Milk Quality and Mastitis Tests

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Abstract

A variety of diagnostic tests are routinely used to evaluate milk quality on dairy farms. Tests such as bulk milk bacterial counts, bulk tank somatic cell count and tests for adulterants such as water, sediment or antibiotics are routinely used by regulatory agencies. Other tests such as individual cow somatic cell count values, *Staphylococcus aureus* milk antibody tests, the California Mastitis test, milk conductivity and milk microbiology are often used diagnostically to investigate milk quality problems. Veterinarians also use various types of antibiotic susceptibility tests to help guide mastitis treatment decisions. The successful use of milk quality and mastitis tests requires knowledge of the methodology and diagnostic capabilities of each test. This paper reviews practical applications and supporting research of tests that are commonly used to investigate and solve milk quality and mastitis problems on dairy farms.

Introduction

Mastitis is usually considered the most costly disease of dairy cattle. Subclinical mastitis is considered the most economically important type of mastitis because of long term effects on total milk yields. Production losses due to subclinical mastitis were recently estimated to cost the dairy industry $1 billion dollars annually.[40] Additional costs of mastitis are due to the failure to receive quality premiums from milk purchasers. While premiums vary regionally, the monthly premium opportunity for a select group of 54 Wisconsin dairy farmers was reported to range from $6.70 to $15.02 per cow per month (depending upon processor and current SCC level).[49] More losses can be attributed to clinical mastitis, the risk of antibiotic residue violations, culling and death.[14] Mastitis remains a concern of most dairy farmers and their veterinarians due to these profound economic consequences.

Numerous diagnostic tests are used to test milk. Some tests are used to define raw milk quality. These tests include bulk tank somatic cell count (SCC), bacterial counts (such as the standard plate count (SPC)) and tests for adulterants such as water or antibiotics. Other tests are used diagnostically to investigate milk quality problems. Diagnostic tests include individual cow SCC values, the California Mastitis Test (CMT), milk conductivity, and milk microbiology. Other tests, such as antimicrobial susceptibility tests are used to guide treatment decisions.

This paper will review the procedures and diagnostic capabilities of milk quality and mastitis tests and highlight the practical application of these tests for dairy farm problem solving.

Testing Milk Quality

Standard Plate Count

Throughout the world, official regulatory standards for milk are based on determination of bacterial numbers present in raw milk. The SPC is the official regulatory test used for estimating bacterial populations of raw milk and milk products and is the official reference method specified in the Grade A Pasteurized Milk Ordinance (PMO).[24] The PMO requires the SPC to be less than 100,000 cfu/ml for Grade A farms; grade B milk regulations require the SPC to be less than 300,000 cfu/ml. Few dairy producers consistently exceed the regulatory limit. Of 804,575 monthly SPC values examined for WI grade A farms between 1994 and 1998, 90% were <34,000 cfu/ml. Of grade B farms, 50% of SPC values were <25,000 cfu/ml but 10% of the values exceeded 170,000 cfu/ml. In New York State, 50% of 855 bulk tank samples were <10,000 cfu/ml.[3] The SPC is a critical control point for milk quality and many milk purchasers have standards that are more rigorous than the official regulations. A reasonable goal for SPC is ≤5,000 cfu/ml and a count of >10,000 cfu/ml is usually indicative of a problem.[47,25] The SPC is an overall measure of milk quality but a single SPC value is not very useful diagnostically.[47] A high SPC is an indication of a milk quality problem usually caused by errors in cooling milk or cleaning milking equipment. Rarely, a high bacterial count can be associated with subclinical mastitis (especially mastitis caused by *Streptococcus spp.).*[19] In many of these instances the SCC and the SPC are both high and the causative organism should be apparent from a bulk tank milk culture.

The SPC is performed following prescribed methods and because of differences in methodology the results should not be compared to qualitative bulk tank cultures. In brief, the procedure is performed by pipetting standard dilutions of milk into petri dishes, adding standard methods agar and incubating the plates at 90F (32C) for 48 hours. Bacterial colonies are then counted using a variety of methods depending upon the colony types present; the SPC is computed based upon the dilution and number of colonies present. There are a number of alternatives to the SPC. The plate loop count (PLC) is an equivalent method but is not considered precise when raw milk bacterial counts exceed 200,000 cfu/ml. The spiral plate count (SPL) requires less technical expertise, is considered equivalent to the SPC and requires no dilution when bacterial numbers are expected to be between 500 and 500,000 cfu/ml. The equipment used for performing the SPL is not widely available. The SPC, PLC and SPL are direct methods based on counting visible bacterial colonies. The **Bactoscan™** method is a recent technological advance that uses continuous epifluorescent microscopy to count bacterial cells stained with acridine orange. Bactoscan™ has compared favorably to traditional bacteriologic methods and is considered to be less variable and more reproducible. [6,29] Bactoscan™ is now used as the official reference method for several countries and the Canadian province of Ontario. The total bacterial count can also be determined using Petrifilm™ or Redigel™ aerobic counting methods.

Laboratory Pasteurized Count

The laboratory pasteurized count (LPC) is usually performed as a diagnostic test when SPC values are high. The LPC is a SPC performed on milk that has been heated to 145F (62.8C) and held for 30 minutes (low temperature-long time pasteurization). The

objective of the LPC is to identify organisms that survive pasteurization (thermoduric bacteria). High LPC are associated with unclean equipment, improper sanitizing practices and milkstone deposits.[36] Typical mastitis causing organisms do not survive pasteurization. Thermoduric bacteria may include Micrococcus, Microbacterium, Lactobacillus, Bacillus, Clostridium and occasional *Streptococci*. Thermoduric organisms are often related to spoilage of pasteurized milk. Poor milking hygiene can result in an elevation of coliform counts and SPC with near normal LPC. The LPC should be below 100 to 200 cfu/ml and a LPC below 10 cfu/ml indicates excellent equipment hygiene.[47] In New York State, 60% of 855 bulk tank samples were <200 cfu/ml and 40% were less than 80 cfu/ml.[3]

