Test used to Diagnose Mastitis on Dairy Farms
Pamela L. Ruegg, DVM, MPVM
University of Wisconsin, Madison

Introduction
Identification of cows infected with mastitis is necessary to make decisions regarding treatment, culling or isolation of infected animals. Common methods used to identify infected cows include: milk microbiology (“cultures”), the California Mastitis Test (CMT), individual cow SCC values and electrical conductivity.

Individual Quarter or Composite Milk Cultures. Microbiologic exam of milk samples may be used for control programs (such as segregation plans) or for detection of new pathogens. Culturing is also used to determine antibiotic susceptibility of mastitis pathogens. Microbiologic examination of milk samples is often considered to be the gold standard for identification of infected quarters. Negative results (no growth of bacteria from samples of animals suspected to be infected) are a common outcome. No bacteria were isolated from approximately one-third of milk samples submitted to a major mastitis laboratory in Wisconsin between 1994 and 2001 (Makovec and Ruegg, 2003). Between 1994 and 2001, the proportion of negative results increased from 22.6% to 49.7% (Figure 1). Potential reasons for negative results include a decrease in the amount of mastitis caused by high shedding organisms (such as Strep ag), increased amount of mastitis caused by organisms that don’t grow using routine laboratory procedures (such as Mycoplasma bovis) and the use of insensitive sampling methods and laboratory techniques. To ensure that meaningful data will be obtained from milk samples taken from cows with suspected subclinical infections, at least 25 quarter samples should be submitted for culture.

The interpretation of results of milk cultures is not straightforward and is dependent on the organism, sampling method and laboratory procedures. For example, bacteria are often shed sporadically and in low numbers from quarters that are infected with Staph aureus in contrast to frequent shedding of large numbers of bacteria from quarters infected with Strep ag. The timing of collection of the milk samples can influence the recovery of mastitis organisms. In one study, more colonies of Staph aureus were recovered from samples obtained before milking as compared to samples obtained after milking (Sears et al., 1991). When pre-milking samples were used, 91% of infected
cows were accurately identified as compared to identification of 81% of infected cows using samples obtained after milking (Sears et al., 1991).

Milk Samples can be obtained from individual quarters (usually performed for clinical mastitis cases) or commingled milk from all 4-quarters (often used as a screening test for subclinical mastitis). When a single quarter sample was used, only 75% of cows subclinically infected with *Sta aureus* were accurately identified; the addition of 2 or 3 additional milk samples increased detection to 94-98%. When highly sensitive methods of identifying cows infected with *S. aureus* are required, quarter milk samples should be obtained from foremilk. The laboratory should be asked to plate a larger volume of milk and incubate or centrifuge any samples that don’t show bacterial growth by 24 hours of incubation. If negative milk cultures are obtained from cows that have chronically high SCC values, additional cultures should be obtained to verify the result.

The use of milk culturing to identify cows infected with *Strep ag* is straightforward. Culture of single milk samples have been found to accurately identify >95% of cows chronically infected with *Strep ag* (Dinsmore et al, 1991). Results do not appear to be influenced by obtaining samples before or after milking, the use of 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.

_Petrifilm™ Selective Medias_

_Petrifilm™* plates (3M Microbiology. St. Paul, MN) are sample-ready selective culture media that are marketed for rapid bacteriological isolation and enumeration of bacteria from food products. *Petrifilm™* products that are potentially useful for diagnosis of mastitis include *Petrifilm™* Aerobic count plates, Coliform count plates and Staph Express count plates. The *Petrifilm™* Staph Express Count Plate contains chromogenic, modified Baird-Parker media that is selective and differential for *Staph. spp*. Confirmation of *Staph aureus* is performed using a disk that contains deoxyribonuclease and a dye that reacts to produce a pink zone around *Staph. aureus* colonies. *Petrifilm™* products may be especially useful for farm based culture systems that are used to guide treatment decisions because they are easy to use and usually result in a diagnosis within a 24 hour period. The sensitivity of the *Petrifilm™* Staph Express plates has been compared to standard and augmented laboratory techniques using milk samples obtained from commercial dairy cows (Silva, et al., 2004). Laboratory methods evaluated included standard microbiological methods (NMC, 1999), preincubation of milk samples before plating and centrifugation (Zecconi et al., 1997).
The total number of quarter samples included samples from cows with high SCC (n = 97), postpartum (n = 161), clinical cases of mastitis (n = 102) and composite duplicate milk samples from known *Staph. aureus* infections (n = 29). The gold standard was defined as isolation of *Staph aureus* from a milk sample by use of any of the microbiological methods. Using this definition, the estimated prevalence of *Staph. aureus* was 8.2%. The sensitivity was significantly lower ($P < 0.05$) for milk samples processed using the standard method (65.6%) as compared to milk samples processed using Centrifugation (75.0%), Incubation (84.4%) or Petrifilm™ (87.5%).

