interpreting the IQR in a spot test format.

The present study found that the $\log_e$(SCC) or the LS are the preferred transformations for the linear model of NAG measurement. The normal distribution of the residuals of the NAG $\log_e$(SCC) regression met the statistical assumptions for a linear model. That is, the requirements for existance, independence, linearity,

homoscedasticity (variance in NAG is fixed for any combination with SCC) and normal distribution of the data (Kleinbaum et al, 1988). The linear model for SCC and NAG could be used to produce conversion scales adapted for on farm use.

The LS may be used as the predictor for subclinical mastitis because it most closely relates yield losses to SCC levels (Shook, 1982). The LS transforms the SCC into single digits followed by one decimal place. The transformation to LS meets the regression requirement for a normal distribution of the data and also simplifies the presentation of the data for the user. The advantage when applied to individual cow samples, is the creation of easily identifiable groups of cows at defined levels of inflammation within the herd. The data generated by the present study demonstrated the relationship of NAG to LS in bulk tank milk. One parameter may be used as a direct measure of the other. The LS transformation of the cell count in milk represents the best conversion factor for a measurement of yield losses in kg of milk (economic factor) in mastitis evaluation and for this reason was included in the analysis. The $\log_e$(SCC) or the LS may be used with equal confidence in the mathematical model generated by regression analysis in this study. This may be of value in the industrial use of the NAG milk test as it is applied to the interpretation of production loss estimates. The NAG test may be a useful BTM test for the estimation of subclinical mastitis at the herd level. The following human example demonstrates the effect of permitting more frequent testing: by increasing the frequency and rapidity of measurement of glucose levels through home monitoring, a patient with diabetes mellitus is able to gain better metabolic control. Similarly,

daily or weekly quarter milk NAG and periodic BTM testing could allow herd mastitis monitoring by a simple, sensitive and rapid method.

The preferred method of the NAG assay determined by this study was the fluorometric method because of its correlation with the somatic cell count and the simplicity of the assay method in milk. The NAG(F) test is rapid and does not require additional preparation of the raw milk sample as stated in Chapter 2. The "relative" sensitivity and specificity of the NAG(F) test was demonstrated for one threshold of somatic cell count and the results showed that the test is able to predict milk cell count reasonably well.

In conclusion, NAG levels were closely related to $\log_e$(SCC) or LS of the SCC in the random pool of bulk tank milk samples used in this study. The mathematical equation relating SCC and NAG described may be used to predict levels of subclinical mastitis in herds. The equation may be used to convert a manual NAG test to the standard SCC levels in order to estimate the level of subclinical disease in herds. Future use of the NAG test in milk may include yield loss estimates based on NAG alone or in combination with SCC.

4. GENERAL DISCUSSION

The NAG test's ability to detect inflammation in the bovine udder is a function of the change in the numbers and types of inflammatory cells in milk, as well as an increase in NAG from other sources. Some of the increase in NAG may come from parenchymal cells. Induced lysosomal parenchymal NAG is released into the milk unaccompanied by PMNs. The reactivity of NAG to infectious stimuli is different from the milk cell count as indicated by the frequency distributions. The NAG activity levels return to normal within 48 hours of the abated stimulus (Anderson, 1977) and have peak activity for 24 to 36 hours (Wilson et al, 1991). The cell count may take considerably longer to return to normal levels. The combined NAG and SCC measurements therefore, may be shown to be more useful than the classification of cell types which has proven difficult in the past due to morphological similarity between many epithelial cells and macrophages (Davies, 1974). It is not clear if in chronic subclinical mastitis, the activated (tissue) macrophage becomes a significant contributor of NAG in milk (Guyton, 1991). Milk from normal cows contain mainly macrophages (Lee et al, 1980). Mammary macrophages have a significant effect on the proliferative response of blood lymphocytes during lactation (Concha and Holmberg, 1990). Enhanced phagocytosis could result from local immunostimulation of lymphocytes by the macrophage. More information may be gained by combining SCC and NAG data because somatic cells may disintegrate or aggregate in milk and therefore may not be counted (Kitchen, 1981).

Berning and Shook (1992) predicted mastitis defined by bacterial status, using NAG and SCC in a model. A high correlation between the natural logarithms of both the NAG and SCC in composite milk samples was reported. In that the two parameters, NAG and SCC, were combined into a single model. The only other factor of significance in the model was 'herd'. The addition of log NAG to the model assisted in differentiating minor from major pathogens (Berning and Shook, 1992). Berning and Shook (1992) concluded that SCC and NAG involve different responses to bacterial status. Their work supports the idea of combining NAG, SCC and herd effects in a predictive model for mastitis.

