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Evaluation of a commercial real-time multiplex PCR assay for the detection of bovine mastitis pathogens directly from milk

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Evaluation of a commercial real-time multiplex PCR assay for the detection of bovine mastitis pathogens directly from milk

by

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DEDICATION

This thesis is dedicated to my parents Kathy and Greg who have blessed me with their guidance to this point over great distance with love and patience. Everything you have taught me has brought me to a place where I have found purpose, passion and endless opportunities. I will never forget how fortunate I am.

This thesis is also dedicated to those whose greatest challenge in life is themselves.

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NOMENCLATURE

ADSA	American Dairy Science Association
ATCC	American type culture collection
BC	Bacterial culture
BTM	Bulk tank milk
BTSCC	Bulk tank somatic cell count
CFU	Colony forming unit
CNS	Coagulase-negative Staphylococcus
Ct	Cycle threshold
DMSCC	Direct microscopy somatic cell count
ESCC	Electronic somatic cell count
FDA	Food and Drug Administration
IAC	Internal amplification control
IMI	Intramammary infection
LOD	Limit of detection
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
mL	Milliliter
N/A	Non applicable
NMC	National Mastitis Council
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline

PCR	Polymerase Chain Reaction
SCC	Somatic cell count
SDS	Sequence detection software
TBC	Total bacteria count
TLR	Toll-like receptor
qPCR	Quantitative Polymerase Chain Reaction
USDA	United States Department of Agriculture

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ABSTRACT

Bovine mastitis, prevalent in dairy cattle, is often caused by a bacterial infection in the mammary gland. Bovine mastitis is costly to the dairy industry for loss of milk production.

Causative bacteria are determined by microbiological culture. Culture remains popular for its low cost, simple procedure and interpretation however, has limitations. Culture based assays are subjective, timely and may not support fastidious organisms. Nearly 30% of clinical mastitis cases are culture-negative especially in cows treated with antibiotics. Treated cows with negative milk cultures may indeed still be infected, shedding low numbers of bacteria that do not appear in culture causing false negative results.

One potential alternative to culture is a commercial assay, PathoProof™ Mastitis PCR Assay (ThermoFisher Scientific, Waltham, MA). PathoProof is capable of detecting 11 mastitis-causing bacteria from milk. To evaluate PathoProof, the analytical specificity and limit of detection (LOD) was determined using 20 culture isolates of target and non-target bacteria. The LOD was determined by inoculating phosphate-buffered saline and milk with three different bacteria. Serial dilutions, standard plate count and PCR were performed. Further evaluation used cows that were treated with antibiotics for mastitis. Milk samples were collected from cows on days 0, 3, 7, 14 and 30 post-treatment. Samples were evaluated using culture and PathoProof PCR.

PathoProof PCR only detected target bacteria from a group of 20 target and non-target isolates resulting in an analytical specificity of 100%. Average LOD ranged from 10^3 to 10^4 CFU/mL and 10^1 to 10^3 CFU per PCR reaction, relatively high values compared to

previous investigations of other mastitis PCR assays. The high LOD suggests concerns about false negative results from cows shedding bacteria at low levels.

Data from 25 cows treated for mastitis were used to compare culture to PathoProof. More cows were PCR-positive on days 3, 7, 14 and 30 post-treatment demonstrating that PathoProof may be helpful in detecting bacteria in milk from treated cows. Information from PathoProof may be useful in evaluating efficacy of treatment and assist veterinarians and producers in making decisions. Further investigation into the assay's sensitivity and quantitative abilities is needed to better determine its value.

CHAPTER I

INTRODUCTION: THESIS FORMATTING

This thesis is organized into 4 chapters. Chapter 1 outlines the format of the thesis. Chapter 2 entitled "Review of bovine mastitis etiology and diagnostic methods" reviews general information about mastitis and current research in mastitis diagnostics. Chapter 3, "Quantitative and qualitative evaluation of real-time PCR in the detection of bovine mastitis pathogens" follows chapter 2. Chapter 4 is titled, "Evaluation of conventional bacteriological culture and real-time PCR for detection of bacteria in milk from cows treated for mastitis" followed by chapter 5 "General Conclusions." Tables and figures adjoining each chapter will be found at the end of the chapter after the list of references.

CHAPTER 2. REVIEW OF BOVINE MASTITIS ETIOLOGY AND DIAGNOSTIC METHODS

Introduction

Mastitis or intramammary infection refers to inflammation of the mammary gland and may affect all mammals. It has particular importance in the dairy industry affecting the quantity and quality of milk produced by infected cows resulting in significant economic losses. There are nearly 9.3 million dairy cows in the United States responsible for 100 million tons of milk (USDA-ERS, 2015) produced yearly. Purported as the perfect food, milk and milk products represent a significant source of protein and minerals required in the human diet. Maximizing milk production while maintaining the welfare of cows is a complex system of balancing animal nutrition, housing, animal reproduction, milking procedures and health.

Bovine mastitis is the most common cause of decreased milk production in dairy cattle which further implies the importance of prevention and control of this costly disease and protection of the global food supply (ADSA and Jones, 2006). The ever increasing need for resources to support the growing global population places the burden on food producers to maximize efficiency to meet increased demands.

