Université de Montréal

Comparison of 3M Petrifilm™ Staph Express, 3M Petrifilm™ Rapid Coliform and 3M Petrifilm™ Aerobic count plates with standard bacteriology of bovine milk

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Ce mémoire intitulé :

Comparison of 3M Petrifilm™ Staph Express, 3M Petrifilm™ Rapid Coliform and 3M Petrifilm™ Aerobic count plates with standard bacteriology of bovine milk

présenté par :
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Résumé

Les objectifs de l'étude étaient d'évaluer les caractéristiques diagnostiques des plaques Petrifilm^{TM} d'identifier ; 1) *Staphylococcus aureus* dans les échantillons de lait provenant de vaches a) en début de lactation, b) avec un comptage des cellules somatiques élevés, c) avec une mammite clinique, en utilisant les plaques de numération express de *S. aureus* (STX). 2) les coliformes lors de mammite clinique, en utilisant les plaques de numération rapide des coliformes (RCC). 3) les streptocoques lors de mammite clinique, en utilisant les plaques de numération des bactéries aérobies (AC). 4) Évaluer l'effet de la congélation des échantillons de mammite frais sur les caractéristiques diagnostiques des STX et RCC. Pour chaque objectif, l'effet d'utiliser un échantillon dilué (1 :10) ou non était comparé. La concordance entre le STX, RCC et AC versus la bactériologie standard était évaluée. Les résultats sont: 1a) un total de 1204 échantillons frais ont été analysés. La sensibilité (Se) et la spécificité (Sp) du STX pour les échantillons non-dilués et dilués étaient 69.2%, 98.3%, et 74.2%, 97.8%, respectivement. 1b) La Se et Sp des 300 échantillons étaient plus élevées pour les échantillons dilués ; 85.1% et 96%, respectivement. 1c) Les valeurs de Se et Sp du STX étaient plus élevées pour les échantillons provenant de lait frais et dilués. Pour les échantillons congelés, la Se et Sp étaient semblables pour les échantillons non-dilués et dilués. 2) La Se (76.4%) du RCC pour identifier les coliformes était plus élevée pour les échantillons mammiteux frais et dilués. 3) Pour l'analyse de AC, les analyses ont démontré une Se et une Sp de 86.5% et 9.8%. La faible valeur prédictive positive (24.6%) indique que l'AC n'est pas utile pour la détermination des streptocoques dans le lait. 4) Quand les échantillons de lait mammiteux frais étaient congelés, il y avait une augmentation de 10.6% de Se pour l'identification de *S. aureus*. Pour l'identification des coliformes, il y avait une diminution de Se de 14.7% suite à la congélation des échantillons. La concordance entre le STX, RCC et AC et la bactériologie standard pour les objectifs 1,2,3 et 4 était supérieur avec les échantillons dilués. En résumé, les résultats obtenus indiquent que les milieux de culture Petrifilm STX et RCC sont comparables à la bactériologie standard pour l'identification de *S. aureus* et des coliformes.

Mots-clés : Petrifilm, *Staphylococcus aureus*, coliforme, Streptococci, microbiologie, lait bovin

Abstract

The objectives of the study were to evaluate the diagnostic test characteristics of the: 1) Petrifilm™ Staph Express Count plates (STX) to identify *Staphylococcus aureus* from cows' milk. Milk samples were taken from cows: a) in the first 30 days in milk; b) with high somatic cell count (SCC) during lactation; c) with clinical mastitis. 2) Petrifilm Rapid Coliform Count plates (RCC) for identification of coliforms from cows with clinical mastitis. 3) Petrifilm Aerobic Count plates (AC) for identification of streptococci from cows with clinical mastitis. 4) Petrifilm plates (STX, RCC) after freezing clinical mastitis milk samples. The effect of an undiluted and a diluted sample (1:10) on the test characteristics of Petrifilm plates was also determined in each objective. The agreement (Kappa) between Petrifilm plates (STX, RCC, and AC) and standard bacteriology was evaluated. The results for part 1a) A total of 1204 fresh milk samples were used in the analysis. The sensitivity (Se) and specificity (Sp) of the STX versus standard bacteriology for non-diluted and diluted samples versus standard bacteriology was 69.2%, 98.3%, and 74.2%, 97.8%, respectively. 1b) The Se and Sp of the STX for 300 fresh milk samples was highest for diluted samples; 85.1%, and 96%. c) The test characteristics of the STX were the highest for diluted fresh samples from mastitis cows. For frozen samples, the Se and Sp were similar for undiluted and diluted samples. 2) The Se (81.6%) of the RCC to detect coliforms was the highest for the diluted fresh samples. 3) For AC analysis, the Se and Sp for diluted fresh samples was 86.5%, and 9.8%, respectively. The poor positive predictive value (24.6%) indicates that the AC is not effective for streptococci determination. 4) When fresh mastitis samples were frozen and then replated, there was an increase in Se of 10.6% in *S. aureus* identification. For coliform identification, the Se decreased by 14.7% after freezing fresh samples. The agreement between the STX, RCC, and AC and standard bacteriology for part 1, 2, 3, and 4 was best with diluted samples. Overall, the results indicate that the Petrifilm STX and RCC culture media are comparable to standard bacteriology for the detection of *S. aureus* and coliforms.

Key Words: Petrifilm, *Staphyloccocus aureus*, coliforms, Streptococci, microbiology, bovine milk

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AC:	Petrifilm™ Aerobic Count plate
BMSCC:	Bulk tank milk somatic cell count
CAMP:	A lytic reaction to differentiate group B streptococci
CC:	Petrifilm™ Coliform Count plate
cfu :	Colony forming unit
CMT :	California mastitis test
CNS :	Coagulase negative staphylococci
DHI :	Dairy herd improvement
EC :	Petrifilm E.coli/coliform count plate
GN :	Gram negative
GP :	Gram positive
HSCC:	High somatic cell count
IDF:	International Dairy Federation
IMI :	Intramammary infection
IMM:	Intramammary
IRCM:	Incidence rate of clinical mastitis
LAL-test:	Limast® test
LSCC:	Low somatic cell count
mL :	Millilitre
NG:	No growth
NPV:	Negative predicative value
OFCS:	On farm culture system
PCR:	Polymerase chain reaction
PPV:	Positive predictive value
RCC:	Petrifilm™ Rapid Coliform Count plate
SCC :	Somatic cell count
Se:	Sensitivity
Sp:	Specificity
STX:	Petrifilm™ Staph Express Count plate,
TSA :	Trypticase soy agar

TSI :	Triple sugar iron
TKT:	Thallium, crystal violet, and toxin in blood agar

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Introduction

Clinical mastitis is the primary reason for antibiotic use in dairy cow (Guterbock, 1994; Erskine et al., 2003). Presently, there is more and more scrutiny on antibiotic usage in agriculture. As public concerns grow, there is a demand for more prudent use of antimicrobials in animals. Ideally, treatment should be based upon culture results. Bacteriological culture of milk is essential in determining the presence and type of pathogen causing mastitis. However, issues associated with collection, shipping and handling of milk samples, cost, and the lag-time in reporting results have made some producers reluctant to have milk bacteriology performed.

Studies have shown that in most cases of mild clinical mastitis, treatment can be delayed for 1 day (while waiting for the culture results) with minimal adverse effects (Silva et al., 2004). Using milk culture and targeted therapy has the potential for less antibiotic use and milk loss. Reason being that approximately 10-40% of clinical cases yield no-growth and do not require antibiotic therapy. Of the Gram negative growth, most approved intramammary products are not effective. Also, there is a high self-cure rate with certain Gram negative infections. On the other hand, intramammary therapy is recommended in Gram positive infections such as *Streptococcus agalactiae*, environmental *Streptococcus*, and *Staphylococcus aureus.*

There is a need for an inexpensive and rapid bacteriologic test that allows veterinarians as well as dairy producers to make real-time decisions to better manage udder health in their herds. Petrifilm^{TM} plates (3M Microbiology) may fill this gap. Petrifilm^{TM} plates are ready-to-use culture media that are used primarily in the food industry. The Petrifilm may enable us to rapidly diagnose and implement a targeted treatment regime (for clinical mastitis) or control program (for contagious mastitis).

Literature Review

1. Introduction

1.1 The impact of mastitis on the dairy industry

Mastitis is considered the most costly disease in the dairy industry (DeGraves and Fetrow, 1993; Blosser 1979; Miller et al., 1993). The economic losses can be characterized into six major categories. They include the cost of treatment, milk withdrawal, loss of production, increased labour costs, decrease in genetic gain and culling or death (Bartlett et al., 1991; Fetrow et al., 2000). Of these categories, milk loss accounted for over 80% of the total cost of mastitis (Lightner et al. 1988). A study in the United States showed that losses associated with clinical mastitis have been estimated at an average of $ 179 per case (Bar et al., 2008). The cost increased to $403 when a high yielding cow was affected with clinical mastitis (Bar et al., 2008). The costs per clinical episode have also been found to vary depending on herd size and the pathogen isolated (Miller et al., 1993). Losses associated with subclinical mastitis are much greater. Studies have shown that 70-80% of the losses due to mastitis are associated with a decrease in milk production due to subclinical mastitis (Dohoo et al., 1984; Gill and Howard, 1990). Cows with subclinical mastitis can also act as a reservoir for mammary pathogens that could potentially infect other cows in the herd (Erskine, 1991).

Production losses depend on the stage of lactation, duration of infection, level of production at the onset of mastitis, and the organism involved (Wilson and Gonzalez, 1997; Degraves and Fetrow, 1993, Anderson et al. 1992; Bartlett et al. 1991). Clinical mastitis episodes in early lactation also result in greater lactational losses than those episodes occurring in later lactation (Bartlett et al., 1991). Losses of 3-6% of production are commonly reported over the entire lactation (Reneau, 1993). Some studies have reported that following the acute milk loss period, milk production tends to return to preinfection predictions (Hoblet, et al., 1991), while other studies observed the opposite (Bartlett, et al., 1991; Lucey, et al., 1984; Rajala-Schultz, et al., 1999). Rajala-Schultz evaluated the effect of

clinical mastitis on milk yield in 24,276 Finnish Ayrshires, and determined that there was an overall loss of 110 to 552 kg of milk over the entire lactation. Cows that experienced clinical mastitis were not able to reach premastitis milk yields after the clinical episode. Barlett et al. (1991) also concluded that cows experiencing a clinical mastitis produced 341 kg less salable milk during 60 days following the onset of clinical mastitis as compared to projected production. These differences may be real or reflect differences in study design. In one study evaluating coliform mastitis, the average production loss was 2,584 pounds of milk (Anderson et al., 1992). Using current US milk price of $17.00 per hundred-weight this loss would be 439$. Conflicting results have also been reported as to whether the agent causing the mastitis episode influences production losses (Anderson, et al., 1992; Bartlett, et al., 1991; Reneau, 1993; Gröhn et al, 2004). Morin et al. (1998c) reported greater decrease in milk yield when clinical mastitis was caused by a Gram negative (GN) organism as compared to Gram positive (GP).

Besides the costs associated with clinical and subclinical mastitis, the costs associated with management programs to prevent mastitis must also be considered. From a study in 50 Ohio dairy herds, it has been estimated that mastitis prevention costs comprised 48% of the total cost of preventing all disease within those herds (Miller et al., 1993). The annual estimated cost for preventing mastitis was $14.50 per cow, whereas the cost to producers for a case of clinical mastitis was $37.91 (Miller et al., 1993). The cost-benefit of a control program for *Staphlyococcus aureus* (*S. aureus*) mastitis, on a 2000-cow dairy herd, was estimated to be extremely beneficial, yielding $2.40 in direct benefits for every dollar invested (Goodger and Ferguson, 1987). The steps in this control program were teat dipping, bacteriologic culturing of all fresh cows and from cows with clinical mastitis, and culling affected cows.

According to data from Québec herds enrolled in VALACTA in 2005, 28 % of cows were involuntarily culled because of mastitis or an elevated SCC (Brisson, 2006). This translates into 18,232 cows eliminated due to mastitis. In addition, 666 cows died from a clinical mastitis episode. After reproduction, mastitis is the second most common reason for culling in dairy herds (Brisson, 2006).

In 2005, the average SCC in Canada was 256,000 (Cloutier, 2005). In Québec, the average weighted SCC average for 2005 was 260 000 (Boyer, 2005). That was an increase of

5.5% from the previous year, placing Québec in 8th place out of 9 provinces. In addition, only 32-43% of producers were able to produce milk with a SCC of 200,000 or less. When the cow level SCC is above 200,000, there is a proportional reduction in milk production (Dohoo et al., 1984). Considering this fact, there are considerable economic gains to be realized by reducing the overall cell count in milk production alone. Not to mention the benefits realized at the cow level.

The major costs associated with mastitis can be easily calculated: the value of discarded milk, antibiotic cost, and veterinary fees if applicable (Fetrow et al, 2000). It is more difficult to estimate the financial benefits or the loss that would occur if no antibiotic treatment was used. Few studies use a negative control, therefore economic data are generally lacking. The economics of treating mild and moderate cases of clinical mastitis with intramammary (IMM) antibiotics compared to an untreated control group were examined (Hallberg et al, 1994). The short term benefit of antibiotic treatment was calculated to be $12.25/cow to the dairyman. The assumptions made to calculate this dollar value may be an over-simplification of the financial benefits of treating with IMM antibiotics. In addition, the costs of mastitis differ between herds and may differ over time within the same herd (Fetrow, et al., 2000). In another study, IMM antibiotic therapy was compared to oxytocin therapy for the treatment of mild clinical mastitis (Van Eenennaam et al, 1995). No financial differences were reported for the cost per episode of clinical mastitis between the two treatment regimes.

Clinical mastitis is the number one reason for antibiotic use in the dairy cow (Guterbock, 1994; Erskine, 2003; Leger et al, 2003). There is increased public and scientific interest in the use of antibiotics in agriculture. This is primarily due to the documented rise in resistance of bacteria known to be human pathogens (Piddock, 1996). As public concern grows, the demand for more prudent use of antimicrobials in food animals should not be ignored.

2. Mastitis

2.1 Definitions

2.1.1 Mastitis

Mastitis is the inflammation of the mammary gland caused by a trauma or injury to the udder, a chemical irritation, or more commonly an infection cause by a pathogenic organism (Harmon, 1994). There are over 137 different agents that cause mastitis (Watts et al, 1988). These include bacteria, *Mycoplasma*, yeast, viruses and algae. An intramammary infection (IMI) occurs after the penetration of a pathogenic agent by the teat canal (Harmon, 1994). However, *Mycoplasma* can also gain access to the mammary gland via the blood system (Gonzalez and Wilson, 2003). After the penetration of mastitis causing pathogens into the teat canal, the micro-organisms can then multiply inside the affected quarter. Bacteria cause damage by releasing toxins that cause swelling and death of milk producing alveolar cells. The cell death releases inflammatory mediators that attract leucocytes to the area of infection. It is this influx of leucocytes that constitutes the inflammatory response (Erskine et al., 2003). If the alveolar cells are destroyed, there is fibrosis of the affected area (scar tissue), leading to reduced milk production in the current and subsequent lactations (NMC, 1999).

2.1.2 Intramammary infection

An IMI can be described as either clinical or subclinical, depending on the degree of inflammation (NMC, 1999). In the literature, an IMI is often defined as milk with the presence of microorganisms demonstrated by microbiological culture (Bartlett, et al., 1992; Ruegg, 2003). With clinical mastitis, there are visible signs of mastitis such as udder swelling, clots in the milk, and systemic signs: fever, pain, and decreased feed intake. Clinical mastitis can be described by evaluating each of the following factors; the milk, udder and cow. Consequently, clinical mastitis can be divided into three categories; Grade 1/Mild: abnormal milk, Grade 2/Moderate:

abnormal milk and inflammation of udder, Grade 3/Severe: abnormal milk, inflammation of udder, and systemic signs (Roberson, 2003). Having a scoring system for clinical mastitis is extremely important for the implementation of on-farm culture systems (OFCS) because treatment will vary depending on the severity of the mastitis.

Subclinical mastitis occurs when there is the presence of inflammatory cells without any visible abnormalities at the cow, udder or milk level. Subclinical mastitis can be determined by bacteriologic analysis or indirect tests (Smith, 1996; NMC, 1999). Indirect tests measure the amount of inflammatory cells in the milk but do not determine the cause of the inflammation. Two examples of indirect tests include the California Mastitis Test (CMT) or somatic cell counts (SCC) (Kirk et al., 1994). A threshold of greater than 200,000 cells/ml is often used to indicate the presence of an IMI (Ruegg, 2003).

3. Pathogens that cause mastitis in the dairy cow

Most of mastitis cases are bacterial in origin. Mastitis pathogens can be classified as either contagious or environmental.

3.1 Contagious pathogens

Contagious mastitis is defined as IMI's transmitted from cow to cow by milking machines or milkers (Rebhun, 1995). Their primary reservoir is the mammary gland (Rebhun, 1995; Radositits, 2000). The most common contagious major pathogens are *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*Str. agalactiae*), and *Mycoplasma* spp.

Staphylococcus aureus is the most common contagious pathogen in dairy herds (Lombard et al., 2008; OldeRiekerink et al., 2006). *Staphylococcus aureus* is mainly found on the teats and udders of lactating cows (Sears and McCarthy, 2003b). *Staphylococcus aureus* causes milk loss, reduced milk quality and chronic infections that are difficult to cure

(Rebhun, 1995; Radostits, 2000; Sears and McCarthy, 2003b). Various virulence mechanisms and characteristics of the organism result in failure to eliminate *S. aureus* from the mammary gland (Sears and McCarthy, 2003b; Radostitis, 2000). The probability of cure of *S. aureus* IMI depends on cow, pathogen and treatment factors (Barkema et al., 2006). Cure rates decrease with higher parity (Sol et al., 1997; Sol et al., 2000), higher SCC (Deluyker et al., 2005; Dingwell et al., 2003), increased colony forming units (Dingwell et al., 2003), chronic infections, such as, multiple *S. aureus* positive samples before treatment (Sol et al., 1997), and when the infection is in the hind quarters or in multiple quarters (Dingwell et al., 2003, Sol et al., 1994).

Streptococcus agalactiae is an obligate inhabitant of the mammary gland and is highly contagious (Rebhun, 1995). However, it is very susceptible to antibiotics and is relatively easy to cure (Erskine, 2001). In contrast, *Mycoplasma* causes a purulent interstitial clinical mastitis and does not respond to antibiotic therapy. It is also highly contagious (Gonzalez, 2003). Transmission of *Mycoplasma* can occur at milking via the milking units and milkers' hands (Radostitis, 2000). It can also disseminate from other body sites to the mammary gland via the blood (Gonzalez and Wilson, 2003). Greater risk of *Mycoplasma* mastitis is associated with increasing herd size, especially if animals are purchased from various sites (Erskine, 2001; Lombard et al., 2008). It is important to identify and cull cows with this type of infection (Erskine, 2001).