Bulk tank milk testing offers a simple method of monitoring herds. The BTM sample is collected routinely and is available for testing with new methods. It needs to be determined if bulk tank NAG values could be used to assist SCC to in order to index herds. Kitchen et al. (1984b) suggests that NAG levels are related to both the presence and type of pathogen and to SCC. Herd prevalence of mastitis is thought to be the least understood and most important factor (management related) affecting the accuracy of the positive SCC test (Reneau, 1986b). A survey conducted in Ontario in 1989 found *Streptococcus agalactiae* and *Staphyloccocus aureus* to be the most commonly isolated organisms in high SCC bulk tank milk samples (Innes, 1990). Some organisms may elicite a greater or lesser NAG response. A recent survey of within herd prevalence of *Staphylococci* and coagulase-negative *Staphylococci* in PEI on 54 randomly selected dairy herds reported prevalence means of 14% and 27% respectively based on composite milk samples (Chibeu, 1992). A

frequency distribution of mastitis prevalences underlies these means which are based on specific pathogens isolated in the population sampled. Establishing herd prevalence would assist interpretation of SCC. Understanding the herd factor important in NAG interpretation, 'stage of pregnancy', as an overall mean may assist BTM interpretation. The frequency distribution of the herds 'stage of pregnancy' underlies the NAG level in the BTM sample. Understanding of the interaction of NAG from serum sources (such as increases that occur with stage of pregnancy) in milk and inflammation as a result of microbial infection in the udder will assist NAG interpretation in bulk tank milk.

In this study, NAG was introduced as a new method of subclinical mastitis detection in bulk tank milk. The colorimetric and the fluorometric methods of the NAG assay were applied to milk that was randomly selected from the regular collection of BTM samples. The colorimetric assay for NAG in pathological urine has been developed into a kit for non-laboratory personnel in clinics and on wards (Yuen et al, 1984). Frozen or freeze-dried standards for the substrate are used to standardize the miniphotometer described by Yuen et al. (1984). The use of the colorimetric method however requires that the milk be cleared. This additional work adds expense and time to the procedure making it less desireable for use in milk testing. In this study, the clarification process resulted in a significant increase in NAG level as observed in the treated-fluorometric group. The colorimetric and fluorometric methods were equally able to detect low levels of NAG in BTM and were highly positively correlated. The milk did not require re-assaying that is often

necessary in pathological urine due to high NAG, 'off-scale', samples. This suggests that the range of NAG in BTM is narrow.

The NAG testing was applied to frozen samples in order to accurately establish a total NAG value and to make the laboratory time more convenient. It has been reported that fresh milk samples give lower NAG levels than stored or frozen samples (Kaartinen et al, 1988). If the assay is used in both fresh and frozen material, different scales and threshold values are needed to evaluate the disease status (Kaartinen et al, 1988). Freezing causes the release of additional sources of NAG into milk mainly from stimulation of PMNs (Kaartinen et al, 1988). It is recommended that the milk be frozen and thawed or let stand in a warm place for a few hours before processing when NAG is used to estimate SCC (Karrtinen et al, 1988). All samples used in this study were frozen and thawed once and processed within one month of collection. The observed effect of DDF was significant in the colorimetrically determined NAG. This may reflect the small mean difference between pairs. In general, a small loss of activity would not be considered of clinical significance.

A range of NAG from 0 to 10 U/mL may be used to describe the pool of bulk tank milk in this study. Since fresh milk levels would be lower than frozen milk levels this range could be applied in a field spot test for use in fresh or frozen samples. Internal subclinical (low and high) masititis milk standards are needed to ensure reliability of the NAG test under variable field conditions. The lyophilized standard for NAG available from EFLab (Espoo, Finalnd) and incorporated into their

microtitre plate system could be used for the above mentioned purpose.

The degree of association in the random pool of BTM was consistent with the findings of Kitchen et al. (1984a). Considerable differences between the two parameters were also demonstrated by the residuals and the frequency distributions. This may prove to be advantageous in future analysis (Kitchen et al, 1984b). Samples with a high NAG and a low SCC could be investigated to see if these samples represent the false negatives that are of concern in the SCC test. The SCC test and NAG test measure independent events in inflammation that are closely related (Kitchen et al, 1984b). Since overall, NAG test performed well in its ability to differentiate between disease positive and disease negative groups, as defined by and relative to the SCC, a manual NAG test may be useful for rapid preliminary screening of milk samples for clinic and farm use.

The mathematical regression models that provided the best linear fit for the fluorometric and colorimetric methods of NAG measurement with the SCC in BTM were defined.

The higher correlation coefficient for fluorometrically determined NAG versus the colorimetric form of the test and logarithmically transformed SCC may not be significant overall but may merely reflect the 'shift' in the NAG data that resulted from the biochemical alteration of the milk samples and the change in substrate.