The U.S. dairy industry

The U.S. dairy industry has experienced dramatic changes due to rapid growth during the past century. The American Dairy Science Association (ADSA) was formed to increase dairy production through advancements in animal health, genetic selection and farm

technologies. Since their establishment in 1906, the ADSA has collected data regarding the status of the dairy industry in the U.S along with government agencies such as the U.S. Department of Agriculture (USDA) and the Food and Drug Administration (FDA).

The previous century's dairy industry was considerably smaller and less efficient when dairy cattle remained in numbers less than ten to provide families with a source of food rather than a source of income. Between 1930 and 2014, the annual per capita consumption of dairy products in the U.S. increased to 614 pounds (USDA-ERS, 2015). Due to increased demands, the average size of dairy herds increased from 5 to 115 cows with an average annual milk production of 4,500 pounds in 1930 to nearly 20,000 pounds per cow in 2006 (ADSA and Jones, 2006). This advancement could not have come without great strides in farming technologies.

Automated milking became standard in the 1940s by use of vacuum milking units that attached to all 4 teats to collect milk through controlled pulsation (ADSA and Jones, 2006). Vacuum pulsation is used in modern milking facilities and reduces milking time and labor per cow. Automatic milking has also increased milk yield through proper emptying of the udder and has helped limit the transfer of microorganisms (Thompson, 1980).

Etiology of bovine mastitis

Since the advent of automated milking and the efficiency it brings, dairy operations have been able to expand herd size and operations by decreasing the time, labor and materials spent milking each cow. These advancements in technology have also improved sanitation methods but even in well-sanitized dairy operations, cows are still constantly exposed to microorganisms making mastitis a common threat.

Clinical mastitis

Clinical mastitis is the presence of inflammation of the mammary gland and may be attributed to injury or infection from any pathogenic microorganism due to invasion through the teat canal. Clinical signs are most often detected during premilking procedures that are performed by farm employees. Premilking procedures include the application of an antimicrobial solution to each teat, removal of excess soil and manure with cloth towels and the removal of foremilk also known as "forestripping" prior to attaching milking equipment (Hogan et al., 1999). In between milking times, bacteria present on the skin or environmental organisms from soil or manure may migrate into the teat canal and possibly further into the mammary gland. To prevent these organisms from entering the bulk tank and potential cross-contamination between cows, stripped foremilk is observed for color and consistency. Visible changes in the milk's consistency may include clotting, flaking and the presence of blood. Indications of clinical mastitis are not limited to changes in the milk. External redness and swelling of the udder, tenderness to the touch, heat and palpable abscesses may also be present in clinical mastitis cases (Hogan et al., 1999).

Subclinical mastitis

Subclinical mastitis is defined as the absence of physical signs of inflammation to the udder and is the most common form of mastitis (Orlandini, 2011). It is primarily detected by increased somatic cell count (SCC) in the milk when increased numbers of white blood cells are recruited to the mammary gland due to mild infection or trauma (Orlandini, 2011). The SCC is expressed in cells per milliliter (mL) of milk and maintains high significance in the dairy industry and is routinely measured on individual cows and from milk collected from the

bulk tank. Somatic cell counts of 200,000 cells per mL or higher is indicative of inflammation (Hogan et al., 1999).

Immunity of the mammary gland

The mammary gland is protected by physical and chemical factors that impede the invasion of microorganisms. Bacteria must enter through the teat orifice that is controlled by sphincter muscles that relax during milk removal or let-down and requires 2 hours to fully contract after completion of milking (Srivastava et al., 2015). If microorganisms pass through the teat orifice they contact the teat canal, a cylindrical duct located inside the teat opening. The stratified epithelium lining the teat canal secretes keratin, a waxy, physical barrier that forms a plug that exits the teat during initial milk ejection and reforms after milking is completed (Rainard and Riollet, 2006). Keratin also provides a chemical defense with bacteriostatic and bactericidal compounds that include long chain fatty acids. A previous study demonstrated an infection rate of approximately 26.3% for quarters that had the keratin removed prior to milking followed by exposure to *Streptococcus agalactiae* after the milking process suggesting the importance of keratin as a preventative against mastitis (Capuco et al., 1992). Despite keratin's protective effect, some bacteria have the ability to migrate into the mammary gland and cause infection.

Innate immune responses are critical during the initial stage of mastitis to respond to infection by different pathogens after they invade the mammary gland. The first to recognize the release of bacterial compounds are toll-like receptors (TLR), proteins embedded in leukocytes and epithelial cell membranes that recognize pathogen associated molecular patterns (PAMPS) such as lipopolysaccharides or peptidoglycan fragments (Rainard and Riollet, 2006; Salyers and Whitt, 2002; Wellnitz and Bruckmaier, 2012). After the

stimulation of TLR's by invading bacteria inflammation is mediated by polymorphonuclear neutrophilic (PMN) leukocytes which are recruited in vast numbers from the blood to the mammary tissue (Wellnitz and Bruckmaier, 2012). PMNs engulf and destroy the bacteria using intracellular granules that contain bactericidal enzymes and proteins such as superoxide ions, hypochlorite, hydrogen peroxide and hydrolytic enzymes (Wellnitz and Bruckmaier, 2012). When expended, PMNs will lyse and are subsequently engulfed by macrophages and destroyed (Salyers and Whitt, 2002).

