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Early lactation extended therapy against *Staphylococcus aureus* intramammary infections in heifers

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Mémoire présenté à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de maître ès sciences (M. Sc.)
en sciences vétérinaires option sciences cliniques

Avril 2017

RÉSUMÉ

Les infections intra-mammaires (IIM) chez les taures causées par *Staphylococcus aureus (S. aureus)* sont difficiles à traiter en suivant les protocoles habituels. Des observations récentes suggèrent qu'un traitement prolongé de pirlimycine serait le protocole le plus efficace pour traiter les infections à *S. aureus* en Amérique du Nord. L<utilisation de milieux de culture à la ferme, tels que les plaques Petrifilm^{TM} Staph Express Count (STX), peut aider à la détection précose des IIM. L'objectif principal de cette étude est d'évaluer le taux de guérison des IIM causées par *S. aureus* et identifiées grâce aux plaques STX, chez les taures en début de lactation, après un traitement prolongé de pirlimycine. Le objectif secondaire est d'évaluer les caractéristiques des Petrifilm quand celles-ci sont utilisées pour un protocole de détection précoce des IIM chez les taures en début de lactation. Les échantillons de lait ont été récoltés chez des taures (n=946) dans les premiers jours suivant la parturition (moyenne= 5 jours), et parmi celles-ci, les taures ayant une IIM causées par *S. aureus* (n=72) ont été divisées en deux groupes de façon aléatoire. Le groupe recevant le traitement (n= 55 quartiers de 39 taures) a reçu 50 mg de pirlimycine en infusion intra-mammaire dans les quartiers affectés pendant 8 jours consécutifs, tandis que le groupe contrôle (n=43 quartiers de 33 taures) n'a reçu aucun traitement. Un taux de guérison de 64% a été obtenu pour les quartiers mammaires dans le groupe traitement; ce taux est statistiquement supérieur à celui obtenu dans le groupe contrôle (33%). Les quartiers traités avec de la pirlimycine étaient 3,6 fois plus susceptibles d'être guéris que ceux du groupe contrôle. La proportion de faux positifs rencontrée en utilisant les Petrifilm était de 38% et le genre bactérien le plus souvent cultivé était du *staphylocoque*. Notre étude démontre qu'un traitement prolongé de pirlimycine mis en place peu de temps après la parturition permet d'atteindre un haut taux de guérison chez les taures laitières. L'utilisation des STX pour la détection des IIM causées pas *S. aureus* chez les taures peut résulter en des traitements inutiles vu le haut taux de faux positifs.

Mots-clés : *Staphylococcus aureus,* infections intra-mammaires, taures, Pétrifilm, pirlimycine, thérapie prolongée.

ABSTRACT

Intramammary infections (IMI) in heifers caused by *Staphylococcus aureus (S. aureus)* are challenging to treat using standard protocols. Recent evidence suggests that an extended treatment protocol with pirlimycin is the most effective way to treat *S. aureus* IMI in North America. Using on farm culture methods with the Petrifilm™ Staph Express Count (STX) plates can help with the early detection of IMI. The primary objective of this study is to evaluate the cure rate of an extended pirlimycin treatment on heifers in early lactation positive for *S. aureus* IMI identified using the STX plates. The secondary objective was to assess Petrifilm characteristics when used in a protocol for early lactation detection of infected quarters in heifers. Milk samples were collected from heifers (n=946) in the first few days of calving (mean= 5 days). Heifers with a laboratory-confirmed IMI caused by *S. aureus* (n=72) were randomly allocated in two groups. The treatment group (n=55 quarters from 39 heifers) received a sterile intramammary infusion of 50 mg of pirlimycin for 8 consecutive days in the infected quarters; the control group (n=43 quarters from 33 heifers) received no treatment. Mammary quarters treated showed a statistically significant cure rate of 64% compared to the control group (33%). Quarters treated were 3.6 times more likely to be cured then the control group. With the STX, a total of 38% of *S. aureus* positive quarters were identified as other staphylococci with standard culture. The study reveals that a high cure rate for *S. aureus* IMI can be achieved in dairy heifers if an extended treatment protocol is used soon after calving. Use of Petrifilm for identification of *S. aureus* infected heifers could lead to many unnecessary treatments because of false positive results.

Keywords : *Staphylococcus aureus*, intramammary infections, heifers, Petrifilm, pirlimycin, extended therapy.

TABLE OF CONTENTS

LIST OF TABLES

Tables present in the literature review

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

CFU :   Colony forming unit

CMT: California Mastitis Test

CNS :   Coagulase negative *Staphylococcus*

DHI :   Dairy herd improvement

DIM :   Days in milk

DNA :   Deoxyribonucleic acid

DNase :   Deoxyribonuclease

E. coli :   *Escherichia coli*

FAO   Food and Agriculture Organisation

FDA :   Food and Drug Administration

IIM :   Infections intra-mammaires

IMI :   Intramammary infection

IRCM :   Incidence rate of clinical mastitis

μL: Microlitre

mL :   Millilitre

MALDI-TOF MS   The matrix assisted laser desorption ionization-time of flight mass spectrometry

PCR :   Polymerase chain reaction

NMC :   National Mastitis Council

RCT :   Randomized control trial

S. aureus :   *Staphylococcus aureus*

SCC :   Somatic cell count

Se :   Sensitivity

Sp :   Specificity

STX :   Petrifilm^{TM} Staph Express Count plate

USD: United States Dollars

I dedicate this work to my grandfather Γιώργιος Βενιέρις. Χορίς εσένα, δεν θα είτανε δυνατόν. Ευχαριστώ για την υπομονή σου και τις διδασκαλίες σου. Σ'αγαπω.

ACKNOWLEDGEMENTS

INTRODUCTION

Staphylococcus aureus (**S. aureus**) is a contagious pathogen that causes persistent intramammary infections (**IMI**). Its prevalence in dairy heifers, in the pre- and post-partum periods, can range between 0 and 15% (Fox, 2009). This bacterium has many virulent factors that make it difficult to treat and contribute to its persistency. *Staphylococcus aureus* is impossible to eradicate because it is part of the cows natural skin flora.

Intramammary infection with *S. aureus* has detrimental economic effects on the producer and the health of the herd. The increased somatic cell count (**SCC**) associated with this pathogen decreases the quality of milk that the animal produces as well as the quantity of milk produced. Its contagious nature makes it very important to identify and treat infected individuals rapidly. It is equally important to implement strict hygiene protocols to avoid the transfer of *S. aureus* from one animal to the next. *S. aureus* IMI is a costly disease and it also decreases the life span of the animals because of early culling. Early identification is key to proper management of this microorganism. On-farm culture methods, such as the Petrifilm^{TM} Staph Express Count plate, are an inexpensive quick diagnostic tool that can be used by veterinarians and producers alike in order to make a rapid decision on antibiotic treatment for a given animal

With the recent scrutiny on antibiotic usage in agriculture, it is important to treat bacterial infections appropriately by implementing a protocol on infected quarters only, based on culture results, with a specific dose in order to keep a sufficiently high concentration of antibiotic in the mammary gland. There have been various studies on cows and heifers positive for *S. aureus* IMI in order to determine the best antibiotic treatment. To date, the best cure rate results were obtained when using an extended 8-day pirlimycin treatment on lactating dairy cows (Deluyker et al., 2005). However there has been no study to date

evaluating treating *S. aureus* infected heifer quarters that have been diagnosed within the first few days of calving.

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LITERATURE REVIEW

Mastitis

Intramammary infections

Intramammary infection (**IMI**) is defined as the presence of microorganisms in the mammary gland, often based on results from bacteriological milk culture (Berry and Meaney, 2006). Researchers have shown that heifers are quite susceptible to IMIs; in fact, > 50% of quarters can be infected before parturition (Fox et al., 1995). The quarter-prevalence of IMIs varies between 29 and 75% before parturition (De Vliegher et al., 2012; Fox et al., 1995; Trinidad et al., 1990b). The number of quarters harbouring an IMI decreases to 12-57% after calving (De Vliegher et al., 2012). Various pathogens can cause IMI and they are discussed in more detail below.

In order to diagnose a quarter as being infected, we most often rely on milk sample bacteriological culture, an imperfect method yielding various sensitivity (**Se**) and specificity (**Sp**) depending on the pathogen investigated and the IMI definition chosen. Unfortunately, there is no gold standard to identify infected and non-infected quarters. A consensus was recently reached in order to develop an IMI definition when interpreting routine milk bacteriological culture of three samples collected at weekly intervals (i.e. a pseudo-gold standard): 2 out of 3 consecutive samples positive for the same organism or a single sample harbouring at least 10 CFU/ 10 μL of milk (Dohoo et al., 2011b). Later on, Se and Sp of different IMI definitions were computed for single sample analyses (Dohoo et al., 2011a). A more detailed account of standard bacteriological culture is described in the diagnosis section.

Clinical and subclinical mastitis

Mastitis is defined as the inflammation of the mammary gland, which can result from any type of trauma to the udder, but more frequently from bacterial infection (Harmon, 1994). In most cases, mastitis is caused by bacteria and their toxins that invade and damage milk producing tissue (Jones and Bailey, 2009). The inflammatory response is the defense mechanism of the animal in which polymorphonuclear cells invade the site of infection and release inflammatory mediators in order to eliminate the infection (Jones and Bailey, 2009). Mastitis can be further categorized as being subclinical or clinical (Olde Riekerink et al., 2008).

Subclinical mastitis is defined as inflammation of the mammary gland without visible physical symptoms such as a modification of milk appearance, visible inflammation of the quarters, or systematic signs (Radostits et al., 2007). In addition, subclinical mastitis can cause reduced milk production (Djabri et al., 2002; Halasa et al., 2009). More often, an elevated somatic cell count (**SCC**) will be used as a screening test to diagnose subclinical mastitis. Somatic cells are a mixture of leukocytes (major percentage) and milk producing cells that are shed from the udder (a minor percentage) (Radostits et al., 2007). With inflammation, the leukocytes will increase in number in the milk. Since udder inflammation is most likely due to a subclinical infection, the SCC helps, veterinarians/ researchers determine if an infection is present within the udder. Therefore, this method of looking at SCC will help us determine indirectly if there is an infection present. The threshold value that is acceptable varies between regions. Confirmation of the presence of an IMI is done using routine milk culture. Somatic cell count can be determined semi-quantitatively by tests such as the California Mastitis Test (**CMT**), or quantitatively using laboratory-based or hand-held cell counter devices (Harmon, 1994; Radostits et al., 2007). These will be described further in the diagnosis section (section 4).

Conversely, clinical mastitis is defined as inflammation of the mammary gland with visible signs of inflammation like abnormal milk (e.g. flakes in the milk, watery milk), redness, pain, swelling of the udder, and systemic signs such as fever, anorexia and lethargy (Harmon, 1994). Clinical mastitis can be further divided in three categories, according to severity, which can be used to determine treatment regimen. Grade 1 is defined as abnormal milk only (e.g. flaking, curdled), grade 2 is the presence of abnormal milk and inflammation of the udder, and grade 3 is all of the above in addition to systemic signs (Roberson, 2003).

Pathogens That Cause Udder Infections

Environmental Pathogens

The 5-point National Mastitis Council (**NMC**) control plan is an udder health program that has been put into place in order to minimize the incidence of infection by contagious pathogens (Ruegg, 2012). This plan was then extended to the 10-point plan in order to encompass the control of environmental pathogens as well. The prevalence of environmental pathogens that affect heifer quarters can range between 4 and 10% prior to calving and 4-13% after calving (Fox, 2009). There are many environmental pathogens that cause mastitis such as coliform bacteria (*Escherichia coli* and *Klebsiella* spp.) and environment streptococci (*Streptococcus uberis* and *Streptococcus dysgalactiae*) to name a few. These bacteria are present in the cow's environment and most are opportunistic microorganisms, hence their limited capabilities of survival in the udder (Ruegg, 2012). However, one must not be misled by the term environmental pathogens since some strains of these microorganisms may behave like contagious IMI and spread from infected quarters to other quarters. Since these bacteria are mostly present in the environment of the cow, proper management and cleanliness of the cow's environment

can greatly reduce the incidence of IMI caused by environmental pathogens (Ruegg, 2012).

Environmental management is the best way to control the incidence of environmental pathogen-associated mastitis. The moisture and the type of bedding can influence the amount of bacteria present in the immediate cow's environment (Hogan et al., 1989; Zdanowicz et al., 2004). Even though approximately 40% of environmental IMIs can be spontaneously eliminated (Hogan and Smith, 1987), it is still important for the producer to have proper management practices such as providing adequate manure removal, decreasing cow density in the herd, ensuring proper ventilation, and having general farm cleanliness. These practices are all important aspects to follow in order to decrease the incidence of IMI caused by environmental pathogens.

Contagious Pathogens

The major reservoir for contagious pathogens is the udder of the infected cow. These pathogens are transmitted from cow to cow during the milking period (Harmon, 1996b). The method of transmission is usually by milking machines, farmers hands or contaminated towels or materials (Harmon, 1996b). There are many contagious pathogens that can infect the cow's udder. However, the ones that are the most detrimental to the udder's health are *Staphylococcus aureus (S. aureus), Streptococcus agalactiae,* and *Mycoplasma* spp. With proper management and mastitis control programs, *S. agalactiae* can be easily eradicated in most dairy herds (Radostits et al., 2007). Clinical mastitis cases caused by *M. bovis* do not respond well to therapy and usually culling is the best way to deal with this pathogen (Radostits et al., 2007). Nevertheless, mastitis cases caused by *M. bovis* are relatively rare in the Northeast USA and Canada (Radostits et al., 2007). Infections caused by *S. aureus,* however, are much more prevalent in Canada than *M. bovis* and can be difficult to treat.

6

Finally, because of the impossibility to precisely speciate coagulase negative staphylococci (**CNS**) using routine milk bacteriological culture, this large heterogeneous group of microorganisms is more often consider as a whole. Nevertheless, some CNS species probably act more like contagious pathogens and others like environmental pathogens (Vanderhaegehen et al., 2014). Due to their larger prevalence in heifers in early lactation, the following sections will focus mostly on *S. aureus* and CNS IMI.

Staphylococcus aureus

In many dairy herds, *S. aureus* can be the most prevalent bacterial cause of mastitis (Olde Riekerink et al., 2008; Waage et al., 1999). In most cases, it causes subclinical mastitis due to the bacteria's virulent factors enabling it to evade the immune system.