Specificity was determined in a separate experiment using quarter milk samples (n = 332) that were obtained from all lactating cows (n = 88) located on a commercial farm with a suspected *Staph aureus* problem. Immediately after collection, a 1 mL aliquot of each milk sample was spread on a Petrifilm™ Staph Express Count Plate following manufacturers directions (3M Microbiology, St. Paul, MN). Samples were incubated at 37°C for 24 H. *Staph aureus* were identified following manufacturers instructions for the 3M™ Staph Express Count Plate and the 3M Staph Express Disk. The disk was applied to all Staph Express Plates with evidence of bacterial growth after 24 h of incubation (n = 320). Petrifilm™ plates with evidence of any zones associated with colonies (n = 127) were recorded (weak or distinct) after 2 more hours of incubation. No further processing was performed for plates (n = 193) that lacked colonies with visible zones. All colonies that formed weak (n = 62) or distinct (n = 62) pink zones on Petrifilm™ were picked and regrown on blood agar plate. *Staphylococcus aureus* were identified by hemolytic pattern, positive catalase reaction, growth on mannitol, and positive tube coagulase reaction.

The occurrence of a distinct pink zone surrounding a colony was highly specific for *Staph. aureus* (Table 1). The use of a weak pink zone to diagnose *Staph. aureus* resulted in a high rate of false positive results. The intensity of the pink zone was strongly associated with the odds of confirming *Staph. aureus* ($P < 0.001$). Colonies exhibiting distinct pink zones were 120 times more likely to be confirmed as *Staph aureus* as compared to colonies that exhibited weak pink zones.

<table>
<thead>
<tr>
<th>Zone Intensity</th>
<th>Staph Aureus</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td>Specificity</td>
<td></td>
</tr>
<tr>
<td>Weak or Distinct</td>
<td>65</td>
<td>59</td>
<td>124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Zone</td>
<td>0</td>
<td>193</td>
<td>193</td>
<td>76.6%</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Results of milk samples by zone intensity
California Mastitis Test. For 50 years the California Mastitis Test (CMT) has been the only reliable cowside screening test for subclinical mastitis. The CMT does not identify the type of bacteria that cause mastitis but is used to identify quarters that have subclinical mastitis. The CMT was developed to test milk from individual quarters but has also been used on composite milk samples and bulk milk samples (Schalm and Noorlander, 1957). 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 before testing because somatic cells 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 2).