Somatic Cell Count

Leukocytes inevitably end up in milk due to their massive numbers in response to infection in the mammary gland. In bovine mastitis leukocytes, macrophages, secretory and squamous cells are collectively referred to as somatic cells that are measured in individual and bulk tank samples, a value called somatic cell count (SCC) (Norman et al., 2011). An SCC level reflects the health of the udder or the health of the herd. SCC also has significance in quality determination by the Food and Drug Administration, the governing body of milk regulation. According to the 2015 Grade "A" Pasteurized Milk Ordinance (PMO), milk of grade "A" status may not have a bulk tank somatic cell count greater than 750,000 cells/mL of raw milk (FDA, 2015). Although somatic cells in milk have not been found to pose a health risk to humans (Hogan, 2005), SCC is a reflection of the hygiene practices of the operation (Moxley et al., 1978).

For the determination of mastitis, limits have been set at 200,000 cells/mL or greater as an indicator of inflammation (Norman et al., 2011). SCC is particularly useful in diagnosing subclinical mastitis as there are no visible clinical signs in the milk and has become a routine practice in mastitis diagnostics. Methods to determine SCC as approved by

the FDA are direct microscopic somatic cell count (DMSCC) or electronic somatic cell count (ESCC). DMSCC is the reference method performed by dispensing milk on a glass slide and staining with methylene blue dye allowing somatic cell nuclei to be visualized and counted (Orlandini, 2011).

Vaccines

There are many reports that have investigated the use of vaccines for prevention of bovine mastitis (Pereira et al., 2011). Currently, few vaccines are commercially available for preventing mastitis. The obvious challenge to developing a broadly efficacious bovine mastitis vaccine is the large variety of bacteria known to cause mastitis (Watts, 1988). Another challenge to develop mastitis vaccines is inducing long term immunity when the target bacterium may change antigenically or with subtypes of bacteria specific to different regions and herds. Vaccine development has primarily targeted mastitis caused by *Staphylococcus aureus* and *Escherichia coli* (Pereira et al., 2011) likely due to the pathogenicity and high prevalence of cases (Makovec and Ruegg, 2003; Oliveira et al., 2013; USDA-APHIS, 2008).

Pereira et al. published an extensive review of *S. aureus* vaccine studies and developed a scoring system to determine the efficacy of published methods. Their findings indicated that the use of recombinant proteins associated with *S. aureus* provided 50% protection in experimentally infected quarters. One of the reviewed studies, Carter and Kerr, 2003, performed this method using protein A, a virulence factor of *S. aureus*, encoded on a staphylococcal plasmid which was then transfected into cells that were injected into animals. Pereira et al. also reported 50% protection using inactivated vaccines known as bacterins or inactivated toxins known as toxoids. One such study (Leitner et al., 2003) achieved success

using a bacterin and toxoid from 3 different strains of *S. aureus* with different hemolysis patterns.

Contagious and Environmental Mastitis

Bovine mastitis is classified into two different types according to the origin of the organism and mode of exposure. Contagious mastitis is associated with several pathogens with the ability to be transmitted between cows and exist in low numbers in the environment. Environmental mastitis is caused by a variety of organisms found in the cow's environment originating from manure, soil, water or bedding. However, once a cow is infected, they will shed the organism into the environment or milking equipment with the ability to transmit the pathogen to another animal.

Contagious Mastitis

Staphylococcus aureus

Perhaps the most significant mastitis pathogen, *Staphylococcus aureus*, has been isolated from individual cow and bulk tank milk samples in 43% of U.S. dairy operations in the top 17 milk-producing states according to a USDA-APHIS study in 2007. Mastitis caused by *S. aureus* may present as clinical, subclinical and chronic infections in cows (Srivastava et al., 2015). *S. aureus* is associated with diseases of several body systems in humans and has become a critical public health concern with its increasing resistance to antimicrobials.

The *Staphylococcus* genus refers to a Gram-positive coccoid bacterium often arranged in groups or clusters. Colony morphology is large, white or yellow mucoid colonies on agar plates. Staphylococci are catalase positive and exhibit gamma or beta hemolysis although *S. aureus* is usually beta hemolytic. *S. aureus* is a common skin inhabitant of humans and the udder of cows. Most *S. aureus* infections affecting the skin are opportunistic,

requiring damage to the epidermis such as lacerations, abrasions and burns, to cause infection. *S. aureus* primarily affects dairy cows by intramammary infection and represents the most economically devastating mastitis pathogen (Oliver et al., 2004).

Some species of staphylococci produce coagulase, which is an important surface protein that is commonly used to differentiate *S. aureus* from other *Staphylococcus* spp. although it is not the only coagulase-positive *Staphylococcus* species. Coagulase proteins produced by some staphylococci are considered a virulence factor although coagulase itself is not directly involved in pathogenesis. Coagulase induces clumping of the blood by forming a complex with fibrinogen and prothrombin, two blood proteins involved in the clotting cascade (Graber et al., 2009). Coagulase mediates adherence to mammalian cells, such as red blood cells, that is thought to disguise the bacterium as a host protein to prevent recognition by the immune system (Salyers and Whitt, 2002). Coagulase status is determined by rinsing pure culture into rabbit sera in a tube or on a slide. The formation of a gel or coagulation of the serum indicates the bacterium contains the coagulase enzyme (Boerlin et al., 2003).