Coliform Count

Coliform counts are performed by culturing dilutions of raw milk on selective media such as violet red bile agar. The plates are incubated at 90F (32C) for 24 hours. The source of coliform bacteria in bulk tank milk is the udders of cows or unsanitary milking practices. The coliform count is an indication of the effectiveness of cow preparation procedures during milking and the cleanliness of the cows' environment.[47] Coliforms can also incubate on residual films of milking equipment. The coliform count should be less than 10 cfu/ml.[47] A coliform count between 100 and 1000 usually indicates poor milking hygiene and a coliform count >1000 suggests that bacterial growth is occurring on milk handling equipment. Thirty percent of coliform counts in 855 samples in New York state were <10 cfu/ml but 20% of bulk milk samples exceeded 100 cfu/ml.[3]

Preliminary Incubation Count

The preliminary incubation count (PI) is used as a measure of raw milk keeping quality and is also used to monitor sanitation practices on farms.[26] The PI is a SPC performed on milk that has been incubated at 70F (21C) for 18 hours (simulating poor refrigeration). The PI is not associated with mastitis pathogens and is used to measure Psychrotrophic bacteria. These bacteria are often associated with off-flavors, milk spoiling and reduced shelf life. Recommended PI counts are <10,000 cfu/ml but up to 50,000 is considered acceptable.[25] In New York State, the PI count was <14,000 for only 30% of 855 herds examined and only 10% of PI counts were <8,000 cfu/ml.[3]

Interpretation of Bulk Milk Bacterial Counts

Many farms consistently produce high quality milk, however sporadic elevations in bacterial counts occur on many farms. Bacteria in raw milk can come directly from the environment or originate as mastitis organisms. Bacterial "spikes" (defined as "transient sporadic increases in SPC values that exceeded a 95% confidence interval for mean SPC and were >10,000 cfu/ml") have been associated with *streptococci* (primarily *Strep uberis*) and gram-negative organisms.[4] The origin of *S uberis* in this study was not determined. Very high shedding has been documented for cows infected with *S uberis* mastitis and *S agalactiae* is known to be an occasional cause of high bacterial counts. Subclinical mastitis problems should be considered when both the SCC and SPC are high.[47] Bacteria that are deposited on milking equipment can multiply and become a major source of contamination if cleaning is not adequate. In general, high LPC are typical of equipment cleaning and sanitation problems. Incubation of bacteria in the

milking system causes elevated coliform (>1000) and LPC counts. Inadequate premilking hygiene can result in coliform counts in the range of 100 to 1000 cfu/ml. Care should be taken when collecting raw milk samples for testing. The samples must be obtained without contamination (never sample from the bulk tank outflow) and stored below 40F (4C) until processing. A series of at least three tests should be performed to reach a confident diagnosis. A comparison of values from multiple tests such as SPC, LPC, coliform and SCC values can be used to help diagnose a problem with high raw milk bacterial counts.[47]

Other Tests of Raw Milk Quality

Milk is also tested to determine if water or sediment have been added. When water is added to the milk, the concentration of salts and lactose is diluted and the freezing point of milk progressively approaches that of pure water. The freezing point of milk is determined using a cryoscope. A freezing point value of $>-.530^{o}$ Hortvet (the scale is named for the individual that developed the testing system) indicates that milk composition has changed. Possible causes of high cryoscope readings include: intentional addition of water, poor system drainage, use of excessive water during milking, backflushing units with the vacuum on, rinsing the top of the bulk tank or freezing of the milk in the bulk tank.[26] Processors are required to test milk for sediment. Acceptable levels are less than 1.5 mg/gal of milk.[26] The combination of excessive udder hair, sand bedding and poor premilking preparation can contribute to unacceptable values for this test.

Testing for Mastitis

Somatic Cell Counts

Somatic cells are composed of white blood cells (WBC) and occasional sloughed epithelial cells. Cells found in normal bovine milk from uninfected glands include neutrophils (1 – 11%), macrophages (66 – 88%), lymphocytes (10 – 27%) and epithelial cells (0 – 7%).[31] The macrophages have an important role in providing surveillance in the uninfected gland. When bacteria invade and colonize the mammary gland, the macrophages respond by initiating the inflammatory response that attracts polymorphonuclear cells (PMNs) into the milk to engulf and destroy the bacteria.[18] The largest factor that influences the SCC of milk is mastitis.[18] The SCC of a cow that is not infected with mastitis is usually less than 200,000 cells/ml and many cows maintain SCC values of less than 100,000 cells/ml.

When infection occurs, the macrophages present in the udder signal the cow's immune system to send neutrophils to engulf and destroy the bacteria. More than 90% of SCC in infected glands are composed of neutrophils and a SCC of greater than 200,000 cells/ml is a strong indicator of mastitis.

SCC thresholds are often used to predict intramammary infections (IMI) at either the quarter or cow level. There are some obvious problems with using composite milk SCC to identify infected cows because of dilution of SCC values with milk from uninfected quarters. Consider the hypothetical situation when a cow is producing 40 lbs of milk per milking evenly distributed between 4 quarters (10 lbs per quarter) but only 1 quarter is infected with subclinical mastitis. If the SCC of the milk from the 3 uninfected quarters

is 100,000 cells/ml, the composite SCC value will not reach a threshold of 250,000 cells/ml until the SCC from the infected quarter exceeds 700,000 cells/ml (Figure 1).