Corynebacterium bovis (*C. bovis*) is also considered a contagious but minor pathogen (Radostitis, 2000). The sources of *C. bovis* are infected udders and teat canal (Radostitis, 2000). In herds with inadequate or no teat dipping, *C. bovis* can be spread rapidly from cow to cow (NMC, 1999). An intramammary infection with C. bovis causes only a moderate inflammation and are infrequently associated with clinical mastitis (Radostitis, 2000; Sears and McCarthy, 2003).

A recent Canadian study estimated the prevalence of contagious pathogens in bulk tank milk in Prince Edward Island (OldeRiekerink et al. 2006). The prevalence of *S. aureus, Str. agalactiae*, and *Mycoplasma* spp., was 74%, 1.6%, and 1.9% respectively. Although, 75% of herds had *S. aureus*, there was a relatively low prevalence of *Str. agalactiae* and *Mycoplasma*. In a National Animal Health Monitoring System (NAHMS) study of US dairy

herds, *S. aureus* was detected in only 43% of bulk tank milk samples, while 3% were positive for *Str. agalactiae* or *Mycoplasma* (Lombard et al., 2008).

3.2 Environmental pathogens

Environmental mastitis can be defined as an IMI caused by pathogens whose primary reservoir is the dairy cows' environment (Radostitis, 2000). Pathogens in this category include environmental streptococci, coliforms, and enterococci (NMC, 1999). Environmental streptococci are defined as species of streptococci other than *Str. agalactiae. Streptococcus uberis* (*Str. uberis*) and *Streptococcus dysgalactiae* (*Str. dysgalactiae*) are common environmental streptococci (Harmon, 1994; Radostitis, 2000). *Streptococcus uberis* is most associated with straw bedding but can also be isolated from the gut, feces and vulva of the dairy cow (Zadoks et al., 2005; Hillerton and Berry, 2003). Both *Str. uberis* and *Str. dysgalactiae* can behave as both an environmental and contagious pathogen (Oliver et al., 1997; Zadoks et al, 2001). Approximately half of the IMI due to environmental streptococci cause clinical mastitis (Todhunter et al., 1995). Fortunately, most clinical cases due to environmental streptococci cause mild to moderate signs, and not systemic signs (Morin et al., 1998; Todhunter et al., 1995).

Escherichia coli (*E. coli*) and *Klebsiella* spp. are the most frequently isolated coliforms cultured from cases of clinical mastitis (Wilson, 2003; Erskine et al., 2003; Radostitis, 2000). Wood shavings are considered to be the main source of *Klebsiella* on dairy farms. However a New York study found that over 80% of fecal samples were positive for *Klebsiella pneumoniae* (Munoz et al., 2006). Cows in early lactation are reported to be more susceptible to clinical mastitis due to *E. coli* (Cebra et al., 1996; Eberhart et al., 1979). Coliform bacteria are found in large numbers in the dairy environment (manure, bedding, soil, water) (NMC, 1999). Approximately 70-80% of coliform infections become clinical and 50% of environmental IMI become clinical (Harmon, 1994).

Other less common types of Gram negative bacteria that are also considered coliforms include *Enterobacter, Serratia, Citrobacter, Proteus* and *Pseudomonas* (Wilson, 2003). These bacteria are found in bedding, manure, and soil (Radostits, 2000).

Exposure of uninfected quarters to environmental pathogens can occur at any time during the life of the cow including milking time, between milkings, and during the dry period (Rebhun, 1995). All organic bedding materials, such as straw or wood shavings, facilitate the growth of environmental pathogens (Radostitis, 2000). Due to their environmental source, milk samples can be contaminated with these organisms during sample collection (Sears and McCarthy, 2003). False positive culture results may therefore complicate the identification of environmental pathogens from mastitis cases. In well managed low SCC-herds, environmental mastitis is the leading cause of clinical mastitis (Radostitis, 2000; Hillerton and Berry, 2003).

Coagulase-negative staphylococci (CNS) are referred to as minor pathogens and cause only mild inflammation and elevated somatic cell counts (Harmon, 1994; Sears and McCarthy, 2003b). Coagulase-negative staphylococci are part of the normal skin flora of cows and are also abundant in the animal's environment (Smith, et al., 1995b; Sears and McCarthy, 2003b). In Finland, CNS are the most common bacterial pathogen isolated (Pitkala et al., 2005). Coagulase-negative staphylococci are the primary cause of IMI in heifers at calving (Hillerton and Berry, 2003; Roy et al., 2007).

Mastitis can also be caused by a number of less common pathogens that include *Arcanobacterium pyogenes*, *Bacillus* spp., *Prototheca* spp., yeast, and the coliforms: *Pasteurella* spp (*Mannheimia), Proteus* spp., or *Pseudomonas* spp. (Radostitis, 2000).

4. Proportions and incidence rates of intramammary infections and clinical mastitis

4.1 Clinical mastitis

The prevalence of mastitis can be defined as the percentage of the population that is affected with mastitis at a given point in time (Martin et al., 1987). Mastitis rates are usually recorded as incidence rates. The incidence rate is the percentage of new mastitis cases over a period of time for a given population. The incidence rate for clinical mastitis (IRCM) is

calculated as the number of quarters with clinical mastitis per 100 cow-years at risk (IDF, 1997). Measuring prevalence may provide useful information in cases of subclinical IMI (Martin et al., 1987). Measuring incidence, instead of prevalence, is more useful for types of mastitis with a short duration of infection either due to a high therapeutic or spontaneous cure rate, or a high probability of culling or death (Radostits, et al., 1994). The International Dairy Federation (IDF) has recommended that the reporting of clinical mastitis data be standardized in order to allow comparisons across studies (IDF Bulletin, 1997). The IDF recommends that incidence rates of clinical mastitis primarily should be reported as an incidence rate of cases and/or cows treated per cow-year at risk. Some studies have reported the incidence rate as the number of clinical quarter cases per 100 cows at risk per year, including the dry period (Miltenburg et al., 1996; Olde Riekerink et al., 2008). Others have used a lactational incidence risk which excludes the dry period (Sargeant et al., 1998). The cow incidence of clinical mastitis has also been explained as the number of cows affected with clinical mastitis at least once per 100 cows per year (Barkema et al., 1998).

Many studies have reported herd-level incidence rates for clinical mastitis and there has been considerable variation in the IRCM reported among herds (Barkema et al., 1998; Erskine et al., 1988; Hillerton et al., 1995; Sargeant et al., 1998; Schukken et al., 1989; Bradley and Greene, 2001; Olde Riekerink et al., 2008). Mean clinical mastitis incidence rates range from 7.2 to 50.7 cases per 100 cow-years at risk (Table 1). A study in Ontario followed 65 herds, over a period of two-years, to determine the lactational incidence rates of clinical mastitis (Sargeant et al., 1998). Overall, 19.8% of cows experienced at least one case of clinical mastitis during lactation. That translates into 1 in 5 cows will have a case of clinical mastitis during lactation. The majority (40%) of these cases occurred in early lactation. In the Netherlands and Canada, the IRCM ranged from 7.3 to 27 per 100 cow-year at risk (Steeneveld et al., 2008) and 0.7 to 97.4 (Olde Riekerink et al., 2008), respectively. In the Canadian study there was a significant difference in the IRCM between provinces. The central provinces, Ontario (32.5) and Québec (30.4), had the highest IRCM as compared to the Western (15.6) and Atlantic (18.7) provinces.

The IRCM for selected major pathogens also varied widely between the studies. *Staphylococcus aureus* was isolated from 6.7%, 9.6%, 18.3%, and 21.7% of samples in Ontario (Sargeant et al., 1998), the Netherlands (Schukken et al., 1989), the United States (Erskine et al., 1988), and Canada (Olde Riekerink et al., 2008), respectively. For the same

series of studies, the percentage of environmental streptococci and coliforms isolated were 14 and 17.1%; 12.8 and 16.2%; 12.6 and 8%, 22.4 and 17.6%, respectively.

The incidence of clinical mastitis is influenced by many factors such as parity, stage of lactation, quarter location, etiologic agent, season, and geographic location. As parity increases, there is an increased IRCM (Sargeant et al., 1998; Barkema et al., 1998; Hogan et al., 1989). Cows in early lactation have the highest IRCM with the peak occurring around calving (Barkema et al., 1998; Sargeant et al., 1998; Schukken et al., 1989). Although in some studies, the IRCM is higher in first lactation cows than multiparious cows in the first month after calving (Miltenburg et al., 1996; Barkema et al., 1998). Rear quarters have a significantly higher IRCM than front quarters (Miltenburg et al., 1996; Dingwell et al., 2003). The predominant microorganism that causes clinical mastitis in herds with low bulk tank SCC is *E. coli* (Barkema et al., 1998; Erskine et al., 1988; Schukken et al., 1989). The IRCM increases during the summer months (Erskine et al., 1988; Hogan et al., 1989; Olde Riekerink et al., 2007). Geographic factors such as climate, breed of cattle, and level of production may also affect the IRCM (Bartlett et al., 1992; Olde Riekerink et al., 2007). In addition, management practices have been shown to influence the IRCM (Barkema et al., 1999; Olde Riekerink et al., 2008). Tie stall barns in Canada had a higher IRCM than freestall herds (Olde Riekerink et al., 2008).

Table I: Incidence rates for clinical mastitis.

Year	Author	Location	Number of herds	Number of CM[1] cases	IRCM[2] cases per 100 cow/year	Most prevalent isolate
1988	Erskine et al.	USA	18		50.7 (LSCC[3]) 34.9 (HSCC[4])	Coliforms (43.0%); *S.aureus* (18.3%)
1989	Hogan et al.	USA	9	646	54.7	Coliforms (29.7%)
1989	Schukken et al.	Netherlands	125	1140	17.9	*E.coli* (16.2%)
1992	Bartlett et al.	Ohio, USA	50		38.4	*E.coli* and CNS
1995	Hillerton et al.	UK	1 (4 years)	305	25.7	*S. uberis* (39%)
1996	Miltenberg et al.	Netherlands	171	1103	12.7	*E.coli* (16.9%)
1998	Barkema et al.	Netherlands	274	8429	26.2	*S. aureus*
1998	Sargeant et al.	Ontario, Canada	65 (2 years)	834	23.7	CNS (28.5%)
2008	Olde Rinerink	Canada	106	3149	23.0	*S. aureus*

[1] CM= Clinical mastitis
[2] IRCM = Incidence rate for clinical mastitis
[3] LSCC = Low somatic cell count
[4] HSCC = High somatic cell count

Bulk tank milk SCC (BMSCC) has been reported to be associated with the incidence of clinical mastitis. Erskine et al. (1988) recorded higher rates of clinical mastitis in low SCC herds (<150, 000 cells/ml). Olde Riekerink et al. (2008) found no association between the overall IRCM and BMSCC. However, when these data were analysed for specific pathogens, low BMSCC herds (<150, 000 cells/ml) had significantly higher IRCM (2.78) than medium (151 to 250) BMSCC herds (2.26) for *S. aureus*. The high (>250, 000 cells/ml) BMSCC herds had the greatest IRCM of 3.97 due to *S. aureus*. This parallels work by Barkema et al. (1998) where there was no difference in the overall IRCM between low, medium and high SCC herds. But high SCC herds had a higher IRCM due to *S. aureus* and *Str. dysgalactiae* and *Str. agalactaie* than low SCC herds. The IRCM varies amongst trials for various reasons including detection bias, definition of clinical mastitis, method of data collection, environmental conditions, housing and season (Bartlett, 1992; Miltenburg, 1996; Sargeant et al., 1998; Lam, 1993).

4.2 Subclinical mastitis

As previously mentioned, subclinical mastitis occurs when there is the presence of inflammatory cells without any visible abnormalities at the cow, udder or milk level (Smith, 1996). It is generally agreed that 70-80% of the estimated loss from subclinical mastitis is due to reduced milk production (Dohoo et al., 1984; Gill et al., 1990). A linear relationship between the increase in the log SCC and a decrease in test day milk production is well documented (Dohoo et al., 1984; Deluyker et al., 1993).

The most predominant pathogens that cause subclinical mastitis are streptococci and staphylococci (Erskine, 2001). Over half of new infections due to environmental streptococci at calving are acquired during the dry period and persist into lactation (Bradley and Green, 2001; Todhunter et al., 1995). Fifty-five percent of the environmental IMI present in the first half of the dry period persisted into early lactation (Todhunter et al., 1995). In the same Ohio study, the incidence rates of an IMI caused by environmental streptococci was 0.00312 IMI/cow-day during the dry period, which was 5.5 times greater than the IMI incidence rate during lactation (0.00054 IMI/cow-day).

Erskine and colleagues (1988) conducted whole herd surveys on 18 farms in Pennsylvania in twelve low SCC herds (< 150,000 cells/ml). Coagulase negative staphylococci (10.4%) were the most predominant pathogens isolated, followed by *C. bovis* (6.8%). In Finland in 2001, a similar pattern occurred but the prevalence was much higher (Pitkälä et al., 2005). The overall herd prevalence of subclinical IMI was 30.6% and among the bacterial isolates, CNS were the most commonly isolated bacteria (49.6%), followed by C. bovis (34.4%) and *S. aureus* (10.2%). In Québec, *S. aureus* (10%) was the most prevalent bacteria isolated at calving, closely followed by CNS (9%) (Bouchard, et al., 2005).

The most common mastitis pathogens identified in primiparous cows post calving were CNS (Trinidad et al., 1990; Roberson, 1994; Fox et al., 1995). In addition the prevalence of *S. aureus* IMI in primiparous cows at parturition in these studies ranged from 1 to 8%.

5. Diagnosing the cause of mastitis

It is important to determine the cause of a clinical mastitis episode in order to implement appropriate therapy decisions (Sears and McCarthy, 2003) Many studies have demonstrated that clinical observations do not allow accurate predictions of pathogens that cause mastitis (Morin et al., 1998; Jones et al., 1990; White et al., 1986). White et al. (1986) compared the accuracy of experienced clinicians to predict the type of pathogen causing clinical mastitis infection. They found that experienced clinicians (more than three years experience) had an accuracy rate of 58% versus 48% for less experienced clinicians. In a similar study by Jones et al. (1990), a decision tree was used to determine whether the organisms causing mastitis was gram negative or gram positive. With this diagnostic scheme, the overall accuracy rate for the predictions of the type of infection was 78%. However, in herds vaccinated with lipopolysaccharide core antigen, the sensitivity of prediction of the cause of clinical mastitis decreased to 58% (Morin et al., 1998).

There are many different methods to diagnose the cause of mastitis in dairy cows. There are indirect and direct measures of udder health. Indirect measures detect the presence of inflammatory cells in the milk while direct tests determine the type of pathogenic agent causing the inflammation.

5.1 Indirect tests

5.1.1 Somatic cell count

Somatic cell count (SCC) is an indirect measure that quantifies the presence of inflammatory cells in milk. The somatic cells found in milk consist of lactocytes and leucocytes. The leucocytes include polymorphonuclear cells (neutrophils and eosinophils), macrophages, and lymphocytes (Miller et al., 1991; Paape et al, 1963). In cows' milk the dominant somatic cells found are the lymphocytes (25-70% of cells) and macrophages (25-50%), and the are very few lactocytes (<10%) (Miller et al., 1991; Paape et al., 1963).

Somatic cell counts are used to identify cows that have potential to have an IMI (NMC, 1999). This test is usually performed monthly by Dairy Herd Improvement (DHI) program; Valacta in Quebec. Earlier research had suggested that SCC are elevated during the first two weeks of lactation, followed by a rapid decrease (Dohoo, 1993). More recent experiments have shown that cow level SCC, in the absence of IMI caused by a major pathogen, declines more rapidly than previously suggested (Barkema et al., 1999; Sargeant et al., 2001). Using a threshold of 200, 000 cells/ml, the Se and Sp of detecting any pathogen responsible for an IMI is only 73% and 86% respectively (Dohoo and Leslie, 1991).

5.1.2 California mastitis test

Another indirect test is the California Mastitis Test (CMT). The CMT is a surrogate measure of SCC in milk, and is a rapid cow-side test for subclinical mastitis. Indirect tests indicate the presence of inflammation but do not determine the type of infection (Erskine, 2001; Schalm et al., 1971).

The California Mastitis Test (CMT) has been used to identify IMI in the first 10-days post-calving (Sargeant et al., 2001). This study determined that the optimal sampling time to select infected quarters for bacteriology was three days post-calving using a CMT threshold of greater than zero. The Se and Sp of the CMT to identify IMI due to a major pathogen, at this cutpoint, was 66.7% and 54.8%, respectively. The results indicated that the CMT could be used to select cows for enrolment into an udder health protocol immediately after calving. Similar work in the Netherlands and Canada support these findings (Barkema et al., 1999; Wallace et al, 2004). In another study on colostral milk, it was determined that CMT was a useful test to indicate an increased SCC, and thus identify an IMI in the early postpartum period (Maunsell et al., 1999).

5.2 Bacteriologic tests

Bacteriologic Diagnosis

There are many methods used to determine the type of pathogen that causes an IMI or clinical mastitis. These include standard bacteriology of milk, the polymerase chain reaction (PCR), the Minnesota Biplate and Triplate system, Petrifilm, and other methods (ColiMast, HyMast, and the Linmast test).

In the literature, in order to confirm that a quarter truly has an IMI, the same organism must exist in two to three consecutive samples taken at least 1 day apart (Sears and McCarthy, 2003). However, multiple cultures are not practical on most commercial dairies. It will increase the culturing costs, requires more labor, and creates a longer lag time before results are available (Sargeant et al., 2001; Leslie et al., 2002). A study by Erskine et al. (1988) found a 92.4% agreement between simultaneous duplicate samples for *S. aureus*. They concluded that a single sample was adequate to identify quarters infected with *S. aureus*. This statement is also supported in a recent study by Dingwell et al. (2007). They demonstrated that there is similar probability of a correct diagnosis when using a single sample as compared to duplicate samples for subclinical mastitis.