The use of the LS with NAG in regression modelling demonstrates the possible future use of NAG in the interpretation of yield losses. The NAG test may be a faster, indirect method of predicting inflammation or SCC.

The recent development of an ELISA standard for the detection of subclinical mastitis by Ball and Greer (1991) offers classification of disease positive samples that is independent of the SCC test. Berning and Shook (1992) used bacteriological status to define mastitis status and found that SCC and NAG responded differently to changes in bacterial status.

The BTM sample is the most dilute and conservative level of milk testing and represents a mean level of the disease status of the herd over the time of the accumulated milk. The limitation of BTM measurement lies in the fact that the BTM sample does not describe the underlying differences between individual herds' BTM NAG/SCC relationship that would be revealed by composite or quarter milk sample testing. In quarter analysis, the quarter found to have the lowest value of NAG may be considered the normal measurement within the cow reflecting her normal physiological level. The difference between the low quarter used as the baseline for that cow can help to identify the outlying quarter(s) if one exists. The difference between quarters may be expressed as percent difference or as a ratio. The lowest quarter could similarly serve as the control in the spot test format. In composite samples, the within-herd subgroups defined by confounders such as age, stage of lactation (dilution effect and pregnancy effect) can be used to define a normal range of the NAG test in individual herds.

5. SUMMARY

The present study determined that NAG measurement by the fluorometric or colorimetric methods can be applied with equal confidence in milk, as an adjunct test to BTSCC. This result is consistent with the findings of Yuen et al. (1984) who compared a colorimetric method with the fluorometric method and found a strong correlation between the two determinations ($r = 0.997$) in pathological urine samples. The correlation between the colorimetric and fluorometric methods was ($r = 0.843$) for paired determinations of NAG in BTM.

The measurement of NAG by the fluorometric method in raw milk was preferred because of the ease and rate of processing. Since no biochemical preparation of the sample is required in fluorometry, the cost and work time are greatly reduced compared to the colorimetric form of the test. The colorimetric NAG assay may be preferred because colorimeters are generally more available and fluorometry is expensive (Yuen et al, 1984). There was no statistical difference between the colorimetric and the fluorometric NAG measurement in milk. However, for use in large volume milk testing, raw milk used in the fluorometric determination of NAG offers an advantage as a facile, rapid test for subclinical mastitis detection.

The time in frozen storage effect on NAG was modified by the assay method applied. The fluorometric NAG measurement was less affected by frozen storage. Fluorometrically determined NAG did not require clarification of the milk as part of the assay greatly reducing the time and materials required. This may be the main

advantage to consider in the development in a manual NAG test that uses the fluorometric substrate with raw milk.

The range of NAG(F) in a randomly selected pool of BTM was from 2-7 U/mL which corresponded to a range of SCC from 100,000 to 1,000,000 cells/mL. The logarithmic transformation of SCC provided the best fit of the data with NAG in a linear regression model. The two tests are highly positively correlated in cases of subclinical mastitis. The differences between the SCC and NAG may be of use in mastitis detection in the future (Kitchen et al, 1984).

Future study includes development of a manual NAG kit for milk to provide producers with a more refined tool for subclinical mastitis detection. This form of the test would permit more frequent monitoring of herd and quarter level mastitis. Secondly, the pregnancy effect on bovine NAG levels in milk and its possible interaction with inflammation must be assessed before the NAG test can be usefully applied in the detection of subclinical mastitis.

APPENDIX A

Calculations Used to Determine the NAG Measurement in Bovine Milk

(1) A standard curve was constructed by serial dilutions of NAG (Jackbean, Sigma Chem. Co., St. Louis, MO) in base milk in U/mL: 0, 2.5, 5.0, 7.5, 10.0. The fresh daily standards accompanied each group of test samples.
(2) The OD was calculated for each point on the standard curve and for each test sample as: final OD = average OD of the duplicates - (RB(OD) + SB(OD)).
(3) The background 'noise' created by the base milk was subtracted from the standards to generate a zero in U NAG per mL of milk.
(4) The resultant ODs of the standard curve (x) of the run and the NAG dilutions in U/mL (y) were entered into the computer and used to generate a linear regression equation using Minitab statistical package (Minitab 7.1). The sample final OD was used to predict the U/mL of NAG in the sample by interpolation using the linear regression equation of the standard curve of the run.

APPENDIX B

Protocol Used in the Fluorometric Determination of NAG in Bovine Milk.