S. aureus has a variety of extracellular enzymes found in bovine mastitis isolates including staphylokinase, hyaluronidase, phosphatase, nuclease, lipase and catalase. These enzymes are thought to assist *S. aureus* in survival and spread in the host by destroying extracellular matrices or making milk components available as an energy source for the bacterium (Salyers and Whitt, 2002; Sutra and Poutrel, 1994). Exact mechanisms of their pathogenesis are not known.

Streptococcus agalactiae

Streptococcus agalactiae is a significant contagious pathogen in bovine mastitis with a high propensity to transmit from cow to cow, lacking the ability to thrive in the

environment. It causes clinical and subclinical mastitis in dairy cattle passing from cow to cow by the hands of milking staff, cleaning rags used on cows as well as milking machines (Hogan et al., 1999). When 530 U.S. dairy operations had milk from bulk tanks sampled, 2.6% cultured positive for *S. agalactiae*, a low percentage compared to *S. aureus* or *E.coli* which were both above 40% (USDA-APHIS, 2008).

The *Streptococcus* genus represents nonmotile, gram-positive coccoid bacteria often arranged in chained patterns. These catalase negative bacteria tolerate oxygen but may prefer anaerobic conditions and perform fermentative metabolism. They exhibit alpha, beta, and gamma hemolysis in culture and present as small to medium sized colonies (Oliver et al., 2004).

Little is understood about the virulence of *S. agalactiae*. One potential virulence factor was observed in human infections was found with the production of maternal antibody to its capsular polysaccharides suggesting the capsular polysaccharides may cause clinical symptoms (Salyers and Whitt, 2002). Bovine isolates contain the gene *hylB* encoding hyaluronidase, an enzyme capable of cleaving the extracellular matrix in tissues (Sukhnanand et al., 2005).

***Mycoplasma* species**

Mycoplasma is a genus of bacteria associated with contagious bovine mastitis. The most prevalent *Mycoplasma* species is *Mycoplasma bovis* however, 11 additional species have been isolated from bovine mastitis (Oliver et al., 2004). *Mycoplasma* spp. is considered a fastidious organism that requires 10% CO_2 in a 37°C incubator for 7 to 10 days on culture media (Hogan et al., 1999). *Mycoplasma* spp. lack a cell wall so are resistant to antibiotics that target the cell structure (Bürki et al., 2015).

Mycoplasma spp. also perpetrate respiratory infections, arthritis, otitis and genital infections in cows thus providing numerous potential reservoirs in a dairy operation (Bürki et al., 2015). Little is understood about the molecular mechanisms of pathogenicity of mycoplasmal species. Their small genomes provide a few processes that allow *Mycoplasma* spp. to adhere and survive in hosts. Adherence to host cells, heavily mediated by surface proteins, is critical for this organism as they lack biosynthetic pathways to acquire essential resources such as amino acids. *M. bovis* has been found to invade alveolar epithelial cells in the bovine mammary gland which may contribute to its ability to disseminate throughout the host (Bürki et al., 2015).

Environmental mastitis

Environmental *Streptococcus* spp.

Mastitis caused by streptococci species are attributed to *Streptococcus dysgalactiae* and *Streptococcus uberis*. Other environmental streptococci known to cause mastitis are *S. acidominus, S. canis, S. equi,* and *S. equinus*. These pathogens have been isolated from the intestinal tract and manure of dairy cattle which may contaminate bedding (Oliver et al., 2004). In dairy operations, concentrations of streptococci in bedding may reach 10^6 CFU/g in wood shavings, recycled manure and pelleted corn (Todhunter et al., 1995).

Pathogenicity of environmental streptococci is not well understood however several mechanisms allow streptococci to thwart host immunity and adhere to and invade host cells. *S. dysgalactiae* and *S. uberis* interact with host proteins fibronectin, fibrinogen, immunoglobulins, collagen and laminin which enables the bacteria to adhere to host cells. *S. dysgalactiae* contains hyaluronidase and fibrinolysin believed to allow the bacterium to disseminate in host tissue. The capsule of *S. uberis* allows it to avoid phagocytosis however,

similar capsule formation has not been observed in *S. dysgalactiae*. Both species have been found inside mammary secretory cells thought to involve host cell kinases and rearrangement of microfilaments (Calvinho et al., 1998).

Coliforms

Coliforms are gram-negative bacteria that ferment lactose to produce gas and are associated with the gastrointestinal system of animals. Coliforms are responsible for animal health and food safety concerns because they can be pathogenic and also reflect the hygienic practices of dairy operations and food processors. The presence of coliforms in any product suggests contamination with fecal material through manure, sewage or run-off. This material is likely to harbor bacteria responsible for foodborne-illness. Grade "A" milk may not contain greater than 10 coliform bacteria per mL of raw milk upon arrival at the processing plant according to the 2015 PMO (FDA, 2015).

Coliform bacteria are abundant in the environment of dairy operations (Hogan et al., 1999). The most common coliforms that can cause mastitis are *E. coli, Klebsiella pneumoniae* or *oxytoca*, and to a lesser extent *Citrobacter* species and *Enterobacter* species (Makovec and Ruegg, 2003; Oliveira et al., 2013). Bacterial isolation from milk is often achieved with selective and differential media including MacConkey's agar which is selective for gram-negative organisms and differentiates them based on their lactose fermentation result. Growth appears at 18 hours when incubated at 37°C. Further identification is often performed with differential biochemical tests for gas production, motility and carbohydrate substrate utilization (Oliver et al., 2004).