Table 2. Interpretation of California Mastitis Test Reaction

<table>
<thead>
<tr>
<th>CMT Score</th>
<th>Visible Reaction</th>
<th>SCC Range (cells per mL)</th>
<th>Approximate SCC midpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Mixture remains liquid – no evidence of precipitate</td>
<td>0 – 200,000</td>
<td>100,000</td>
</tr>
<tr>
<td>Trace</td>
<td>Slight precipitate, best seen by tipping, disappears with continued movement</td>
<td>150,000</td>
<td>400,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500,000</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Distinct precipitate but no tendency toward gel formation</td>
<td>400,000-1,500,000</td>
<td>800,000</td>
</tr>
<tr>
<td>2</td>
<td>Mixture thickens immediately, moves toward center</td>
<td>800,000</td>
<td>1,600,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,000,000</td>
<td>3,200,000</td>
</tr>
<tr>
<td>3</td>
<td>Gel forms and surface becomes convex</td>
<td>&gt;5,000,000</td>
<td>6,400,000</td>
</tr>
</tbody>
</table>
The use of the CMT to identify quarters infected with contagious mastitis has been extensively evaluated (Barnum and Newbould, 1961, Brookbanks, 1966, Painter and Schnepper, 1965, Wesen et al., 1968). In general, as CMT reactions increase, the likelihood of recovering pathogenic bacteria increases. The ability of the CMT to detect infected quarters of fresh cows has been recently reported (Sargeant et al, 2000). In that study, CMT was performed on quarter milk samples each day after calving until 10 days postpartum. When a positive CMT was defined as a reaction of $\geq 1$, about 57% of infected quarters were accurately identified (43% were missed). Another study used the CMT to test 7,431 composite milk samples obtained from herds in which about 35% of the cows were subclinically infected with \textit{Sta aureus} and \textit{Str ag} (Brookbanks, 1966). When a CMT value of trace or greater was used, 92% of infected cows were correctly identified. When a CMT value of $>1$ was used only 72% of infected cows were correctly identified. To minimize the number of false negative results, the test should be read as positive when at least a trace reaction is apparent.

\textbf{Somatic Cell Counts.} Subclinical mastitis is often diagnosed based on SCC values that exceed a threshold (such as 250,000 cells/ml). It is important to remember that most available SCC values (for example: monthly DHIA tests) are performed on milk that has been commingled from all 4 quarters. There are some obvious problems with using composite milk SCC to identify infected cows because of dilution of somatic cells with milk from uninfected quarters. Consider the hypothetical situation when a cow is producing 20 kg of milk per milking evenly distributed between 4 quarters (5.0 kg 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 200,000 cells/ml until the SCC from the infected quarter exceeds 700,000 cells/ml (Figure 2).

New subclinical mastitis infections are usually diagnosed when the monthly SCC of a cow exceeds a threshold (often 250,000 cells/ml) for the first time in that lactation. There is no simple way to estimate the amount of subclinical mastitis, the development of new infections or the result of mastitis control procedures without access to monthly individual cow SCC values. Common industry goals for subclinical mastitis are: \textbf{85\% cows with somatic cell counts $\leq 250,000$ and less than $<5\%$ of cows developing new subclinical mastitis infections per month.} In Wisconsin herds that subscribe to a large DHIA provider, approximately 30\% of the cows have SCC values that indicate the presence of subclinical mastitis.

\textbf{New Cowside Tests for Rapid Diagnosis of Sublclinical Mastitis.} Many mastitis control programs would be improved with the use of a rapid diagnostic test for subclinical mastitis. The Delaval Direct Cell Counter (DCC) is a new device that is designed to be used on farms for rapid enumeration of somatic cells. Small cassettes
designed to be used on farms for rapid enumeration of somatic cells. Small cassettes are filled with approximately 1µl of fresh milk, stained automatically in the cassette and inserted into a small battery operated optical cell counter. The DCC produces a somatic cell count in less than 1 minute within the range of 10,000 to 4,000,000 cells/ml. In one experiment we determined the test characteristics of the Direct Cell Counter (Ruegg, Hulland and Reith, unpublished). Quarter milk samples (n = 800) were obtained from cows (n = 200) during days 3-9 post-calving. Study personnel performed the CMT and DCC on the farm and submitted additional duplicate quarter milk samples for laboratory determination of SCC and for microbiological analysis.

Infections with major pathogens (S. aureus, env. Strep. E. coli) or minor pathogens (coagulase negative Staph., corynebacterium) were defined based on isolation from both duplicate quarter samples. There was no significant difference between the log_{10}SCC (5.1) and the log_{10}DCC (5.1; P = 0.76). The correlation between the log_{10}SCC and log_{10}DCC was 0.92 (P<0.001). When subclinical mastitis was defined based on a threshold of 200,000 cell/ml, there was 95.4% observed agreement between the SCC and DCC and kappa was 0.895. The log_{10}DCC was higher for milk samples from which major (5.8) or minor (5.5) pathogens were recovered as compared to milk samples that were contaminated (5.1) or negative (5.0) (p <0.001). The DCC appears to be an accurate method to rapidly determine SCC values.