The sensitivity and specificity of using a SCC threshold of 200,000 cells/ml as the cut point for IMI have been evaluated in several studies.[11,33,58] Reported sensitivities range from 73 – 89% with corresponding specificities of 75 – 85%. The sensitivities are relative sensitivities because the "gold standard" was bacterial culture, which is not a perfect test. A SCC threshold of 100,000 cells/ml for quarter samples had the maximal sensitivity and specificity for detecting IMI in fresh cows that were tested on day 5 post-calving.[51] The probability that a cow over the threshold will actually be infected (the positive predictive value) or the probability that a cow under the threshold is actually uninfected (the negative predictive value) are useful values for on-farm problem solving. Positive and negative predictive values are a function of the underlying prevalence of disease in the tested herd. This concept is somewhat self evident in that 100% of test positive animals are truly positive in a herd with 100% prevalence, whereas 100% of test negative animals are truly negative in a herd with zero prevalence. The impact of prevalence on predictive values at several SCC thresholds and levels of herd prevalence has been estimated (Table 1).[11]

In a herd with a low prevalence of subclinical mastitis, raising the SCC threshold to 250,000 improves the PPV (57% of cows above 250,000 are actually infected) but doesn't significantly affect the NPV (only 3% of cows with infections are incorrectly identified). The use of likelihood ratios to predict the probability of subclinical mastitis based upon SCC ranges eliminates the need to set a strict threshold and incorporates information on herd prevalence. Likelihood ratios can be easily calculated using spreadsheets and can be used on a practical basis to plan and evaluate mastitis control programs. A full description of the methodology of using likelihood ratios to predict IMI has been recently published.[9]

Bulk tank somatic cell count (BTSCC) is the most frequent reference point for milk quality. All dairy farms, have periodic BTSCC and bacterial count data supplied by their milk purchaser. BTSCC vary regionally, seasonally and with herd size. Many dairy farmers consistently produce high quality milk. Of 168,989 monthly grade A SCC values from official regulatory records of all WI dairy farms in 1998 more than 1,800 WI dairy farms had average BTSCC of <130,000 cells/ml and over 4,500 dairy farms obtained annual average BTSCC of <200,000 cells/ml.[50] The median BTSCC was 290,000 for grade A dairy farms and farms with average BTSCC values that exceeded 400,000 cells/ml were ranked in the bottom 25% of herds. The risk of having a violative antibiotic residue increases after BTSCC levels exceed 400,000 cells/ml.[50] BTSCC values verify the existence of a mastitis problem but individual cow SCC values are needed to define the problem on a herd basis. Bulk tank SCC values often differ considerably from herd SCC values estimated by DHIA. DHIA SCC values are usually estimated as a weighted average of the milk sample SCC multiplied by the individual cow milk yield. The error associated with both measures contributes to error in estimating BTSCC. Additional reasons for the disparity include differences in methodology and sampling and differences in animals contributing to the bulk tank versus DHIA reports.

There is no simple way to estimate the prevalence, incidence or effect of mastitis control procedures without individual cow SCC values. Common industry goals for subclinical mastitis are: **85% cows with somatic cell counts ≤250,000** and less than ***<5% of cows developing new subclinical mastitis infections per month.***[59] While many herds achieve these goals, many other herds experience considerably more subclinical mastitis. In December 2000, there were >7000 WI dairy herds (of approximately 18,500 total WI dairy herds at that time) that processed records with a leading WI DHI provider (AgSource CRI) and no production category had <90 herds (Figure 2). About 40 – 50% of the cows were infected with subclinical mastitis in low producing herds and 26% of cows were infected in high producing herds. Less than 5% of cows were infected with subclinical mastitis in the top 10% these herds. A new IMI is defined by that processing center as any cow with a linear score greater than or equal to 4.0 for the first time in the *current lactation*. This definition underestimates the rate of new infections as a cow can only experience one new infection per lactation (subsequent infections would be classified as chronic even if intervening SCC values were <4.0 for many months). Other herd management software and DHI centers define new IMI differently and the definitions for this value should always be confirmed.

A popular method used to monitor subclinical mastitis is the creation of **scatter graphs** using 2-consecutive months of linear score data (Fig. 3). The cows with new subclinical infections are shown in box "D." These are the cows that have developed new subclinical infections since the last SCC test. The total number of infected cows is the sum of "B"+"D." These plots are often used to classify milk quality problems as environmental or contagious in nature. For example, the large number of newly infected cows and relatively lower number of chronic infections in herd "Y" is highly suggestive of an environmental mastitis problem caused by pathogens such as *E. coli* or environmental *streptococci*. Contagious mastitis pathogens (such as *Strep ag*, *Staph aureus* and *Mycoplasma* spp.) should be suspected in herds with a large proportion of chronic infections (Box B) and relatively few animals with spontaneously cured infections (Box A). The sensitivity of using changes in SCC values is considered relatively low but the specificity appears to be quite acceptable (Table 2). Users of scatterplots should remember that the relatively low sensitivity underestimates new infections and results in overemphasis of chronic infections. Mastitis control programs that utilize segregation to control contagious mastitis should not rely exclusively on changing SCC values to identify newly infected cows. The use of SCC values for mastitis problem solving has been addressed in numerous other publications and is beyond the scope of this paper.

California Mastitis Test

The California Mastitis Test (CMT) remains the only reliable screening test for subclinical mastitis that can be easily used at the cowside. The CMT was developed to test milk from individual quarters but has also been used on composite quarter milk samples and bulk milk samples.[53] Fresh, unrefrigerated milk can be tested using the CMT for up to 12 hours, reliable readings can be obtained from refrigerated milk for up to 36 hours. If stored milk is used, the milk sample must be thoroughly mixed prior to testing because somatic cells tend to segregate with the milkfat. The CMT reaction must be

scored within 15 seconds of mixing because weak reactions will disappear after that time. The CMT reagent is simply a detergent plus bromcresol purple (used as an indicator of pH). The degree of reaction between the detergent and the DNA of cell nuclei is a measure of the number of somatic cells in milk. The relationship between SCC values and CMT is not precise because of the high degree of variability in SCC values of each CMT score (Table 3).

The use of the CMT to identify infected quarters has been extensively evaluated.[1,5,43,63] The type of mastitis pathogens in the populations of animals used in these studies were primarily contagious organisms typical of the predominant mastitis problems of herds when the CMT was developed. Results vary between studies probably because of differences in microbiologic techniques (loop size, sample handling etc.). In general, as CMT reactions increase the likelihood of recovering pathogenic bacteria increases.