5.2.1 Standard bacteriology of bovine milk

Standard bacteriology of bovine milk is currently the gold standard for the identification of pathogens in milk (Ruegg, 2005; Sears and McCarthy 2003). The diagnostic procedures to identify mastitis-causing pathogens are published in the *Laboratory Handbook on Bovine Mastitis* (NMC, 2004). This method consists of inoculating 0.01 mL of milk onto a blood agar plate. Examination of the blood agar plates is performed 18-24 hrs after inoculation. The final diagnosis is normally obtained at 48 to 72 hrs. From a bacteriology point of view, the organisms that cause mastitis can be divided into five groups: gram-positive cocci, gram-negative bacteria (coliforms), *Corynebacterium, Mycoplasma,* and others (*Nocardia, Prototheca,* and yeast) (Sears and McCarthy 2003). The majority of these pathogens will grow on blood agar, except for *Mycoplasma* (Sears and McCarthy 2003).

The Gram stain is the first procedure used to classify bacteria into the five groups (NMC, 2004). The bacterial colonies are smeared onto a slide, stained and then bacteria are differentiated under a microscope. The stains used are Crystal violet (blue) and Safranin (red) (NMC, 2004). Gram positive (GP) bacteria will stain blue (*Bacillus*, *streptococcus*, and *staphylococcus*). Gram negative (GN) bacteria (coliforms and *Pseudomonas*) will appear red. Sears and colleagues (2003) have described the following method for bacteria identification. A catalase reaction is used to differentiate staphylococci (catalase positive) and streptococci (catalase negative). A tube coagulase test will classify staphylococci as coagulase-positive (e.g. *S. aureus*) or coagulase negative (CNS). Partial or complete hemolytic zones around the colonies on blood agar are also used to identify *S. aureus*. *Streptococcus agalactiae* and Streptococci spp are identified based on their appearance on blood agar, a CAMP reaction, and esculin hydrolysis. Streptococci other than *Str. agalactiae* are generally CAMP negative and esculin positive. Hippurate, inuline and raffinose are used to further identify Streptococcal species. Gram negative bacteria can be differentiated based upon their appearance on blood and MacConkey agar and an oxidase test. Coliforms are oxidase negative, and *Pseudomonas spp* and *Pasteurella spp* are oxidase positive. The MacConkey agar contains lactose. Lactose fermenters will turn the media pink. *E. coli* produce dry colonies with a pink halo on MacConkey agar, *Klebsiella* appear mucoid and pink-yellow in color, and *Enterobacter* appear pink and dry. Non-lactose fermenters will appear colorless (Proteus) or greyish (*Pseudomonas* and *Serratia*) on this media.

The sensitivity of a standard culture will vary depending on the organism and the sampling method used. The Se for detecting *S. aureus* from a composite milk sample in subclinically infected cows is only 58-63% (Lam et al., 1996). The low Se is thought to be due to the dilution effect of the uninfected quarters. A study perform by Sears et al. (1990) demonstrated that the sensitivity of a single quarter milk sample was 75% from experimentally infected quarters. Sensitivity increased to 94% and 98% by collecting a second and third culture, respectively. Also, quarters with a low shedding cycle have a higher risk of false negative when a single sampling method was used. With naturally occurring infections, cows with high shedding cycles had a Se of 100%. In the case of clinical mastitis due to coliform infections, milk samples will culture negative approximately 20% of the time (Gonzalez et al.,, 1990). For the isolation of *Str. agalactiae*, quarter samples are

recommended. The Se and Sp of quarter and composite milk samples is 98.8 % and 100%, and 96.5% and 100%, respectively (Dinsmore et al., 1992).

Preculture incubation, enrichment and centrifugation are methods used to increase the sensitivity of detecting pathogens in milk (Dinsmore et al., 1992; Lam et al., 1996; Sol et al., 2002; Zeconni et al., 1997). The preculture incubation method is performed by placing a milk sample in a water bath at 37°C for 4 to 6 hours. This method has also been shown to increase the recovery rate of bacterial pathogens as compared to standard culture (Dinsmore et al., 1992). Milk samples that are negative on culture can be recultured in an enrichment broth. This method can allow recovery of pathogenic bacteria in 10% of such samples (Dinsmore et al., 1992). Centrifugation of milk samples is another method that can increase the recovery rate of *S. aureus*, however this method is time consuming (Zecconi et al., 1997).

The major advantage to bacteriologic culture is the complete identification of bacteria from the milk sample, including the less common pathogens (Prototheca, Norcardia, and yeast). *Mycoplasma* can also be identified using a modified Hayflick medium (Gonzalez and Wilson, 2003).

The disadvantage to bacteriologic culture that often leads to the underutilization of this method is the lag-time for results (Ruegg, 2005; Sargeant et al., 2001). Often there is a 4-5 day delay from sample submission to receiving results, therefore making targeted treatment decisions for clinical mastitis based on bacteriology difficult. Other disadvantages to bacteriologic culture, which also apply to other culture based methods, include failure to recover pathogens, and contaminated samples. (Buelow et al., 1996; Sears and McCarthy, 2003). There are various reasons for "no growth" results, they include spontaneous clearance (Radostitis, 2000; Sears and McCarthy, 2003), and the cyclical shedding pattern of *S. aureus* infections (Sears et al., 1990). The minimum detection limit when plating 0.01 mL of milk is about 100 cfu/mL (Anderson et al, 1991). Recovery of few cfu/ml may be due to low shedding of *S. aureus* or the dilution of an affected quarter by the collection of a composite culture (Sears et al., 1990; Buelow et al., 1996). Negative milk culture results may also occur if the bacteria present were engulfed by phagocytes (Hill et al., 1978). Antibiotics may have killed or suppressed the level of organisms or storage can reduce the numbers of viable organisms (Anderson et al., 1991). Careful sample collection and interpretation of

bacteriologic results is also necessary to prevent false positives from contamination with environmental pathogens (Sears and McCarthy, 2003).

5.2.1.1 Inoculum volumes

Increasing the inoculum volume is one method that can be used to increase the sensitivity of detecting pathogens from milk samples. When inoculum volume sizes increased to 0.1 mL several studies demonstrated that the sensitivities for *S. aureus* were higher (Buelow and Norland 1999; Dinsmore et al., 1992). When a single composite sample and a milk inoculum of 0.01 mL were used, the Se and Sp were 78% and 86% respectively (Lam et al., 1996). The Se and Sp are 90% and 95%, respectively when 0.1 mL of milk was plated. Anderson et al. (1991) also evaluated inoculum volumes in the diagnosis of mastitis from clinical quarters. There was no significant difference in cultural outcomes between 0.1 versus 0.01 mL inoculum volumes. There was also no significant difference in cultural outcomes between 0.05 and 0.01 mL volumes.

5.2.2 Polymerase chain reaction

Polyermase Chain Reaction (PCR) is a molecular method to identify the pathogenic agent by the amplification of specific parts of it's DNA (Zadoks and Schukken, 2003). The PCR can be performed directly on milk or an enrichment step can be performed before the test (Gillespie et al., 2005; Khan, 1998). Bacteria can be first grown in pure culture (standard bacteriology) to generate DNA for isolation. A study by Gillespie et al. (2005) used an enrichment step on milk before performing the PCR. Using this method, the PCR could correctly identify 96.4% of samples (91.7% *S. aureus*, 98.2% *Str. agalactiae*, 100% *Str. uberis*). Maxwell and colleagues (2008) found the PCR to be somewhat less effective for the identification of *E. coli*, with a sensitivity of only 82.6%. Riffon et al. (2001) attempted to eliminate the pre-PCR enzymatic lysis step of bacterial cells because their aim was to make the test as simple and cheap as possible. They evaluated a PCR test that could be performed in one day with no culture step. They determined that with no pre-enzymatic step the detection limit was $5x10^3$CFU/mL as compared to $3.12x10^2$ CFU/mL. They concluded that

there was reduced sensitivity with the PCR with no pre-enzymatic step, but the detection limit was still sensitive enough to be used as a diagnostic tool for bovine mastitis. This allowed for the elimination of expensive reagents and lengthy cultivation steps. Therefore, direct detection of *E. coli, S. aureus, Str. agalactiae, Str. dysgalactiae, a*nd *Str. uberis* by PCR can be performed within four and a half hours. The PCR can also be used for the detection of *Mycoplasma* spp in milk. Cai et al. (2005) determined that the detection limit of a real time PCR for the detection of *Mycoplasma bovis* was 550 cfu/mL for bovine milk. The relative sensitivity and specificity was 100% and 99.3%, respectively.

Advantages of the PCR include: milk samples can contain antibiotics, the bacteria can be alive or dead, and the results are available within 24 hours (Riffon et al., 2001). Simultaneous detection of multiple mastitis pathogens directly from milk is also possible. A major disadvantage is that this test can be performed only in a laboratory setting and it is relatively expensive (approximately $22 per sample) as compared to other methods (Biovet, 2008).

5.2.3 Minnesota Easy Culture System II: Biplates & Triplates

The University of Minnesota has developed the Easy Culture System, which offers two types of selective media culture systems; the Bi-plate and Tri-plate. The Bi-plate system is intended to identify a quarter as infected with GP or GN organisms. The culture media consists of half blood agar with 1% esculine and half MacConkey media (Godden et al., 2007). The Tri-plates give more complete identification by having an additional media selective media (with TKT) that allows just streptococci growth (Sears and McCarthy, 2003). The test is performed by swabbing the milk sample onto the plate, incubate and read at 24 hours. If there is no growth observed, plates are rechecked at 48 hours.

The Bi-plates were evaluated for their ability to differentiate between GP and GN pathogens in mild to moderate cases of mastitis and in fresh cow samples (Lago et al., 2006; Hochhalter et al, 2006). The sensitivity and specificity of the Biplate was 83% and 90% respectively for mastitis cases and 88% and 70% in fresh cow samples, respectively, as compared to bacteriologic culture (Lago et al., 2006). In Hochhalter's study, the Se and Sp were higher at 100% and 92.3%, respectively. The Bi-plates were also evaluated in herds

using the on-farm culturing system (OFCS). There were minimal adverse effects, in cows treated immediately after diagnosis of mild to moderate mastitis, as compared to those cows treated after waiting 24 hours for OFCS results (Wagner et al., 2007).

The test characteristics of the Tri-plates were evaluated by Jones et al. (2006) for their ability to differentiate between gram positive versus gram negative growth and CNS versus Streptococcus spp. as compared to standard bacteriology. In 101 quarter samples of mild to moderate clinical mastitis cases the sensitivity and specificity to determine GP growth versus GN growth was 94.1% and 100%, respectively. When the Tri-plate was evaluated on its ability to differentiate CNS versus streptococci spp., the Se and Sp was 78% and 67%, respectively. In 210 quarter samples taking at calving, the test characteristics for the same series of comparisons was 95.7% and 73.9%, and 83% and 74%, respectively. These values are similar to those of the Bi-plate and allow for more complete microbialogical analysis for streptococci spp.

The Minnesota Easy culture system allows for relatively simple microbiological analysis to be performed on-farm in 24 to 48 hours. However, it is important to note that this system does not intend to replace commercial laboratories nor does it identify all the organisms present (such as *Mycoplasma*, yeast etc.) (Minnesota Easy Culture System II Handbook. 2000). The Bi-plates and Tri-plates have a shelf-life of approximately six weeks (under ideal handling conditions).

5.2.4 Petrifilm™

Petrifilm™ plates (3M Microbiology) are sample-ready selective culture media that are used to the rapid identification and numeration of bacteria. They are currently widely used in the food industry (Tassinari et al., 2005; Ingham et al., 2003). The Petriflim plates that have potential for use as diagnostic tests in the dairy industry are the the Petrifilm™ Staph Express count plate, the Coliform count plates, and the Petrifilm Aerobic count plates. The Petrifilm culture system requires 1 mL of milk be added to the Petrifilm plate, incubated at 37°C and results are available in 22-24 hours.

5.2.4.1 Petrifilm™ Staph Express Count plate

The Petrifilm™ Staph Express Count plate (STX) contains chromogenic, modified Baird-Parker media that is selective and differential for Staphlycocci. After 22-24 hours, a positive Petrifilm will present with red-violet colonies. If there are more than one type of colony present, confirmation of *S. aureus* is performed by using a Staph Express Disk that contains deoxyribonuclease and a dye that reacts to produce a pink zone around the *S. aureus* colonies. The disk will also occasionally react with *Staphylococcus hyicus* and *Staphylococcus intermedius*.

The STX was compared to the Baird-Parker agar method for the detection of *S. aureus* in naturally contaminated poultry and raw milk and artificially contaminated cheeses and smoked fish (Ingham et al., 2003). Both methods performed similarly for the enumeration of *S. aureus*. However, in another study evaluating *S. aureus* growth in ham, the baird-Parker method recovered significantly greater numbers of *S. aureus* than the STX method (Ingham et al., 2004). The Petrifilm STX 24 hour reading was also similar to those of the standard 72 hour method for the detection of *S. aureus* in dairy foods (Silbernagel et al., 2003).

The Petrifilm STX was evaluated for detection of *S. aureus* in bovine milk (Silva et al., 2005). The gold standard used to comparison was the isolation of *S. aureus* from any of the four microbiological techniques of standard culture, centrifugation, incubation, and the Petrifilm. The sensitivity for *S. aureus* detection was 87.5%, which was significantly higher than standard microbiological techniques (65%). The specificity of the STX was evaluated using different interpretation parameters. The specificity of the STX when a distinct pink zone was present was 98.5%. Using weak pink zones to diagnose *S. aureus*, the specificity was only 77.6% which would result in a high false positive rate. They also concluded that the interpretation of the Petrifilm was highly dependant on the reader's ability to identify colony colour and distinct pink zones after application of the Staph Express disk plates.

The Petrifilm STX was evaluated on a commercial dairy in Wisconsin (Silva et al., 2004). The test characteristics were low (Se (56%) and Sp (78%)) when plates were interpreted by the farm personnel. Further training of the personnel improved these values.

The main advantages of the Petrifilm STX are that *S. aureus* can be confirmed in 24-26 hrs and requires less labor and expertise (Silva et al., 2005).

5.2.4.2 Rapid Coliform Count plates

Rapid Coliform Count plates (RCC) are used for the rapid detection of coliforms. It is currently the fastest approved coliform test for the enumeration of coliforms in food (3M, 2001). This Petrifilm can detect high levels of coliform contamination (>1000 plate) as early as six hours during incubation. Total confirmed coliform counts can be available in 14 hours as indicated by a color change around the potential colonies. At 24 hours, coliform colonies will appear red and have gas bubbles. The RCC uses a modified violet red bile lactose nutrient base. It is currently approved as the AOAC official methods of analysis for foods. The Petrifilm RCC was evaluated for the detection of coliforms in foods as compared to the Standard Methods for the Examination of Dairy Products (Kinneberg and Lindberg, 2002). The results at 14 and 24 hours were not significantly different between the two methods. The RCC has not been evaluated for detection of coliforms in bovine milk.

5.2.4.3 Petrifilm E. coli/Coliform Count plate

Petrifilm E.coli/Coliform Count plates (EC) are used for the detection of coliforms. The difference between this plate and the RCC is that the EC does not have a color change indicator at 14 hours. Final results are only available in 24 hours. The Petrifilm RC count plates have been compared with standard methods for enumeration of fecal coliforms and *E.coli* in water (Schraft and Watterworth, 2005). The Petrifilm EC plates had almost identical results for the numeration of fecal coliforms and *E. coli* in water as compared to the standard membrane filtration method.

5.2.4.4 Aerobic Count plate

The nutrient base used for the aerobic count plate (AC) is a modified standard method. All colonies that grow on the AC appear red. The AC is currently approved as the AOAC official methods of analysis for raw/pasteurized milk, dairy products, and foods. The AC proved to be an alternative method for testing the microbiological quality of acidic fruit juices (Ramazotti-Ferrati, et al., 2005).

5.2.5 Other Methods

5.2.5.1 ColiMast™

The ColiMast™ was developed in New Zealand and is a rapid test for the detection of coliforms in milk. The test is performed by adding 2 ml of milk into a tube that contains enrichment media. Reading of the test is between 9 and 12 hours. A colour change indicates that the sample is positive for coliforms (ICP, 2000). The sensitivity and specificity of the test is 76% and 81 % respectively (Gawrylash, et al., 2002). Presently this test is not available in Canada.

5.2.5.2 HyMast™

The Hymast test (Pharmacia & Upjohn Animal Health, Kalamazoo, Michigan) was a test with selective media for the detection of GN and GP bacteria in milk samples. The test consists of a plastic vial with a paddle attached to a screw cap. The paddle is embedded with a selective media for the detection of GP growth on one side and GN growth on the other. Milk is added to the vial, inverted a couple of times and the excess is discarded. The vial is incubated for 36 hours, but should be verified for the presence of bacterial growth at 8-12 hour intervals. A study performed by Leslie et al. (1995) determined that the sensitivity and specificity of the Hymast were 80% and 76% for the detection of GP, 60% and 98% for GN

and 13% and 91% for *S. aureus*. Because of these average results, the test was not very popular and is no longer available in Canada.

5.2.5.3 Limast® test

The Limast® test is the commercial name of the LAL-test. The LAL-test is a rapid test for the detection of endotoxin from coliform bacteria (Waage et al., 1994). This test is used on-farm primarily in Europe. The test consists of a series of transfers and dilutions of the milk sample and ultimately the diluted sample is transferred to a specialized glass bottle and placed into a 37-40°C water bath for 15 minutes, a color change to yellow indicates a positive test. The Se and Sp of the LAL-test for detecting endotoxins of Gram-negative bacteria were 63% and 97%, respectively (Waage et al., 1994). The positive predictive value of this test was 75%, while the negative predictive value was 95%. Waage et al. (1994) also state that this test requires at least 10^4 -10^5 CFU/mL of Gram negative bacteria for a positive test. Waage et al. (1994) determined that the LAL-test was a valuable cow-side test that can help the practitioner in selecting the adequate antimicrobial for the initial treatment of clinical mastitis. This test is not currently available in North America.

5.2.5.4 Other tests

For the identification of *S. aureus* in bovine milk, commercial agglutination systems from human medicine have been evaluated. One study compared six commercially available slide agglutination tests to the conventional coagulase tube tests for *S. aureus* (Zschöcket, et al., 2005). All six tests provided moderate sensitivity (ranging from 75% to 87%) for *S. aureus* detection. The Se in bovine milk were lower than previously recorded for *S. aureus* detection of human origin. The authors reasoned that the low Se may be due to the lack of detection of certain strains of S. aureus that lacked protein A, clumping factor, or capsule type 5 or 8 (Zschöcket, et al., 2005). Consequently, these tests are not widely used for diagnosis of *S. aureus* in bovine milk.