S. aureus has evolved in such a way that it causes persistent infections of the udder. Firstly, it needs to invade the teat canal in order for the bacteria to adhere to the mammary epithelia cells (Trinidad et al., 1990b). The teat canal is considered part of the primary defense system against IMI (Trinidad et al., 1990b). Keratin, which is found near the orifice of the teat canal, is known for its inhibitory effects on certain microorganisms. However, some pathogens, such as *S. aureus*, can survive and colonize keratin, and thus can damage the milk secreting epithelium (Nickerson, 2009; Trinidad et al., 1990b). In addition, such colonization can serve as a reservoir for contagious pathogens that cause mastitis (Nickerson, 2009). The risks of infection increase when there are lesions or damage to the teat skin and teat canal keratin (Dufour et al., 2012a). The ability of this bacteria to adhere to epithelial cells allows it to stay within the gland during milking instead of being washed out (Middleton, 2013). Moreover, the bacteria have developed many defense mechanisms in order to decrease neutrophil phagocytosis, which is the primary defense mechanism in the mammary gland (Middleton, 2013). Some other

virulent factors include hiding within phagocytic cells, the capacity to resist oxidative bursts, adhering to epithelial cells within the teat canal, and by avoiding the immune system's Toll-Like Receptors (Zecconi and Scali, 2013). Most of the virulent factors are shown in figure 1 taken from Liu's review of the pathogenesis of *S. aureus* (Liu, 2009). Not only does *S. aureus* evade the immune system with its capsules and proteins, but the concentration of opsonizing antibodies in the milk are low as well (Sutra and Poutrel, 1994). These factors all contribute to the chronicity seen with *S. aureus* IMI. These virulence mechanisms can lead to persistent IMI, which is why *S. aureus* can be very detrimental economically for the producer.
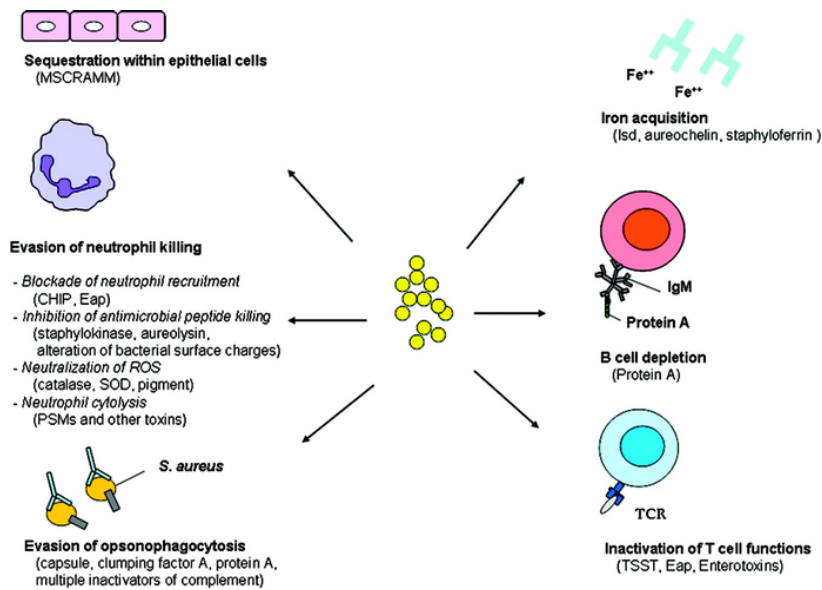
Figure 1. *Staphylococcus aureus* survival mechanisms during infection (Liu, 2009)

Unlike environmental pathogens, *S. aureus* main reservoir is the mammary gland, thus transmission occurs via milking units, producers' hands, towels, suckling calves, and any other sources that can potentially transmit the bacteria from one udder to the next (Harmon, 1996a). In addition, infected replacement heifers may also contribute to the introduction of the pathogen within a herd (Middleton, 2013). Nevertheless, the most common source is the milk of the lactating cows (Roberson et al., 1998). Moreover, various studies demonstrated that flies might contribute to the transmission of *S. aureus* from cow to cow by acting as vectors (Nickerson et al., 1995; Piepers et al., 2011). Herds

that have stringent fly control programs have lower risks of mastitis and IMIs caused by contagious pathogens like *S. aureus* (Nickerson et al., 1995).

S. aureus is a challenging pathogen to control (Barkema et al., 2006) and it is known to cause IMI that are difficult to cure (Barkema et al., 2006; Oliver and Mitchell, 1983; Roberson et al., 1994). Cure rates for IMI caused by *S. aureus* depend on various factors such as the SCC, the duration of infection, resistance to antimicrobials, the bacterial colony counts, and the number of quarters infected (Barkema et al., 2006). The different cure rates observed for different treatments will be discussed in a later section (Treatment of Heifer Mastitis). In addition, different strains of *S. aureus* may have different shedding patterns, which makes it harder to identify and, consequently, harder to control (Sears et al., 1990). Sears et al. (1990) stated that *S. aureus* with a low shedding cycle has a high risk of false negatives when a single sample is taken (Se of 74.5%). However, methods such as duplicate sampling, centrifugation and increasing the inoculum volume can increase the Se up to 94.2% (Zecconi, 2010). Regardless, shedding patterns should always be taken into consideration when identifying *S. aureus* to avoid false negative results and to allow us to have a better control over this pathogen.

Coagulase negative staphylococci

Many studies have shown that CNS are the most prevalent pathogens in dairy cows immediately prior to and at calving (Barkema et al., 1999; De Vliegher et al., 2012; Fox, 2009; Nickerson, 2009; Oliver et al., 1992; Oliver et al., 2003; Pankey et al., 1991; Pyörälä and Taponen, 2009; Sampimon et al., 2009). Some studies showed that even though CNS are frequently isolated in many mastitis cases, not many were found to cause severe clinical mastitis (Lam et al., 1997; Makovec and Ruegg, 2003; Piepers et al., 2010; Pyörälä and Taponen, 2009; Supre et al., 2011).

The prevalence of CNS-associated subclinical mastitis in heifers at parturition differs between studies; the percent of mammary quarters infected range between 5.2 and 39.0% (De Vliegher et al., 2012). The prevalence of CNS mastitis in the post-partum period in primiparous cows can be as high as 27.8% compared to 12.3% in the second week of lactation (Matthews et al., 1992). The prevalence of CNS in pre-calving heifers can reach up to 55% in some cases (Trinidad et al., 1990b). The most common species isolated in heifers is *Staphylococcus chromogenes* and *Staphylococcus hyicus* (Matthews et al., 1992; Trinidad et al., 1990b). The differences seen across studies may be due to the fact that there are many factors that will influence the prevalence of certain species of CNS in dairy heifers. For instance, factors such as management procedures, virulence of specific species or strains of CNS, and resistance of the pathogen against the immune system all play a role in the incidence of infection (Barkema et al., 2006). The fact that CNS IMI definitions often differed between studies can also explain an important part of this variation (Dufour et al., 2012a).

The SCC is often slightly elevated right after parturition compared to later on throughout lactation and it was initially thought to be physiological in heifers (Dohoo, 1993; Harmon, 1994). However, many studies show that a large percentage of heifer quarters have IMI at the time of parturition, which would explain the increased SCC in early lactation (Fox et al., 1995; Nickerson et al., 1995; Trinidad et al., 1990b). These studies show that elevated SCC above a certain threshold during early lactation is not physiological, but rather an indication of IMIs (Barkema et al., 1999; De Vliegher et al., 2004). The SCC of heifers often gradually decreases in the first two weeks after parturition (Dohoo, 1993). As mentioned, many studies concluded that CNS are commonly isolated in primiparous heifers during the peri-partum period with prevalence ranging from 20 to 55% (Fox et al., 1995; Oliver et al., 1992; Oliver and Mitchell, 1983; Pankey et al., 1991; Trinidad et al., 1990b) and this prevalence decreases after calving without treatment (Fox et al., 1995; Oliver et al., 1992; Oliver and Mitchell, 1983). These results suggest that CNS IMI have a high probability of being eliminated spontaneously after parturition, which would correlate with the gradual decrease of SCC seen in some heifers.

Heifer Mastitis

Prevalence and Incidence

The prevalence of mastitis is defined as the percentage of a given population that is affected with mastitis at a given point in time (Dohoo et al., 2009). The incidence rate of mastitis is the number of new mastitis cases over a period of time for a given population (Dohoo et al., 2009). The prevalence is used in order to evaluate the cases of subclinical mastitis that affect the herds on a given day (i.e. a snapshot). Measuring the incidence rate provides additional useful information and is an important, if not the most important, predictor for future herd prevalence (Dufour et al., 2012a). The incidence is an important tool for mastitis control programs; it is often reported as the number of cows (or quarters) infected per cow-years at risk.

Prevalence

The prevalence of IMI in heifers before parturition has been shown to be quite elevated ranging from 30% and reaching up to 97% in some cases. (Fox et al., 1995; Middleton et al., 2005; Oliver et al., 1992; Oliver and Mitchell, 1983; Trinidad et al., 1990b). In addition, prevalence of *S. aureus* infected quarters in the pre- and post-partum period can range from 1 to 15% in heifers in North America (Tables 1 & 2) (Andersen et al., 2010; Fox et al., 1995; Middleton et al., 2005; Myllys, 1995; Oliver et al., 1992, 1997; Owens et al., 2001; Pankey et al., 1991; Roberson et al., 1994; Trinidad et al., 1990b). The large variation seen between studies may be explained by the fact that many factors can influence the prevalence of IMI such as season, location, time of gestation, contact between suckling calves and heifers, fly-control programs, number of infected quarters, teat abrasions, and udder edema (Fox et al., 1995).

In a Louisiana study, four herds were observed in order to determine the prevalence of mastitis in unbred and gravid heifers (Trinidad et al., 1990b). The study was conducted on Jersey heifers (n=116) in which teat canal keratin (n = 461) and secretion samples (n= 370) were collected and analyzed. Trinidad et al. found that 93.1% of heifers and 70.7% of quarters had teat canal colonized by pathogens such as *S. aureus* (16.8% of quarters), *Staphylococcus chromogenes* (42.9% of quarters), *Staphylococcus hyicus* (25.2% of quarters), other staphylococci species (5.7% of quarters), as well as various streptococci species.

Trinidad et al. demonstrated that 96.9% of heifers and 75% of quarters had an IMI (Trinidad et al., 1990b) which is in concordance with other findings as well (Oliver et al., 2004). Trinidad et al. (1990) found that *S. aureus* was isolated in up to 37.1% of Jersey heifers and 14.9% of quarters. Twenty-nine percent (29%) of *S. aureus* infected heifers showed clinical signs of mastitis (Table I). This is in contrast with Oliver's study in which, *S. aureus* was responsible for only 8% of IMI in Jersey heifers (Oliver et al., 2004). Nevertheless, the prevalence of *S. aureus* IMI in Holstein heifers was found to be 30% by Oliver's group. In this study, the included cattle of both breeds (Jersey and Holstein) originated from different herds, therefore, the differences between breeds in pathogen causing IMI may have been due to differences in herds and management practices, and thus, the authors couldn't conclude that these variations were related to breed differences.

Some studies have shown that there is a difference in the prevalence of IMI between seasons. The prevalence of IMI has been shown to be the greatest during the summer months after parturition, but it doesn't seem to be the case for heifers of breeding age (Fox et al., 1995).

Table I. Prevalence of intramammary infections in dairy heifers caused by *Staphylococcus aureus* and coagulase negative staphylococci in the pre-partum period.

Study	Location	Number of Heifers	Number of quarters	Quarters infected by *S. aureus* (%)	Quarters infected by CNS (%)
Trinidad et al., 1990b	LA, USA	94	370	14.9	52.9
Oliver et al., 1992	TN, USA	115	460	1.7	52.8
Fox et al., 1995	USA	1,583	4,950	2.8	21.8
Oliver et al., 1997	TN, USA	82	314	3.2	55.1
Middleton et al., 2005	MO, USA	183	663	3.9	37.3
Roy et al., 2007	QC, Canada	428	2,140	10.3	59.3

Table II. Prevalence of intramammary infections in dairy heifers caused by
Staphylococcus aureus **and coagulase negative staphylococci after calving.**

Study	Location	Number of heifers	Number of quarters	Quarters infected by S. aureus	Quarters infected by CNS
Trinidad et al., 1990b	LA, USA	93	372	4.4	53.8
Pankey et al., 1991	VT, USA	382	1,533	0.7	11.4
Oliver et al., 1992	TN, USA	41	164	0.6	39.0
Fox et al., 1995	USA	1,583	4,950	2.8	21.8

Incidence

Dutch researchers have conducted a study in the Netherlands to estimate the incidence of subclinical mastitis in dairy heifers in early lactation using SCC (in the first 100 days in lactation) (Santman-Berends et al., 2012). After questioning 189 farmers, they estimated that the incidence of subclinical mastitis in dairy heifers was 25.5% in the first 100 days in milk (**DIM**) and correlated a higher incidence with various risk factors. They have shown that housing close-to-calving heifers with lactating cows could decrease the incidence of subclinical mastitis. The authors belie that outing the heifers with lactating cows before calving, allows them time to transition and is less stressful then if they are transferred after calving. Additionally, conventional milking systems rather than automatic milking systems also decrease the incidence of subclinical mastitis in dairy heifers (Santman-Berends et al., 2012).

There are no studies that focus on the incidence rate of clinical mastitis in heifers. There is a Canadian study that compares incidence rates of clinical mastitis in dairy cows. They mentioned that the incidence rate of clinical mastitis (**IRCM**) was higher in heifers than in cows in the first two weeks of lactation: approximately 118 IRCM vs. 100 IRCM per 100-cow years, respectively (Olde Riekerink et al., 2008). Another study conducted in the Netherlands also demonstrated that the incidence rate of clinical mastitis in the first 2 weeks of lactation was over 30% in heifers as compared to 13% in older animals (Barkema et al., 1998).

Impacts (Economic/ Productivity Losses)

The major problem associated with elevated SCC and mastitis is the milk production losses caused by either low quality milk or a decrease in milk production. In addition, there are economical losses due to the costs for the diagnosis and for the treatment of the infection. There is an exponential growth of the mammary parenchyma that occurs during heifers' first gestation, especially during the last trimester. Infections that take place during the growth of the mammary gland can have deleterious effects on future milk yield (Nickerson et al., 1995; Trinidad et al., 1990a). Milk yield in unbred and primiparous heifers was found to be 18% less in infected quarters compared to non-infected quarters (Nickerson et al., 1995). Experimental *S. aureus* IMI in heifers yielded a large amount of inter-alveolar connective tissue deposition instead of secretory tissue in epithelial and luminal areas (Trinidad et al., 1990a). This histological change may explain the low milk yield seen in heifers with IMI because the scar tissue formation potentially interferes with the development of milk producing cells.

A study conducted in Belgium on 117 496 heifers, demonstrated that heifers with SCC over 200 000 cells/ml after calving (i.e. between 5 to 14 days in milk) also had higher SCC throughout the first lactation (De Vliegher et al., 2004). The researchers did not look at the pathogens responsible for the increase in SCC, but their study emphasized that more than 27% of 14 766 dairy heifers had an elevated SCC in early lactation, which

suggests an IMI in the peri-partum period. Once again, this emphasizes the fact that heifers are at great risk of having IMIs early on in their productive life and that this can affect the SCC and their general health throughout their first lactation and possibly in subsequent lactations.

Increased SCC, which is associated with IMI, is coupled to decreased milk productivity especially during the first lactation (De Vliegher et al., 2005). In various studies it has been shown that infections by *S. aureus* yielded SCC of up to 9.2 x 10^6 cells/ml in heifers (Nickerson et al., 1995). In addition, heifers with an elevated SCC in early lactation have an SCC that remains elevated throughout the first lactation (De Vliegher et al., 2004). The increased SCC is due to the large amount of leukocyte infiltration seen with *S. aureus* IMI (Nickerson et al., 1995). Elevated SCC prior to and after calving will affect milk-producing tissue and subsequently affect future milk yield (De Vliegher et al., 2005). De Vliegher et al. (2005) looked at 14 243 Belgian dairy heifers and showed that heifers with 50 000 cell/mL produced 0.26 kg more milk per day than heifers with a first test day SCC between 51 000-200 000 cells/mL. They also deemed that the difference was on average 1.44 kg/day for heifers in the lowest SCC class (< 50 000 cells/mL) compared with heifer in the highest SCC class (> 1 000 000 cells/mL) (De Vliegher et al., 2005). These findings stress the importance of proper udder health management during the pre- and peri-partum periods in order to control IMI and subsequently to avoid drops in milk production.

Another study, however, did not show a significant difference in milk production in *S. aureus* or CNS infected quarters in the first month of lactation (Paradis et al., 2010). The authors of this later study believed that the lack of a significant difference might have been due to the small numbers of IMI observed and the regrouping of mild and severe infections into one category. Looking at the production differences in severe cases may have yielded different conclusions.