The PortaSCC™ (PortaScience, Portland ME) is another rapid test that is being developed for cowside testing of somatic cells. This test is adapted from a product used by human cancer patients to monitor white blood counts. The test measures only white blood cells (not epithelial cells) and has an upper limit of detection of 3,500,000 cells/ml. The test consist of a small strip that is inoculated with a drop of milk and a reagent. The test strip requires a 45 minute room temperature incubation and is read in a small handheld meter. We recently used this product to evaluate 300 quarter milk samples obtained from cows located on 10 separate dairy farms. There was no significant difference in Log_{10} SCC between the SCC and the results of the PortaSCC™ (SCC = 4.98 & PortaSCC = 4.86, P <0.001). The correlation between the tests was 0.81 (P<0.001). When subclinical mastitis was defined based on a threshold of 200,000 cell/ml, there was 88.0% observed agreement between the SCC and the PortaSCC.

Both of these tests appear to be significant improvements over the CMT because they are able to more accurately enumerate somatic cells at much lower thresholds than the CMT test is able to do and both remove the subjective aspect of visual observation.

Tests of Electrical Conductivity (EC). The use of EC has generated considerable interest because it forms the basis of detection of abnormal milk in automated milking systems and because several hand-held EC tests are available. In milk, electrical conductivity (EC) is determined by the concentration of Na^+, K^+, and Cl^- . Typical EC of milk from an uninfected cow varies between 4.0 and 5.5 mS/cm. During
EC of milk from an uninfected cow varies between 4.0 and 5.5 mS/cm. During infections with mastitis, the milk concentration of lactose and K+ are decreased and concentrations of Na⁺ and Cl⁻ are increased because of changes in the permeability of cells and blood vessels (Kitchen et al., 1980). Mastitis is not the only circumstance that causes changes in milk EC and non-mastitis related variation in EC is a major drawback. Non-mastitis factors influencing EC include milk temperature, stage of lactation, fat percentage, milking interval, and breed.

EC can be measured using either quarter or composite milk samples and is reported as an absolute value or as a comparison of EC between quarters (often expressed as a ratio). Both absolute thresholds (a quarter or cow is considered to have mastitis when EC exceeds the threshold) and within-cow quarter comparisons of EC (a quarter with EC ≥16% above the lowest quarter is considered to have 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 (Hamman and Zeconci, 1998). EC did not perform well as a screening test for either clinical or subclinical mastitis. Of 100 positive EC tests only 58 would truly have clinical mastitis and 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.”

Comparisons of EC applied to quarter samples have also 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 variation. The use of differential EC has been shown to improve the ability of EC to detect mastitis (Neilen et al., 1992). When differential values were used rather than an absolute threshold, the rate of false positives decreased from 43% to 32% and the rate of false positives decreased to 4%. The use of differential quarter sample EC values is probably the best current use of this technology.

Several handheld EC tests are available. 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 ≥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 (Musser et al, 1998). Results of EC were compared to culture results obtained from single milk samples of 425 cows. 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 each cow.
based on the difference between the highest and lowest EC scores for the 4-quarters of each cow. Neither of the interpretation methods achieved enough accuracy to be recommended as screening tests. At the manufacturers recommended cut point, 71% of EC positive samples would be negative on culture and major mastitis pathogens would be isolated from 11% of test negative samples.

Other handheld screening tests have also been evaluated. Under U.K. conditions, conductivity increased in cows subclinically infected with *Sta aureus* infections but was not detectably increased in IMIs caused by *Str uberis* Hillerton and Walton, 1991; Milner et al, 1996). Australian researchers evaluated a hand-held resistance meter and concluded that the predictive value of the method was generally poor (Segura and Mansell, 2000). With current technology and diagnostic methods, 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.

**Summary**
The detection and diagnosis of subclinical mastitis is an important component of mastitis control. A number of tests, each with its own strengths and weaknesses, are available to aid in the diagnosis of mastitis. When test results disagree, it is prudent to resample cows and confirm the results. The use of monthly individual cow SCC values is fundamental for control of mastitis and the recent development of new rapid tests is promising for identification of subclinical quarter infections.

**References**