5.2.6 The Effects of Freezing on Milk Samples

There have been many studies evaluating the effect of freezing on bacteriologic culture of milk samples (Schukken et al., 1989; Murdough et al., 1996; Sol et al., 2002; Godden et al., 2002). Schukken *et al.* (1989) determined that freezing decreased the number of positive *E. coli* samples while CNS increased in numbers. In this study, freezing had no significant effect on *S. aureus* or streptococci. The implications of these findings stress the importance of having fresh samples if a coliform mastitis is suspected. Murdough et al. (1996) had contradictory findings. Freezing had no effect on viability of any pathogens (*S. aureus, S. hyicus, Str. dysgalactiae, Str. uberis, C. bovis, and E. coli.*). When the effects of freezing on the isolation of *S. aureus* was evaluated, Sol et al. (2002) demonstrated that using incubation in broth combined with freezing resulted in the highest *S. aureus* isolation percentage. As well, Godden and colleagues (2002) added to these findings by determining that the maximal sensitivities for the detection of *S. aureus* were obtained fresh or frozen pre-milking samples and frozen post-milking samples. *Mycoplasma* species lack a cell wall, thus they are fragile and susceptible to drying and changes in pH (Gonzalez and Wilson, 2003). It is recommended to send fresh milk samples to the laboratory for analysis (Biddle et al., 2004).

6. Evaluation of Diagnostic Tests

Diagnostics tests are an integral part of food animal veterinary practice. Diagnostic tests for mastitis can be used to identify the causative organism of a clinical mastitis episode or as a screening test for subclinical mastitis (Erskine, 2001). When choosing a diagnostic test, it is important to realize the limitations of the test (Kelton, 2006; Slenning, 2001) No test is infallible. Therefore, when diagnostics tests are used, certain criteria of the test must be understood before making a final decision or action plan based on the result of the test. Ideally, the study used to determine the characteristics of the diagnostic test should be critically evaluated to ensure the validity of the results (McKenna and Dohoo, 2006). These include: having an appropriate reference test used for comparison or more commonly known as the "gold standard", determining if there are any biases in the study, evaluating the agreement between tests, and finally evaluating test characteristics. The test characteristics of

a diagnostic test are the Se, Sp, positive predictive value (PPV), and the negative predictive value (NPV) (Martin et al., 1987).

6.1 Gold standard

In diagnostic testing, a gold standard refers to a procedure that can always identify the true condition, such as infected or non-infected animals (Dawson and Trapp, 1994). In many situations, there is no gold standard available or one that is completely free of error (Dawson and Trapp, 1994; Kelton, 1996). For milk culture, the gold standard is presently considered to be standard bacteriologic culture (Erskine, 2001, Ruegg, 2005). However, we know that this method is not completely effective at isolating the causative organisms in every situation (Sears and McCarthy, 2003; Sol et al., 2002). Methods have been developed at the bacteriology level and via sampling procedures to increase the likelihood of detection (Dinsmore et al., 1992; Zecconci et al., 1997; Buelow et al., 1999; Lam et al., 1996) Therefore, when comparing a new diagnostic test to the existing gold standard there are certain limitations and bias. For example, for the detection of *S. aureus*, if the "gold standard" is negative and the new test is positive, then this would be considered a false positive result.

6.2 Bias

The definition of bias is "the error related to the ways the targeted and sampled populations differ" (Dawson and Trapp, 1994). Bias is unwanted as it threatens the validity of the study (Gay, 2006). For bacteriologic tests, a diagnostic bias can occur when the gold standard used to detect the pathogen is not perfect (Schukken and Deluyker, 1995). This becomes an issue in a study since the more a test is imperfect, more the underestimation of the difference between the gold standard and the new test is great. If a perfect gold standard does not exist, often one be can created by including/combining existing tests (Dawson and Trapp, 1994).

6.3 Agreement

Kappa (k) is the measure of agreement between two tests that adjusts for the amount of agreement that could be obtained by chance alone (Martin et al., 1987). Kappa can be used to determine how well two tests agree or how well a new test compares to an existing test. A k value of 1 indicates perfect agreement, while a k value of 0 indicates no agreement beyond what would be expected by chance (Mckenna and Dohoo, 2006). There are proposed guidelines when interpreting k (Dawson and Trapp, 1994):

0.93-1.0	Excellent agreement
0.81-0.92	Very good agreement
0.61-0.80	Good agreement
0.41-0.60	Fair agreement
0.21-0.40	Slight agreement
0.01-0.20	Poor agreement
<0.00	No agreement

6.4 Sensitivity and Specificity

Determining the Se and Sp of a diagnostic test implies that the true state of infection is known or can be determined by a gold standard (Martin et al., 1987). The Se of a test is the ability of a test to correctly classify animals as infected. It is the proportion of infected animals that test positive as compared to the positive individuals detected by the gold standard in the study population (Slenning, 2001). The Sp of a test is the ability to correctly classify animals as uninfected or negative. It is the proportion of animals that test negative as compared to negative individuals by the gold standard in the study population (Slenning, 2001).

Diagnosing intramammary infection is subject to error. In order to determine the true infection status of a quarter, adhering to aseptic sampling techniques, and proper handling and storage of milk samples is of utmost importance (Sears and McCarthy, 2003). False

positive samples occur when a pathogen is isolated in a culture but the quarter is not truly infected. This happens when a sample is contaminated during sampling or processing. False negative samples occur when the culture is negative for a pathogen when the quarter is truly infected (Sears and McCarthy, 2003)

In a clinical setting the true prevalence of a disease is not known. Therefore we need to know the probability that an animal is truly infected if it has a positive test. This value is called the positive predictive value (PPV) (Martin et al., 1987). Conversely the probability that an animal is truly negative if it has a negative test is the negative predictive value (NPV). The predictive values are affected by both the prevalence in a population and the Se and Sp (Slenning, 2001).

7.0 Mastitis control programs

The goal of mastitis control programs is to produce high quality milk. As well, there is an economic motivator for producers to maintain the herd SCC below 200,000 to avoid losses in milk production due to subclinical infections (Fetrow et al., 1994; Dohoo and Leslie, 1991). In Canada, the current regulatory limit for milk SCC is 500, 000 cells/ml.

The isolation and identification of mastitis-causing pathogens is the fundamental aspect to milk quality and udder health control programs (Ruegg, 2005). Diagnostic methods for determining mastitis include SCC and microbiological analysis (Kirk et al., 1994, Ruegg, 2005). The long term objective of mastitis control programs is to prevent new infections. A short-term objective is to evaluate the effectiveness of current protocols or to find the cause of a mastitis outbreak (Kelton and Godkin, 2000). Mastitis control programs vary depending on the type of mastitis; contagious or environmental (Kelton and Godkin, 2000). For contagious mastitis, control programs have concentrated on cow and milker hygiene during milking, while the control of environmental mastitis has focused on dry cow programs, bedding and a clean environment (Kirk et al., 1994). Fetrow et al. (1994) have described a mastitis monitoring program having two components; 1- a surveillance system designed to detect mastitis "problems" as soon as possible; 2- a status monitoring protocol to assess the efficacy of implemented changes.

The surveillance system for contagious mastitis is based on routine evaluation of somatic cell counts. This can be done at the bulk tank and individual cow level. Once a change in SCC has been detected, there is a need to collect and culture milk samples in order to determine the type of pathogens involved (Kelton and Godkin, 2000).

For environmental mastitis, the surveillance system is based on periodic review of clinical mastitis records (Kelton and Godkin, 2000). Monthly SCC evaluation is less effective for environmental control programs because the duration of clinical mastitis is often of short duration (Kirk et al., 1994). Consequently, elevations in SCC due to environmental pathogens are not always detected by monthly SCC.

7.1 Systematic sampling and milk cultures

There has been a variety of culture programs suggested and implemented for dairy herds (Leslie, 1994; Gonzalez and Wilson, 2002; Kelton and Godkin, 2000). These programs include 1- periodic culture of all milk cows; 2- strategic culturing of new additions and all cows and heifers at dry-off and/or freshening; 3- culturing all clinical mastitis cases; 4- periodic culture of bulk tank milk samples. Which program or combination of programs used in a specific herd will depend on the goals and udder health objectives of that farm.

Periodic culture of all milking cows will estimate the prevalence of major pathogens in the herd and identify cows with contagious pathogens. However, it is costly to culture all cows especially in large herds (Sargeant et al., 2001). When dealing with contagious mastitis (*S. aureus* or *Mycoplasma*) repeat sampling may be necessary due to low sensitivity of a single composite culture (Sears and McCarthy, 2003b; Gonzalez and Wilson, 2003).

Strategic culturing of high risk cows and at critical points during lactation is an alternative to whole-herd culture. From a biosecurity viewpoint, new additions should be cultured prior to or shortly after arrival to the herd to reduce the chance of introducing contagious pathogens to the milking herd (Wilson and Gonzalez, 1997). Somatic cell counts can be used to identify high risk cows. If a threshold of 200, 000 cells/ml is used to select cows for culture one must keep in mind that the Se and Sp of detecting any pathogen

responsible for an IMI is only 73% and 86% respectively (Dohoo and Leslie, 1991). Dry-off and calving are also convenient times to collect milk samples for culture. Dry-off cultures will help indicate the efficacy of contagious mastitis control programs during lactation (Kelton and Godkin, 2000). Milk samples collected after calving are useful to monitor the effectiveness of the dry cow programs and the quality of the environment of the cow before and after parturition (Dingwell et al., 2003). The results of those milk samples help the veterinarians to propose treatment, readjust the dry cow program, segregate or cull some cows based on the organism identified.

Culturing milk of clinical mastitis cases should be an important component of environmental control programs. Contagious pathogens may also be identified through this process (Kelton and Godkin, 2000). These samples can be used to identify the most common mastitis-causing pathogens in the herd (Roberson, 2003). These samples are not used to guide therapy decisions for the current case of clinical mastitis but used to help base future case therapy decisions because it is difficult to receive results from the diagnostic laboratory quickly enough to implement a therapy decision (Sears and McCarthy, 2003).

For contagious mastitis control program success, early detection of infected cows is of primordial importance (Barkema et al., 2006). *Staphylococcus aureus* positive animals need to be identified and dealt in such a way to reduce spread of the pathogen within the herd (Zadoks et al., 2002). Primiparous cows that have an IMI due to *S. aureus* at calving can also act as reservoirs of this contagious pathogen to herd mates as well as increase the prevalence rate in the herd (Roberson et al., 1994).

Bulk tank cultures are a useful tool for monitoring udder-health status in a herd especially with respect to contagious pathogens (Jayarao and Wolfgang, 2003). A single bulk tank sample is not recommended but serial or monthly samples are (Gonzalez and Wilson, 2002; Jayarao and Wolfgang, 2003). From herds in New York and Northern Pennsylvania, the sensitivity for a single bulk tank culture for *S. aureus*, *Str. agalactiae*, and *Mycoplasma* is 59%, 71%, and 33% respectively (Gonzalez and Wilson, 2002).

7.2 Mastitis control programs based on culture results

The National Mastitis Council (NMC) lists ten fundamental elements that they recommend as a part a mastitis control program (Table II). Guideline five, recommends appropriate treatment of clinical mastitis during lactation. Ideally, therapy should be based on culture results (Sears and McCarthy, 2003). By knowing the causative organism, targeted therapy can be implemented. Treating all cases of clinical mastitis is costly and inappropriate (Roberson, 2003). Culture results can provide valuable information for the implementation of a targeted treatment regime for clinical or subclinical mastitis. With cases of mild to moderate clinical mastitis, waiting 24 hours for OFCS results or treating immediately, had no significant difference in the incidence of recurrent mastitis cases (Wagner et al., 2007). Blindly treating all cases of clinical mastitis is costly and inappropriate (Roberson, 2003; McCarron and Keefe, 2008). Ten to 50 % of milk cultures from cases of clinical and subclinical mastitis yield no growth (Makovec and Ruegg, 2003; Bartlett et al., 1992; Erskine, 1991). Milk samples with positive growth may be divided into categories such as GP, GN and other (yeast, algae, etc.). Antibiotic treatment of most mastitis caused by GP bacteria has been shown to be effective and profitable (Cattel et al., 2001, Morin et al., 1998b, Roberson, 2003). In an ongoing study by the National Mastitis Research Network, of 500 cases of clinical mastitis only 55% were GP (Table III). Of these GP organisms, not all should be treated with antibiotics such as chronic *S. aureus* (Barkema et al., 2006). A small percentage of clinical cases are also caused by less common pathogens that are refractory to antibiotic therapy (yeast and algae).

Table II. The National Mastitis Council 10 Guidelines for a Mastitis Control Program

1.	Establish goals for udder health
2.	Use proper milking procedures
3.	Maintain a clean and comfortable environment for cows
4.	Properly maintain milking equipment
5.	Use appropriate treatment of clinical mastitis during lactation
6.	Provide effective dry-cow management
7.	Maintain biosecurity for contagious pathogens and cull chronically infected cows
8.	Keep good records
9.	Regularly monitor udder health status
10.	Periodically review the mastitis control program

Table III. Results for the category of treatment from 500 cases of clinical mastitis.

Catergory of Treatment	n (%)
No Growth	116 (23.3%)
Gram Positive	277 (55.4%)
Gram Negative	92 (18.4 %)
Other (yeast/algea)	15 (3%)
Total	500

G. Keefe, (Mastitis Research Network for Bovine Mastitis, National Cohort study) Sept 2007.

The effects of using OFCS to guide treatment decisions for clinical mastitis was recently evaluated (Lago et al., 2006). Treatment groups consisted of either immediate treatment of all mild to moderate clinical mastitis cases with an intramammary (IMM) antibiotic or waiting for culture results before treatment. The cultures were performed using the Minnesota Easy Culture System II – Biplate. Using the OFCS, quarters with GP growth were treated with IMM antibiotics, while GN and no growth did not received IMM antibiotics. The culture based group had a significant reduction in antibiotic use with only 43% of clinical mastitis cases receiving IMM antibiotics. When bacteriological cure rates were evaluated there was no significant difference between the treatment groups. The effects on cow health and the cost-benefit of adopting an OFCS were not yet evaluated.

Another study performed in a laboratory setting evaluated the test characteristics of two potential OFCS; the Minnesota Easy Culture System II – Biplate (University of Minnesota, St.Paul, MN) and the Petrifilm™ (3M Microbiology, London, ON) (McCarron and Keefe, 2008). The gold standard for comparison was standard bacteriologic culture. The Biplate was evaluated for the presence of GP, GN or no growth (NG). For the Petrifilm, two plates were used to detect the presence of GP, GN or NG; the total Aerobic Count (AC) and the Coliform Count (CC). In this targeted treatment protocol, the tests were evaluated on their ability to differentiate appropriate treatment groups. The Biplate had a sensitivity of 98.6% to correctly identify GP growth and the proportion of clinical cases that would need to be treated with antibiotics was 67.9%. With the Petrifilm system using a cut-point of more

than 5 colonies on the AC and more than 20 colonies of the CC, the sensitivity to correctly identify GP growth was 95,5% and the proportion of clinical cases that would need to be treated with antibiotics was 66.8%.

A retrospective cohort study surveyed dairy producers that used an OFCS and evaluated the impact of the OFCS for bacteriologic culture from cows with low-grade mastitis (Neeser et al., 2006). Ninety five percent of respondents stated that they used an antimicrobial based on the type of bacteria cultured. Farms that used on-farm culture systems and treatment protocols had a significant reduction in the rates of antimicrobial use.

The use of the Petrifilm in an on-farm culture and treatment protocol was evaluated by Silva et al., (2004) on a 600-cow commercial dairy in Wisconsin. Their objective was to use Petrifilm^{TM} microbiological products in a protocol to appropriately use antibiotics to treat clinical mastitis caused by GP pathogens. The 3M microbiological products used in the protocol were the Staph Express, Coliform Count, and Aerobic culture media. Milk samples were collected from clinical cases of mastitis. One sample was frozen for laboratory analysis, and the second sample was used to inoculate three separate Petrifilm plates (STX, coliform, and AC). Plates were read 24 hours later and treatments were applied according to protocol. Intramammary antibiotic treatment was indicated for *S. aureus* positive cases for a new infection (first case, single quarter, and lactation 1 or 2), and for probable streptococci infections (if the AC plate was positive and STX negative). Coliform positive and no growth cases received no IMM treatment. When the 3M culture protocol was used, there was a significant reduction in days out of the tank, number of IMM tubes used per case of mastitis, and cases receiving IMM tubes. In addition, when the costs of antibiotic tubes and milk discard were considered, the cost per case of mastitis when the protocol with the Petrifilms was implemented was 90$ per case as compared to cases without a protocol at 264$ per case.

Objectives of the Study

The objectives of the study are to determine if the Petrifilm™ bacteriological plates are effective at bacteriological determination of bovine milk samples. There were four main objectives to the trial. The first objective was to evaluate the diagnostic test characteristics (Se, Sp, positive (PPV) and negative predictive values (NPV)) of the Petrifilm™ Staph Express count plates (STX) for identification of *S. aureus* from non-diluted and diluted (1:10) cows' milk. Milk samples were taken from cows: a) in the first 30 days in milk; b) with high somatic cell count (SCC) during lactation; c) with clinical mastitis.

The second objective was to evaluate the diagnostic test characteristics of the Petrifilm™ Rapid Coliform count plates (RCC) for identification of coliforms in clinical mastitis (non-diluted and diluted samples). As well as evaluate the effectiveness of the 6-12-hour color change of the RCC plate as an indicator of coliform mastitis.

Objective three was to to evaluate the diagnostic test characteristics of the Petrifilm™ Aerobic count plates (AC) for identification of streptococci in clinical mastitis (non-diluted and diluted samples).

The final objective was to evaluate the test characteristics of Petrifilm plates (STX, RCC) after freezing fresh clinical mastitis samples. In each of these objectives, the agreement (Kappa) between Petrifilm plates (STX, RCC, and AC) and standard bacteriology was evaluated.

The overall objective of the study was to determine if the Petrifilm™ bacteriological test system has potential to be an effective and rapid decision-making for a targeted approach to therapy of clinical and subclinical mastitis.

Materials and Methods

Sampling

All milk samples received at the bacteriology laboratory at centre hopitalier universitaire vétérinaire de Montréal for standard bacteriology from the bovine ambulatory clinic of the Université de Montréal, Faculté de Médecine Vétérinaire were used for this project. Some clinical mastitis samples also arrived from herds from the Ormstown Veterinary Hospital and from a project of Dr. Paul Baillargeon. Submitted milk samples were taken during monthly veterinary herd health visits or from cows with clinical mastitis. Milk samples were collected by both veterinarians and dairy producers. Most of these samples were plated the day of collection. If the milk samples could not get to the laboratory within 24 hours, the samples were frozen. The trial commenced in June 2006 and was completed in April 2007.