The losses associated with decreased milk yield and costs of treatment can be detrimental to a producer. An increased risk of early culling is also associated with IMI

(Myllys and Rautala, 1995). In the United States, the treatment of IMI in heifers has been shown to be economically beneficial if milk prices were above $0.029/kg USD (Oliver et al., 2003). In this study, heifers with an IMI were treated with an intramammary infusion of 200 mg cephapirin. The treatment costs were $15.60 USD per heifer and the milk production averaged 5 195 kg for untreated heifers and 5 726 kg for treated heifers. The authors took into account the costs of treatment and the revenue obtained by the increased milk production and concluded that prepartum antibiotic treatment was indeed economically beneficial by increasing the net revenue by $200.64 USD per heifer (Oliver et al., 2003).

Risk Factors

It is important to determine risk factors associated with heifer mastitis since this disease has been shown to cause major health issues and negatively impacts milk quality. Knowing the risk factors would be beneficial for producers and veterinarians in order to improve management and to prevent IMI in heifers.

There has been some research stating that horn flies may be a risk factor for the spread of contagious pathogens (Fox et al., 1995; Gillespie et al., 1999; Owens et al., 2002; Owens et al., 1998; Piepers et al., 2011). The examination of DNA profiling of *S. aureus* isolated in horn flies and *S. aureus* isolated from heifers revealed that there is a high possibility that the transmission of *S. aureus* can occur by horn flies (Gillespie et al., 1999). Another group found that pour-on insecticide reduced the prevalence of IMI (Owens et al., 2002) suggesting that controlling flies in the herd can reduce risk of IMI. It was also shown that exposure of non-infected heifers with horn flies colonized by *S. aureus* can result in IMI acquisition (Owens et al., 1998).

As mentioned previously, season and location may be factors that influence the prevalence of IMI. Fox et al. (1995) looked at numerous herds from different states and the largest prevalence after parturition was seen in Louisiana (58%). The same study also

demonstrated that the prevalence was increased during the winter months. The differences seen across studies suggest that management has an impact on IMI prevalence.

Udder edema is a factor to consider when dealing with clinical mastitis in heifers (Compton et al., 2007; Waage et al., 2001). Udder edema has been shown to increase the chances of heifer pre-partum clinical mastitis by 80% (Compton et al., 2007). It is thought that udder edema impairs the flushing effect and, therefore, causes an increased concentration of the pathogen in the udder; thus, leading to clinical mastitis (Compton et al., 2007). In addition, udder edema is thought to affect the local blood circulation impeding pathogens' clearance by the immune system (Waage et al., 2001).

Teat abrasions or scabs have been shown to have a correlation with increased IMI. The prevalence of IMI caused by *S. aureus* was shown to be almost twice as high (7% to 12%) in heifer quarters with scabs or abrasions compared to healthy quarters (Owens et al., 2001). Likewise, another group showed that 70% of heifers with teat abrasions had an IMI compared to only 40% of heifers without any scars or abrasions (Nickerson et al., 1995).

During the final trimester of gestation, prevalence of IMI in heifers by environmental pathogens seems to be at its highest compared to other stages of gestation (Fox et al., 1995)In the spring, *S. aureus* and CNS infected 5.6% and 20.6% of heifers, respectively, in their first trimester. This number increases to 8.5% and 37.7% in their last trimester of gestation. The fact that the prevalence is increased during the end stages of gestation may be explained by the physiology of the mammary gland. During the last trimester, the mammary gland is at a point where its growth rate is at its maximum, which would make the mammary gland more susceptible to bacterial infections (Trinidad et al., 1990a).

Milking procedures play a role in good managing practices as well. It is highly suggested that workers should wear gloves during the milking procedures, use automatic

milking unit take-offs, and apply a post-milking teat disinfectant (Dufour et al., 2011). In addition, appropriate triage should be implemented by milking clinical mastitis cases and animals with high SCC last.

Based on the different studies on risk factors, there are different levels in which we can intervene in order to reduce the prevalence and incidence of mastitis caused by a contagious pathogen. Therefore, implementing fly control programs, good cow and personnel hygiene, and constant udder examinations are some of the many ways in which a producer can minimize prevalence of mastitis within the herd.

Diagnosis Of Mastitis

Introduction to Different Diagnostic Techniques

Culture and proper identification of microorganisms causing heifer mastitis is imperative for a proper mastitis control plan. Identifying the infection and the bacteria that caused the infection can help veterinarians decide on the appropriate intervention for the heifer (Sears and McCarthy, 2003).

Indirect Methods

There are indirect methods for evaluating udder health. One of the methods mentioned previously is the SCC test that measures the concentration of inflammatory cells (neutrophils, macrophages, and lymphocytes) and desquamated epithelial cells present in the milk sample. This test can be done using an automated electronic counter, which will measure cell concentration (Radostits et al., 2007).

The California Mastitis Test (**CMT**) is an indirect test that can determine the SCC semi-quantitatively. The test consists of taking milk samples from quarters into a four-plate container and adding a reagent. The reagent contains a detergent that will react to DNA and also has a pH indicator. The CMT is a very fast, reliable and inexpensive test where the results can be read within 15 seconds. Scores from 0-3 can be given depending on the thickness of the milk and reagent solution (Radostits et al., 2007). A score of 0 or negative is given when the mixture remains homogenous and a score of 3 is given when the mixture is a gelatinous mass. The score of + or A is added if the reagent is alkaline (purple) or acidic (yellow: pH <5.2), respectively (Levesque, 2004). The CMT is a common test used cow-side in order to assess the individual cow's milk quality. The Se (66.7%) and the Sp (54.8%) of the test to identify an IMI in early lactation caused by a major pathogen are relatively modest (Sargeant et al., 2001). Still, this test is important for screening animals in order to determine whether they have an IMI or, following a SCC elevation, to identify which quarter is affected. Since it is only an indicator of inflammation, bacterial cultures are still necessary to identify pathogens involved. There are a few limitations to the test such as the fact that it does not provide an exact value of SCC and the interpretation is dependent on the experience of the person executing the test (CBMRN, 2015).

The measurement of electrical conductivity of the milk is an indirect method for the detection of subclinical or clinical mastitis. The test is based on the sodium and chlorine ion concentrations in the milk. With an infection, cells are damaged and this will release ions present within the cell into the interstitial space. The ions released will cause the concentration of those ions to increase in the milk (will end up in the milk ducts via diffusion) and consecutively, increase the electrical conductivity of the milk. The electrical conductivity test requires looking at every quarter and making a comparative analysis between quarters since there are no threshold values to compare to. This test measures the injury of the udder and not necessarily the immune response, as does the

CMT (Radostits et al., 2007). Hence this test would be better for the detection of clinical mastitis and less useful for the detection of IMI without substantial udder injury.

Out of the three indirect tests described above, the SCC is the most widely used tool for the detection of inflammatory cells. The method is precise and can be used to evaluate individual cows and, sometimes, individual quarters.

Direct Methods

Polymerase Chain Reaction

Polymerase-Chain Reaction (**PCR**) is a method of diagnosis that is not commonly used because of its costs, despite its very high analytical sensitivity to detect microorganisms present in a milk sample. In one study, pathogens were identified in 70% of milk samples from clinical mastitis using standard bacterial culture whereas pathogens were identified in 92% of milk samples from clinical mastitis cases using real-time PCR (Keane et al., 2013). However, PCR results can be quite difficult to interpret. The technique has a very high analytical sensitivity and can detect the presence of just one bacterium, and the presence of solely one bacterium does not necessarily mean there is an infection; the presence can be due to contamination from the teat skin, or the sampler's hands. Furthermore, PCR detects not only living bacteria but dead ones (i.e. DNA remnants) too, which can falsify the results. Lastly, PCR requires primers that will only detect one strain. Many primers can be added to a tube sample, however each additional primer is charged to the client. If this technique were to be used to detect many bacteria, the amount of primers required can become very costly.

Culture-dependent methods

Routine bacterial milk culture

Standard aerobic bacterial culture of bovine milk samples is commonly used in the diagnosis of mastitis. Bacterial culture is currently the gold standard for the identification of pathogens because blood agar is capable of supporting the growth of almost any microorganism (Sears and McCarthy, 2003). The inoculate volume recommended is 0.01 mL of milk, which is to be streaked onto a blood agar plate (NMC, 2004). The blood agar plate is then incubated at 37 °C with 5% CO_2 for 24 to 48 hours before examination.

Once colonies are observed on the plate, their color, form, and haemolytic capacities are evaluated. *S. aureus* colonies, for instance, are usually creamy, white, about 3-5 mm in diameter, and have a double zone of hemolysis (NMC, 2004). This distinct haemolytic zone is composed of a complete beta-hemolysis around the colony and a broader alpha-haemolytic zone surrounding the prior zone (NMC, 2004). However, very rarely, some *S. aureus* species may be non-haemolytic and further tests are required to identify them. These tests consist of a Gram-stain, catalase tests, and the coagulase test (NMC, 2004). Figure 2 shows a flow chart of the method to identify *S. aureus* from a milk sample at the Faculty of Veterinary Medicine of the Université de Montréal.
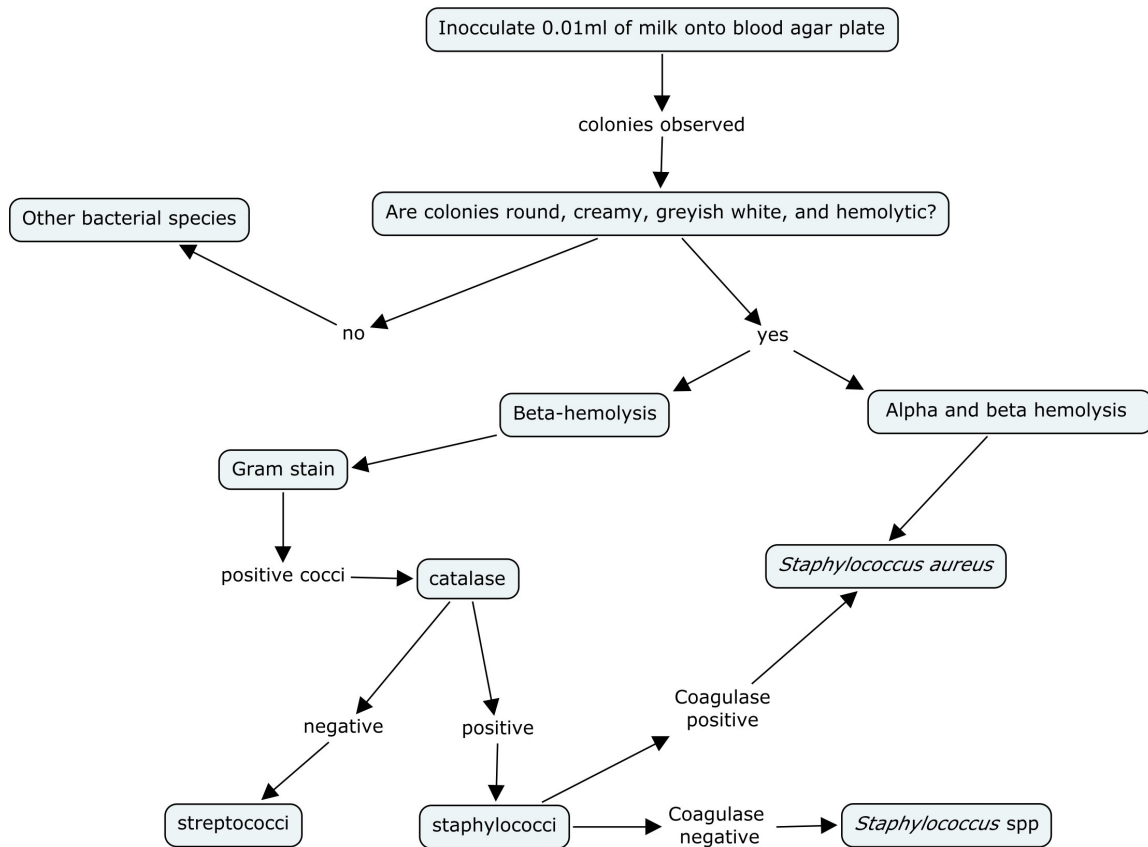
Figure 2. Routine bacteriological method for identifying *Staphylococcus aureus* from a milk sample at the Faculté de médecine vétérinaire of the Université de Montréal

In order to obtain higher Se for *S. aureus* detection, various techniques are used. One group suggests that milk samples should be from fresh or frozen pre-milking samples or frozen post milking samples (Godden et al., 2002). The reason for a higher Se when the milk is frozen may be due to the fact that freezing has the potential to lyse the phagocytic cells and release the pathogen for better detection (Villanueva et al., 1991). Godden's group show that there is no difference in Se when taking a frozen pre-milking sample compared to a frozen post-milking sample (Godden et al., 2002) even though some speculate that a post-milking sample will be less likely to be contaminated due to the teat canals being washed out during the milking process (Sears et al., 1991). In addition to this finding, another research group state that the best results for *S. aureus* detection were obtained when freezing the milk sample in an incubation broth (Sol et al.,

2002). Centrifugation of quarter milk samples has been shown to increase the number of *S. aureus* positive samples by 94% (Zecconi et al., 1997). Moreover, Silva's group (2005) found that they obtained the highest Se when they centrifuged the milk sample prior to incubation.

Despite all these findings, a more recent research group showed contradictory results (Artursson et al., 2010). This Swedish group investigated eight methods for the isolation of *S. aureus* in 204 quarter-samples throughout 41 dairy herds. These methods included: a standard method of incubation on blood agar, an enrichment method where the milk was added to an equal volume of nutrient broth, a larger volume of 0.1mL instead of the standard 0.01mL, a sedimentation method where the sample was centrifuged, a freezing method, a freezing combined with an incubation method where the thawed milk samples where incubated for about a day before culturing, and an incubation method where the fresh milk sample was incubated for about a day before culturing it. The only methods that seemed be better at identifying the microorganism was the freezing/incubation method and the fresh milk incubation method. The authors state that the only reason that the freezing with incubation resulted in a higher Se is mainly because of the incubation technique. This technique allows *S. aureus* to multiply to greater numbers in the milk before culturing on a plate and is thought to be an important method to detect infections when there is a low *S. aureus* concentration (Artursson et al., 2010).

There is always the question on whether one milk sample is sufficient in order to diagnose the quarter as infected or if multiple milk samples are required. The United States FDA guidelines suggest duplicate milk samples should be used in series to correctly identify an IMI, though it has been suggested that a single sample is enough to identify most contagious pathogen (Dohoo et al., 2011a; Erskine and Eberhart, 1988). In Dohoo et al. study (2011a), they suggested that a triplicate sample had the best combination of Se and Sp, however it was not significantly different to the Se and Sp of a single sample. Erskine and collaborators showed that when comparing single and

24

multiple milk samples, there was a very high agreement between the samples for contagious pathogens like *S. aureus* (94.2%) compared to the agreement seen with environmental pathogens such as coliforms (55.6%).

When analyzing the milk culture results, one has to take into account the Se and Sp of the standard culture. If we were to use multiple samples, analyzing the results in series (both samples need to be positive for a pathogen to deem a quarter as infected), would lower the Se but increase the Sp (Dohoo et al., 2011a). The opposite holds true if we were to interpret multiple samples in parallel (only one sample needs to be positive for the quarter to be deemed infected). Sensitivity would be increased and Sp decreased.

When defining IMI based on culture results, it is important to understand that the Se can vary depending on whether composite or quarter milk samples are being used. Composite milk samples have relatively low Se, which may be due to the fact that milk from the infected quarter is diluted with milk from the other healthy quarters (Lam et al., 1996; Reyher and Dohoo, 2011). However, when using single quarter milk samples for confirming experimentally induced *S. aureus* infections, Se of 75% can be achieved. Sensitivity would increase with parallel interpretation of two (Se=94%) or three (Se=98%) consecutive cultures (Sears et al., 1990). The same researchers showed that the Se could approach 100% with three samples interpreted in parallel when investigating naturally occurring *S. aureus* infections in cows with high shedding cycles.