The sensitivity and specificity of using the CMT to detect IMI in fresh cows has been recently reported.[51] The CMT was performed on quarter milk samples daily from calving through day 10 post calving and compared to bacteriologic results from samples obtained on days 1 and 3 post-calving. The herd had a quarter prevalence of IMI of 36%. The test characteristics of CMT thresholds of trace/1, 2+ and 3 were compared in this study. A CMT threshold of trace on day 3 resulted in the highest sensitivity (66.7%) and specificity (54.8%) for detection of major pathogens. A positive CMT was defined as $\geq$1. The overall sensitivity and specificity on day 3 were 56.5% and 56.1% respectively. The sensitivity of CMT used on day 3 varied by pathogen: *E coli* (50%), *Klebsiella* (80%), *S aureus* (60%), environmental *streps* (84%). These values are comparable to test characteristics that can be calculated using data from CMT values reported for composite milk samples in herds with contagious mastitis problems. One study used the CMT to test 7,431 composite milk samples.[5] The prevalence of IMI caused by *S aureus* and *Strep ag* was 35% in the survey population. Using CMT values of trace or greater to define a positive test, calculated relative test characteristics were: .92(sensitivity), .41(specificity), .46(PPV) and .91(NPV). If the test characteristics are recalculated using a CMT test threshold of $\geq$1, the sensitivity drops to .72 and the specificity increases to .64. The PPV is relatively unchanged at .52 but the NPV drops to .81. If the objective of using the CMT is to minimize the rate of false negatives, the test should be read as negative versus positive with trace scores recorded as positive. If the CMT is to be used in culling decisions a threshold with a lower rate of false positives may be desirable.

The CMT has been investigated as a tool to identify cows for selective dry cow therapy.[44,48] The objective of these studies was to treat only cows or quarters infected with major mastitis pathogens. The CMT test correctly identified 75% - 80% of infected cows depending upon the study and the type of pathogens present. However, both studies concluded that a large percentage of "uninfected quarters" would receive dry cow therapy because 23% - 46% of animals without infections with "major pathogens" were detected by CMT. The use of CMT to screen animals for a selective dry cow therapy program in herds that have not successfully controlled contagious mastitis would result in many infected cows not receiving appropriate dry cow therapy.

Individual Cow Cultures

Most mastitis control programs include the use of individual cow cultures to determine which mastitis pathogens are present on a farm. Culturing can be used in a targeted fashion for specific control programs (such as segregation plans for contagious mastitis) or for surveillance to detect the presence of new or emerging pathogens. Culturing is also used to evaluate treatment efficacy and to establish susceptibility patterns to aid in the development of rational treatment strategies. The success of culture programs varies depending upon the type of organism, sampling methodology and laboratory procedures.

There have been a number of studies examining the test characteristics of sampling strategies. The use of pre-milking versus post-milking samples was evaluated in a herd with a high prevalence of *Staph aureus* IMI.[56] The relative sensitivity of premilking samples as compared to postmilking samples was higher for *S aureus* (91% versus 81%), coagulase-negative *Staph* (CNS; 91% versus 45%) and environmental *Streptococci* (97% versus 58%). Colony counts of *S aureus* were significantly higher in premilking samples. The rate of contaminated samples (defined as samples with >1 isolate) was higher in premilking samples resulting in lower specificities. The relative sensitivities reported in this study were higher than other studies have reported. Isolation of *S aureus* from a single quarter milk sample has been estimated to be only 75% sensitive but the addition of 2 or 3 consecutive samples can increase the sensitivity to 94-98%.[55]

Composite milk samples are often used rather than quarter samples to reduce the cost of culturing. The relative sensitivity of a single composite milk sample used to detect *S aureus* has been estimated to be 63% (95% CI, 0.51 to 0.82).[30] The relative sensitivity increased with increasing number of infected glands per cow from 0.58 for cows with 1 infected gland to 0.89 for cows with 4 infected glands. Using multiple composite samples can increase the sensitivity. The overall probability of obtaining a negative composite sample from a cow with at least 1 infected gland can be decreased from 37% (single composite sample) to 14% (2 consecutive samples) to 5% (3 consecutive samples). Sensitivities for *S aureus* can also be improved by utilizing a greater inoculum volume (Table 4).[30]

The use of consecutive milk samples is considered cost-prohibitive for many dairy farmers. However, failing to identify infected cows in a herd with a moderate to low prevalence of contagious mastitis can lead to a continuous source of infection within the herd. Centrifugation and sedimentation of quarter milk samples has been studied in an attempt to improve the frequency of recovery of *S aureus*.[65] Quarter milk samples were centrifuged at 2000 x g for 15 minutes, the supernatant discarded and the sediment resuspended in 0.05 ml of saline. The resuspended solution was then plated on blood agar and incubated. The sedimentation technique successfully identified greater numbers of cows that were positive for *S aureus* (Table 5). More infected cows were identified by waiting until 7-10 days post-calving as compared to obtaining samples within the first 5-days after calving.

When highly sensitive methods of identifying cows infected with *S aureus* are required, quarter milk samples should be obtained from foremilk and centrifuged at approximately

1-week post-calving. A larger inoculum volume or consecutive samples should be obtained if composite milk samples are used.

In contrast to the high degree of variability and limited sensitivity found in identifying *S aureus* infected cows, the use of milk culturing to identify cows infected with *Strep ag* is straightforward. The sensitivity of single cultures for chronic *Strep ag* infections ranges between 95 and 100% and specificities are around 95%.[8] Test characteristics are not improved by obtaining samples before or after milking, quarter versus composite milk samples or differences in inoculum volume. When single cultures are used, about 10% of culture positive and culture negative animals will be misclassified. Therefore, repeated culturing is necessary to fully eradicate *Strep ag* from the dairy herd.

Bulk Tank Culture

The microbiologic exam of bulk tank milk is a standard element of mastitis control programs. Bulk tank cultures (BTC) are used as an inexpensive screening test for mastitis pathogens in herds (or groups) of lactating dairy cows. The methodology of BTC varies immensely. The sampling interval, sample collection, microbiologic methods and reports have not been standardized across the industry and it is difficult to compare results of different laboratories. The test characteristics of single bulk tank cultures have been assessed in several studies (Table 6).[2,15,64] All the studies used individual herd cultures as the "gold-standard" but the inoculum size was not always specified.