E. coli is the most common cause of coliform mastitis (Makovec and Ruegg, 2003; Oliveira et al., 2013) with the ability to induce mild to severe symptoms for a short duration.

The pathogen is often eliminated by the cow's immune system without treatment (Döpfer et al., 1999; Wenz et al., 2006). *E. coli*, like other gram negative organisms, possess an outer membrane composed of antigenic lipopolysaccharide (LPS) the component that induces the initial immune response and inflammation after infection (Salyers and Whitt, 2002). There are few consistent virulence factors among *E. coli* bovine mastitis isolates (Fernandes et al., 2011; Wenz et al., 2006) but they all have components that cause an immune reaction and allow better colonization of the organism (Shpigel et al., 2008). One of these is the ability to form biofilms on the mammary alveolar epithelial cells (Shpigel et al., 2008) thought to be mediated by type I fimbriae (Fernandes et al., 2011). Fernandes et al. further concluded that the majority of *E. coli* isolates analyzed from bovine mastitis were of a genotype associated with commensal *E. coli* found in the GI tract indicating cows are exposed when they come into contact with manure or soiled bedding.

Klebsiella is another opportunistic bacterial species found in a dairy cow's environment. *Klebsiella pneumoniae* has become significant in human medicine in the ongoing battle with multidrug resistant bacteria in hospital environments (Diancourt et al., 2005). *Klebsiella pneumoniae* resists attack by PMNs due to a thick polysaccharide capsule production that surrounds the outer membrane of the bacterium (Kanevsky-Mullarky et al., 2014). This capsule also makes *K. pneumoniae* distinguishable on an agar plate due to the mucoid appearance of the colonies (Oliver et al., 2004).

Enterococcus species

Enterococcus species were originally thought to be members of the *Streptococcus* genus and innocuous to dairy cows. *Enterococcus* species has risen in clinical occurrence in hospital environments. Like streptococci, *Enterococcus* spp. are gram positive and catalase

negative. Where the two genera differ is their reservoirs in animals where enterococci are found in the intestinal tract and manure of cows (Oliver et al., 2004) at concentrations near that of *E. coli* (Salyers and Whitt, 2004).

Viruses

With nearly 30% of bovine milk samples submitted to diagnostic laboratories for bacteriological culture yielding a no growth result (Makovec and Ruegg, 2003; Oliveira et al., 2013) it is possible that a portion of these cases are attributable to viral infections. Most viral bovine pathogens do not produce an infection local to the mammary gland but are shed through milk due to a systemic viral infection. Viral pathogens associated with dairy cattle and isolated from milk include bovine herpes virus (BHV), foot and mouth disease (FMD), parainfluenza (PI), bovine leukemia virus, vaccinia, cow pox, vesicular stomatitis, bovine papilloma virus, bovine viral diarrhea virus, rinderpest virus and bovine enterovirus (Wellenberg et al., 2002). Clinical mammary symptoms present as classic inflammation such as swelling and tenderness of the udder, fever and failure to thrive. Subclinical signs include an increased SCC and decreased milk production. Viruses BHV1, FMD, PI3 have induced clinical mastitis after experimental introduction through the intramammary route and BHV4 has induced subclinical mastitis. However, natural induction through the mammary gland is not common due to their susceptibility to the environment (Wellenberg et al., 2002).

Diagnosis of bovine mastitis

Dairy practitioners and herd managers use a variety of tools to diagnose mastitis in an animal before the next step of pathogen detection. Most often, somatic cell count (SCC), which can be determined by several methods, is used to detect mastitis. Other methods such

as metal conductivity, visual inspection and animal records assist clinicians in identifying mastitis cases.

PCR/nucleic acid tests for the detection of bovine mastitis

The characterization of polymerase chain reaction (PCR) in 1985 by Kerry Mullis incited a substantial change to virtually all areas of biological science. PCR allowed scientists to choose a segment of deoxyribonucleic acid (DNA) and exponentially increase the amount of DNA in a sample for further analysis. The first nucleic acid assays for use with bovine milk were developed to detect pathogens that are difficult to cultivate in the laboratory. Various PCR assays were used to detect *Mycobacterium avium* ssp. *paratuberculosis* (Johne's disease) (Over et al., 2011), *Mycobacterium bovis* (Antognoli et al., 2001; Sreevatsan et al., 2000), *Brucella* spp. (Romero and Lopez-Goñi 1999; Sreevatsan et al., 2000), *Coxiella burnetii* (Muramatsu et al., 1997), *Cryptosporodium* spp. (Laberge et al., 1996), and *Mycoplasma* spp. (Baird et al., 1999).

Before nucleic acid assays can be performed the genetic material must be extracted and purified from the sample. Protocols developed for bacterial DNA extraction directly from clinical samples must reduce or eliminated inhibiting substances naturally occurring in the sample that can reduce the sensitivity of the assay. Milk contains fat, carbohydrates, protein and minerals that impede the isolation of bacterial DNA. Calcium ions in milk can affect DNA replication by interfering with the magnesium cofactor of polymerase. Milk proteins impede DNA polymerase by acting as a physical barrier by sequestering the target DNA and primers. Bacterial cellular debris and polysaccharides have shown similar ability to physically block DNA polymerase by the target DNA and primers (Wilson, 1997). Raw milk

samples are often combined with enrichment media to dilute these factors (Gillespie and Oliver, 2005; Rossen et al., 1992).