There are some disadvantages to using routine bacterial culture to identify a microorganism. An important one is the time required to get results (Sargeant et al., 2001). Pathogens are rarely speciated based on colony appearance alone; other tests are usually conducted, which can delay the process of obtaining a definitive result and for treating the infected animal. Moreover, there are chances of contamination due to either non-proper collection of the milk sample or even human laboratory error. Once again, this would delay the time for the results to come in. There is also the fact that many clinical mastitis cases yield no growth in culture media. Reasons for no growth can be

due to infections by slow-growing bacteria or by bacteria that cannot grow on regular medium. For instance, *Mycoplasma* is a microorganism that does not grow easily on blood agar and requires other specific culture media (Sears and McCarthy, 2003). However, since infection by the *Mycoplasma* pathogens are rare, there are other more plausible reasons for the no growth. Sometimes it can be due to the fact that there are very few pathogens present in the milk and techniques with higher sensitivities should be used such as PCR (Botaro et al., 2013; Keane et al., 2013; Taponen et al., 2009). There is also the possibility of cyclical shedding patterns which can result in no growth if a sample was taken during the low shedding period (Sears et al., 1990). Lastly, there is the possibility that the pathogen was spontaneously eliminated by the host or even if an antibacterial treatment was already put in place prior to milk collection (Sears and McCarthy, 2003).

Bacterial culture and microbiological techniques are the most common and reliable way to identify mastitis causing microorganisms. Data analysis and the sampling method used are dependent on the objective pursued. As with any technique, there are pros and cons. Despite the lag times associated with bacterial culture, the efficiency, accuracy, and relatively low price associated with these techniques make it a favourable choice for identifying the pathogen causing an IMI.

The MALDI-TOF MS

The matrix assisted laser desorption ionization-time of flight mass spectrometry (**MALDI-TOF MS**) is a new tool for microbial identification and diagnosis. The principals are based on the conversion of peptides into ions by addition or loss of one or more protons without affecting the integrity of the sample (Singhal et al., 2015). Once the sample is charged, the protons are accelerated to a fixed potential and they are separated from each other on the basis of their mass-to-charge ratio (Singhal et al., 2015). With the MALDI-TOF-MS, this ratio is measured by determining the amount of time it is required to travel the flight tube (Singhal et al., 2015). This will eventually give a peptide mass

fingerprint, which is used to identify microbes. The proteins used are mostly the ribosomal complexes within the microorganisms, which are species specific (Barreiro et al., 2010). This system allows for the identification of microorganisms from its genus to its species and, potentially, its strain (Singhal et al., 2015). This new diagnostic technique is faster at identifying infectious organisms then the conventional diagnostic techniques (Barreiro et al., 2010). The results obtained by the MALDI-TOF MS can be as quick as 24 hours which is a great improvement from the average of 5 days with standard bacterial culture (Barreiro et al., 2010). With quick results, a proper antibacterial protocol can be put in place to treat animals that have bacterial infections quicker.

On farm culture methods

The 3M Petrifilm^{TM} is a ready made dehydrated culture medium used to quickly identify specific microorganisms (3M, 2010). Different plates can be used to identify different microorganisms: the Aerobic Count plate is used to detect aerobic bacteria, the Petrifilm *E. coli*/Coliform Count plate is used for the detection of coliforms, the Rapid Coliform Count plate is for the quick detection of coliform bacteria (i.e. 4-24 hours), and finally the Petrifilm Staph Express Count (**STX**) plate is a medium selective for staphylococci.

The STX plate is made of a culture medium that contains a chromogenic modified Baird-Parker medium (3M, 2010). The inoculum volume required is 1 mL of sample, which is incubated for 22-24 hours at 37 °C. The presence of *S. aureus* in the milk sample is indicated by the presence of red-violet colonies on the STX (3M, 2010). The company suggests that observing red-violet colonies may be sufficient to diagnose the sample as positive for *S. aureus*. If there are no red-violet colonies, a Petrifilm Staph Express disk is available to detect DNase reactions specific to *S. aureus*. The disk has toluidine blue-O stain to facilitate the detection of the reaction. Once the disk is placed on

the STX plate, it is incubated for 1-2 hours at 37°C. If *S. aureus* is present, a pink halo (i.e. a zone of DNase reaction) is observed surrounding the colony.

The inoculum volume necessary for the Petrifilm plate is 1 mL. This volume is 100 times greater than the volume used in standard bacterial culturing (0.01 mL). It is expected that the increase in volume for the Petrifilm plates increase the Se of this technique as compared to the standard routine culture. Research has demonstrated that this larger volume actually impedes the proper reading of the plate in some cases (i.e. too many CFU), making it difficult to correctly interpret the results (Wallace et al., 2011). This later group compared Petrifilm results to standard bacterial culture results and also compared non-diluted and diluted (1:10) milk samples. Wallace et al. (2011) demonstrated that for postpartum milk samples, the highest Se for the STX plate was observed with diluted milk samples (93.7%). The greater Se for diluted samples holds true for elevated SCC samples and clinical mastitis samples as well. The dilution allows for better reading and interpreting the results. Too many colonies on one plate can hinder the reader's capabilities from correctly identifying *S. aureus* colonies. However the 1:10 dilution is still 10 times higher than the standard bacterial culture volume required (0.01 mL) and this still potentially leads to a greater Se than the standard test.

A study evaluating the STX plate for the isolation of *S. aureus* from milk was conducted in 2005 (Silva et al., 2005). The group conducted many experiments to assess the STX plate. In the first experiment, they compared results obtained from the Petrifilm to standard microbiological procedures (gold standard). When comparing the Petrifilm to other microbiological techniques, Silva et al. (2005) found that the Se for detecting *S. aureus* was much higher (87.5%) compared to the standard techniques (65.0%). The second experiment's objective was to compare results from composite and quarter milk samples on the Petrifilm. No significant difference was observed. They also investigated the inter-reader agreement of the STX. They demonstrated that the probability of obtaining positive *S. aureus* results increased when there was a distinct pink zone surrounding the colony (Se increased to 98.5%). In addition, Silva's group determined

that the reader influenced the STX interpretation since the reader's assessment varied in number of colonies, size, and recognition of the pink zone (Silva et al., 2005). Conversely to manufacturer's recommendations, the group suggested that the DNase disk is absolutely necessary for the confirmation of the presence of *S. aureus*. They showed that out of 35 plates that were considered to have red-violet colonies prior to the DNase disk, only eight were confirmed to be *S. aureus*.

The main advantage for using the STX plate is that the results can be obtained within 22-24 hours (Silva et al., 2005). This is very favourable because, the faster the results are obtained, the faster a proper intervention or control program can be established. The rapidity of the results is comparable to the MALDI-TOF MS. The main advantage the STX would have over the MALDI-TOF MS would be the fact that no expensive equipment is required for the STX. The increased inoculum volume also increases the Se for *S. aureus* detection. The increased Se may help diagnose infections when the bacterial concentration is relatively low (Lam et al., 1996). Proper training and experience are, however, essential for dealing with the plates since the reader's interpretation can affect the Se of the STX plate.

Treatment Of Heifer Mastitis

Antibiotic Therapies

Infection of the mammary gland with *S. aureus* can cause damage to the milk producing tissue, which can have long term detrimental effects on milk productivity if not treated right away (Owens et al., 2001). Previous studies showed that delaying treatment of dairy heifers infected with a major pathogen often result in persistent infections, since the rate of spontaneous cure for these is quite low (Owens et al., 1991, 1994; Trinidad et

al., 1990b). Immediate treatment of IMI caused by major pathogens in heifers during early lactation is a better option then delaying treatment to drying-off in order to avoid losses in milk production, in addition to preventing chronic carriers in a herd..

Various antimicrobial therapies can be used against mastitis causing pathogens. The preferred route of administration is usually intramammary infusion. Parenteral and subcutaneous injections of antibiotics often result in relatively lower antimicrobials concentration in the mammary glands compared to intramammary infusion (Nickerson, 2009). This is mostly explained by antimicrobials failure to transfer from blood to milk (Nickerson, 2009). Quite a few approved commercial antimicrobial products formulated specifically for intramammary infusion in lactating and dry cows are available to Canadian veterinarians and have beneficial effects on cure rates at least for some pathogens.

Intramammary infections caused by *S. aureus* are relatively difficult to eliminate compared to other microorganisms. Many studies were conducted to investigate use of various antimicrobials (Owens et al., 2001; Roy and Keefe, 2012; Watts and Salmon, 1997) and vaccines (Nickerson et al., 1999; Smith et al., 2006) for treatment or prevention of *S. aureus* IMI, respectively. In the following paragraph, these studies will be looked at in greater detail.

Use of antimicrobials in animal production has become a concern for public health because of the emergence of resistant strains. The use of antimicrobial agents creates a selective pressure for microorganisms to acquire multi-resistant genes (Tenover, 2006). Mutations in genes and the exchange of antimicrobial resistant genes from one bacterium to the next are mechanisms by which resistance to antimicrobial agents can be acquired. Using the appropriate drug at the proper dosage and for the right duration is imperative in order to reduce the risk of antimicrobial resistant strains to emerge (Tenover, 2006). It is therefore very important to base treatment protocol on

microorganism identification when dealing with an infected animal. Important questions to contemplate are: is the condition worth being treated (considering imperatives such as economics, animal health, and animal welfare), which antibiotics should be used, what dose is required, and what is the appropriate treatment duration (Tenover, 2006). For instance, heifers with an elevated SCC or with clinical mastitis are more likely to be culled prematurely (De Vliegher et al., 2012; Myllys and Rautala, 1995). These heifers are more likely to have chronic established IMI that are not very prone to respond to treatment. The infected heifers can consequently act as reservoir and infect their herd mates which can increase the prevalence of the disease within the herd (De Vliegher et al., 2012). Furthermore, many of these infected animals will later on experience flare-up of clinical mastitis, which will, in most instances, require antimicrobial treatment (De Vliegher et al., 2012). Therefore, it is vital to treat infected animals quickly following onset of the IMI, as few times as possible, and with a high enough dose to avoid the occurrence of resistant strains.

Pre-partum heifer treatment

Cure rates

Many researchers observed that pre-partum antimicrobial treatment of infected quarters significantly reduce subsequent SCC and IMI prevalence in heifers (Borm et al., 2006; Middleton et al., 2005; Oliver et al., 2003; Owens et al., 2001; Owens et al., 1994; Roy et al., 2007; Sampimon et al., 2009; Trinidad et al., 1990b). Pre-partum antimicrobial treatment is a valued option because of the reduced amount of discarded milk. However, there is the risk of introducing microorganisms into the udder and causing new IMI when treating animals in this period (Roy et al. 2007).

Various antibiotics were tested at different times (0 to 90 days, 90 to 180 days or 180 to 270 days pre-partum) in order to determine the best treatment plan for *S. aureus*

IMI in heifers (Owens et al., 2001). Owens et al. (2001) observed that cure rates were similar between treatment groups regardless of the time of administration and the antibiotics used. They observed, however, that heifers treated during the last trimester of gestation did not acquire new *S. aureus* IMI. As discussed previously, the last trimester of gestation is the time where heifers are most susceptible to becoming infected with *S. auerus* (Trinidad et al., 1990a). These results suggest that the last trimester is possibly the best time, during the pre-partum period, to treat *S. aureus* IMI with intramammary antimicrobials.

Studies using a dry cow treatment with 300 mg cephapirin benzathine, a first generation cephalosporin that targets the synthesis of the bacterial cell wall, administered to heifers 10 to 12 weeks prior to calving, demonstrated that 100% of experimentally induced IMI (13 Jersey heifers) and 96% of natural IMIs (24/25 quarters) with *S. aureus* were cured at calving (Owens et al., 1991, 1994). Even though cure rates were elevated, antimicrobial residues could be detected for up to 5 weeks in mammary secretions of non-gravid Jersey heifers treated with cephapirin benzathine (Owens et al., 1991). Moreover, the same study on experimentally induced *S. aureus* IMI showed that a very little proportion (1/9) of infected quarters had cured spontaneously at calving (Owens et al., 1991). The results observed in this study emphasize the need to treat infected animals in order to prevent economic losses for the producer since self-cure is uncommon.

A study showed that treating heifers with 200 mg of cephapirin sodium 2-3 weeks prior to calving reduced the prevalence of IMI at calving (Borm et al., 2006). They tested 561 North American heifers during the pre-partum period and observed that 24.5% of prepartum infections were caused by coagulase positive staphylococci (hypothesized to be mainly *S. aureus*), coliforms and streptococci. They demonstrated that treatment 2-3 weeks prior to calving of infected mammary quarters resulted in cure rate of 79.9% compared to 31.7% in the control group. In addition, the group observed that the cure rate for coagulase positive pathogens were significantly greater with antibiotic treatment (72%) then without (25%). A related study in Tennessee treating Jersey heifers with 200 mg cephapirin sodium one week prior to calving obtained similar results with only 2.1%

of quarters being infected in early lactation after treatment compared to 44.5% in the control group (Oliver et al., 1992). The same group also compared treatment with 200 mg of sodium cloxacillin, a β-lactamase- resistant penicillin, which was not as affective as the prior antibiotic (8.6% of quarters remained infected) but nevertheless increased cure rates compared to the control group (44.5% of quarters remained infected). Despite the fact that this herd demonstrated a relatively low quarter prevalence of IMI caused by *S. aureus* (1.7%), they observed that there was a decrease in the number of infected quarters when treated with antibiotics.

A study conducted in four herds in Louisiana, evaluated the effectiveness of treating 35 heifers with an intramammary infusion of penicillin dihydrostreptomycin approximately 60 days prior to calving (Trinidad et al., 1990b). The proportions of infected heifers (97%) and quarters (73%) were reduced to 40% and 34%, respectively, after treatment. Trinidad et al (1990) isolated 11 *S. aureus* infected quarters before treatment and observed only one infected quarter after calving compared to 11 quarters (6 heifers) that were still infected out of the original 18 quarters (10 heifers) in the control group.

Pirlimycin hydrochloride, a lincosamide that targets the bacterial ribosomal 50s subunit, is effective at curing IMI in heifers. In a study conducted by Oliver et al. (2004) on two different dairy herds, quarters were treated 14 days prior to expected parturition with either penicillin-novobiocin, pirlimycin hydrochloride, or were left untreated (control group). Cure rate was significantly higher when treating with an antibacterial compared to the control group (Oliver et al., 2004). Eighty seven percent and 59% of infected quarters were cured following pirlimycin treatment in Jersey and Holstein heifers, respectively. Following the penicillin-novobiocin treatment, 75 and 76% of infected quarters were cured in Jersey and Holstein heifers, respectively. In the untreated control group, 57% and 26% of infected quarters were cured after parturition in Jersey and Holstein, respectively. Coagulase negative staphylococci IMI prevalence is known to decrease significantly after calving (Oliver and Mitchell, 1983) and since this pathogen was very prevalent in the Jersey herd included in that study, we can assume that the high

33

cure rate observed in the untreated control group may be due to spontaneous CNS IMI cure.