The positive predictive values (detection of at least 1 positive cow when the organism was found using BTC) were estimated for *Strep ag* (98%), *S aureus* (97%) and Mycoplasma spp (80%).[64] The relatively low sensitivity of a single bulk tank culture for screening herds for contagious pathogens has led to several modifications. Incubation of milk samples (95F for 18 h) prior to plating was assessed using triplicate milk samples from 56 herds; an inoculum volume of 0.1 ml was used in this study.[27] Pre-incubation resulted in identification of *S aureus* in 23 of 24 farms that were negative on the primary (non-incubated) plating. The use of commingled samples that have been independently collected for 4 consecutive days is recommended to overcome the daily variation in shedding rates for *S aureus*.[13]

The inoculum volume used for BTC varies from 0.01 ml to 0.2 ml depending on lab procedures. BTC is a screening test that is often used to detect low numbers of organisms, therefore the use of the largest volume of milk possible will increase the probability of recovering organisms.

The interpretation of BTC can be confusing because isolates can arise from either IMI or environmental contamination. The number of organisms isolated using BTC does not correspond to the prevalence of infected cows in the herd therefore BTC should not be used to monitor results of a control program. The interpretation of BTC must consider characteristics of the individual organisms. Recommendations for interpretation of BTC have been published but the scientific validity of the recommendations have not been documented under field conditions.[13,25] The presence of obligate intramammary

pathogens such as *Strep agalactiae* in bulk tank milk are indicative of IMI and should be sufficient evidence to initiate a control program. Many other pathogens such as coagulase-positive *staphylococci* can originate from infected udders and contaminated bedding or skin. Non-agalactiae *streptococci* are usually present in the environment of the cow. While IMI can contribute to high levels of environmental *streps*, poor premilking hygiene should always be investigated when excessive numbers of these organisms are found. The natural duration of IMI caused by coliform organisms is short, therefore excessive numbers of coliforms suggests poor premilking hygiene or environmental contamination.

ProStaph©

The ProStaph© milk antibody test is an ELISA test that detects *S aureus* antibody in milk samples. This test has been available from DHIA processing centers but its use has diminished in recent years. The test was developed as an alternative to culturing and does not require the collection of sterile milk samples. The performance of the ProStaph© test has been compared to standard microbiologic methods.[12,16,20,32] The test was demonstrated to be highly repeatable in a study that used 30 composite milk samples (obtained from a pool of samples submitted from 5 states) tested at 4 separate laboratories.[32] Only 6 of 720 classifications were not in agreement resulting in 99.2% agreement between test laboratories. Of the 6 discordant results, 5 were obtained from a single milk sample. There are several potential sources of disagreement between the ProStaph© test and microbiologic tests. A cow in early stages of infection can be culture positive but antibody negative. A cow can be antibody positive but culture negative because of the intermittent shedding pattern of cows with chronic *S aureus* mastitis or because milk from a single infected quarter was not included (or diluted) in a composite milk sample. Additionally, the test is not considered accurate for cows that are <30 days in milk or producing <30 lbs of milk per day. Finally, differences in sampling and laboratory techniques can influence the outcome of microbiologic tests.

The sensitivity of ProStaph© has been reported to range from 69% to 90% (Table 7). The highest sensitivity was obtained from a trial that included only 20 cows with confirmed chronic *S aureus* infections as the positive samples.[16] The lowest sensitivity and specificity was obtained when a very rigorous "gold standard" was applied.[20] In that study *S aureus* was confirmed only when 2 of 3 consecutive milk samples in a 4-week period tested positive for *S aureus*. That study also defined "suspicious" ProStaph© results (optical density between 85-100% of the positive control) as negative. In any case, it is likely that the false negative rate of a single ProStaph© test ranges between 10-25%. In some herds, the ProStaph© test may be a useful tool in a surveillance and control program for *S aureus* but a single test should not be exclusively relied upon to estimate prevalence of *S aureus* mastitis in the dairy herd.

Conductivity

In-line Electrical Conductivity Tests

An accurate method to automatically detect subclinical and clinical mastitis soon after infection has been desired for many years. There are a number of parameters related to mastitis that can be automatically detected during milking.[39] Deviations in milk

temperature, animal activity, daily milk yield and milk electrical conductivity (EC) can be recorded automatically using various milking systems. The use of EC has generated considerable interest because it forms the basis of detection of abnormal milk in automated milking systems and because there are several hand-held EC tests marketed internationally. On-line EC is measured on quarter or composite milk samples (as performed using most in-line systems such as the Afikim® system) and can be reported as an absolute value or as a comparison (often expressed as a ratio) of EC between quarters. Electrical conductivity is a measure of the resistance of milk to an electric current; conductivity is the reciprocal of the resistance. The unit of measurement for EC is millisiemens per centimeter (mS/cm). In milk, EC is determined by the concentration of anions and cations, primarily Na^+, K^+, and Cl^-. Typical EC of milk from an uninfected cow varies between 4.0 and 5.5 mS/cm at 25^o C. During infections with mastitis, the milk concentration of lactose and K+ are decreased and concentrations of Na^+ and Cl^- are increased because of increased blood capillary permeability, the destruction of tight junctions, and the destruction of action ion-pumping systems.[28] Mastitis is not the only circumstance that causes the ionic content of milk to change and non-mastitis related variation in EC is a major drawback to the diagnostic value of EC. Non-mastitis factors influencing EC include milk temperature (EC increases 0.113 mS per degree C in a linear fashion as the temperature of the milk sample increases[41]), stage of lactation, fat percentage (fat is a nonconductor), milking interval, and breed (Table 8).