Each milk sample was immediately inoculated onto TSA plates, then onto Petrifilm plates (Figure 2). One millilitre of milk was placed on the first Petrifilm plate and a 0.1 mL of milk with 0.9 mL of Butterfield's Phosphate Buffer solution (1: 10 dilution) on the second plate. A sample was considered positive for bacteria "gold standard" if standard bacteriology (primary or incubated) was positive for the bacteria or if Petrifilm culture was positive for the bacteria and identification of the Petrifilm isolate was confirmed by standard bacteriology.

Bacteriological Procedures

Bacteriological analysis was performed by the clinical bacteriology laboratory at the Faculté de Médecine Vétérinaire of the Université de Montréal according to NMC guidelines (NMC, 2004). Once samples arrived at the laboratory, fresh milk was plated immediately and frozen samples were allowed to thaw. Samples were vortexed before being streaked onto trypticase soy agar plate enriched with 5 % sheep's blood (TSA) (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) using 0.01 mL disposable plastic loops. Plates were

then incubated at 35°C for 24 h (standard method). All samples were also incubated. The incubated milk samples were plated onto TSA halves. After incubation, plates were examined, colonies tentatively identified based on morphologic features, pattern of haemolysis, Gram stain reaction, and catalase test, and the colonies enumerated (Figure 1). A second reading was made at 48 h. Gram stain positive and catalase positive cocci were submitted to a coagulase test or if necessary a DNase test to distinguish between *S. aureus* and coagulase CNS. Catalase-negative Gram positive cocci presumptively identified as streptococci were submitted to streptococcus identification tests: CAMP reaction, esculine hydrolysis, hippurate hydrolysis, inulin and raffinose fermentation (Fortin et al., 2003). Identification using an API20S system (BioMérieux, Marcy L'Étoile, France) was done when confirmation was necessary. Gram-positive bacilli were classified according to their microscopic morphology and the results of the catalase test. Gram-negative bacilli were re-inoculated onto a McConkey agar (Difco) and identified using the following tests: oxidase, triple sugar iron, urea, citrate, indole, and motility. Other bacteria or yeast were identified according to morphology with the Gram's stain.

Figure 1. Diagnostic procedures used by the bacteriology laboratory at the Faculté de médecine vétérinaire de l'Université de Montréal for bacteriologic milk cultures.

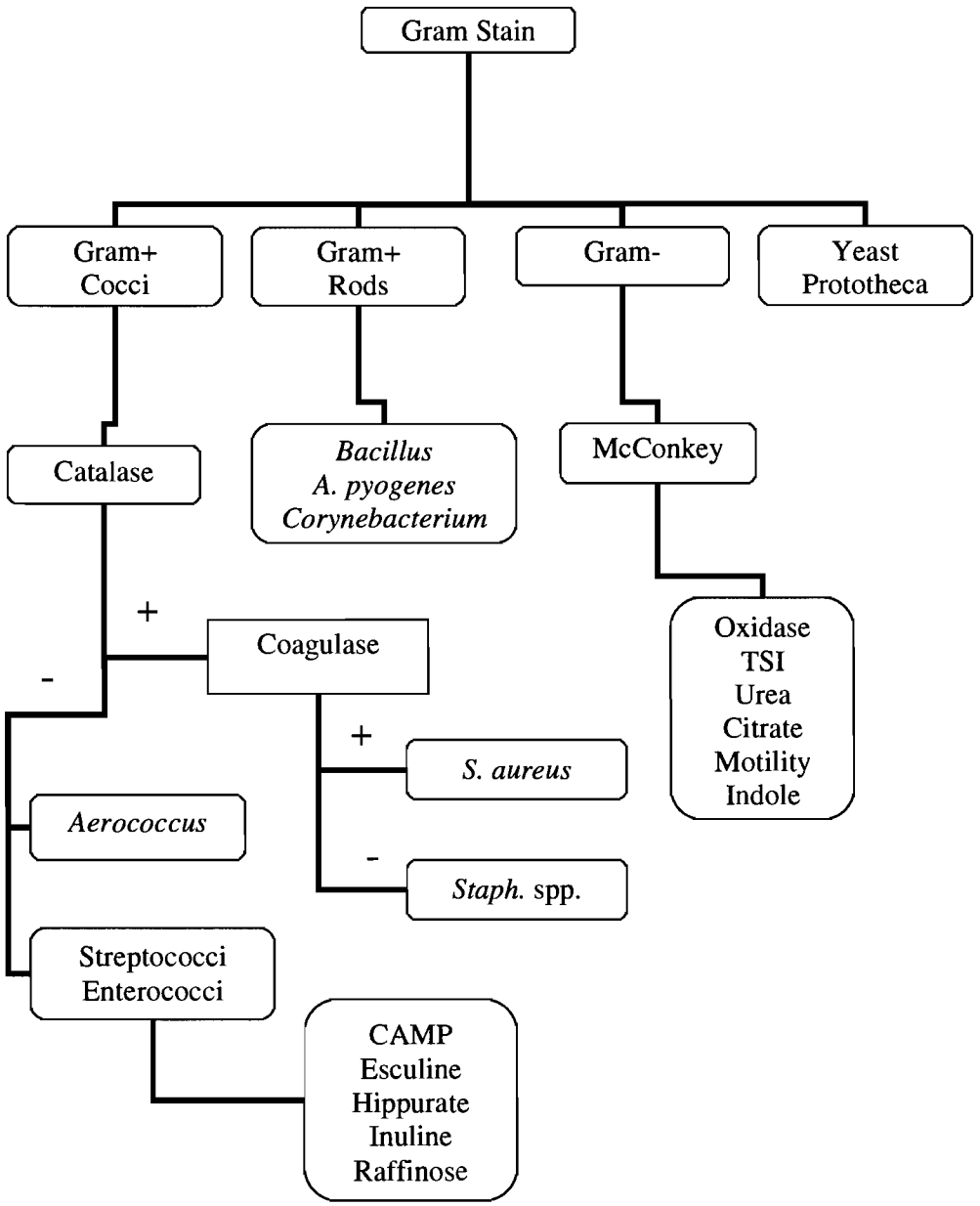
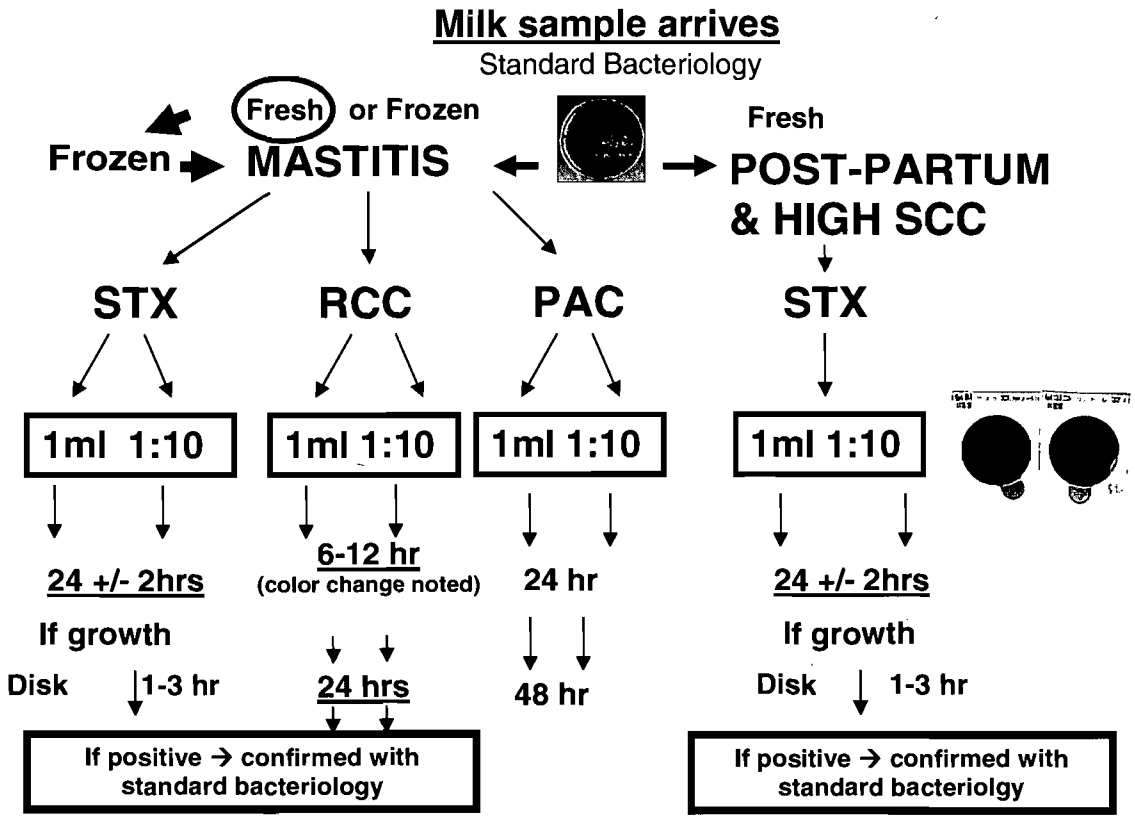


Figure 2. Petrifilm procedures used by the bacteriology laboratory at the Faculté de médecine vétérinaire de l'Université de Montréal for milk samples in Petrifilm study.



Petrifilm Procedures

Immediately after inoculation onto TSA plates, each mastitis milk sample was placed on duplicate STX, RCC, and AC plates: a) a 1 ml aliquot b) a 1:10 dilution using Butterfield's Phosphate Buffer. A 3M representative spent two days training the laboratory staff about the Petrifilm procedures and interpretation prior to the commencement of the trial. The 3M Petrifilm plates were used by trained technicians according to 3M Microbiology instructions. Reading and interpretation of the STX plates was performed at 24 ± 2 h at the bacteriology laboratory. If any colonies appeared on the STX plate, a Staph Express Disk was used and read one to three hours later. If 3M Petrifilm STX was positive for *S. aureus*, the colony was picked, regrown, and confirmed using standard methods. Reading of the RCC plates was done 6-12 hours after plating and a final reading was performed at 24 hours. If the RCC was positive for a coliform, the colony was picked, regrown, and confirmed using standard methods. The AC plates were read at 24 and 48 hours after plating in the 3M Plate Count Reader. The Plate count reader is a machine that counts the colonies on the AC plates. If AC is positive, but RCC and STX are negative, the isolate will be considered as streptococci. This result was compared with standard bacteriology to calculate sensitivity, specificity, positive and negative predictive values of the AC.

Fresh milk samples from clinical mastitis cases were frozen after analysis. After being frozen for seven days, milk samples were thawed and plated again on STX, RCC, and AC. These results were compared to the fresh sample results.

Milk samples from cows after calving or who had an elevated cell count during lactation were received fresh and inoculated on STX plates only. The criteria for an elevated SCC during lactation were a SCC over 200, 000 cells/mL, and 50% in SCC from the previous month, or a SCC over 1, 000,000 cells/mL. Two STX plates were used for each milk samples; a non-diluted (1 mL) and diluted (0.10 mL). The reading and interpretation procedures were the same as mentioned above for mastitis samples.

Statistical Analysis

Statistical analysis was performed using NCSS statistical package (Dawson and Trapp, 1994). The Se, Sp, PPV, NPV were evaluated using the Chi-square analysis versus the Gold standard. The Gold standard was positive when the bacteria was isolated from standard bacteriology (primary or incubated) or through the identification of the Petrifilm isolate as confirmed by the bacteriology lab. In order to evaluate the agreement (kappa), the analysis was performed by comparing the Petrifilm tests, diluted and non-diluted, to standard bacteriology.

Both quarter and composite milk samples were used in the study. However, in the majority of the milk samples submitted, the type of samples was not indicated therefore sample type was not included in the analysis. Contaminated samples (three of more type of bacterial growth) were not removed from the data set since this study is not a prevalence study but the evaluation of a diagnostic test.

Article

A study was conducted to determine whether the 3M Petrifilm Staph Express, Rapid Coliform, and Aerobic Count plates are effective bacteriological media for bovine milk. The Se of the Petrifilm STX for mastitis, calving and high SCC diluted milk samples was 67 %, 75%, and 85%, respectively. The Petrifilm RCC system had the highest Se (76.4%) for coliforms on fresh samples when diluted versus undiluted (72.7%). The Petrifilm Aerobic Count plate had low PPV (24.6%) to differentiate Streptococci in mastitic milk samples. Freezing mastitis milk samples increased the Se of the STX and decreased the Se of the RCC. The Petrifilm culture system (STX and RCC) is comparable with standard bacteriologic culture for the isolation of *S. aureus* and coliforms.

MASTITIS DIAGNOSIS IN DAIRY COWS

Comparison of 3M Petrifilm™ Staph Express, Petrifilm™ Rapid Coliform and Petrifilm™ Aerobic count plates with standard bacteriology of bovine milk

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ABSTRACT

The objectives of the study were to evaluate the test characteristics of the: 1) Petrifilm™ Staph Express Count plates (STX) for identification of *S. aureus* from cows' milk. Milk samples were taken from cows: a) in the first 30 days in milk; b) with high somatic cell count (SCC) during lactation; c) with clinical mastitis. 2) Petrifilm Rapid Coliform Count plates (RCC) for identification of coliforms from cows with clinical mastitis. 3) Petrifilm Aerobic Count plates (AC) for identification of streptococci from cows with clinical mastitis. 4) Petrifilm plates (STX, RCC) after freezing clinical mastitis milk samples. The effect of an undiluted and a diluted sample (1:10) on the test characteristics of Petrifilm plates was also determined in each objective. Also, the agreement (Kappa) between Petrifilm plates (STX, RCC, and AC) and standard bacteriology was evaluated. The results for part 1a) A total of 1204 fresh milk samples were used in the analysis. The sensitivity (Se) and specificity (Sp) of the STX versus standard bacteriology for non-diluted and diluted samples versus standard bacteriology was 69.2%, 98.3%, and 74.2%, 97.8%, respectively. 1b) The Se and Sp of the STX for 300 fresh milk samples was highest for diluted samples; 85.1%, 96%, respectively. c) The test characteristics of the STX were the highest for diluted fresh samples from mastitis cows. For frozen samples, the Se and Sp were similar for undiluted and diluted samples. 2) The Se (76.4%) of the RCC to detect coliforms was the highest for the diluted fresh samples. 3) For AC analysis, the Se and Sp for diluted fresh samples was 86.5%, and 9.8%, respectively. The poor positive predictive value (24.6%) indicates that the AC is not effective for streptococci determination. 4) When fresh mastitis samples were frozen and then replated, there was an increase of 10.6% in *S. aureus* identification. For coliform identification, the Se decreased 14.7% after freezing fresh samples. The agreement between the STX, RCC, and AC and standard bacteriology for part 1, 2, 3, and 4 was best with diluted samples. Overall, the results indicate that the Petrifilm STX and RCC culture media are comparable to standard bacteriology for the detection of *S. aureus* and coliforms.

(Key Words: Petrifilm, *Staphyloccocus aureus*, coliforms, microbiology, bovine milk)

INTRODUCTION

Isolation and identification of mastitis pathogens is one of the fundamental aspects of milk quality control programs (Ruegg, 2005). However, standard culture in commercial laboratories often requires two to five working days to obtain results. This leads to under-utilization of this important diagnostic method (Sargeant et al., 2001). There is a need for a rapid and precise method for identifying pathogens on-farm for either clinical or subclinical mastitis control programs (Leslie et al., 2002). Petrifilm™ plates (3M Microbiology) may fill this gap.

Currently bacteriologic culture is the gold standard for diagnosing the etiologic agent causing an intramammary infection (IMI) or clinical mastitis (Sears and McCarthy, 2003). The sensitivity of standard culture will vary depending on the organism and the methods used for sampling and culturing (Lam et al, 1996; Sears et al, 1991; Dinsmore et al., 1992; Godden et al., 2002). The sensitivity (Se) for detecting *S. aureus* from a composite milk sample in subclinically infected cows is only 58-63% (Lam et al, 1996). The low Se is thought to be due to the dilution effect of the uninfected quarters. The Se increases (75%) when quarter milk sample are used (Sears et al, 1990). There are a variety of methods used to increase the Se of detecting pathogens from milk samples such as larger inoculum volumes, preculture incubation, enrichment and centrifugation (Dinsmore et al., 1992; Lam et al., 1996; Sol et al., 2002; Zeconni et al., 1997).

Petrifilm™ plates are ready-to-use culture media that are used primarily in the food industry, for the rapid identification and numeration of bacteria (Tassinari et al., 2005; Ingham et al., 2003). The Petrifilm bacteriologic results are available in 12 to 24 hours. The Petriflim plates that have potential for use as diagnostic tests in the dairy industry are the Petrifilm™ Staph Express count plates, the Coliform count plates, and the Aerobic count plates.

The Petrifilm™ Staph Express count plate (STX) contains chromogenic, modified Baird-Parker media that is selective and differential for *Staphlococcus aureus* (*S. aureus*). After 22-24 hours, confirmation of *S. aureus* is performed by using a Staph Express Disk that contains deoxyribonuclease and a dye that reacts to produce a pink zone around the *S. aureus*

colonies (3M Microbiology). The Petrifilm STX was evaluated for detection of *S. aureus* in bovine milk (Silva et al., 2005). The Se for *S. aureus* detection was 87.5%, which was significantly higher than standard microbiological techniques. It was also concluded that the interpretation of the Petrifilm was highly dependant on the readers' ability to identify colony colour and distinct pink zones after application of the Staph Express disk plates. Another study evaluating the STX on a commercial dairy resulted in a low Se (56%) and specificity (Sp) (78%) when plates were interpreted by the farm personnel. Further training of the personnel improved these values. When on farm milk quality protocol included culturing with Petrifilms and treatment protocols there was a significant monetary savings per case of clinical mastitis (Silva et al, 2004).

Petrifilm rapid coliform count plates (RCC) are used for the rapid detection of coliforms. It is currently the fastest approved coliform test for the enumeration of coliforms in food (3M Canada, 2001). The Petrifilm RCC can detect high levels of coliform contamination as early as six hours of incubation. Total confirmed coliform counts can be available in 6 to 14 hours as indicated by a color change around the potential colonies. Final results are obtained in 24 hours.

The Petrifilm aerobic plate count (AC) are used for the detection of aerobic bacteria. The nutrient base used for the AC is a modified standard method. All colonies that grow on the AC appear red. The AC is currently approved as the AOAC official methods of analysis for; raw/pasteurized milk, dairy products, and foods.