In another study, Holstein–Friesian heifers with natural occurring IMI were given one dose of pirlimycin 10 to 14 days prior to calving (Middleton et al., 2005). In this later study, pre-partum treatment with pirlimycin hydrochloride did significantly reduce the prevalence of early lactation IMI. Among the 220 studied quarters in one herd (herd A), 58.6% had a prepartum IMI. Among the 443 quarters observed in the other herd studied (herd B), 42.6% had a prepartum IMI. The percentage of quarters cured in herd A was 67% compared to 35% in the untreated control group. In herd B, the percentage of quarters cured was 89% in the treatment group compared to 74% in the control group. Herd B had 88% of its infected quarters in the treatment group infected with CNS and 52% of the quarters in the control group as well. As discussed previously, CNS have a very high cure rate whether it be with treatment or with spontaneous cure, which would explain the high numbers seen in this study. There were no *S. aureus* infected quarters in the treatment group of herd B. However, in Herd A, there was a significant difference seen between the treatment group in *S. aureus* infected quarters (78%) vs. the control group (8%).

Alternatively, treatment of nulliparous heifers with pirlimycin hydrochloride no more than 7 days prior to expected calving date had a significant effect in decreasing IMI by gram-positive bacteria and preventing new IMI (Roy et al., 2007). When comparing heifer IMI prevalence reduction prior to and after parturition, a decrease of 55.7% in heifers compared to 34% in the untreated control group was observed. Roy et al. (2007) also demonstrated a positive association between treatment and environmental bacteria (gram-negative and yeast) IMI incidence; thus, emphasizing the importance of aseptic techniques when treating heifers with intramammary antibiotics.

In summary (Table III), use of various antibiotics at different times prior to parturition has been investigated to control IMI prevalence in heifers during the early lactating period.

Table III. Cure rates following pre-partum antibiotic treatment on heifers with intramammary infections caused by different pathogens

Study	Location	Number of Heifers & Breed	Number of quarters	Antimicrobial treatment	Time of treatment (in days before calving)	Cure rate (%)		Quarters infected after treatment (%)	
						Treated	Control	SA[6]	CNS[7]
Owens et al. 1991	LA, USA	15 Jersey	21	Cephapirin[1]	84	100.0	0.11	0.0[8]	N/A
			9	Control					
Oliver et al. 1992	TN, USA	115 Jersey	152	Cloxacillin[2]	7	91.4	55.5	0.6	39.0
			144	Cephapirin[3]	7	97.9	55.5	1.3	6.6
Oliver et al. 2004	TN, USA	55 Holstein	25	Novobiocin + penicillin[4]	14	76.0	26.0	N/A	N/A
			29	Pirlimycin[5]	14	59.0	26.0	N/A	N/A
			19	Control					
		70 Jersey	72	Novobiocin + penicillin[4]	14	75.0	56.0	N/A	N/A
			70	Pirlimycin[5]	14	87.0	56.0	N/A	N/A

			57	Control					
Middleton et al. 2005	MO, USA	63 Holstein	108	Pirlimycin[5]	14	67.0	35.0	22.0	40.0
	IA, USA	120 Holstein	242	Pirlimycin[5]	14	89.0	74.0	N/A	12.0
Borm et al. 2006	North America	561	374	Cephapirin[3]	10-21	79.9	31.7	N/A	42.5
Roy et al. 2007	QC, Canada	428	876	Pirlimycin[5]	7	55.7	33.9	1.9[9]	6.3[10]

1. 300mg Cephapirin Benzathine

2. 200mg Sodium cloxacillin

3. 200mg cephapirin sodium

4. 150mg novobiocin sodium + 100,000 IU penicillin G

5. 50mg pirlimycine hydrochloride

6. Presence of *Staphylococcus aureus*

7. Presence of Coagulase negative staphylococci

8. Infused experimentally with 10^4 CFU *S. aureus*

9. At least 100CFUmL of *S. aureus* for an IMI

10. At least 1000CFU/mL isolated in pure culture or 2000 CFU/mL isolated in mixed culture for an IMI

Milk yield

Effect of pre-partum antimicrobial treatment on milk yield varied from one study to another. Borm et al (2006) investigated the milk production of 561 heifers receiving cephapirin sodium 10-21 days prior to calving. They studied nine North American herds, with a minimum and maximum milk production (kg/heifer per day) of 14.2 to 33.7 kg of milk per day. They observed that treated heifers produced on average 28.1 kg/day and control animals 27.1 kg/day. In this study treated animals milk yield was not significantly higher (Borm et al., 2006). Borm et al. observed that 56% of the heifers in the control group produced more milk than the average heifer in the treated group. The authors stated that treatment alone couldn't increase milk yield.

Conversely, treatment with cephapirin sodium two weeks prior to parturition was associated with an increased milk yield (Oliver et al., 2003). In this later study, Oliver et al. investigated impact on actual and mean 305-day milk production and demonstrated a difference of 531 kg of milk between treated (mean= 5 726kg/305 days) and control heifers (mean= 5 195 kg/305 days).

In another study, treating heifers with pirlimycin 14 days prior to calving did not yield any significant increase in milk production (Middleton et al., 2005). In this later study 305 days milk production for two herds, one with 53 heifers (herd A) and one with 105 heifers (herd B), was monitored. They concluded that, over a 305 days period, no significant differences were observed in milk yield. Yet, if the group would have focused on the immediate three weeks after calving, a more profound difference between treated and control groups may have been observed.

Roy et al. (2007), conversely, observed an increase of 302 kg/305 days of milk when pirlimycin was infused once 7 days prior to calving. Heifers that received treatment less than 7 days prior to calving, however, did not demonstrate any significant increase in milk production. Moreover, in such a case, a negative effect was observed on milk production.

These results suggest that antibiotic would potentially only be beneficial for milk production when treating within a certain window of time prior to calving.

Somatic Cell Count

The impacts of treatment on SCC varied from one study to another. One group that studied North American dairy heifer response to treatment with cephapirin sodium did not show a significant reduction in the linear SCC in the first 200 DIM even though the treatment increased IMI cure rate (Borm et al., 2006). Owens and al. (1994), however, demonstrated that heifers treated with cephapirin 8-12 weeks pre-partum had lower SCC in the cured quarters compared to the quarters that were still infected at 2 months post-partum (Owens et al., 1994). In concordance with the latter study, results obtained when treating heifers with cephapirin 1-2 weeks prior to parturition showed a decrease in the lactation average SCC (Oliver et al., 2003). In a study investigating treatment with 50 mg pirlimycin in Holstein heifers, there was a significant reduction of SCC in early lactation (i.e. 3, 7, 14, and 21 DIM) in the treated group compared to the untreated group (Middleton et al., 2005). There, is no clear consensus on the positive impact on SCC of prepatum antibiotics and, therefore, the use of prepartum antibiotics cannot be universally recommended as a mean to reduce SCC (De Vliegher et al., 2012). There are other and perhaps more important factors such as breed, age, pathogen, and time of infection that could impact SCC (de Haas et al., 2002).

The inconsistencies between the relatively good cure rate obtained and the mitigated effect on milk production and subsequent SCC, raises some doubt regarding usefulness of treating all pre-partum IMI. These results suggest that not all pre-partum IMI have detrimental consequences on heifers productivity and that we should, perhaps, focus on a subset of these IMI: those that actually impede heifer's productivity. We can hypothesize that better host-adapted pathogens are more likely to persist and have detrimental effect on heifer's productivity. These later pathogens would potentially still be found in the mammary gland

after calving while other less well adapted, and possibly irrelevant, strains would be eliminated shortly after calving.

Post-Partum Heifer Treatment

There are not many studies conducted on efficacy of treating infected heifers immediately after calving. This could be an interesting approach since lactating heifers still harbouring IMI after calving could relatively easily be identified at that time using bacteriological culture or other techniques. Early treatment of infected heifers in the post-partum period could be beneficial. We can hypothesize, for instance, that curing IMI early during the first lactation would result in better milk quality and increased milk production and revenue. When heifers are treated in the pre-partum period, usually all heifers are treated since it is very difficult to select only infected animals. Treating only the infected animals post-partum, reduces the use of antibiotics and reduces the costs of treatment as well when compared to universal pre-partum treatment. Furthermore, it would avoid unnecessary treatment of IMI that would be spontaneously eliminated following calving.

In a recent study, the cure rates of heifers treated after calving with an intramammary infusion of 50 mg pirlimycin hydrochloride or combination of novobiocin and penicillin G (Oliver et al., 2007) were compared. Cure rates for all bacterial species for Jersey and Holstein heifer quarters were significantly higher (76.6% and 57.1%, respectively) when treated with pirlimycin compared to the untreated control quarters for Jersey and Holsteins (39.6% and 23.1%, respectively). The treatment of infected Jersey and Holstein quarters with penicillin-novobiocin was less effective but still significant in Jerseys with a cure rate of 61.8% compared to 39.6% for the control. This study however, did not show significant results for Holsteins treated with penicillin-novobiocin compared to the control, and the authors state that it may be due to the low number (n=36) of individuals surveyed (Oliver et al., 2007).

Extended Pirlimycin Treatment

Studies have shown that extended (i.e. treatment duration > 5 days) treatment with pirlimycin during lactation can have a significant effect in treating chronically infected dairy cows (Gillepsie et al., 2002, Deluyker et al., 2005, Barlow et al., 2013). For instance, it has been demonstrated that treatment of dairy cows with an eight day pirlimycin therapy was much more effective at treating IMI by environmental streptococci and *S. aureus* compared to a five day treatment, a two day treatment, or a control group (Gillespie et al., 2002). This later study showed that cure rates for IMI caused by *S. aureus* with pirlimycin treatment were 13.3%, 31.3% and 83.3% for the two, five and eight-day treatment. Additionally, another study showed similar results: using an eight-day pirlimycin treatment for IMI caused by *S. aureus,* increased cure rates (86% for eight day treatment compared to 56% and 6% for two day treatment and untreated groups, respectively), and reduced the SCC significantly (decreased by 69% in the eight day treatment compared to 37% in the two-day treatment), suggesting that an extended treatment is better at curing IMI (Deluyker et al., 2005). A more recent study in New York conducted on two dairy herds observed that an extended eight-day treatment with pirlimycin on dairy cows infected with *S. aureus* (n=18), resulted in high cure rates (78%), in significant reduction of the duration of infections (93 days and 256 days infected in the treatment (n=22) and control group (n=16), respectively), and lowered the rate of new IMIs (42 new IMI/501,774 quarter-days) (Barlow et al., 2013).

Despite the fact that there is no studies on the effects of extended pirlimycin treatment on heifers after calving, the recent literature on dairy cows, suggest the same overall conclusion: extended pirlimycin treatment has a higher cure rate for treating IMI compared to standard 2-day treatment protocols.

Factors Affecting Cure Rates

Regardless of treatment regimen and bacterial species isolated, cure rate is usually higher in younger animals and when fewer colonies are isolated (Deluyker et al., 2005). Likewise, it has been demonstrated that cure rate is greater in lower parity cows, when a lower number of quarters are infected on a given cow, and when SCC is less than 500,000 cells/ml (Sol et al., 1997). Reasons that cure rate may be greater in younger animals is that younger cows are more likely to be infected for a shorter period of time and there is less scar tissue and abscess formation in heifers as compared to older cows, which may influence drug distribution (Trinidad et al., 1990a).

Deluyker's group (2005) confirmed that eight-day pirlimycin treatment increased cure rate for *S. aureus, Streptococcus uberis* and other streptococci as compared to two-day treatment or control groups. Conversely, there was no difference between the two-day and eight-day treatment plan for the CNS group which suggests that cure rates differ between different types of pathogens (Deluyker et al., 2005). In addition, Deluyker (2005) showed that depending on treatment regiment, cure rates decrease as lactation progressed. For instance, an eight-day treatment with pirlimycin did not increase cure rate past 100 DIM, indicating the importance to treat early on in the lactation period. A longer and early treatment plan, like an eight-day post-partum pirlimycin treatment, seems to be a good choice to increase cure rates of subclinical mastitis in heifers caused by *S. aureus*. Early detection of IMI, type of IMI, and SCC of the heifer are important factors that can influence the cure rates for heifer IMI.

Spontaneous Cure Rates

Researchers studying pre-partum treatment of intramammary infections in dairy heifers obtained spontaneous quarter cure rates for *S. aureus* that ranged between 10.5% and 28%.

(Owens et al., 2001; Owens and Ray, 1996; Trinidad et al., 1990b). A spontaneous cure rate for *S. aureus* as high as 37% was observed in one study; however, that result was claimed to be non-statistically significant (Roy et al., 2007). Post-partum spontaneous cure rates seem to decrease as lactation progresses with the highest percentage being at the very beginning of lactation (Deluyker et al., 2005).

Conclusion

One of the most economically important diseases to the dairy industry is mastitis. Recent literature all point to the fact that heifers are equally, if not more so, susceptible to having IMI as dairy cows. The prevalence of infected quarters can be as high as 97% in some cases.

There are many bacterial pathogens that have been isolated from infected quarters that can cause severe problems and affect future milk yield and SCC of these heifers. The most common pathogen isolated prior to calving are CNS; however, the prevalence declines after parturition suggesting that they can be eliminated spontaneously. The IMIs caused by *S. aureus* are known to be more persistent and can cause severe cases of clinical mastitis. This results in increased economic losses for the producers because of treatment costs, veterinary costs, lower milk yield, discarded milk, and early culling of the infected animal. *S. aureus* IMI are also difficult to control, as prevalence often remains high despite implementation of mastitis control programs.

Over the years many diagnostic tools have been developed to identify pathogens causing IMI. The most widely used tool is the routine bacterial culture because of its low costs and high accuracy at identifying the pathogen of interest. Bacterial isolation of *S. aureus* is fairly easy because of its distinct haemolytic capacities. However, in herds with an elevated prevalence of heifer IMI, a faster method is sometimes necessary in order to treat the infected

animal quickly and avoid future udder problems, such as dried off quarters and elevated SCC throughout subsequent lactations. The Petrifilm$^{\text{TM}}$ Staph Express Count media culture system is an effective way to get results within 24 hours. As with any tool, there are pros and cons associated with it. One of the most important disadvantages is that the identification of the *S. aureus* that can be tricky depending on the experience of the reader.

The inappropriate use of antibiotics is an important public health concern especially with the emergence of new "superbugs" that can be resistant to numerous antibiotics. It is of utmost importance to use antibiotics in an informed manner in order to limit emergence of multi-resistant strains while maintaining animal health and welfare. Since *S. aureus* is a pathogen that is relatively hard to treat, there have been many studies on the efficacy of various antibiotic treatments. Most of these studies, however, are on pre-partum treatments, which are universal therapies that require the treatment of all the animals. Post-partum treatment has the benefit of allowing for selection and treatment of infected heifers, hence reducing the amount of antibiotics used. With that being said, *S. aureus* are usually very sensitive to antibacterial products. The antibacterial products used for IMI may not necessarily reach therapeutic concentrations because of poor diffusion due to the inflammation and fibrosis in infected tissues. In addition, as mentioned in the literature review, *S. aureus* has developed mechanism to evade the immune system such as hiding within cells, and these same mechanisms can help the microorganism evade antimicrobial products.

The duration of treatment also plays a significant role. Some may argue that extended treatment is classified as antibiotic overuse. However, the literature demonstrates that higher cure rates are achieved with extended treatment. Treated animals, therefore, have a higher probability of being and remaining healthy, hence avoiding subsequent re-treatment. Pirlimycin has been tested as an eight-day treatment during lactation and the cure rates against *S. aureus* are substantial. There have been no studies to date that investigated cure rates for extended pilimycin treatment of dairy heifers post partum.

Our hypothesis is that an extended treatment with pirlimycin on heifers early in lactation will result in elevated cure rates when compared to no treatment. The purpose of this

study is to evaluate the effect of a post-partum extended pirlimycin treatment on heifers naturally-infected with *S. aureus*. A secondary objective is to evaluate efficacy of Petrifilm Staph express plates to correctly identify *S. aureus* infections during the early post-partum period

ARTICLE

Early lactation extended pirlimycin therapy against naturally acquired *Staphylococcus aureus* intramammary infections in heifers: A randomized controlled trial.