Both absolute thresholds (a quarter or animal has mastitis when EC exceeds the threshold) and within-cow quarter comparisons of EC (a quarter with EC $\geq$16% above the lowest quarter has mastitis; also referred to as "differential EC") have been used to diagnose mastitis. An expert panel assembled by the International Dairy Federation performed a meta-analysis of EC (using absolute thresholds) from a selection of published papers.[17] EC did not perform well as a screening test for either clinical or subclinical mastitis (Table 9). The low PPV for clinical mastitis indicates that out of 100 positive tests only 58 would truly have clinical mastitis. The relatively low NPVs indicate that 15-30% of animals identified as mastitis free would be truly infected. These results led the IDF panel to conclude that: "The published information is too varied to justify a claim that mastitis, especially subclinical mastitis, can be detected by means of electrical conductivity measurements in milk."

Within-cow comparisons of quarter EC have been reported. The principle behind differential EC is that sources of variation in EC other than mastitis would be the same for all for quarters, so a comparison of EC values between quarters should reduce extrinsic variation. The use of differential EC has been shown to improve both sensitivity and specificity of EC.[38] The sensitivity increased from 57% to 68% and specificity increased from 91% to 96% when differential values were used rather than an absolute threshold.[38] The use of differential quarter sample EC values is probably the best current use of this technology.

Handheld Electrical Conductivity Tests

Several handheld EC tests are available internationally. The devices accurately measure conductivity of milk samples and are designed for use on quarter milk samples. In the

U.S., **Mas-D-Tec®** (Wescor, Logan Utah) is marketed as a portable hand held milk analyzer that can be used to detect subclinical mastitis. The manufacturer of Mas-D-Tec® suggests that absolute EC scores of $\geq$5 indicate the presence of subclinical mastitis. One study evaluated the use of Mas-D-Tec® to detect subclinical mastitis on farms in Costa Rica.[37] Microbiologic results from single milk samples obtained from 425 cows were used as the gold standard. The prevalence of subclinical mastitis in the study herds was 20.2%. Results were interpreted based on both absolute values (as recommended by the manufacturer) and by calculation of a differential score based on the difference between the highest and lowest EC scores for the 4-quarters of each cow. The test characteristics of both methods were determined (Table 10). Neither of the diagnostic methods achieved sufficient accuracy to be recommended as screening tests. At the manufacturers recommended cut point, 71% of test positive samples would be microbiologically negative and major mastitis pathogens would be isolated from 11% of test negative samples.

Other handheld screening tests have also been evaluated.[21,35,57] Under U.K. conditions, conductivity increased in cows subclinically infected with *S aureus* infections but was not detectably increased in IMIs caused by *S uberis*.[21,35] Australian researchers evaluated a hand-held resistance meter and concluded that the predictive value of the method was generally poor.[57] With current technology and diagnostic algorithms, other screening tests (individual SCC values, CMT and individual cow milk cultures) continue to be more useful in mastitis control programs than the use of hand-held EC meters.

Antibiotic Susceptibility Tests

Antimicrobial susceptibility tests are used to guide the selection of mastitis treatments. In recent years, antimicrobial susceptibility testing has come under scrutiny because of concerns about antimicrobial resistance, changes in methodology and the relationship between in-vitro results and on-farm clinical outcomes. Antimicrobial susceptibility tests are based upon inhibition of bacterial growth (not killing of bacteria) and the end-points of the various testing methods can be either qualitative (sensitive, intermediate or resistant) or quantitative (minimal inhibitory concentration "MIC").[60] The standard test used in most veterinary diagnostic reference laboratories has been the Kirby-Bauer disk diffusion (KBDD) test. The KBDD method is widely used in veterinary clinics because it is easy to perform and relatively inexpensive. The underlying principle of KBDD is the inverse linear relationship between the log MIC and the diameter of the zone of inhibition of growth of a standard inoculum of bacteria (approximately 1×10^8 cfu) around a filter paper disk containing a standard amount of antibiotic on standardized growth media. The diffusion of antibiotic results in a drug concentration gradient and when the concentration of antibiotic becomes too dilute to inhibit growth the zone of inhibition is formed. A significant source of error in this test is the failure to standardize the bacterial inoculum. Veterinary reference laboratories routinely run quality control strains and standardize inoculums by incubation of selected colonies in broth until they reach a turbidity of 0.5 McFarland standards. The **Prompt©** system (Becton-Dickson) is a rapid standardization system that has shown 96% agreement with traditional inoculation systems using common mastitis pathogens.[61] This system can be easily used in field laboratories to reduce error associated with inoculum volume. The KBDD separates isolates into 3

populations: sensitive, intermediate or resistant based upon the zone size surrounding the antibiotic disk.[45] Results indicate if an isolate is thought to be sensitive to an antibiotic assuming that tissue concentrations reach the in-vitro breakpoint. Many of the zones of inhibition of older classes of drugs were based upon serum levels in human patients and veterinary clinicians sometimes question the clinical relevancy of this data. The appropriate use of KBDD may be to indicate drugs that are clearly inappropriate rather than to indicate in-vivo susceptibility.

Quantitative susceptibility testing is generally performed using broth microdilution (MD) tests. Broth MD is performed in microtiter plates, using antibiotics in progressive 2-fold dilutions in similar concentrations to those obtained in serum or tissue. An MIC is recorded as the lowest concentration of an antimicrobial that completely inhibits the growth of the isolate. MIC data is more useful than qualitative results because it can more precisely define the degree of susceptibility (and required drug dosage). The pharmacokinetic parameters correlated with drug efficacy differ between classes of drugs.[46] For example, serum concentrations should continuously exceed the MIC for beta-lactams whereas the peak serum concentration should be 8-10 times the MIC for aminoglycosides. MIC values for a number of common antibiotics have been reported for bacteria isolated from bovine mastitis.[7,62] For *S aureus* the overall level of resistance was low but there was considerable variation in MIC values.[7] Antimicrobial susceptibility was variable for *Strep* sp and only ceftiofur and enrofloxacin were reported to be effective against enterococci.[62] The clinical efficacy of intramammary mastitis therapy using penicillin-novobiocin (Albacillin®, Pharmacia Co.) for treatment of subclinical IMI has been compared to the results of susceptibility tests obtained using MIC values (Table 11).[42] This study defined clinical cure as the absence of bacteria in duplicate quarter milk samples collected 28 days post treatment. Only isolates susceptible to penicillin-novobiocin were treated. The relationship between in-vitro susceptibility results and bacteriologic cure was higher for *Strep* spp than *Staph* spp. and was lowest for chronic *S aureus* infections. The authors concluded that in-vitro testing was a good predictor of therapy for IMI caused by *Staph spp*, new *S aureus* infections, *S uberis*, *S dysgalactia*, and *S agalactiae* but not for IMI caused by chronic *S aureus*.