There were four main objectives to the trial. The objectives were to evaluate 1) the Se, Sp, positive (PPV) and negative predictive values (NPV) of the 3M Petrifilm™ Staph Express count plates (STX) for identification of *S. aureus* from non-diluted and diluted (1:10) cows' milk. Milk samples were taken from cows: a) in the first 30 days in milk; b) with high somatic cell count (SCC) during lactation; c) with clinical mastitis. 2) The Se, Sp, PPV and NPV of the 3M Petrifilm Rapid Coliform count plates (RCC) for identification of coliforms in clinical mastitis (non-diluted and diluted samples). 3) The 3M Petrifilm Aerobic count plates (AC) for identification of streptococci in clinical mastitis (non-diluted and diluted samples). 4) The test characteristics of Petrifilm plates (STX, RCC) after freezing fresh clinical mastitis samples. The agreement (Kappa) between Petrifilm plates (STX, RCC, and AC) and standard bacteriology was evaluated.

MATERIALS & METHODS

Sampling

All milk samples received at the bacteriology laboratory at the Université de Montréal for standard bacteriology from the bovine ambulatory clinic of the Université de Montréal, Faculté de médecine vétérinaire were used for this project. Some clinical mastitis samples were also collected in herds from the Ormstown Veterinary Hospital and from a project of Dr. Paul Baillargeon. Submitted milk samples were taken aseptically during monthly veterinary herd health visit or from cows with clinical mastitis by a veterinarian or a trained animal health technician. Most of these samples were plated the day of collection. If the milk samples could not get to the laboratory within 24 hours, the samples were frozen. Each milk sample was submitted for standard bacteriology and simultaneously placed on Petrifilm plates. The trial commenced in June 2006 and was completed in April 2007.

Bacteriological Procedures

Bacteriological analysis was performed by the clinical bacteriology laboratory at the Faculté de Médecine Vétérinaire of the Université de Montréal according to NMC guidelines (NMC, 2004). Once samples arrived at the laboratory, fresh milk was plated immediately and frozen samples were allowed to thaw. Samples were vortexed before being streaked onto trypticase soy agar plate enriched with 5 % sheep's blood (TSA) (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) using 0.01 mL disposable plastic loops. Plates were then incubated at 35°C for 24 hours (standard method). All samples were also incubated as an enrichment method. The incubated milk samples were plated onto TSA halves. After incubation, plates were examined, colonies tentatively identified based on morphologic features, pattern of haemolysis, Gram stain reaction, and catalase test, and the colonies enumerated. A second reading was made at 48 h.

Gram stain positive and catalase positive cocci were submitted to a coagulase test or if necessary a DNase test to distinguish between *S. aureus* and coagulase negative staphylococci (CNS). Catalase-negative Gram stain positive cocci presumptively identified as streptococci were submitted to streptococcus identification tests: CAMP reaction, esculine hydrolysis, hippurate hydrolysis, inulin and raffinose fermentation (Fortin et al., 2003).

Identification using an API20S system (BioMérieux, Marcy L'Étoile, France) was done when confirmation was necessary. Gram-positive bacilli were classified according to their microscopic morphology and the results of the catalase test. Gram-negative bacilli were re-inoculated onto a McConkey agar (Difco) and identified using the following tests: oxidase, triple sugar iron, urea, citrate, indole, and motility. Other bacteria or yeast were identified according to morphology with the Gram's stain.

A milk sample was positive for the bacteria for standard bacteriology when the primary or incubated sample isolated the bacteria. Since this study is not a prevalence study but the evaluation of a diagnostic test, the presence of any bacteria was considered positive.

Petrifilm Procedures

Immediately after inoculation onto TSA plates, each mastitis milk sample was placed on duplicate STX, RCC, and AC plates: a) a 1 mL aliquot of milk; b) a 1:10 dilution, using 0.1 mL of milk and 0.9 mL of Butterfield's Phosphate Buffer. 3M Petrifilm plates were used by trained technicians according to 3M Microbiology instructions. Reading and interpretation of the STX plates was performed at 24 ± 2 hours he bacteriology laboratory. If any colonies appeared on the STX plate, a Staph Express Disk was used and read one to three hours later. If 3M Petrifilm STX was positive for *S. aureus*, the colony was picked, regrown, and confirmed using standard methods.

Reading of the RCC plates was done 6-12 hours after plating and a final reading was performed at 24 hours. If the RCC was positive for a coliform, the colony was picked, regrown, and confirmed using standard methods. The AC plates were read at 24 and 48 hours after plating in the 3M Plate Count Reader. The Plate count reader is a machine that counts the colonies on the AC plates

Fresh milk samples from clinical mastitis cases were frozen after analysis. After being frozen for seven days at -20°C, milk samples were thawed and plated again on STX, RCC, and AC. These results were compared to the fresh sample results.

Milk samples from cows after calving or who had an elevated cell count during lactation arrived fresh and were inoculated on duplicate STX plates only. The criteria for an elevated SCC during lactation were a SCC over 200, 000 cells/mL, and 50% in SCC from the previous month, or a SCC over 1, 000,000 cells/mL. The reading and interpretation procedures were the same as mentioned above for mastitis samples.

Statistical Analysis

Statistical analysis was performed using NCSS statistical package (Dawson and Trapp, 1994). The Se, Sp, PPV, NPV, and Kappa of the Petrifilm tests (diluted and non-diluted) were evaluated using the Chi-square analysis versus the standard milk bacteriology results. The STX and RCC plates were compared to standard bacteriology and a gold standard. A gold standard was created to help reduce bias. This was done because the new test, the Petrifilm, was shown to have better sensitivity than the existing bacteriological tests (Silva et al., 2005). For bacteriologic tests, a diagnostic bias can occur when the gold standard used to detect the pathogen is not perfect (Schukken and Deluyker, 1995). This becomes an issue in a study since the more a test is imperfect, the greater the underestimation of the difference between the gold standard and the new test. If a perfect gold standard does not exist, often one can be created by including or combining existing tests (Dawson and Trapp, 1994). In the current study, the gold standard was positive when the bacteria was isolated from standard bacteriology (primary or incubated) or through the identification of the Petrifilm isolate as confirmed by the bacteriology lab.

For part 3 of the trial, if the AC was positive, but RCC and STX were negative, the isolate was considered as streptococci. This result was compared with standard bacteriology to calculate the test characteristics of the AC.

Contaminated samples (three of more different organisms) were not removed from the data set since this study is not a prevalence study but the evaluation of a diagnostic test.

RESULTS

The first objective was to evaluate the Se, Sp, PPV, NPV and Kappa of the 3M Petrifilm™ Staph express Count Plates (STX) for identification of *S. aureus*. 1) a) A total of 1208 milk samples from fresh cows were submitted for analysis. There were 4 samples removed because positive colonies on the STX were not picked and regrown for confirmation. A total of 1204 fresh milk samples were used in the analysis. The proportion of *S. aureus* isolated from these samples was 9.8% and 10.9% for standard bacteriology and the gold standard, respectively. The Se and Sp of the STX versus standard bacteriology for identification of *S. aureus* was highest with diluted samples at 74.2% and 97.8% respectively (Table 1). The agreement (Kappa) between diluted and non-diluted samples of the STX and standard bacteriology was good at 0.73 and 0.74 (Table 1). The false positives of the STX versus standard bacteriology are evaluated by using the results of the re-isolation of positive STX colonies. There were 18 false positives (10 CNS and 8 *S. aureus*) for the non-diluted samples and 24 false positives (9 CNS, 12 *S. aureus*, and 3 no growth) for the diluted samples. In the diluted samples, 50% of the false positives were due to *S. aureus*. When compared to the gold standard, the PPV of the STX for non-diluted and diluted samples was higher at 90.1% and 89.4%, respectively (Table 2). For this comparison, there were 10 false positives for the non-diluted STX, all due to CNS. For the diluted STX, there were 12 false positive results; 9 due to CNS, and three no growth.

Table 1. Test characteristics of the 3M Petrifilm Staph Express count plate versus the standard bacteriology for fresh milk samples from fresh cows.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa	n
Non-diluted	69.2	98.3	82.2	96.6	.726	1204
Diluted (1 :10)	74.2	97.8	78.8	97.2	.739	1204

Table 2. Test characteristics of the 3M Petrifilm Staph Express count plate versus the gold standard for fresh cow samples.

Type of sample	Sensitivity	Specificity	PPV	NPV	n
Non-diluted	67.9	99.1	90.1	96.1	1204
Diluted (1 :10)	75.4	98.9	89.4	97.0	1204

b) A total of 303 milk samples were submitted from high SCC cows. There were three records removed because positive colonies on the STX were not picked and regrown for confirmation. For the final analysis, 300 fresh milk samples from high SCC cows were used.

The proportion of *S. aureus* isolated from these samples was 15.7% and 19.0% for standard bacteriology and the gold standard, respectively. The Se and Sp of the STX for non-diluted and diluted samples of the STX for identification of *S. aureus* versus standard bacteriology was 76.6%, 95.3% and 85.1%, 96%, respectively (Table 3). The PPV was slightly higher with diluted samples. When compared to standard bacteriology, for the non-diluted samples and diluted samples, there 12 (8 *S. aureus*, 2 CNS, and 2 no growth) and 10 (6 *S. aureus*, 1 CNS, and 3 no growth) false positives, respectively. The agreement between the Petrifilm STX and standard bacteriology was good (k=0.790) for diluted samples (Table 3). When the STX was compared to the gold standard the test characteristics were similar with the diluted samples having a slightly higher Se (Table 4). The highest PPV (94.0%) and NPV (96.0%) were also obtained with the diluted samples. For the non-diluted samples, there were 4 false positives; 2 CNS, and 2 no growth. For the diluted samples there were only 3 false positive results; 2 CNS, and 1 no growth.

Table 3. Test characteristics of the 3M Petrifilm Staph Express count plate versus standard bacteriology for fresh milk samples from high SCC cows.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa	n
Non Diluted	76.6	95.3	75.0	95.6	.712	300
Diluted (1 :10)	85.1	96.0	80.0	97.2	.791	300

Table 4. Test characteristics of the 3M Petrifilm Staph Express count plate versus the gold standard for 300 samples from high SCC cows.

Type of sample	Sensitivity	Specificity	PPV	NPV	n
Non Diluted	77.2	98.4	91.7	94.8	300
Diluted (1 :10)	82.5	98.8	94.0	96.0	300

c) A total of 536 fresh and frozen mastitis milk samples were tested. There were 19 records removed because positive colonies on the STX were not picked and regrown for confirmation, and three records were removed due to incomplete results. Therefore, 514 records were used in the analysis; 320 fresh, and 194 frozen samples. The proportion of *S. aureus* isolated with standard bacteriology and the gold standard was 14.6% and 17.3% respectively. The test characteristics of the STX were the highest for diluted samples from mastitis cows (Table 5). Similar Se (66.7%) were obtained using diluted fresh or frozen milk samples. The highest PPV (84.2%) and best agreement (k=0.71) was achieved using fresh diluted samples. For the combined fresh and frozen milk, there were 18 (4 CNS, 10 *S. aureus*, 2 no growth, and 2 other) and 12 (1 CNS, 9 *S. aureus*, and 2 no growth) false positive results for the Petrifilm STX non-diluted and diluted samples, respectively. For the non-

diluted samples 55% were due to *S. aureus* and 22% to CNS. When the STX was compared to the gold standard the Se was also the highest (67.9%) with fresh diluted milk samples (Table 6). For the non-diluted STX, there were seven false positives; 4 CNS, 1 no growth, and 2 other. For the diluted STX there were 1 CNS and 2 other growth that appeared as false positives.

Table 5. Test characteristics of the 3M Petrifilm Staph Express count plate versus standard bacteriology for mastitis samples.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa	n
Non diluted : fresh	60.4	96.0	72.5	93.2	.605	320
Diluted : fresh	66.7	97.8	84.2	94.3	.705	320
Non diluted :frozen	59.3	95.8	69.6	93.6	.587	194
Diluted : frozen	66.7	96.4	75.0	94.7	.661	194
Non diluted: fresh & frozen combined	60.0	95.9	71.4	93.3	.598	514
Diluted : fresh & frozen combined	66.7	97.3	80.6	94.5	.688	514

Table 6. Test characteristics of the 3M Petrifilm Staph Express count plate versus the gold standard for mastitis samples.

Type of sample	Sensitivity	Specificity	PPV	NPV	n
Non diluted Fresh	62.3	97.4	82.5	92.9	320
Diluted Fresh	67.9	99.3	94.7	94.0	320
Non diluted Frozen	63.9	100.0	100.0	92.4	194
Diluted Frozen	63.9	99.4	95.8	92.4	194
Non diluted Fresh & Frozen Combined	62.9	98.4	88.9	92.7	514
Diluted Fresh & Frozen Combined	66.3	99.3	95.2	93.4	514

2) The 3M Petrifilm Rapid Coliform count plates (RCC) were evaluated for identification of coliforms in clinical mastitis samples. A total of 536 fresh and frozen mastitis milk samples were tested. There were 13 records removed because positive colonies on the RCC were not picked and regrown for confirmation, and two records removed due to incomplete data. Therefore, 521 samples were used in the final analysis; 319 fresh and 202 frozen. The proportion of coliforms isolated from standard bacteriology and the gold standard was 22.8% and 27.8%, respectively. Of the 22.8% from standard bacteriology, 16.7% were *E.coli*, 3.4% *Klebsiella*, 1.7% *Enterobacter*, 0.4% *Serratia* and 0.7% other coliforms. The Se (76.4%) and PPV (80.1%) of the RCC was the highest for the diluted fresh samples (Table 7). The highest agreement (k=0.740) was achieved with diluted fresh samples. For the combined fresh and frozen mastitis samples, 97% of the false positives were due to coliforms, as confirmed by

replating positive samples. There were 17 false positives (3 *Klebsiella,* 9 *E. coli,* and 5 coliforms) for the non-diluted samples and 24 (4 *Klebsiella,* 11 *E. coli,* 8 other coliforms, and 1 *S. aureus*) for the diluted samples as compared to standard bacteriology. There was a higher proportion of false negatives for the non-diluted samples as compared to the diluted; 35 versus 9. When the RCC was compared to the gold standard, the Se was the highest with diluted fresh samples Table 8). There was only one false positive for the non-diluted samples; 1 *Bacillus*. For the diluted samples, 1 *S. aureus* was the cause of the false positive results. The false negatives were also much higher for the non-diluted samples as compared to the diluted samples; 42 versus 9.

Table 7. 3M Petrifilm Rapid Coliform count plate test characteristics versus standard bacteriology for mastitis samples.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa	n
Non diluted Fresh	72.7	95.5	76.9	94.4	0.697	319
Diluted Fresh	76.4	96.2	80.1	95.1	0.742	319
Non diluted Frozen	68.8	94.2	84.6	86.7	0.662	202
Diluted Frozen	71.9	89.9	76.7	87.3	0.628	202
Non diluted Fresh & Frozen Combined	70.6	95.0	80.8	91.6	0.686	521
Diluted Fresh & Frozen Combined	73.9	94.0	78.6	92.4	0.694	521

Table 8: 3M Petrifilm Rapid Coliform count plate test characteristics versus gold standard for mastitis samples.

Type of sample	Sensitivity	Specificity	PPV	NPV	n
Non diluted Fresh	75.0	99.6	98.1	93.6	319
Diluted Fresh	76.5	100	100	94.0	319
Non diluted Frozen	67.5	100	100	83.3	202
Diluted Frozen	76.6	99.2	98.3	87.3	202
Non diluted Fresh & Frozen Combined	71.0	99.7	99.0	89.9	521
Diluted Fresh & Frozen Combined	76.6	99.7	99.1	91.7	521

The ability of the RCC to detect coliforms, as early as 6-12 hours after incubation, was evaluated. Positive coliform growth was indicated by a color change and gas formation around the potential colony. Of the 521 RCC samples, a total of 379 were evaluated at 6-12 hours after incubation for their ability to predict coliform growth (Tables 9 & 10). The Se of the RCC was low when compared to standard bacteriology and the gold standard. However, the NPV was very good at 92.3% for diluted fresh samples (Table 9).

Table 9. The test characteristics of the 3M Petrifilm Rapid Coliform count plate versus standard bacteriology to indicate coliform growth by a color change at 6-12 hours after incubation.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa	n
Non diluted Fresh	54.8	93.0	62.2	90.8	0.501	243
Diluted Fresh	61.9	95.5	74.3	92.3	0.615	243
Non diluted Frozen	17.1	93.7	53.8	72.4	0.133	136
Diluted Frozen	22.0	93.7	60.0	73.6	0.191	136
Non diluted Fresh & Frozen Combined	36.1	93.2	60.0	83.9	0.343	379
Diluted Fresh & Frozen Combined	42.2	94.9	70.0	85.4	0.433	379

Table 10. The test characteristics of the 3M Petrifilm Rapid Coliform count plate versus the gold standard to indicate coliform growth by a color change at 6-12 hours after incubation.

Type of sample	Sensitivity	Specificity	PPV	NPV	n
Non diluted Fresh	56.9	95.8	78.4	89.3	243
Diluted Fresh	58.8	97.4	85.7	89.9	243
Non diluted Frozen	18.0	95.3	69.2	66.7	136
Diluted Frozen	28.0	98.8	93.3	70.2	136
Non diluted Fresh & Frozen Combined	37.6	95.7	76.0	80.9	379
Diluted Fresh & Frozen Combined	43.2	97.8	88.0	82.7	379

3) The AC plates were evaluated for their ability to differentiate streptococci in mastitis milk samples. A total of 536 samples were tested. Forty-seven records were removed due to incomplete data. Samples that were negative for *S. aureus* on the STX, negative for coliforms on the RCC, but had growth on the AC were assumed to be Streptococcus spp. Therefore, for the anaylsis, positive STX and RCC results were removed to determine the ability of the AC plate to detect streptococci. For the non-diluted and diluted samples, there were 318 and 312 samples that had growth on the AC plate but were negative on both the STX and RCC plates, respectively. The proportion of Streptococcus spp from the non-diluted and diluted samples was 22.1% and 22.6% respectively. The 24 and 48-hour results are presented in Table 11. Although the Se are good, ranging from 65.0% to 100% at the 48 hour reading, the PPV's are very low, and there is very poor to no agreement between the AC and standard bacteriology.

Table 11. 3M Petrifilm Aerobic count plate test characteristics versus standard bacteriology for fresh and frozen mastitis samples at 24 and 48 hours with positive STX and RCC results removed.