(1) Pipette 0.4 mL (400 uL) substrate into sample test tubes and into the reagent blank (RB). Pipette 0.4 mL of citrate buffer into each sample blank (SB) test tube.
(2) Set all samples and assay reagents into a 37°C waterbath to equilibrate. Cover with parafilm.
(3) Mix the sample by lightly vortexing and at timed intervals, add 100 uL of milk sample to each of two corresponding test tubes and SB. Add 100 uL of deionized water to the RB. Shake the reaction mixture after the addition of the milk sample to ensure rapid and thorough mixing.
(4) Incubate the reaction mixture for 15 min at 37°C.
(5) Add 1.5 mL glycine buffer to all test tubes in order to stop the reaction. Manually shake after the addition of the stop buffer.
(6) Measure the intensity of fluorescence for each sample and its blank without delay. Use an excitation (slit width 5) and emmission (slit width 3) settings of 355 and 460 nm wavelengths respectively, on low gain.

Diagnostic Chemical Limited (DCL) protocol

APPENDIX C

Reagents Used for the Fluorometric NAG Assay in Bovine Milk

(1) **Reagent Buffer:** The reagent buffer is a 250 mM citrate buffer, pH 4.60. For 100 mL of buffer add 4.470 g citrate trisodium salt dihydrate (Sigma Chem. Co., St Louis, MO) (FW 294.1) to 2.059 g citric acid monohydrate (Sigma Chem. Co., St Louis, MO) and make up to 100 mL with deionized water. Add a small amount of 1 N HCl to achieve a pH of 4.60. This buffer may be stored at 4°C for 3 days.

(2) **Stop Buffer:** The stop buffer is a 2M glycine buffer, pH 10.7. For 100 mL of buffer add 1.501 g glycine (freebase ammonium free) (Sigma Chem. Co., St Louis, MO) (FW 75.07) to a flask and make up to 100 mL with deionized water. Adjust the pH to 10.7 by the addition of a few mL of supersaturated NaOH solution.

(3) **Substrate Solution:** This is a light sensitive solution. Mix 2.25 mM 4MeUNAG (Sigma Chem. Co., St Louis, MO) (FW 379.4 g/M) in Reagent Buffer. This solution is made up daily to ensure quality.

All chemicals are of analytical grade.

APPENDIX D

Sample and Solution Preparation Used for the Colorimetric Determination of NAG with PNP-NAG as Substrate in Bovine Milk.

(1) Sample Preparation: Add 50 uL of chloroform for each mL of raw milk in the sample, cover with parafilm and vortex on high speed for 20 sec. To this mixture add 35 uL of 1 N HCl per mL of raw milk sample to lower the pH to 4.50 units. Vortex again for 20 sec on medium speed. Centrifuge the mixture for 8 min at g=16,000 (Eppindorf). Decant the supernatant. Check the pH, add additional HCl as necessary and repeat the centrifugation step. The clarified sample is the test material for the colorimetric procedure. (This material may be refrigerated overnight.)

(2) Solution Prepatation: The solutions differ from the Fluorometric Buffers only in the pH of the citrate buffer and in the pH and concentration of the glycine buffer. The buffers may be stored under refrigeration for 3 days.

Reagent Buffer: 250 mM citrate buffer, pH 4.50.
Substrate Reagent: 6.2 mM PNP-NAG dissolved in the reagent buffer. This solution is light sensitive and must be covered with foil.
Stop Buffer: 2 M glycine buffer, brought to pH 11.3 with the addition of a supersaturated solution of NaOH.

(DCL protocol)

APPENDIX E

Protocol used for the Colorimetric Determination of NAG in Bovine Milk.

(1) Pipette 0.75 mL of the substrate reagent into sample test tubes in duplicate and into the RB test tubes.
(2) Pipette 0.75 mL of distilled/deionized water into each sample blank.
(3) Allow the solutions and test samples to equilibrate in a 37°C waterbath. Cover with parafilm.
(4) At timed intervals, add 0.75 mL of the prepared test material (supernatant) into the duplicate substrate and accompanying blank test tubes. Incubate for exactly 15 min at 37°C.
(5) Stop the reaction with the addition of 0.5 mL of the stopping buffer to each test tube and mix on the Vortex at low speed for 5 sec.
(6) Centrifuge (g=16,000) the samples and blanks for 5 min in a microcentrifuge (Eppindorff).
(7) Decant the supernatant and determine the NAG measurement by reading the optical density (OD) of PNP at 405 nm on a spectrophotometer.

(DCL protocol).

ADDENDUM

As a result of the Master Defence of the study, the ommission of internal milk standards within the runs was raised as a concern. It is the desire of the student that this be stated to avoid future errors. I, the student, gratefully acknowledge the importance of this control to improve the precision of the data and thank my examiners for their advice. Standards could be made by freezing aliquots of low, medium and high levels of subclinical mastitis or by spiking normal milk and freezing them. These standards could be lyophilized. RAH.

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