For bovine mastitis diagnostics, PCR was originally used for the detection of *Mycoplasma* spp., an optimal candidate for PCR diagnostics due to its fastidious nature in culture. In 1999, Baird et al. developed a nested PCR assay to detect *Mycoplasma* spp. DNA present in bulk tank milk, individual cow milk and enriched liquid cultures with success. In a 2001 study by Riffon et. al. a multiplex PCR assay able to detect several mastitis pathogens was evaluated. *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis, S. parauberis* and *E. coli* cultures were inoculated into UHT (ultra-high temperature) pasteurized milk where samples were exposed to methods including a pre-enzymatic lysis and methods without a pre-enzymatic lysis step. Two different primer sequences were used for each pathogen target representing the16s and 23s rRNA genes to ensure specificity between related species. High specificity was achieved in this analysis with all targets properly amplified and detected and non-targets presenting no amplification. Analytical sensitivity of the assay was $3.12x10^2$ CFU/mL of milk with the pre-PCR enzymatic lysis and $5x10^3$ CFU/mL of milk from the process that did not include a pre-PCR enzymatic lysis step. The authors chose to investigate the effect on sensitivity without a pre-PCR lysis step to reduce the costs of reagents. It is evident that without a pre-PCR lysis step some sensitivity is sacrificed and will have to be considered with the assay's use.

Another multiplex PCR analysis was used for the detection of *S. agalactiae, S. uberis, S. dysgalactiae,* and *S. aureus* in bulk tank milk samples in Australia. Phuektes et al. sampled bulk tanks every 10 days for 5 collections from 42 farms for 176 samples comparing PCR to bulk tank somatic cell count (BTMSCC) and total bacteria count (TBC). Although the study

did not directly compare culture and PCR for the detection of *S. agalactiae, S. uberis, S. dysgalactiae,* and *S. aureus,* results indicated that PCR was able to detect the four organisms from bulk tank milk according to their methods.

Real-time multiplex PCR was first studied for use with bovine milk for mastitis pathogens in 2005 by Gillespie and Oliver. Species-specific primer/probe sets were designed based on separate studies validating individual real-time PCR assays for the detection of *S. agalactiae, S. uberis* and *S. aureus.* The study compared 2 commercial methods and one additional method of extracting bacterial DNA from milk samples. The most reproducible results came from the use of the enzyme pronase which acts on the casein protein, dismantling the micelle that forms with calcium and phosphorus ions, which can interfere with the PCR reaction. Analytical specificity was 100% performed with 47 non-target ATCC strains of 31 target bacterial species. Limit of detection (LOD) or analytical sensitivity was determined using UHT pasteurized milk inoculated with the three organisms with subsequent serial dilutions. The LOD of *S. aureus* was 10^3 CFU/mL and the LOD for *S. agalactiae and S. uberis* was 10^2 CFU/mL. All 3 organisms achieved an LOD of 10^0 CFU/mL with an overnight enrichment step. The assay was then tested against 192 mastitis quarter milk samples with conventional culture results indicating an analytical sensitivity of 95.5% and specificity of 99.6% for target and non-target pathogens. Increased sensitivity was observed when 20 *S. aureus and S. uberis* positive milk samples were enriched with culture broth and incubated overnight and compared to non-enrichment PCR results. It was proposed that the improvement was due to dilution of the inhibitory substances found in milk and the increased growth due to the addition of nutrient broth and incubation time. The potential concern with enriching milk samples is the high likelihood of milk samples being contaminated from the

environment or the udder skin. Enrichment allows for the propagation of environmental contaminants that are in low numbers in the original sample and when tested with PCR could deliver a false positive result prolonging testing time.

PathoProof Mastitis PCR

PathoProof™ Mastitis PCR was the first commercial multiplex, real-time PCR assay capable of simultaneously detecting eleven bovine mastitis pathogens and one resistance gene. The test was developed by Finnzymes Oy of Espoo, Finland and is currently manufactured by ThermoFisher Scientific Inc. of Waltham, Massachusetts. The assay is contained in a kit providing all the reagents needed to extract bacterial DNA from bovine milk and perform real-time PCR. The assay is offered in several forms designated the major-3, complete-12 and complete-16, each identifying significant bovine mastitis pathogens. All kits are compatible with different extraction equipment and thermocycler systems including the KingFisher 96 deepwell automatic extraction using magnetic particle processing and the Applied Biosystems® 7500 Fast thermocycler (both manufactured by ThermoFisher Scientific Inc., Waltham, MA). The complete-12 assay is performed in 4 separate reactions containing 3 different primer/probe sets to detect their respective targets. Included in each reaction is an internal amplification control (IAC), which is a fragment of DNA that in the absence of PCR inhibition should amplify during the reaction and act as a positive control. The 4 separate reactions are as follows: primer/probe set 1 detects *Staphylococcus aureus*, *Enterococcus* species (including *E. faecalis* and *E. faecium*), *Corynebacterium bovis* and an IAC primer/probe; set 2 detects *Staphylococcus blaZ*, the gene encoding beta lactamase responsible for resistance to beta-lactam antibiotics, *Escherichia coli*, *Streptococcus dysgalactiae* and an IAC; primer/probe set 3 detects *Staphylococcus* species (including

coagulase-negative *Staphylococcus* spp.), *Streptococcus agalactiae, Streptococcus uberis* and an IAC; primer/probe set 4 detects *Klebsiella* species (including *K. oxytoca* and *K. pneumoniae*), *Serratia marcescens, Trueperella pyogenes* and/or *Peptoniphilus indolicus* and an IAC.