The **MASTiK™** test (ImmunCel, Portland ME) is marketed as a rapid on-site mastitis antibiotic susceptibility test kit. The MASTiK™ test is promoted as a "milk microdilution test" and does not require the isolation and identification of bacterial colonies prior to determining the susceptibility. The procedure for this test is quite simple. A sample (1 ml) of milk from the mastitic gland is incubated with reagent and then pipetted into antibiotic-coated wells on a 96-well microtiter plate. The wells are observed for color changes after incubation for 6-24 hours. Growth of lactose fermenting bacteria in the presence of the antibiotic results in the production of lactic acid that changes the color in the well from purple to yellow. The absence of color change is interpreted to mean that the organism is susceptible. Under carefully controlled laboratory conditions, susceptibility results obtained by MASTiK™ were compared to KBDD.[22] Agreement between MASTiK™ and KBDD varied by antibiotic, concentration and organism but generally exceeded 80%. No indication of the relationship between susceptibility results obtained using MASTiK™ and clinical

efficacy has been reported. There are a number of potential errors that could be associated with this procedure. The most important potential error is the failure to isolate a single pathogen from the milk sample. Milk samples can contain multiple bacterial isolates from contaminates and it would be impossible to confirm that the susceptibility pattern was related to a true mastitis pathogen.

Conclusion

The veterinary practitioner has a wide variety of diagnostic options for solving milk quality problems. High milk bacterial counts generally suggest problems with milking equipment or cooling but practitioners should be aware of the ability of *Streptococci spp*. to cause transient increases in bacterial numbers of bulk milk. Investigation of increased bacterial counts should include a series of tests to help isolate the source of the bacteria. The use of bulk milk and individual cow SCC values is fundamental to the production of high quality milk. It is important however that veterinarians understand the limitations of these tests when they are used to identify intramammary infections. The CMT has been used for more than 50-years and continues to be the most accurate cowside screening test for subclinical mastitis. Methodological differences contribute to confusion in interpretation of individual and bulk tank milk cultures and antibiotic susceptibility testing but the use of these techniques is necessary to fully understand mastitis problems.

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Table 1. Effect of changing SCC threshold on positive (PPV) and negative (NPV) predictive values.

			Herd A; Prevalence =40%		Herd B; Prevalence = 5%	
Threshold	Sensitivity[a]	Specificity[a]	PPV	NPV	PPV	NPV
200,000	.726	.855	77	82	21	98
225,000	.630	.932	86	79	33	98
250,000	.547	.962	91	79	43	97

[a]from Dohoo and Leslie, 1991

Table 2. Sensitivity and specificity of criteria used to detect new IMI based on changing SCC over two consecutive tests.[a]

Criterion	Level	Pathogens	Sensitivity	Specificity	Reference
<200 to >200	Cow	All	38.8	91.9	Dohoo, 1991
<200 to >250	Quarter	Major	39.6	95.7	Schepers, 1997

[a]adapted from Dohoo, 2001

Table 3. Interpretation of CMT scores and approximate corresponding SCC values.

CMT Score	Visible Reaction	SCC Range (cells per mL)	Somatic Cell Score	Approximate SCC midpoint
Negative	Mixture remains liquid – no evidence of precipitate	0 – 200,000	0	12,500
			1	25,000
			2	50,000
			3	100,000
			4	200,000
Trace	Slight precipitate, best seen by tipping, disappears with continued movement	150,000 – 500,000	5	400,000
1	Distinct precipitate but no tendency toward gel formation	400,000-1,500,000	6	800,000
2	Mixture thickens immediately, moves toward center	800,000 – 5,000,000	7	1,600,000
			8	3,200,000
3	Gel forms and surface becomes convex	>5,000,000	9	6,400,000

Table 4. Relative test characteristics for diagnosis of S aureus mastitis in composite milk samples by inoculum volume.

Sample volume (ml)	Relative Sensitivity (95% CI)	Relative Specificity (95%)
0.01	0.78 (0.70-0.85)	0.95 (0.89-0.98)
0.05	0.86 (0.78-0.92)	0.93 (0.84-0.92)
0.10	0.90 (0.83-	0.86 (0.78-0.92)

Table 5. Identification of cows infected with S aureus by culture technique and time of sampling.

Technique	Time of Sampling	
	1 to 5 days post-calving (n=160)	7 to 10 days post-calving (n=276)
Quarter milk (% positive)	8.8%	9.4%
Centrifuged samples	13.8%	19.2%
Percent increase	57.1%	103.8%

Table 6. Test characteristics of single bulk tank culture.

Study	Herds	Prevalence (%)		Sensitivity (%)	
		Strep ag	Staph aureus	Strep ag	Staph aureus
Bartlett (1991)	46	35	69	35.3	41.2
Godkin (1990)	56	55	76	20.5	9.2
Wilson (1997)	>1700	20	81	77	58.0

Table 7. Relative test characteristics of ProStaph© test.

Study	Sample	Number of Samples	Sens.	Spec.	Gold Standard
Grove et al, '92	Composite, 5 herds	97 milk samples; 20 chronic; 77 uninfected	90%	97%	Micro; .05 ml single culture
El Rashidy et al, '92	Quarter & comp., 1 herd	10 samples from chronically infected cows, 9 from uninfected	83%	99%	Micro; .10 ml single culture
Hicks et al, '94	Composite, 5 herds	185 cows	69%	61%	Micro; 2 of 3 samples positive;

Table 8. Evaluation of physiological parameter of EC in foremilk samples from uninfected quarters.[a]

Parameter	Extent of Influence	Interference with Udder Health Determination
Stage of lactation	>10%	Yes
Lactation number	Not significant	No
Breed	>10%	Yes
Nutrition	<10%	Questionable
Milking interval	>10%	Yes
General cow status (such as estrus)	>10%	Yes

[a]adapted from, Hamann J, Zecconi A., 1998.