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Abstract

Intramammary infections (IMI) caused by *Staphylococcus aureus* (*S. aureus*) are difficult to treat using standard protocols. Recent evidence suggests that an extended treatment protocol with pirlimycin is an effective way to treat *S. aureus* IMI. The primary objective of the current study was to evaluate cure rate following an early lactation extended intramammary pirlimycin treatment on heifers naturally infected by *S. aureus*. The secondary objective was to assess Petrifilm characteristics when used in a protocol for early lactation detection of infected quarters in heifers. Milk samples were collected from heifers (n=946) in the first few days following calving (mean=5 days). Heifers with laboratory-confirmed *S. aureus* IMI (n=72) were randomly allocated into two groups. The treatment group (n=55 quarters from 39 heifers) received an intramammary infusion of 50 mg of pirlimycin once per day for eight consecutive days in infected quarters. The control group (n=43 quarters from 33 heifers) did not receive any treatment. Treatment success was defined as having negative culture results for *S. aureus* in all three post-treatment quarter milk samples collected on days 17, 24, and 31 post-treatment. Treatment group mammary quarters showed a statistically significant higher cure rate (63.6%) compared to the control group (32.5%). When comparing the Petrifilm to bacterial culture, 38% of the *S. aureus* positive Petrifilm were in fact other staphylococci. The current study demonstrates that a relatively high cure rate for *S. aureus* IMI can be achieved in dairy heifers if an extended treatment protocol is put in place soon after calving. Use of Petrifilm for identification of *S. aureus* infected heifers could lead to many unnecessary treatments because of false positive results.

Key words: dairy heifer, mastitis, *Staphylococcus aureus,* pirlimycin, extended therapy, Petrifilm

Introduction

Staphylococcus aureus (**S. aureus**) is a contagious pathogen that causes persistent intramammary infections (**IMI**). Its prevalence in heifers, in the pre- and post-partum periods, can range between 0 and 15% (Fox, 2009). *Staphylococcus aureus* has many virulence factors that contribute to its persistency. In addition, *S. aureus* is part of the cow's natural skin flora making this pathogen impossible to eradicate from herds. The ability of this bacteria to adhere to epithelial cells allows it to stay in the gland during milking instead of being washed out (Middleton, 2013). Other virulence factors include immune cell evasion (avoiding the system's Toll-Like-Receptors), hiding within phagocytic cells, and the capacity to resist oxidative bursts (Zecconi and Scali, 2013). Furthermore, different strains of *S. aureus* may have different shedding patterns which makes it harder to identify, and thus harder to control (Sears et al., 1990). Sears et al. (1990) stated that *S. aureus* IMI with low shedding cycles have a high risk of testing negative when a single sample is collected (Sensitivity [**Se**] of 74.5%). Increasing the inoculum volume, using duplicate sampling, centrifugation, freezing milk samples, and milk incubation can all increase the probability of detecting *S. aureus* in standard bacterial culture; hence, allowing prompt implementation of an appropriate treatment protocol (Zecconi, 2010).

On-farm culture methods can be used for detection of mastitis-causing pathogens. The 3M Petrifilm Staph Express (**STX**) count plate is one of these methods. The main advantage of the STX is the fact that the milk samples do not need to be sent to a diagnostic laboratory; thus, shipping delays are avoided and results can be obtained within 22-24 hours (Silva et al., 2005). In addition, the standard inoculation volume is 1 mL as compared to 0.01 mL for a standard bacterial culture; this higher volume can increases the likelihood of identifying bacteria in the milk culture especially when there are low numbers of *S. aureus* present (Silva et al., 2005). Some have demonstrated, however, that this high volume may impede the proper reading of the STX plate because of the high number of CFU present (Wallace et al., 2011). The best sensitivity (**Se**) results for identifying *S. aureus* using STX plates were obtained when

diluting the milk in a 1:10 saline solution for post-partum cows (Se of 92% *vs.* 83% in undiluted milk) (Wallace et al., 2011). Research has shown that frozen diluted samples yielded highest sensitivity (78%) for detection of *S. aureus* on STX plates as compared to frozen non-diluted (70%) and fresh diluted samples (74%) (Wallace et al., 2011). The disadvantage to the STX plates is the fact that reading and interpreting the results can be quite difficult if the reader is not properly trained or lacks experience in identifying *S. aureus* colonies. For example, the use of a weak pink zone (i.e. a weak DNase reaction on the Petrifilm) to identify *S. aureus*, resulted in 22.4% false positives and interpretation of the STX plate varies as a function of the readers' skills and that readers sometimes disagree in their assessment of colony numbers, size, and the intensity of the pink zones around the colonies (Silva et al., 2005).

Many studies were conducted in dairy cows and heifers to investigate use of various antibiotics for treating *S. aureus* IMI (Owens et al., 2001; Roy and Keefe, 2012). Results from short-term antibiotic treatments are often unsatisfactory (Barkema et al., 2006; Oliver et al., 2007; Owens et al., 2001), which is why extended treatment regimens have been proposed to obtain higher cure rates (Deluyker et al., 2005; Gillespie et al., 2002; Roy and Keefe, 2012). Research suggests that the highest cure rates for *S. aureus* IMI in dairy cows in North America were obtained with pirlimycin hydrochloride and with extended treatment protocols (Roy and Keefe, 2012). In addition, having a younger animal increases the chances of cure compared to older animals (Barkema et al., 2006) and treating early on can increase milk quality and production throughout the remaining lactation (De Vliegher et al., 2012). To our knowledge, there has been no study to date evaluating the cure rates obtained from an extended antibiotic treatment protocol on recently calved heifers with *S. aureus* IMI.

We hypothesized that high cure rates could be obtained in infected first parity cows if they are treated promptly during the early lactation period. In addition, we believe that using Petrifilm STX plates in early lactation with frozen milk samples can be a valuable tool for prompt identification of *S. aureus* IMI. Specific objectives were to: 1) evaluate *S. aureus*

quarter and cow cure rates in quarters treated or not with an extended intramammary pirlimycin formulation; 2) assess test characteristics of the Petrifilm STX when used on quarter-milk samples to identify infected quarters in early lactating heifers; and 3) evaluate if composite samples would be a valuable option to identify *S. aureus*-infected heifers using the Petrifilm.

Material And Methods

The chosen study design was a randomized controlled trial (**RCT**). The REFLECT statement was used as a guideline for planning the study and throughout the manuscript for reporting. The study protocol was accepted by the Animal Ethics Committee of the Université de Montréal (14-rech-1737).

Herd and Heifer Selection

Herds constituting the source population for this study were clients of the collaborative veterinary practice involved in the research project (Clinique vétérinaire Centre-du-Québec, Notre-Dame-du-Bon-Conseil, QC, Canada). A convenient sample of dairy herds was selected from this source population. Selection criteria were: 1) participating in Dairy Herd Improvement (DHI) program; 2) having a historical prevalence of *S. aureus* in heifers at calving $\geq$ 5% in the previous 12 months; and 3) willingness to participate in the study. All recently calved heifers from the chosen herds between April 2014 and December 2015 were evaluated for enrollment in the RCT. Quarter milk samples were collected within the first few days of calving (median = 4 DIM, min = 0, max = 17 DIM). Heifers were enrolled in the RCT when at least one quarter was diagnosed positive for *S. aureus* on the Petrifilm.

Sample size was determined *a priori* using a two-sample comparison of proportions power calculation. It was determined that, 36 heifers (18 in each group) were needed for detecting a difference in cure rate of 65% versus 15% with a power of 80%; therefore, an initial plan for recruitment of 50 heifers was made. However, during preliminary data analysis conducted prior to terminating the RCT, a higher than expected proportion of spontaneous cure rates (30%) was observed in untreated heifers. The sample size was adjusted to be able to detect a smaller difference in cure rate (65% versus 30%) with a power of 80%. The RCT was pursued with the objective of enrolling 76 heifers: 38 heifers in each group. A total of 72 *S. aureus*-infected heifers with complete records were identified during the study period (39 heifers in the treatment group and 33 heifers in the control group).

Sampling and Petrifilm Bacteriologic Procedures

The dairy producer (trained for sample collection) or the veterinarian of the herd collected quarter milk samples aseptically according to National Mastitis Council (**NMC**) procedures (NMC, 2004) within the first few days after calving (mean=5 days from all first lactation heifers with four functional quarters. Following collection, milk samples were kept at 4°C during transportation to the veterinary practice on the same day and then frozen at -20°C until subsequent bacteriological analysis the following day, or immersed in liquid nitrogen for 1 minute if the analysis was to be conducted on the same day. Whether the analysis where conducted the same day or a following day depended on the time the samples arrived at the clinic (i.e. samples arriving at the end of a work day were analyzed the following day).

All Petrifilm bacteriological analyses were conducted at the veterinary practice by two trained technicians or one veterinarian. Prior to analyses, the quarter milk samples were thawed and a composite milk sample was produced with 1 mL of milk from each quarter milk sample. All quarter-milk samples were diluted 1:10 in saline solution (0.1 mL of milk was diluted in 0.9 mL of saline). The composite sample was not further diluted in saline unless, when reading the Petrifilm, the veterinarian or technician deemed that there were too many

colonies to make an appropriate diagnosis. A total of 1 mL of the diluted milk (for quarter samples) or of undiluted milk (for composite samples) was inoculated on the STX plate, covered by the film and gently pressed down with the Petrifilm platter to spread the milk on the entire STX surface and clear out any air bubbles. The Petrifilms were placed in an egg incubator, the Hova-Bator 1602n thermal air incubator (GQF manufacturing company, Savannah, GA), at 37°C and incubated for 24 hours. After 24 hours, if colony growth was observed, a DNase disc was added on top of the colonies on the Petrifilm and the STX was incubated again for 1-4 hours. A trained technician or a veterinarian read each Petrifilm; if a colony was surrounded by a pink halo, which would indicate a positive reaction to the DNase test, a presumptive diagnosis of *S. aureus* IMI was made. The veterinarian or technician identified the presumed positive colonies on the Petrifilm by encircling up to 5 colonies believed to be *S. aureus* on the Petrifilm with a pen marker. The positive Petrifilm and the frozen milk samples were then sent to the bacteriology laboratory of the Faculté de médecine vétérinaire of the Université de Montréal, QC, Canada, to perform a standard bacteriological culture on the marked colonies and, if needed, on the milk sample in order to confirm the *S. aureus* positive results (see details below).

In addition, the veterinarian or technician counted the *S. aureus*- like colonies on the Petrifilm individually. When the count was greater than 50 colonies, they were estimated (e.g. identified as > 50 or > 100 CFU). The pink zones were measured using a standard ruler and only the zones surrounding *S. aureus* colonies were taken into account. Pink zones covering the entire Petrifilm were not given a numerical value and were not considered in the analysis. At the beginning of the study, pink zones were not initially measured for the first three months (until July 2014) because Petrifilm characteristics were not the initial focus of this study. Throughout the course of the study, the decision to measure these pink zones was taken in order to better understand factors that could lead to misidentification of *S. aureus* CFU on Petrifilm.

Standard Bacterial Culture Procedures

The bacterial culture of the Petrifilm and milk samples was performed according to the NMC guidelines (NMC, 2004). Petrifilm-identified colonies were inoculated on a Columbia agar with 5% sheep's blood. The plates were incubated at 35°C ± 2°C with 5% CO_2 for 18-24 hours. Presence of *S. aureus* was suspected if a double zone of hemolysis (alpha-beta hemolysis) was detected on the blood agar. If no *S. aureus* CFU were detected, another of the identified colonies from the Petrifilm was inoculated. The Petrifilm have no need to be conserved once colonies have grown, therefore they were kept at room temperature until the end of the study. If *S. aureus* was, once again, not detected, 10 μL of milk from the corresponding milk sample was inoculated on Columbia agar with 5% sheep's blood. The milk samples were kept frozen at -20°C until needed. The plates were incubated at 35 ± 2°C with 5% CO_2 for 18- 24 hours. The steps followed for identification of the microorganism, regardless of whether it was a colony from the Petrifilm or the milk, were as follows: if only a beta-hemolytic zone was detected around the colonies, a coagulase test was performed; a coagulase positive result led to classification of the microorganism as a *S. aureus*. If the coagulase test was negative, the microorganism was classified as a coagulase negative *Staphylococcus* (**CNS**). If the colonies were non-hemolytic, a Gram-stain, a catalase test, and a DNase test were performed to identify the microorganism as either a *Streptococcus* species or other *Staphylococcus* species.

Treatment Groups

Heifers identified as infected by *S. aureus* based on Petrifilm results, were randomly allocated in two groups using the randomization function in Microsoft Excel. Information on the allocation group was then provided to the veterinarians at the clinic who informed the producers on whether a given heifer would be treated. The producer and the veterinarian were not blind to the allocation groups, as the negative control group heifers received no antibiotic treatment. The treatment group heifers received an intramammary infusion of 50 mg of

pirlimycine hydrochloride / infected quarter (Pirsue - Zoetis Canada Inc., Kirkland, QC, Canada) once a day for eight consecutive days. The producer administered the treatment aseptically in all the infected quarters (i.e. if a heifer had more than one quarter infected, all infected quarters were treated). Uninfected quarters, however, were left untreated.

Follow-Up

Quarter milk samples were aseptically collected from all initially infected quarters from all heifers on day 17, 24 and 31 following treatment (day 1) and sent frozen to the laboratory of the Faculté de médecine vétérinaire of the Université de Montréal for bacteriological culture. Milk samples were analyzed using standard laboratory procedures as previously described. After the end of the follow-up period, control heifers could be treated if desired by the producer. The laboratory technicians analyzing the quarter milk samples were blind to the allocation group.

Cure Definitions

Presence or absence of a *S. aureus* IMI was confirmed with follow-up milk samples taken on days on day 17, 24 and 31 and analyzed using the standard laboratory bacteriological methods described above. Milk samples were considered contaminated if three or more phenotypically different bacterial species were observed on the plate. *Staphylococcus aureus*, however, were still identified and accounted for if suspected (based on phenotypical considerations) in contaminated samples. At the quarter-level, treatment failure was defined as observing *S. aureus* in any of the three post-treatment samples. Quarters with all three post-treatment milk samples negative for *S. aureus* were considered cured. Quarters with ≥ one post-treatment contaminated sample or with a missing post-treatment sample were considered of unknown cure status and excluded from subsequent analyses. At the heifer-level, a heifer was defined as cured if all her initially infected quarters were cured. A treatment failure was

53

defined if any of her initially infected quarters were positive for *S. aureus* at any of the post-treatment samplings. Heifers with ≥ one quarter with unknown status were excluded from subsequent analyses.

Furthermore, quarters or heifers were excluded from subsequent analyses if: 1) they developed clinical mastitis or another sickness that required the use of other antibiotics during the follow-up period; 2) the heifer was culled; or 3) a quarter was dried.

Use of Composite versus Quarter Milk Samples

One mL of non-diluted composite milk sample was inoculated on the Petrifilm as previously described and was sent to the bacteriological laboratory along with the other samples. Descriptive statistics were used to describe the proportion of quarter milk samples, which were positive for *S. aureus*, but had a concurrent composite sample for which *S. aureus* could not be retrieved on the Petrifilm.

Statistical Analysis

Odds of Cure. Effect of treatment on cure probability was analyzed both at quarter (i.e. cure of the quarter) and heifer (i.e. cure of the heifer) levels using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). An alpha value of 0.05 was chosen for significance. Odds of bacteriological cure in treatment and control groups were compared using a logistic regression model, using the GENMOD procedure of SAS, with cure status as the outcome and treatment group as the main predictor. Only quarters and heifers confirmed positive to *S. aureus* pre-treatment using standard laboratory bacteriological procedures were used for cure rate analyses (i.e. quarters or cows identified as positive pre-treatment using Petrifilm, but negative by laboratory procedures, were excluded). To account for clustering of observations by heifers and herds (in

the quarter level model) or simply by herd (in the heifer level model), robust variance was used to compute standard errors (Dohoo et al., 2009).