PathoProof assays include the Norden Lab Mastitis Studio software that provides interpretation of the data collected by thermocycler systems. The Applied Biosystems® 7500 Fast system collects data using sequence detection software (SDS) which can be uploaded into the Norden software. The data is reported in a standard format provided by Norden that includes a list of targets that were determined positive in the assay and their corresponding Ct values. The process is simple and produces results in a report suitable for a client. Currently, there are publications describing the use of Norden software for analysis with the PathoProof assay (Keane et al, 2013; Cervinkova et al, 2013) but not including in-depth discussion of its use and result interpretation leaving question as to its accuracy and utility.

The first published investigation of PathoProof PCR was in 2007 by Pitkälä et al. when it was used in a comparison of different assays to detect beta-lactamase-producing *Staphylococcus* species. One hundred and seventy-five *Staphylococcus* spp. isolates were used in the comparison including *S. aureus, S. intermedius*, and coagulase-negative species including *S. epidermidis, S. chromogenes, S. cohnii, S. xylosus, S. hyicus, S. haemolyticus, S. warneri, S. saprophyticus* and *S. simulans* all originating from cases of clinical bovine and canine mastitis samples. The assays evaluated in this study were compared to a PCR reference method for the detection of the beta-lactamase gene sequence. All methods, except for PathoProof PCR, compared in this analysis were designed to detect the beta-lactamase enzyme while PathoProof PCR was the only assay included to detect the blaZ gene.

Compared to the reference method, PathoProof PCR detected all isolates with the beta-lactamase gene and did not detect the isolates that were determined negative by the reference method. PathoProof PCR was the most successful method in detecting beta-lactamase producing *Staphylococcus* spp. of all the methods compared in the study. The authors recommended PathoProof for routine beta-lactamase producing *Staphylococcus* testing however given that the assay is multiplexed and developed to extract bacterial DNA from bovine milk samples making it was impractical for the detection of single pathogens isolated from pure culture (Pitkälä et al., 2007).

An extensive validation study of PathoProof PCR was published in 2009 by Koskinen et al. with the title, "Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens." Koskinen et al. collected 643 culture isolates originating from bovine, human and companion animal mastitic milk samples from diagnostic laboratories dispersed over a wide geographical range. Targets included 525 isolates including 72 *blaZ* gene-positive staphylococci isolates used and validated by Pitkälä et al., 2007. One-hundred and eighteen isolates were non-target species. The original culture results obtained by the respective laboratories were compared to PathoProof PCR results that became the basis of Koskinen et al. specificity and sensitivity analysis. In cases where target isolates did not match their PathoProof PCR result, 16s rRNA sequencing and comparison was completed.

PathoProof PCR was successful in properly identifying 634 of the 643 isolates including all isolates originating from bovine mastitis with no false negative results. Nine isolates were found falsely positive by PathoProof PCR as confirmed by 16s rRNA sequencing including *Streptococcus pyogenes, Streptococcus sanguis, Streptococcus*

salivarius identified as *S. uberis*, and 1 *Shigella* spp. isolate identified as *E. coli*. The authors of the study reported a 100% analytical specificity for all PathoProof targets except for *S. uberis* at 99% and *E. coli* at 99.5% and a 100% analytical sensitivity for all targets using false positive and negative equations. It should be noted that this study did not apply a consistent, standard method to identify all culture isolates.

The objectives in this study were to define the analytical sensitivity and specificity of the PathoProof assay however analytical sensitivity is defined as the lowest concentration that is detectable by the assay, expressed in a numerical value (Saah and Hoover, 1997). Such figure was not described in this study despite its title. What was performed in this study more closely resembled a diagnostic sensitivity analysis by testing closely-related, non-target species however diagnostic sensitivity for PathoProof should be determined using clinical milk samples according to the definition of diagnostic sensitivity (Saah and Hoover, 1997). Analytical sensitivity is important to diagnosticians as some samples may have low concentrations of target. A threshold concentration of bacteria in a sample that signifies bovine mastitis has not been established in any literature therefore an established analytical sensitivity of PathoProof PCR may not directly translate into a diagnosis. However, analytical sensitivity is still valuable when evaluating an assay and that has yet to be determined for the PathoProof PCR.

PathoProof PCR research continued with "Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing" published in 2009. The objectives in this study were to address PathoProof PCR as a potential solution to the frequent culture-negative results in cows with clinical mastitis. Using 79 milk samples that were negative or no-growth in culture, the

authors used PathoProof PCR to detect bacteria in 43% of the milk samples tested. The Ct values obtained for the bacterial targets ranged from 22.2 to 36.7 with an average of 32.3. Detected targets included *S. uberis* (10), *S. dysgalactiae* (2), *S. aureus* (3), *T. pyogenes* (1), *E. faecalis/faecium* (1), *E. coli* (1), *Staphylococcus* species (9) and *C. bovis* (5) with some samples positive for 2 targets. The average Ct was relatively high indicating low concentration of target which is consistent with the negative culture results that are attributed to low concentrations of bacteria in a sample. Of the positive targets discretion may be needed for culture-negative samples that are positive for *Staphylococcus* spp. and *C. bovis* as they are 2 common skin inhabitants making them common contaminants in milk collections. A separate Ct threshold of significance for these 2 organisms may improve interpretation.