Type of sample		Sensitivity	Specificity	PPV	NPV	Kappa	n
24 hr	Non diluted Fresh	82.7	18.3	25.6	75.7	0.006	205
	Diluted Fresh	82.4	15.3	24.0	72.7	-0.013	208
	Non diluted Frozen	65.0	24.7	15.7	76.7	-0.046	113
	Diluted Frozen	66.7	23.3	15.4	76.9	-0.043	104
	Non diluted Fresh & Frozen Combined	77.8	20.7	22.3	76.1	-0.008	318
	Diluted Fresh & Frozen Combined	78.3	18.1	21.3	74.6	-0.018	312
48 hr	Non diluted Fresh	86.5	9.8	24.6	68.2	-0.020	205
	Diluted Fresh	98.0	10.8	26.3	94.4	0.023	208
	Non diluted Frozen	80.0	19.4	17.6	80.8	-0.003	113
	Diluted Frozen	100	16.3	20.0	100	0.063	104
	Non diluted Fresh & Frozen Combined	84.7	13.9	22.3	75.0	-0.009	318
	Diluted Fresh & Frozen Combined	98.6	12.8	24.3	96.9	0.054	312

4) The test characteristics of Petrifilm plates (STX, RCC) after freezing fresh clinical mastitis milk samples were evaluated versus standard bacteriology. Not all of the fresh clinical mastitis samples were frozen, therefore complete records for 223 STX and 229 RCC were available for analysis. With the STX plates, there was an increase of 10.6% in *S. aureus* identification after freezing fresh non-diluted samples (Table 12). However, when fresh milk samples were frozen and re-evaluated using a dilution, *S. aureus* recovery decreased by 2.2%. For the RCC, freezing fresh milk samples resulted in a 6.9% and 14.7% reduction in coliform identification for non-diluted and diluted samples, respectively (Table 13).

Table 12. 3M Petrifilm Staph Express count plate test characteristics for 223 fresh mastitis samples that were frozen and re-evaluated versus standard bacteriology.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa
Non diluted fresh	61.3	94.8	65.5	93.8	0.576
Non diluted fresh then frozen	71.9	95.8	74.2	95.3	0.686
Diluted fresh	71	97.4	81.5	95.4	0.723
Diluted fresh then frozen	68.8	97.4	81.5	94.9	0.707

Table 13. 3M Petrifilm Rapid Coliform count plate test characteristics for 229 fresh mastitis samples that were frozen and re-evaluated versus standard bacteriology.

Type of sample	Sensitivity	Specificity	PPV	NPV	Kappa
Non diluted fresh	68.4	97.4	83.9	93.9	0.710
Non diluted fresh then frozen	61.5	97.9	85.7	92.5	0.669
Diluted fresh	73.7	98.4	90.3	94.9	0.778
Diluted fresh then frozen	59.0	98.4	88.5	92.1	0.661

DISCUSSION

The Petrifilm STX count plates had a Se of 85.1% for the detection of *S. aureus* in fresh milk samples from cows with elevated SCC in this study. This parallels results from a similar study where the Se was 87.5% (Silva et al, 2005). The Se of the STX was lower in milk samples from fresh cows (74.2%) and mastitis cases (66.7%). The PPV for the STX versus standard bacteriology was good and ranged from 70% to 84%. The implications of a positive *S. aureus* test will depend on the herd udder health protocols and policies. With a high PPV, you will be very sure that a positive test means that the cow is truly infected. The high PPV is important in herds that cull and or segregate positive animals. The PPV's were lower when the STX was compared to standard bacteriology versus the gold standard. In the evaluation of the STX versus standard bacteriology for fresh cow samples, there were 24 false positive cases for the diluted samples. Of these, 50% were due to *S. aureus*. The STX isolated twelve more *S. aureus* samples than indicated by standard bacteriology. If the Petrifilm was compared to standard bacteriology alone, then it would appear to have a high rate of false positives. Using the gold standard, it was determined that the false positives were due to CNS and not *S. aureus*. Considering that the Petrifilm STX has been shown to be more sensitive than standard bacteriology (Silva et al., 2005), the "gold standard" helps evaluate the test characteristics by reducing the bias of an imperfect test.

The RCC was very effective at coliform determination in mastitic milk samples for the 24-hour reading. The best Se and Sp was a result of using fresh diluted milk samples when compared to standard bacteriology. There was also very good agreement between the RCC and standard bacteriology. When the RCC was compared to standard bacteriology, 97% of the false positives were due to coliforms. When compared to the gold standard, the PPV was excellent, ranging from 98 to 100%. The predictive values of the RCC reflect how well this test will work in the field. The PPV indicates the likelihood that a milk sample with a positive RCC is infected with a coliform.

The RCC 12-hour color change for coliform determination did not prove to be sensitive with a Se of only 61.9% for diluted fresh samples and had moderate agreement with standard bacteriology. For the frozen samples, the PPV was very low and there was poor agreement

between the RCC color change and standard bacteriology. This may be explained by the nature of the RCC plate. The color change of the RCC is based upon pH. Mastitis milk samples tend to have a higher pH than normal milk (Dohoo and Meek, 1982). This may negatively affect the color change of the RCC plates. The benefits of the 12 hour color change indicator is the potential to have results available before the next milking in order to make appropriate therapy decisions for clinical mastitis. The drawback to the Petrifilm RCC plates is the cost. The RCC plates are approximately over double the cost ($2.60 versus $1.00) of the Petrifilm *E. coli*/Coliform count plates that have only the 24 hour results.

The AC plates were not effective for streptococci spp. differentiation in mastitis milk samples in our protocol. In our study, positive STX and RCC plates were removed to eliminate *S. aureus* and coliform cases. This OFCS was adapted from previous work by Silva et al (2004) where mastitis milk samples were plated on the STX, CC, and the AC. Samples that were negative for *S. aureus*, negative for coliforms, but had growth on the AC were assumed to be Streptococci spp. The problem with this assumption is that other pathogens like CNS are not accounted for. In the current study the proportion of streptococci spp and CNS isolated was 22% and 32% respectively. Over 80% of the AC plates were positive for growth. This resulted in very poor predictive values for this test. The AC plates have shown potential in a study evaluating OFCS (McCarron and Keefe, 2008). McCarron and Keefe used the AC plate in combination with EC count plates for clinical mastitis samples. The objective was to differentiate Gram positive from Gram negative growth. Clinical mastitis samples that were negative on the EC but positive on the AC were considered Gram positive and appropriate therapy was implemented. The Se and NPV of the Petrifilm OFCS, to differentiate Gram positive and Gram negative growth, was 96% and 92%, respectively.

Silva et al. (2005) determined that the test characteristics of the STX were highly dependant on the reader's ability to interpret the pink zone. In that study, the zone reaction was quantified as weak or distinct. The Sp and the PPV for *S. aureus* detection increased from 87.2% and 31%, to 96% and 57.1%, respectively, when distinct pink zones were seen. The current study made no attempt to classify the pink zones. In addition, reading of the Petrifilm plates was performed by trained laboratory technicians and veterinarians. This was done to reduce variability between readers.

In order to evaluate agreement between the tests, the Petrifilm tests were compared to standard bacteriology and not the gold standard. Standard bacteriology results included both primary and incubated samples. The best agreement was seen with diluted samples for the Petrifilm STX. The highest agreement between tests was seen with diluted samples from cows with high SCC, with a good agreement of 0.790. There was also good agreement between the RCC and standard bacteriology. The highest agreement was with diluted fresh samples. For the detection of Streptococci spp., there was almost no agreement between the AC and standard bacteriology. Therefore, level of agreement was acceptable only between the Petrifilm STX and RCC tests and standard bacteriology.

Inoculum volumes are thought to play an important role of the Se and Sp of a diagnostic test (Anderson et al., 1991; Buelow et al., 1999; Lam et al., 1996). A study evaluating different inoculum sizes showed that both the Se and Sp for *S. aureus* were higher when a 0.1 mL inoculum was plated (Buelow et al., 1999). When a single composite sample and a milk inoculum of 0.1 mL was used, the Se and Sp were 92% and 86% respectively (Lam et al., 1996). Anderson et al. (1991) also evaluated inoculum volumes in the diagnosis of mastitis from clinical quarters. They found that there was no difference in cultural outcomes between 0.10 versus 0.01 mL inoculum volumes. Their experiment also demonstrated that there was no difference in cultural outcomes between 0.05 and 0.01 mL volumes. Their experiment was performed on frozen mastitis quarter samples. In the current study, inoculum volumes were 1 mL and 0.1 mL of milk. The test characteristics of the Petrifilms varied depending on the dilution. For the STX plates there was a small difference in the proportion of false negatives between non-diluted and diluted samples. For STX mastitis, fresh cow, and high SCC samples the proportion of false negatives were 55:45, 48:52, and 56:44, respectively. However, for RCC mastitis samples the proportion of false negatives is much higher for non-diluted versus diluted samples, 80:20. This may be due to the fact that too many colonies on the Petrifilm disk makes reading and interpretation more difficult. As well, when colonies are too numerous to count on the Petrifilm plates, and the growth media readily used making ideal growth of the organism difficult. The authors suggest that diluted samples be used when using the Petrifilm system especially for RCC. One could argue that it is more practical and economic to plate non-diluted samples. There would be no need for additional costs of dilution materials (pipettes and sterile solution), and a dilution could be made if reading is difficult because too many colonies grown on the Petrifilm. That decision should be made in consideration of the objectives of the OFCS. Milk

samples should always be kept in the freezer and sent to the regional bacteriology laboratory in case the Petrifilm results are inconclusive or if the cow was unresponsive to the implemented therapy.

There have been many studies evaluating the effect of freezing of bacteriologic culture of milk samples (Schukken et al., 1989; Murdough et al. 1996; Sol et al., 2002; Godden et al., 2002; Villanueva et al. 1991). Schukken et al. (1989) determined that freezing decreased the number of positive *E.coli* samples while CNS increased in numbers. Also, in that study freezing had no effect on *S. aureus* or streptococci. The implications of these findings stress the importance of having fresh samples if a coliform mastitis is suspected. Murdough et al. (1996) had contradictory findings. They found that freezing had effect on viability of any pathogens (*S. aureus, S. hyicus, Str. dysgalactiae, Str. uberis, Corynebacterium bovis,* and *E. coli.*). When the effects of freezing on the isolation of *S. aureus* was evaluated, Sol et al (2002) demonstrated that using incubation in broth combined with freezing and incubation resulted in the highest *S. aureus* isolation percentage. As well, Godden et al. (2002) added to these results by determining that the maximal sensitivities for the detection of *S. aureus* were obtained when milk samples were fresh or frozen pre-milking samples and frozen post-milking samples. The results of the present study also support these findings. Freezing fresh mastitis samples increased the Se for *S. aureus* detection by 10.6%, while decreasing the Se for coliform detection by 14.7%.

The Petrifilm systems are not intended to replace a high quality laboratory or identify all organisms that may be present. Certain organisms such as *Mycoplasma* will not grow on these media. However, the Petrifilm's do allow for quicker diagnosis than standard bacteriology. As such, it has a definite advantage for use with OFCS where rapid decisions are required for implementation of therapy for clinical mastitis cases and segregation programs for *S. aureus.*

CONCLUSION

There has been an important shift away from always using intramammary antibiotic therapy at the first sign of clots in the milk. Rapid on-farm culture systems can be useful in

the implementation of therapy protocols and the overall herd milk quality control program. For ideal treatment, timely and accurate tests are needed to differentiate cases caused by gram-positive organisms, gram-negative organisms or those yielding no growth. For mastitis control programs, the ability to rapidly identify *S. aureus* and coliforms would be a valuable asset. The Petrifilm culture system (STX and RCC) have the ability to rapidly identify pathogens in milk. The highest test characteristics were obtained when milk samples were diluted (1:10) and fresh. However, freezing milk samples increased *S. aureus* detection, and decreased coliform detection. The results demonstrated that the Petriflm STX and RCC are comparable with standard bacteriologic culture for the isolation of *S. aureus* and coliforms.

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General Discussion

Descriptive data

A total of 2040 milk samples were submitted for analysis for the study. There were 536 clinical mastitis samples. Each clinical mastitis sample was plated on six Petrifilm plates; a non-diluted (1 mL) and diluted sample (0.1 mL milk and 0.9 mL Butterfield's Phosphate Buffer sterile solution) onto the STX, RCC, and AC plates. There were 223 fresh mastitis samples that were frozen, then thawed and replated. These again were plated on six Petrifilm plates, as described above. There were 1204 milk samples from fresh cows and 300 from cows with an elevated SCC during lactation. Each of these samples were plated only on the STX plates, as a non-diluted and diluted sample.

Bacteriology results from samples populations

All milk samples were submitted for bacteriological culture. The proportion of *S. aureus* of standard bacteriology of milk samples from calving and high SCC during lactation was 9.8% and 15.7%. For the 536 clinical mastitis samples the proportion of *S. aureus*, coliforms, and Streptococci spp. isolated from standard bacteriology was 14.6%, 22.8%, and 17.3% respectively. This is higher than previously reported in an Ontario study (Sargeant et al., 1998). Sargeant et al. reported the frequency of *S. aureus,* coliforms, and Streptococci spp. in clinical mastitis causes to be 6.7%, 17.1%, and 14.0%. Québec has been reported to have the highest incidence rate of clinical mastitis due to *S. aureus* in Canada (Olde Riekerink et al., 2008). The proportion of coliforms from mastitis samples from standard bacteriology in the current study was 22.8%. This is higher than the 15% reported by Olde Riekerink et al. (2008). This may be a result of 61% of the samples arriving fresh. All samples in the studies from Olde Riekerink et al. (2008) and Sargeant et al. (1998) arrived frozen. The implication of freezing milk samples is discussed below.

Choice of a Gold standard

Presently, standard bacteriology is considered the "gold standard" for bacteriologic culture ((Ruegg, 2005; Sears and McCarthy 2003). For bacteriologic tests, a diagnostic bias can occur when the gold standard used to detect the pathogen is not perfect (Schukken and Deluyker, 1995). It has been demonstrated that the sensitivity of a single bacteriologic culture to detect *S. aureus* can range from 58% to 72% in composite and quarter milk samples (Lam et al, 1996; Sears et al, 1990). Therefore, as the perfect gold standard does not exist, one can be created by including/combing existing tests (Dawson and Trapp, 1994). The Petrifilm STX was shown to have a higher Se than standard bacteriology (Silva et al., 2004). In this case, if the Petrifilm detects more *S. aureus* colonies, they are considered false positives versus standard bacteriology. Consequently, the test characteristics of the Petrifilm will appear inferior to standard bacteriology. In the current study, a gold standard was created to help reduce this bias. The gold standard was considered positive if *S. aureus* was isolated from either method, standard bacteriology or a positive Petrifilm (as confirmed by standard bacteriology). This gold standard is similar to that used by Silva et al. (2004) for the evaluation of the sensitivity of the Petrifilm STX. By comparing the Petrifilm to standard bacteriology, this allows for the correct evaluation of the agreement between the current (standard bacteriology) and new (Petrifilm) tests. Using a gold standard, allows for a more unbiased comparison of the two tests.

A diagnostic test is used to detect pathogens in milk should ideally have a high sensitivity (no false negatives). A high Se for the STX would allow the detection of the majority of samples with *S. aureus* which is important for segregation and culling programs. However, it is also important to limit the number of false positives (high specificity). It would be very costly to cull false positive cows. When the STX was compared to standard bacteriology the microbiological results of the false positives were evaluated. In diluted calving, high cell count, and mastitis milk samples, 46%, 60%, and 55%, respectively, of the false positives were due to *S. aureus*. For calving samples, the STX isolated eleven more *S. aureus* samples than indicated by standard bacteriology. This demonstrates that the STX may be more sensitive for *S. aureus* identification than standard bacteriology. Some of the false positives on the STX, as confirmed by reculturing positive colonies, resulted in no growth. One

explanation may be that these colonies were fragile and not viable upon replating. False negative reactions may also occur with the STX. This may be due to inexperience reading and interpreting growth on the plate, too many colonies on the plate or insufficient colony forming units.

Baysian analysis is an alternative method to analyse data when a perfect gold standard does not exist. This option was discussed for the current study. However, Baysian analysis is very cumbersome, requiring specialized statisticians to perform the analysis. As well, this method requires a large sample size and a known prevalence of the disease from two or more populations.

Agreement of the Petrifilm

In order to evaluate agreement between the tests, the Petrifilm tests were compared to standard bacteriology and not the gold standard. This reduces bias as the gold standard results include positive Petrifilm results. A kappa value does not indicate which test is superior, but a high kappa value means the two tests agree most of the time and can be used interchangeably. In the current study, the highest agreement between the Petrifilm STX and standard bacteriology was seen with diluted samples from cows with high SCC. The kappa value was good at 0.79. There was also good agreement between the Petrifilm RCC and standard bacteriology. The highest agreement was with diluted fresh samples, with a kappa of 0.742. For the AC plates, the agreement was very poor to negative. The kappa values ranged from -0.005 to 0.086. Therefore, it can be concluded the Petrifilm STX and RCC tests are good alternatives to standard bacteriology. While for the AC tests, there is no agreement between the tests.

Test characteristics of the Petrifilm

The Se of the STX for the detection of *S. aureus* in fresh milk samples from cows with elevated SCC was 83%. For milk samples from fresh cows and mastitis cases, the sensitivity of the STX was lower at 75%, and 68%, respectively. These sensitivities are

slightly lower than the 87.5% Se previously demonstrated by Silva et al. (2005). However, Silva et al. had a smaller sample population for each group of high SCC, fresh and mastitis samples numbering only 97, 163, and 102 milk samples, respectively. As well, 29 known *S. aureus* milk samples were added to the sample set to increase the prevalence of *S. aureus*. Even then, the prevalence of *S. aureus* in that study was only 5.4% for standard bacteriology and 8.2% for the gold standard. Consequently, to evaluate the predictive values of the STX in that study, a different sample set with a *S. aureus* prevalence of 19.5% was used. In the current study, the PPV for the STX were very good at 94%, 89%, and 95% for high SCC, fresh, and mastitis samples, respectively. The implications of a positive *S. aureus* test will depend on the herd udder health protocols and policies. With a high PPV you will be very sure that a positive test means that the cow is truly infected. The high PPV is important in herds that cull and or segregate positive animals.

A diagnosis of *S. aureus* was made when pink zones were observed around colonies after the Staph Express Disk was placed on the sample and incubated for one to three hours. Silva et al. (2005) determined that the test characteristics of the Petrifilm were highly dependant on the reader's ability to interpret the pink zone. In that study, the zone reaction was quantified as weak or distinct. The Sp and the PPV for *S. aureus* detection increased from 87.2% and 31%, to 96% and 57.1%, respectively, when distinct pink zones were seen. The current study made no attempt to classify the pink zones. At the beginning of our study, we had more difficulty differentiating between the weak pink zones and a truly positive colony. My experience with the weak zones around colonies is that they appeared more grayish than pink and were probably CNS and not *S. aureus* colonies. Also when milk samples on the STX plates were diluted and there was an average number of colonies on the plate, the larger colony size as well as a distinct colored zone was a good indicator of *S. aureus*.