Petrifilm's characteristics. Descriptive statistics were used to determine the number of Petrifilm that were not in agreement with the standard laboratory bacteriological results. In addition, descriptive statistics were used for the evaluation of the phenotypical characteristics (i.e. colony shape and DNase zone) of the different bacteria leading to Petrifilm's misinterpretation. A Pearson's χ^2 test was used to determine if there was an association between the colony's phenotypical characteristics on the Petrifilm and the actual bacterial species identified using standard laboratory bacteriological culture.

Results

A total of 946 heifers from 27 herds were evaluated for enrollment in the RCT. A total of 248 quarters from 164 heifers were diagnosed infected by *S. aureus* using Petrifilm and were initially enrolled in the study. However, out of those, only 156 quarters from 110 heifers (60 in the treatment group; 50 in the control group) were confirmed as infected by standard culture at the laboratory and were available for further analyses. A total of 38 animals were excluded from the study because of various reasons: ≥ one post-treatment contaminated milk samples (n = 8 in treatment group; n = 6 in control group); missing follow-up samples (n = 4 in treatment group; n = 5 in control group); received other antibiotics (n = 7 in treatment group; n= 5 in control group); dried off quarter (n = 2 in treatment group); culling (n = 1 in control group).

Heifer-Level cure rate

A total of 72 heifers with 98 infected quarters from 20 different herds were included in the analysis. Fifty-nine percent (59%; 23/39) of heifers treated with pirlimycin and 33% (11/33) of heifers in the control group were cured (Table 1). The odds of eliminating the infection in the treated group were 2.8 (95% CI: 1.1, 7.3) times higher than in the control group. There was an unequal distribution of heifers among treatment groups in regards to the number of quarters infected. There were two times more heifers with more than one quarter infected in the treatment group (n=18 out of 39) as compared to the control group (n=9 out of 33). Furthermore, unconditional association between the number of infected quarters (categorized as one or > one infected quarters) was evaluated using the described logistic model and robust variance estimation. Having multiple infected quarters was associated with 8.0 (95% CI: 3.2, 19.5) times lower odds of cure at the heifer level. The number of infected quarters was added as a predictor to the logistic model to account for the confounding of the association between treatment and cure by the number of infected quarters. When adjusting for confounding by numbers of infected quarters, the odds ratio obtained was greater, as expected. The odds of cure for a heifer were 3.7 (95% CI: 1.3, 10.5) times higher in the treatment group than in the control group after controlling for the number of infected quarters.

Quarter level cure rate

Sixty-four percent (64%; 35/55) of quarters were cured in the treatment group compared to 33% (14/43) in the control group (Table 1). Odds of cure were 3.6 (95% CI: 1.5, 8.2) times greater in the treatment group compared to the control group. The effect of the number of colonies (categorized as < 100 CFU/mL versus ≥ 100 CFU/mL) observed on the Petrifilm during diagnosis on odds of cure was assessed using the Pearson's χ^2 test. There was no significant association found between the outcome and the number of colonies observed on the Petrifilm (*P*-value = 0.06). If the number of colonies on the Petrifilm was < 100 CFU/mL, the cure rate was numerically higher (69.6%, 16/23 *versus* 59.3%, 19/32).

Petrifilm diagnostic characteristics

Out of the 946 heifers sampled, 164 heifers were positive on the Petrifilm for *S. aureus*, which resulted in an apparent prevalence of 17%. Out of the positive Petrifilm, 110 were confirmed positive by standard bacteriological culture, which resulted in an apparent prevalence of 12%. The proportion of false positive heifers (when compared to laboratory-based bacteriological culture) observed on the Petrifilm was 33% (54/164). At the quarter level, there were 248 positive Petrifilm and only 154 of these were confirmed with bacterial culture, which resulted in 38% false positives (again, when compared to laboratory-based culture). The bacterial species isolated from the false positive Petrifilm were mainly other *Staphylococcus* species.

To evaluate whether there was an association between the shape of the colony (i.e. star-shaped *versus* round-shaped) on the Petrifilm and the bacteria type, information that could possibly be used to improve diagnostic characteristics, a Pearson's χ^2 test was performed using all milk samples harboring either *S. aureus* or other staphylococci (n=369). There was a significant association between actual bacterial species (as identified by the laboratory) and shape observed on the Petrifilm (*P*-value <0.01). There were 7% of *S. aureus* isolates compared to 25% for other *Staphylococci* that formed a star-shaped colony on the Petrifilm (Table 2).

The association between the bacterial species (as identified by the laboratory) and the size of the DNase zone on the Petrifilm (categorized as ≤ 2mm or ≥3mm) was also assessed using a Pearson's χ^2 test, and a significant association was observed (*P*-value <0.01). There was 67% of *S. aureus* isolates producing a zone of lysis equal to or greater than 3 mm This proportion was only 29% for other staphylococci. (Table 3).

Quarter vs Composite Samples

The possibility of using only composite milk samples, rather than four quarter-milk samples, for detection of *S. aureus*-infected heifers was assessed. There were a total of 164 heifer composite-milk Petrifilm samples sent to the bacteriological laboratory in the current study. There were nine composite samples that were negative on the Petrifilm but had at least one *S. aureus* positive quarter. There was a 95% (155/164; 95% CI: 89.9, 97.1) agreement between composite samples and quarter positive samples when using the Petrifilm (Table 4).

Discussion

The apparent *S. aureus* prevalence obtained in the current study of 12% was within the range of 0-15% reported during the pre- and post-partum period in other studies across North America (Fox, 2009). We observed an absolute quarter cure rate difference of 31% between the treatment group and control group. The quarter cure rate observed in the current study (64%) was numerically higher but similar with another study using a standard two-day post-partum pirlimycin treatment in heifer quarters (57.1%) (Oliver et al., 2007). When comparing to a study looking at extended eight-day pirlimycin treatment on lactating dairy cows (which observed cure rates of 36 to 88%), we observed a cure rate that lies within the same range in our study (Deluyker et al., 2005).

Antimicrobial resistance for microbial pathogens is a great concern for public health. The emergence of resistance has coincided with the decrease in new antimicrobial development from pharmaceutical companies, which means we have fewer tools available to combat microorganisms that have tremendous powers of adaptability in any environment (Spellberg et al., 2008). Our role is to minimize use of antibiotics in agriculture (i.e. to promote growth of food animals), using an appropriate antibiotic for a specific pathogen, prescribing a dose and a time interval that has the best efficacy against a pathogen (i.e.

limiting sub-lethal concentrations), and limiting their use when necessary. In Canada, over 80% of dairy herds treat all quarters of all cows during the dry period with an intramammary antibiotic regardless of the bacteriological status of the animal (Dufour et al., 2012b). This type of practice for managing mastitis is not a wise use of antibacterial products and can lead to the development of resistant microbes (Spellberg, 2008). In our study, we are implementing a protocol on infected quarters only based on culture results. The dose used keeps a sufficiently high concentration of pirlimycin in the mammary gland for a period of eight days and has been proven by studies to be effective at eliminating *S. aureus* in infected quarters (as compared to a standard two day treatment or with other antibiotics). Applying this to young animals, early in lactation, can potentially decrease the prevalence of *S. aureus* within a herd and, in addition, decrease the overall use of antibiotics.

Researchers studying pre-partum treatment of *S. aureus* IMI in dairy heifers obtained spontaneous quarter cure rates that ranged between 10.5% and 28% (Owens et al., 2001; Owens and Ray, 1996; Trinidad et al., 1990b). In regards to post-partum treatment in dairy cows, one group observed only 6% of spontaneous cure rate for *S. aureus* infected quarters for first parity cows, having between 1 and 10 CFU/uL, and > 200 DIM (Deluyker et al., 2005). The spontaneous cure rate decreased as the CFU increased in the sample. Deluyker et al. (2005) observed a spontaneous cure rate in first parity cows > 200DIM, of 3.4% and 1.7% for samples containing 11 to 100 CFU/uL and > 100 CFU/uL, respectively. One potential hypothesis for the high spontaneous cure rate observed in our study, is that heifers may be infected with strains that are less adapted to the host and are not as persistent as strains usually observed in adult cows; hence, making self-elimination easier. Barkema et al. (2006) observed, using multilocus sequence typing, that the majority of IMI on a given herd are usually caused by a limited number of *S. aureus* strains that belong to a specific clonal complex. However, in order to identify a strain as being part of a specific clonal complex, we would have needed to type the strains identified in the milk culture, which was beyond the scope of this study.

Moreover, evidence suggests that higher CFU per mL of milk plays a role in cure rate as well (Barkema et al., 2006; Deluyker et al., 2005). In the current study, no significant association between number of CFU and the outcome was observed. The colony count in our study was performed using the Petrifilm samples included in the study (n= 98) instead of bacteriological culture. We noticed that spontaneous quarter cure rate was 50% (11/22) for quarters having < 100 CFU/mL of milk compared to 14.2% (3/21) for quarters having ≥100 CFU/mL of milk on the Petrifilm. Using these findings, we could potentially implement a protocol for heifers that require treatment only when the colony count is over a certain threshold, since having a low colony count tended to (even if not statistically significant) increase the chances that the heifer eliminates the *S. aureus* infection without antibacterial intervention. It would be interesting to do more research with a larger sample size to re-evaluate the trend we saw.

In the present study, we evaluated early detection methods to identify *S. aureus* infected heifers and these methods included using Petrifilm STX plates and assessing if a composite sample could be used as screening test. The specificity of STX conducted in one study was 98.5% and this was associated with distinct pink zone around the colony (Silva et al., 2005). If there was a weak pink zone, the rate of false positives observed was 22.4%. Silva et al. (2005) stated that colonies surrounded by a distinct pink zone were 120 times more likely to be *S. aureus*. There were a relatively high number of false positives quarters associated with the STX Petrifilm in our study (38%). At the beginning of the study, all Petrifilm that had a pink zone surrounding a colony were sent to the laboratory as positive *S. aureus* Petrifilm. The distinction between strong or weak pink zones was not taken into consideration. During the course of the study, an adjustment was made in regards to better identify *S. aureus* positive Petrifilm. The colonies with pink zones that had a diameter of ≤ 2 mm were less likely to be classified as positive *S. aureus*. In addition, colonies that were star- shaped were also less likely to be classified as *S. aureus*. After the adjustment was made, there were 27% (26/98) of Petrifilm sent to the laboratory that were in fact not *S. aureus* based on bacteriological results. Therefore, identifying certain characteristics on the Petrifilm, such as colony shape and DNase reaction diameter, could possibly improve the proper diagnosis of *S. aureus* infected animals

and decreases the chances of over diagnosing *S. aureus* in heifers. Analyzing the Se, the specificity, the positive predictive value , and the negative predictive value was beyond the scope of this study since this was already reported in the literature (Wallace et al., 2011).

Five percent (5%) of positive heifers would have been missed if only the composite milk sample was used as a screening test using the Petrifilm in our study. The question now lies whether missing 5% of infected heifers is enough to warrant screening for all four quarters instead of simply using a composite sample. There are many factors to consider especially since our results showed that low numbers of *S. aureus* in the milk was associated with high self-cure rates. There is also the fact that *S. aureus* is a contagious pathogen and very difficult to treat once established in the mammary gland. Perhaps herds which have a high prevalence of *S. aureus* and which have difficulties in eliminating the disease should consider testing all four quarters in order not to miss any infected animals. Herds in which the prevalence is relatively low could rely more on composite samples and CFU in order to determine whether the animal should be treated. If an animal has low numbers of *S. aureus* on the Petrifilm, the veterinarian could potentially monitor the cow's subsequent somatic cells count (SCC) and, if needed, collect additional samples before instigating a treatment. We did not evaluate whether it is economically beneficial to treat, neither did we assess the subsequent changes in SCC or milk production since the producers treated some of the infected heifers in the control group after completion of the follow-up period.

We did not stratify the randomization by herds in our study. Occasionally, we had a small number of infected animals per herd; hence, in some herds, the majority or all of the animals fell into one treatment group. In addition, we had some herds with only one animal being used in the analyses due to the fact that the other heifers were excluded from the study. These issues could potentially bias our results since pathogenic strains can differ from farm to farm. Some strains may be easier to treat in some herds and if there were more treated animals in these herds, it can potentially affect the cure rates observed. Furthermore, this complete separation of data precluded the use of generalized linear mixed models for estimating odds of

cure in treated and untreated heifers. To evaluate statistical significance, we had to opt for a robust variance estimation instead, a very valid, but less efficient method (i.e. in terms of power), which does not require specific assumptions regarding the exact clustering of observations (Dohoo et al., 2009). It is important to understand that, while this methodology can be used to provide valid and accurate standard error estimates for assessing statistical significance using 95% CI or *P*-values, it does not, however, correct the OR point estimate obtained.

Conclusion

An extended treatment protocol (8-day treatment) on quarters of dairy heifer infected with naturally occurring *S. aureus* in the post-partum period resulted in greater odds of cure compared to untreated quarters. We also found that there was a high spontaneous cure rate in untreated quarters and further research in that area is required. Proper training for technicians or veterinarians is needed in order to interpret the Petrifilm and reduce the amount of false positives observed. Composite milk samples may miss up to 5% of positive *S. aureus* cases, which is something to consider when dealing with *S. aureus* problematic herds.

Acknowledgements

Conflict Of Interest

This study was partly founded by Zoetis Animal Health

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Tables

Table I. Cure rate at the quarter- and heifer-level for 72 heifers and 98 quarters enrolled in a randomized controlled trial evaluating effect of an eight day intramammary pirlimycin treatment for treating intramammary infections due to *Staphylococcus aureus* in early lactation.

	Heifer-level			Quarter level		
	Treated	Control	Total	Treated	Control	Total
Diseased	16	22	38	20	29	49
Cured	23	11	34	35	14	49
Total	39	33	72	55	43	98
% Cured	59.0	33.3		63.6	32.5	

Table II. Colonies shape on the Petrifilm Staph Express as a function of bacterial species involved in 369 isolates from milk samples coming from dairy heifers in early lactation.

Colony shape	*S.[1] aureus* (%)	Other staphyloc (%)
Round	226 (92.6)	116 (74.8)
Star	18 (7.4)	39 (25.1)
Total	244	155

1. *Staphylococcus*

2. Other staphylococci species: Coagulase-negative staphylococci, *Staphylococcus* spp.

Table III. Size of DNase reaction zones on the Petrifilm Staph Express plate as a function of bacterial species involved in 210 milk samples coming from dairy heifers in early lactation.

Pink zone (mm)	S.[1] aureus (%)	Other staphylococci.[2] (%)
≤ 2	38 (33.0)	67 (70.5)
≥ 3	77 (67.0)	28 (29.4)
Total	115	95

1 = *Staphylococcus*

2= Other staphylococci: Coagulase-negative staphylococci, *Staphylococcus* spp.

Table IV. Comparing the agreement between composite samples and quarter samples for the detection of *Staphylococcus aureus* using Petrifilm from milk samples coming from dairy heifers in early lactation.

		Petrifilm composite samples		
Petrifilm		Positive	Negative	Total
quarter	Positive	155	9	164
samples	Negative	0	782	782
	Total	155	791	946

GENERAL DISCUSSION

In the current study, we were able to demonstrate that an extended eight-day protocol using pirlimycin on recently calved heifers resulted in an elevated cure rate compared to the control group. We also observed relatively high spontaneous cure rates, which we can potentially associate with the number of CFU present in the milk. In addition, Petrifilm can possibly be used as a quick diagnostic tool to identify *S. aureus* IMI, though further evaluation on their characteristics is necessary to prevent over diagnosing infections.