Table 9. Comparison between EC and mastitis outcomes.[a]

Outcome	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
Clinical Mastitis ($\bar{x}$ ±SD %)	68.2 ±23.9	81.9±9.6	58.1±27.2	81.5±15.5
Subclinical Mastitis (measured by SCC)	68	88	72	85
Subclinical Mastitis (measured by IMI)	61	66	55	70

[a]adapted from, Hamann J, Zecconi A., 1998.

Table 10. Test characteristics of Mast-D-Tec® used to detect subclinical mastitis.

Method	Cut point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Absolute value	5	74	53	29	89
	6	55	69	32	86
	7	43	83	39	85
	8	30	89	41	83
Differential[a]	1	81	26	22	85
	2	53	77	37	87
	3	30	90	44	84

[a]difference between highest and lowest quarter

Table 11. Efficacy of intramammary penicillin-novobiocin for treatment of subclinical IMI in isolates considered susceptible by broth microdilution (from Owens et al, 1997).

Organism	Number Isolates	Percent bacteriologic cure	MIC 90% (μg/ml)
S aureus (chronic)	20	35%	0.015
S aureus (induced)	20	70%	-------
Staph spp.	21	71%	0.0035
Strep ag (induced)	20	90%	-------
Strep uberis	22	91%	0.007
Strep dysgalactia	20	90%	0.007
Other Streptococci	13	77%	0.06

Figure 1. Composite Milk SCC if only 1 Quarter is Infected and the baseline SCC in uninfected quarters is 100,0000 cells per ml.

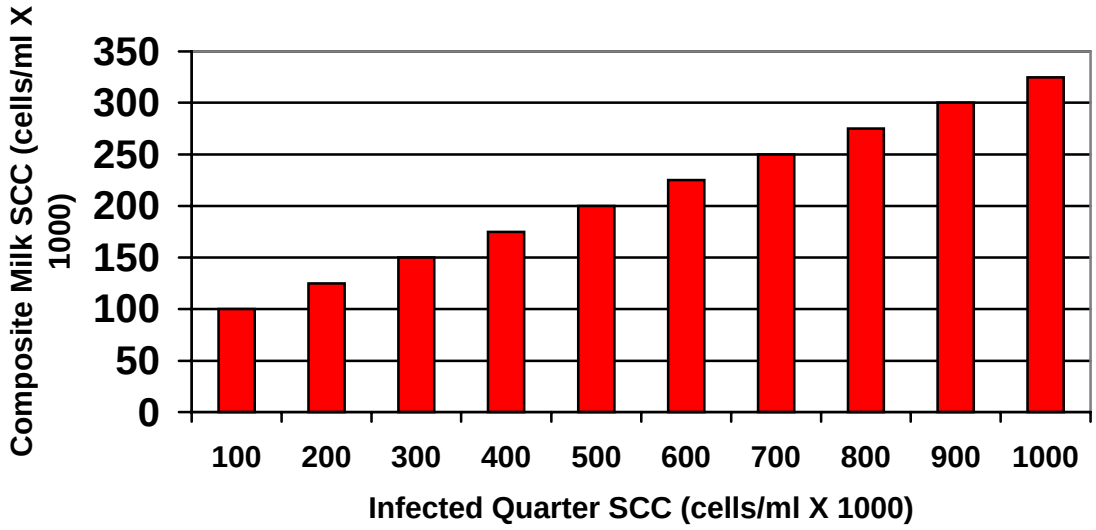

Figure 2: DHI Herd Summary Data by Production Level for Wisconsin Dairy Herds, Dec 2000

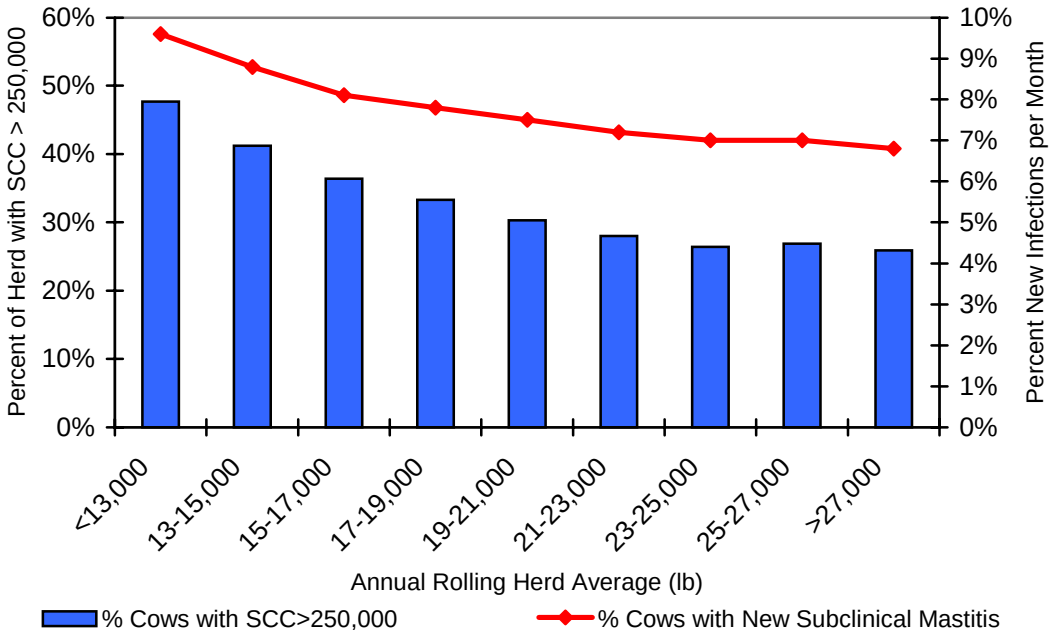

Fig 3. Scatterplot of Linear Scores for two consecutive Months.