For coliform determination in mastitis milk samples, the RCC was very effective. The highest Se (76.4%) and Sp (96.2%) was a result of using fresh diluted milk samples when compared to standard bacteriology. There was also good agreement between the RCC and standard bacteriology. When the RCC was compared to standard bacteriology, 97% of the false positives were due to coliforms. There was one false positive due to *S. aureus*. *Staphylococcus aureus* can grow on the RCC media but usually do not produce gas bubbles,

as they are not lactose fermenters. Therefore, one explanation of the aberrant *S. aureus* growth on the RCC plate may be that the *S. aureus* colony was beside a coliform colony and inadvently picked and regrown. When compared to the gold standard, the PPV was excellent, ranging from 98 to 100%. The predictive values of the RCC reflect how well this test will work in the field. The PPV indicates the likelihood that a milk sample with a positive RCC is infected with a coliform.

Reading and interpretation of the RCC plates was straight forward and simple. A colony was considered positive at 24 hours if there was a dark colony surrounded by air bubbles. The 12-hour color change for coliform determination did not prove to be as sensitive. For the frozen samples, the PPV was very low and there was poor agreement between the RCC color change and standard bacteriology. This may be explained by the nature of the RCC plate. The color change of the RCC is based upon pH. Mastitis milk samples tend to have a higher pH than normal milk (Dohoo and Meek, 1982). This may negatively affect the color change of the RCC plates. The benefits of the 12 hour color change indicator is the potential to have results available before the next milking in order to make appropriate therapy decisions for clinical mastitis. The drawback to the Petrifilm RCC plates is the cost. The RCC plates are approximately over double the cost ($2.60 versus $1.00) of the Petrifilm *E. coli*/Coliform count plates that have only the 24 hour results. Regardless of cost, the RCC may be very useful for veterinary practitioners as well as dairy producers, when faced with a case of acute clinical mastitis. Portable incubators and immediate plating of milk samples on farm will allow for the most rapid diagnosis possible. Preliminary results could be reported as soon as 8-12 hours so that appropriate therapy could be implemented.

The AC plates were not effective for Streptococci spp. differentiation in mastitis milk samples. In our study, positive STX and RCC plates were removed to eliminate *S. aureus* and coliform cases. This protocol was adapted from previous work by Silva et al. (2004) where mastitis milk samples were plated on the STX, coliform count, and the AC. Samples that were negative for *S. aureus*, negative for coliforms, but had growth on the AC, were assumed to be Streptococci spp. The problem with this assumption is that CNS are not accounted for. In the current study the proportion of Streptococci spp. and CNS isolated was 23% and 32%, respectively. The PPV for this test was very poor at 29% for diluted fresh

samples. However, the AC plates have shown potential in a different study evaluating OFCS (McCarron and Keefe, 2008). McCarron and Keefe used the AC plate in combination with EC count plates for clinical mastitis samples. The objective was to differentiate GP from GN growth. Clinical mastitis samples that were negative on the EC but positive on the AC were considered GP. The Se and NPV of the Petrifilm OFCS, to differentiate GP and GN growth, was 96% and 92%, respectively.

There are different methods for improving the Se or Sp of a test. One method is to use multiple tests simultaneously to an individual (Martin et al., 1987). The resulting Se and Sp will depend on how the results are interpreted. When high Se is required, the tests can be interpreted in parallel. With parallel interpretation, the animal is considered positive if it is positive on one or the either or both tests (McKenna and Dohoo, 2006). Parallel interpretation increases Se but tends to decrease Sp. This method of interpretation is useful if the goal of testing is to identify all *S. aureus* positive cows for a herd segregation program. The second method of interpretation is called series intreperetation. This method is used when high Sp is required (Martin et al., 1987). An animal is considered positive only if it is positive on all tests. Series interpretation would be useful for herds that culled positive *S. aureus* cows as a part of their contagious mastitis control program. Generally, the more tests used the greater the increase in Se or Sp depending on the method used (Martin et al., 1987). For many dairy farmers the use of consecutive milk samples is considered cost-prohibitive (Ruegg, 2003). However, failing to identify infected cows in a herd of contagious mastitis can lead to a continual reservoir of infection.

Inoculum volumes

The effect of various inoculum volumes on the test characteristics of the Petrifilm was evaluated. Inoculum volumes are thought to play an important role of the Se and Sp of a diagnostic test (Anderson et al., 1991; Buelow et al., 1999; Lam et al., 1996). A study evaluating different inoculum sizes showed that both the sensitivities and specificities for *S. aureus* were higher when a 0.1 mL inoculum was plated (Buelow et al., 1999). When a single composite sample and a milk inoculum of 0.1 mL was used the Se and Sp were 92% and 86%, respectively (Lam et al., 1996). Anderson et al. (1991) also evaluated inoculum volumes in the diagnosis of mastitis from clinical quarters. They found that there was no

difference in cultural outcomes between 0.10 vs 0.01 mL inoculum volumes. Their experiment also found no difference in cultural outcomes between 0.05 and 0.01 mL volumes. Their experiment was performed on frozen mastitis quarter samples.

In the current study, inoculum volumes were 1 mL and 0.1 mL of milk. The test characteristics of the Petrifilms varied depending on the dilution. For the STX plates there was a small difference in the proportion of false negatives between non-diluted and diluted samples. For STX mastitis, fresh cow, and high SCC samples the proportion of false negatives were 55:45, 48:52, and 56:44, respectively. However, for RCC mastitis samples the proportion of false negative is much higher for non-diluted versus diluted samples, 80:20. This may be due to the fact that too many colonies on the Petrifilm disk makes reading and interpretation more difficult. As well, when colonies are too numerous to count on the Petrifilm plates, and the growth media readily used making ideal growth of the organism difficult. The authors suggest that diluted samples be used when using the Petrifilm system especially for RCC. One could argue that it is more practical and economic to plate non-diluted samples. There would be no need for additional costs of dilution materials (pipettes and sterile solution), and a dilution could be made later if reading is difficult because too many colonies grown on the Petrifilm. That decision should be made in consideration of the objectives of the OFCS. Milk samples should always be kept in the freezer and sent to the regional bacteriology laboratory in case the Petrifilm results are inconclusive or if the cow was unresponsive to the implemented therapy.

Effects of freezing milk samples on Petrifilm test characteristics

In objective four of the study, the effects of freezing clinical mastitis milk samples on the test characteristics of Petrifilm plates (STX, and RCC), was evaluated. With the STX plates, there was an increase in *S. aureus* identification of 10.6% after freezing fresh non-diluted samples. In objective 4 of our study, the freeze-thaw cycle was controlled in the laboratory. Freezing is postulated to increase *S. aureus* recovery by rupturing milk macrophages and neutrophils, releasing engulfed bacteria (Villanueva et al, 1991). Another

possibility is that the timing of sampling (eg. premilking or postmilking) was unknown. Godden et al. (2002) demonstrated that *S. aureus* recovery was maximized with fresh or frozen premilking samples or frozen postmilking samples. This type of information was not known in the current study.

There have been many studies evaluating the effect of freezing of bacteriologic culture of milk samples (Schukken et al., 1989; Murdough et al. 1996; Sol et al., 2002; Godden et al., 2002). Some studies have demonstrated that freezing has no effect on *S. aureus* recovery (Schukken et al., 1989; Murdough et al., 1996). Others have had contradictory findings (Sol et al., 2002; Godden et al., 2002). When the effects of freezing on the isolation of *S. aureus* was evaluated, Sol et al. (2002) demonstrated that using incubation in broth combined with freezing and incubation resulted in the highest *S. aureus* isolation percentage.

Schukken et al. (1989) determined that freezing decreased the number of positive *E.coli* samples while coagulase negative staphylococci increased in numbers. Also, in that study freezing had no effect on *S. aureus* or streptococci. Results of the current study support these findings. The Se of the RCC for coliform identification decreased by 6.9% and 14.7% for non-diluted and diluted samples, respectively. Freezing affected the viability of the coliform bacteria. The implications of these findings stress the importance of having fresh samples if a coliform mastitis is suspected.

The ultimate objective of the study was to determine if the Petrifilm culture system was comparable to standard bacteriology. The agreement and the PPV values of the Petrifilm STX and RCC were very good. The Petrifilm systems are not intended to replace a high quality laboratory or identify all organisms that may be present. Certain organisms such as *Mycoplasma* will not grow on these media. However, the Petrifilms do allow for quicker diagnosis than standard bacteriology. As such, it has a definite advantage for use with OFCS where rapid decisions are required for implementation of therapy for clinical mastitis cases and segregations programs for *S. aureus*.

One of the mandates of the Canadian Bovine Mastitis Research Network is to reduce the prevalence of *S. aureus* in Canadian dairy herds (Barkema, 2008). Québec has the highest IRCM due to *S. aureus* and the highest prevalence of *S. aureus* at calving in Canada (Olde

Riekerink et al., 2008; Bouchard et al., 2005). The Petrifilm STX may be very useful in this situation as it allows for simple and rapid diagnosis of *S. aureus*. The Petrifilm plates also have a long shelf-life of 12 to 18 months as compared to other culture media such as Bi-plates and Triplates (approximately 6 weeks). In small (40-200 cow) Canadian dairy herds, the logistics of maintaining culture media current is an issue. Therefore, the Petriflm media may fill this need.

Evaluation of the Protocol

There are a few points in the current protocol that should be modified if this study was to be repeated.

There should be a longer and more intense training period for reading and interpretation of the Petrifilm plates. As Silvia et al. (2005) demonstrated, there was a difference in the specificity of the Petrifilm STX depending on the level of training of the people who read the plates. For our study, a 3M representative spent two days training the laboratory staff about the Petrifilm procedures and interpretation prior to the commencement of the trial. However, many questions arose after about the interpretation of positive colonies on the STX and RCC plates. The 3M handbook did not have enough pictures similar to the samples we were reading, such as the difference in the appearance of the STX plates for CNS and *S. aureus* colonies. I would recommend plating the STX plates with known CNS and *S. aureus* milk samples at different dilutions to familiarize the readers with the potential outcomes.

The interpretation criteria for the 12-hour color change for RCC plates should have been modified. In our study, any color change was counted as positive. However, some mastitis samples caused the entire plate to change color instead of a small area around a potential coliform colony. Therefore, the type of color change should have been noted such as small spots of color change or total plate color change.

I am very optimistic about the future of the Petrifilm culture system in the dairy industry. Petrifilm's represent a new technology that can aid in the detection of *S. aureus* and coliforms in dairy cows. As well, by incorporating the Petrifilm into mastitis control and

culturing programs, targeted mastitis therapy can be implemented. In Québec, because the herd size is relatively small, the Petrifilm culture system would be best suited for veterinary clinics. A clinic could have a trained technician to plate and read samples.

Questions that remain to be answered include how well the Petrifilms will work when interpreted by farm personnel; whether the RCC or EC plates are more beneficial on farm, and whether the Petrifilm OFCS can help improve overall milk quality. Future research should focus on the economic benefits of an OFCS using the Petrifilm's, the benefits of early targeted therapy on cure rates, milk production, and herd SCC, and the antibiotic use patterns when using the Petrifilm culture system.

General Conclusion

There has been an important shift away from always using IMM antibiotic therapy at the first sign of clots in the milk. For ideal treatment, timely and accurate tests are needed to differentiate cases caused by gram-positive organisms, gram-negative organisms or those yielding no growth. For mastitis control and treatment programs, the ability to rapidly identify *S. aureus* and coliforms would be a valuable asset.

The Petrifilm culture system (STX and RCC) have the ability to rapidly identify pathogens and has potential as an important on-farm diagnostic tool. The highest test characteristics were obtained when milk samples were diluted (1:10) and fresh. The results demonstrated that the Petrifilm STX and RCC are comparable with standard bacteriologic culture for the isolation of *S. aureus* and coliforms. Petrifilm™ bacteriological test system has potential to be an effective and rapid decision-making tool on farm or in the veterinary clinics, for a targeted approach to therapy of clinical and subclinical mastitis. The long shelf-life and simplicity of use compared to standard microbiological techniques make the Petrifilm system appealing for on farm culture programs.

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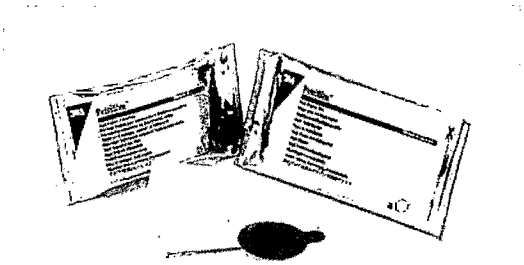
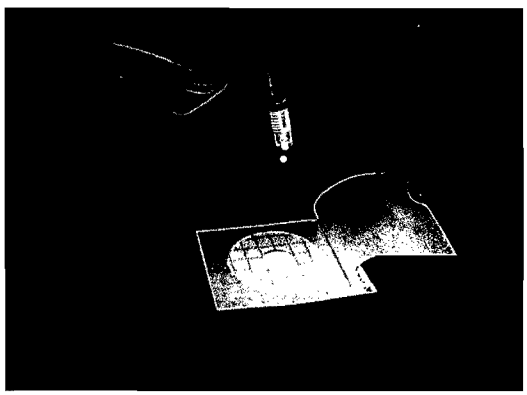
Annexe 1

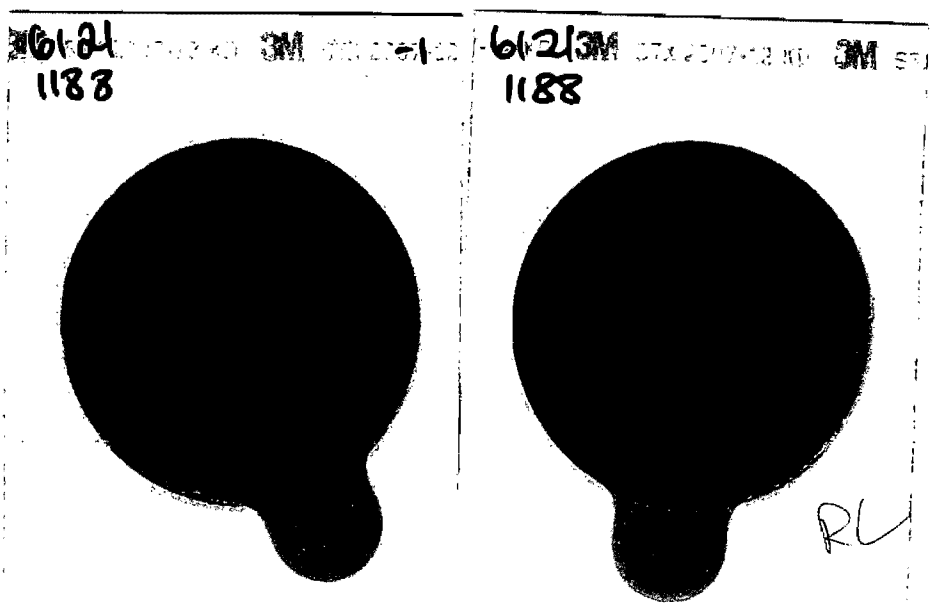
Pictures of the Petrilm plates:



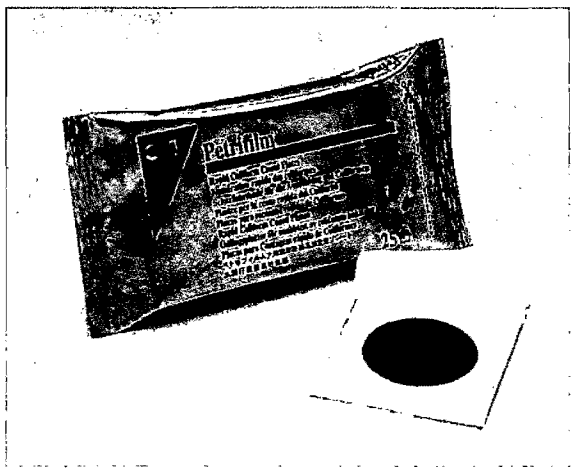
Petrifilm Staph Express plate (STX) and Staph Express disk.



Plating 1 mL of milk onto the STX plate.

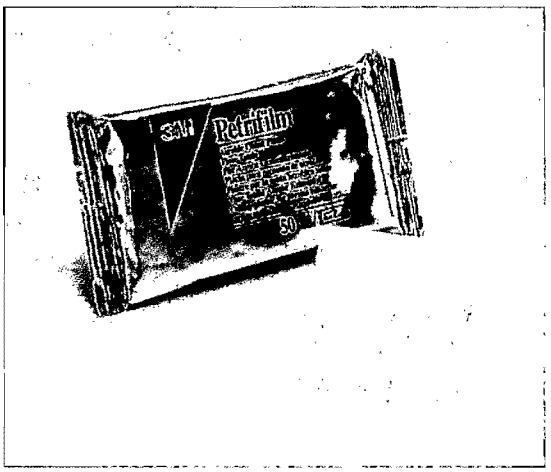


Petrifilm STX plates: diluted and non-diluted samples, both positive for *S. aureus.*

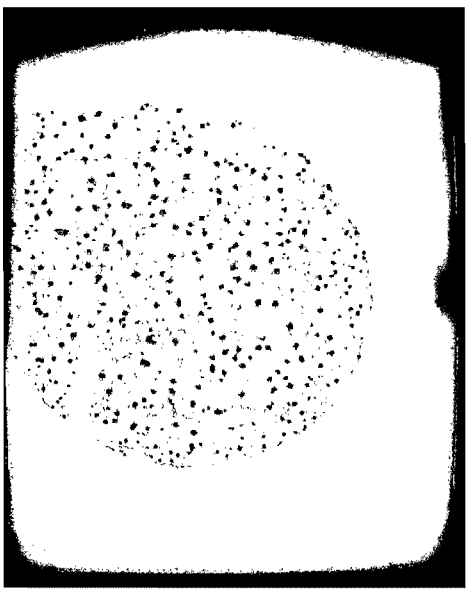


Petrifilm Rapid Coliform Count (RCC) plate.

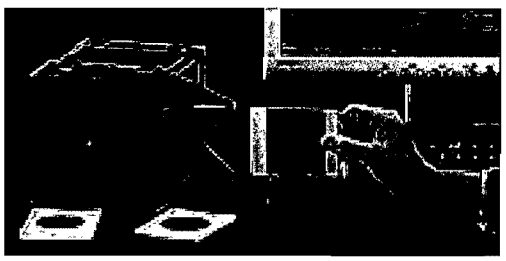
Petrifilm RCC plates: diluted and non-diluted samples, both positive for coliforms.



Petrifilm Aerobic count plate.



Petrifilm Aerobic count plate, positive for bacteria.



Petrifilm Plate Count Reader, for the numeration of bacteria on the Petrifilm Aerobic Count plate.