In our study, we observed an absolute quarter cure rate difference of 31% between the treatment group and control group. There are many factors that can influence the cure rate obtained such as the strains of *S. aureus* in the herd or the meticulousness in administering the treatment for eight consecutive days. The latter point could have been controlled in an experimental setting if there was an individual (ex: a trained technician) that would have been able to administer the treatment to the animals every day at the same time aseptically. In our study, it was not an applicable option; therefore, we cannot be absolutely sure that the producer gave the medication every day aseptically according to our standards. Regardless, I believe the numbers obtained in our study are close to the reality since, in normal day-to-day practice, it is the producer who administers the treatment.

We also speculated that there was a possibility that the original infection was resolved with the antibiotics but there was potential reinfection throughout the sampling period. The reason we assumed this to be a possibility was because we had 12 out of the 20 quarters in the treatment group that were positive for *S. aureus* only in the second or third sampling post-treatment and only eight were positive on the first sampling post-treatment. Conversely, in the control group, we observed 23 out of the 29 quarters still infected with *S. aureus* during the

first sampling post-treatment and only 6 were infected solely on the second or third sampling post-treatment. Was it possible then to have had a cure in our treatment group but the quarters were re-infected afterwards? In order to answer this question, we sent 13 pre- and post-treatment samples from the treatment group to the Université de Sherbrooke, which looked at the characterization of *S. aureus* strains to determine if the colonies were really a non-cure or a re-infection. Only one out of the 13 samples sent out was confirmed to be a different strain from the pre-treatment sample. Can we completely exclude our hypothesis? Most of the *S. aureus* strains were indeed the same strain in the pre- and post-treatment samples; however, these strains may be the predominant strains in the herd. We can confirm with certainty that we have an infection with the same strain pre- and post- treatment, yet we cannot confirm if the quarter was cured and re-infected since it may be possible to be re-infected with the same strain.

Researchers studying pre-partum treatment of *S. aureus* IMI in dairy heifers obtained spontaneous quarter cure rates that ranged between 10.5% and 28% (Owens et al., 2001; Owens and Ray, 1996; Trinidad et al., 1990b). In one study, the cure associated with the control group was the highest at the beginning of lactation and decreased as lactation progressed (Deluyker et al., 2005). Our study was done at the very beginning of lactation and this may be the reason why our spontaneous cure rates were more elevated (33%) then other studies, which focus later on in lactation. Another potential hypothesis for the high spontaneous cure rate observed in our study, is that heifers may be infected with strains that are less adapted to the host and are not as persistent as strains usually observed in adult cows, hence making self-elimination easier. This would be something interesting to look into in future research in order to really figure out what is the difference between *S. aureus* IMI in heifers and adult cows.

In the current study, no significant association between number of CFU and the outcome was observed. We noticed that spontaneous quarter cure rate was 50% (11/22) for quarters having < 100 CFU/mL of milk compared to 14.2% (3/21) for quarters having ≥100

CFU/mL of milk on the Petrifilm. With these findings, we could potentially implement a protocol for heifers that require treatment only when the colony count is over a certain threshold, since having a low colony count tended to increase the chances that the heifer can eliminate the *S. aureus* infection without antibacterial intervention. Veterinarians can rely on CFU in order to determine whether the animal should be treated. If an animal has low numbers of *S. aureus* on the Petrifilm, the veterinarian could potentially monitor the cow's subsequent somatic cells count (SCC) and, if needed, collect additional samples in the following weeks before instigating a treatment. It is important to follow the cow closely in such a case and assure that there are no colonies found in the following week. Waiting too long to take a subsequent sample in such cases, can result in the bacteria multiplying, getting well established in the mammary gland, and, consequently, causing an elevated SCC. These cases, if not followed and treated, can also become chronic carriers for the rest of the herd. Conversely, milk cultures with over 100 CFU /mL should be treated accordingly.

Antimicrobial resistance

We live in an era where antimicrobial resistance for microbial pathogens is a great concern for public health. The emergence of resistance has coincided with the decrease in new antimicrobial development from pharmaceutical companies, which means we have fewer tools available to combat microorganisms that have tremendous powers of adaptability in any environment (Spellberg et al., 2008). Increased selective pressure put on the microbes by chronic antibiotic exposure at sub-lethal concentrations, increase the production of reactive oxygen species that, in turn, cause an increase in point mutations on the bacterial DNA and on chromosomal recombination (IOM, 2010). There is also the acquisition of exogenous antimicrobial genes via horizontal transfer. The blame of antibiotic resistance is not solely put on the use of antibiotics, since the microorganisms have been using resistant genes for their survival for millions of years. However, antibiotic use increases the selective pressure of the microorganisms and it is, therefore, of the utmost importance to use antibacterial products in a cautious manner. Our role is to minimize use of antibiotics in agriculture (i.e. to promote

growth of food animals), using an appropriate antibiotic for a specific pathogen, prescribing a dose and a time interval that has the best efficacy against a pathogen (i.e. limiting sub-lethal concentrations), and limiting their use when necessary. In our study, we are implementing a protocol on infected quarters confirmed with bacterial culture. The dose keeps a sufficiently high concentration of pirlimycin in the mammary gland for a period of eight days to eliminate chronic carriers of *S. aureus* at an early age, and decrease the prevalence of IMI and potentially new IMI in a herd. By doing so, we theoretically decrease the overall use of antibiotics. Assuring that the antibiotic is given for eight consecutive days, which was proven to have a higher efficacy at eliminating *S. aureus* IMI then a two-day treatment, we are in fact keeping a therapeutic concentration for a specific time frame to maximize the chances that the microorganisms are being purged adequately; thus, decreasing the chances of resistance. In addition, we have observed that heifers have a very high spontaneous cure rate when infected with *S. aureus*. As mentioned previously, we can limit the use of pirlimycin in animals and omit its use when there are low bacterial counts, since these animals have a very good chance of eliminating the infection naturally and building up their own immunity to *S. aureus*. A recent study has shown that the use of pirlimycin in lactating cows increased the odds (OR: 2.07; CI95%: 1.38 to 3.09) of developing a resistance to pirlimycin (Saini et al., 2012) Even though generally speaking, *S. aureus* resistant strain in milk are relatively low, the fact that there are resistances developing to important drugs such as pirlimycin makes it important not to over diagnose and to avoid treatment when it is not absolutely necessary (Saini et al., 2012). In Canada, over 80% of dairy herds treat all quarters of all cows during the dry period with an intramammary antibiotic regardless of the bacteriological status of the animal (Dufour et al., 2012b); there are no samples taken for bacterial culture. This type of practice for managing mastitis may not be a wise use of antibacterial products and can lead to the development of resistant microbes. The Food and Agriculture Organisation states that diseases can be prevented by using good husbandry and by potentially ending factory farming. The FAO believes that the use of antibiotics prophylactically has a direct impact on antimicrobial resistance seen in human medicine; hence they are against using antibiotics to prevent disease in farm animals.

Petrifilms

There were a numerically high number of false positive quarters associated with the STX Petrifilm in the current study. At the beginning of the study, all Petrifilm that had a pink zone surrounding a colony were sent to the laboratory as positive *S. aureus* Petrifilm. The distinction between strong or weak pink zones was not taken into consideration. It was reported that colonies with a distinct pink zone were 120 times more likely to be *S. aureus* colonies (Silva et al., 2005). During the course of the study, an adjustment was made in order to better identify *S. aureus* positive Petrifilm. Colonies with pink zones that had a diameter of ≤ 2 mm were less likely to be classified as positive *S. aureus.* In addition, colonies that were star- shaped were also less likely to be classified as *S. aureus*. Therefore, identifying certain characteristics on the Petrifilm, such as colony shape and DNase reaction diameter, could possibly aid in the proper diagnosis of *S. aureus* infected animals and decreases the chances of over diagnosing *S. aureus* in heifers; hence, avoiding overuse of antibiotics. We state that we had "false positive Petrifilm" because our gold standard is a bacterial culture. However, as mentioned in the literature review, Petrifilm's are more sensitive than a bacterial culture and that is because the inoculation volume for Petrifilm is 10 to 100 times greater than bacterial culture. It is possible that the STX can detect *S. aureus* IMI when the CFU count is much lower in the affected quarters, whereas it is a greater possibility that a bacterial culture may miss those low CFU IMI. It is something to keep in mind when comparing the two methods.

In our study, the Petrifilm was read and interpreted by very few people (one veterinarian and two trained technicians). It is very important that there are few people responsible for the interpretation of the Petrifilm since its interpretation may vary between individuals (Silva et al., 2005). As mentioned previously, it is very important to have proper training in reading the STX because they can be difficult to interpret if one is not used to reading them.

The use of Petrifilm STX is still not common practice regardless of their high sensitivity. It could be due to the fact that many veterinarians are not comfortable with their use. However, rotating at the farm animal hospital during my final year in veterinary studies has made me realize that receiving results from a bacterial milk culture can take up to 5 days. Many times, 5 days can be too long to wait for results when an animal is critically ill. Coming back to the proper use of antimicrobials, we would want to know the identification of the bacterial species affecting the animal to put in place a proper treatment protocol with the proper antibiotics in order to avoid their misuse. Perhaps a pre-emptive culture on a Petrifilm can guide the veterinarian on the proper tract while waiting for the bacteriological results to come in. Evidently, using the two methods can become costly, therefore not all cases may warrant the two. Feasibly, one would use both on a critical case that needs an antibiotic treatment put in place promptly. The Petrifilm can give a good idea on what organism is causing the illness and the bacterial culture can confirm the results. Nevertheless, a Petrifilm culture can be a diagnostic tool on it's own if veterinarians are comfortable with their use and a milk culture can be sent out for bacteriological analysis if the results obtained by the Petrifilm are doubtful.

Costs

As in any situation, we must assess the cost of treatment over their benefit. Let's take a hypothetical example of a 100-cow herd in which there is 30% of heifers with a prevalence of *S. aureus* of 15% for a total of 4 to 5 infected heifers per year. If a post-partum composite sample is taken for each heifer in addition to sampling each quarter of the positive heifers (15%), we would have a cost of $480 to identify infected heifers and quarters if the bacterial culture costs $10. If we assume that on average one quarter was infected per heifer, then the cost of an 8-day treatment with pirlimycin once a day ($6.50/ tube) would be $234. The cost of milk loss during the treatment would be around $630 if we assume that in 10 days, the heifer would produce 200kg of milk and that milk costs are around $0.70/L (140$ loss per heifer x 4.5 heifers). This comes out to an estimated total of $1,344 for the herd or $299 per

infected heifer. Are these costs worth it? It all depends on the situation. As mentioned throughout the manuscript, it is important to control IMI due to *S. aureus* because of it's chronicity and the large economic losses it can cause if it becomes highly prevalent in the herd. Treating effectively and early on can potentially decrease the costs throughout the heifers' lactation and maybe throughout subsequent lactations as well. Treatment can lower the prevalence of *S. aureus* IMI in a given herd, resulting in less overall treatment costs, diagnostic costs, and costs associate with loss of milk production and quality of milk produced due to a high SCC, which is usually the case with *S. aureus* infections. With that being said, a heifer that has an infection in several quarters or with high number of *S. aureus* CFU present in the quarters, will most probably have a poor treatment outcome and, in that case, may or may not be worth to treat (depending on the genetic value of the animal). In addition, as we have seen with our study, a low colony count may not warrant investing in treatment right away either. The heifer can potentially eliminate the infection on her own. Therefore, it is more of a case-by-case assessment in order to determine whether the treatment is really worth it or not.

Evaluation of the project

There were many obstacles throughout the course of the project that I must address. The first and major one was the dataset. There were two sets of data: one from the clinic, and one from the laboratory and both were not fit for statistical analysis. It was very time consuming to separate all the results from the merged cells, into separate cells in order to be analyzed by a stats computer program. We should have established the table for both the clinic and the laboratory before the start of the study.

The other major problem was the fact that the clinic did not strictly follow the protocol for sampling the heifers' milk 5-days post-partum. We only found out near the end of the study (second summer of the Master's program) when the analyses were being done. Many

times, the veterinarian would go pick up the milk sample that the producer or the veterinarian collected when he or she was following up on the herd. There was no date on the milk tube if the producer took the sample (therefore it was an estimated date) or the sampling date was taken many days after calving (max 17 days). The main objective of the study was to implement a treatment protocol in *recently* calved heifers; not respecting protocol resulted in discarding a few samples and having to present the results in a different manner (i.e. calculating the mean of the sampling dates rather then stating the max). There is no way of knowing if this affected the cure rates obtained. We would have to re-do the study in order to be absolutely sure.

This brings me to the subject of doing a joint Masters and Doctorate in veterinary medicine degree. I absolutely enjoyed my time doing this project, however, in hindsight, I don't believe it was an appropriate project to have done in a joint degree since it's an ongoing project and I only had the summer months to work on it. It would have been perfect if I could have gone to the farms and sampled the recently calved heifers or sampled the heifers after their treatment. If I could do it over again, I would have taken a sabbatical year in order to take care of the sampling and the dataset instead of only having the summer months. The joint program works best for projects that can be stopped and picked up again like in a laboratory setting (freezing cells and using them the following year). However, having done fundamental research in the past, I wanted a clinical project in which I can see results at the very end, and this was the perfect project for that.

Future research

Finally, it would be interesting to know what are the effects of an early treatment protocol on milk production and SCC. In our study, it was very difficult to evaluate because the heifers in the control group were given the option to be treated after the study period and there was no specific date put in place to evaluate the milk production and SCC prior to and

after treatment. Also, as mentioned previously, it would be worth taking a look to see what are the differences in the strains seen in heifers and adult cows in order to better adjust a treatment protocol that is suited for each. We can potentially look at the effect of treating heifers with low colony counts or not, and evaluating the colony counts in the untreated heifers in the following weeks. What percentage of heifers with low CFU have a spontaneous cure and what percentage go on to develop a higher number of CFU. We can potentially discover factors that differ between these two groups in order to figure out why some heifers will eliminate the organism while others do not, and subsequently, adjust treatment regiments for *S. aureus* positive IMI with low CFU. We can also further evaluate Petrifilm characteristics (based on DNase reaction zones and colony shape) to allow for a better interpretation by veterinarians and decrease the chances of over diagnosing *S. aureus* infections.

GENERAL CONCLUSION

A prolonged treatment protocol using an intramammary infusion of pirlimycin on recently calved heifers positive for *S. aureus* IMI results in a significantly greater cure rate when compared to no treatment at all for both the quarter level and the heifer level. At the heifer level, 59% of heifers were cured following treatment compared to 33% in the control group. At the quarter level, 64% of quarters were cured following treatment compared to 33 % in the control group. These results supported our hypothesis.

Early detection of heifer mammary infections using the Petrifilm is a quick and easy way to screen for infected individuals. Petrifilm have a potential to being a great first line diagnostic tool to quickly identify IMI causing pathogens. The dehydrated culture media is easy to use without the need of specialised equipment. However, due to their high sensitivity and the difficulty associated with reading them when not properly trained, makes them more susceptible to over-diagnose IMI caused by *S. aureus* and may result in unnecessary treatments due to the high number of false positives; 38% of *S. aureus* positive Petrifilm were not identified as *S. aureus* using standard bacterial culture methods. .

The apparent prevalence of *S. aureus* in heifer IMI around Centre du Quebec was 12% which is the average prevalence seen with other studies. The apparent prevalence was 17% when using only the Petrifilm as a diagnostic tool.

There was a relatively high spontaneous cure rate seen within this study. This may be associated with strain specific characteristics or the bacterial charge present within the mammary quarters.

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