This study also investigated the quantitative abilities of PathoProof PCR by creating a standard curve to find the concentration of copy numbers in a sample. The authors used the kit's amplification standard, DNA concentration, amplicon length and mass to calcluate the amount of genome copies per mL of milk. The lowest Ct value among all of the samples was 22.2 for *Staphylococcus* spp. which corresponded to $1.7x10^3$ copies in the original milk sample. The results of this study are helpful for interpretation of Ct values for diagnostic purposes however similar work with empirical methods to determine the LOD is necessary to understand PathoProof PCR abilities.

Another study using bovine milk samples was published in 2010 by Koskinen et al. comparing culture to PathoProof PCR. The authors collected 780 quarter milk samples from clinical mastitis cases as well as 220 samples from cows without clinical indications of mastitis. Culture methods consisted of selective and differential media to isolate and identify

bacteria according to the standards of the National Mastitis Council. The authors did not further specify their culture identification methods.

The results showed culture able to detect bacteria in 77% of samples and PathoProof detected bacteria in 89% of the clinical milk samples. Of the 220 samples from cows with no clinical signs of mastitis, 46 of them were found to have subclinical mastitis with SCC ranging from 296,000 to 4,011,000 cells/mL. Of the 46 subclinical milk samples, culture detected bacteria in 83% of samples and PathoProof PCR detected bacteria in 92%. The authors reported high agreement between PCR and culture identifications. As seen in the previously discussed study, a significantly higher amount of the culture negative/PCR positive samples were positive for *C. bovis* and *Staphylococcus* spp. which could be a result of environmental contamination.

Future use of PathoProof PCR

It is clear that PathoProof PCR requires further research in order to assess its value as a diagnostic aid for bovine mastitis. As discussed previously, an analytical sensitivity must be established to determine whether PathoProof is able to detect low concentrations of bacteria, an ability that may be compromised in culture testing. An analytical sensitivity value would also assist diagnosticians in understanding the relative quantity of bacteria based off of Ct values. Although, some quantitative analysis in copies per mL has been performed by Taponen et al., 2010 the study did not find the minimum and maximum limits the assay was able to detect. Genomic copy number/mL may not directly translate into CFU/mL and CFU/mL is the most relevant to bovine mastitis. LOD values for this assay can be found by inoculating milk with known concentrations of bacteria then serially diluting the milk and performing standard plate count to get an accurate concentration in CFU/mL. PCR should

then be performed on all of the dilution samples to find the last dilution that is detected by the assay; this dilution should have the highest Ct value.

According to Makovec and Ruegg, 2003 and Oliveira et al., 2013 nearly one-third of clinical milk samples submitted for culture are negative or no-growth. Several potential causes have been proposed such as low concentration of bacteria in a sample, fastidious organisms with nutritional requirements beyond what typical culture provides, inhibition by milk compounds or milk from cows receiving intramammary antimicrobial treatment. Taponen et al., 2009 and Bexiga et al., 2011 reported finding bacterial DNA in 43% and 47% of clinical milk samples with negative culture results suggesting PathoProof PCR may be able to overcome some of the circumstances that cause clinical milk samples to be negative in culture. The use of PathoProof in these circumstances has not been investigated. Because molecular assays are typically more costly to perform, PathoProof PCR must find its niche in bovine mastitis diagnostics to make it worth the extra cost. One potential niche may be the use of PathoProof PCR with milk from cows undergoing antimicrobial treatment.

References

American Dairy Science Association., and C. M. Jones, 2006, One hundred years of inquiry and innovation : an illustrated history of the American Dairy Science Association: Long Prairie, MN, Banta, x, 138 p. p.

Antognoli, M. C., M. D. Salman, J. Triantis, J. Hernández, and T. Keefe, 2001, A one-tube nested polymerase chain reaction for the detection of mycobacterium bovis in spiked milk samples: an evaluation of concentration and lytic techniques: J Vet Diagn Invest, v. 13, p. 111-6.

Bexiga, R., M. T. Koskinen, J. Holopainen, C. Carneiro, H. Pereira, K. A. Ellis, and C. L. Vilela, 2011, Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing: J Dairy Res, v. 78, p. 49-55.

Bürki, S., J. Frey, and P. Pilo, 2015, Virulence, persistence and dissemination of Mycoplasma bovis: Vet Microbiol, v. 179, p. 15-22.

Baird, S. C., J. Carman, R. P. Dinsmore, R. L. Walker, and J. K. Collins, 1999, Detection and identification of Mycoplasma from bovine mastitis infections using a nested polymerase chain reaction: J Vet Diagn Invest, v. 11, p. 432-5.

Boerlin, P., P. Kuhnert, D. Hüssy, and M. Schaellibaum, 2003, Methods for identification of Staphylococcus aureus isolates in cases of bovine mastitis: J Clin Microbiol, v. 41, p. 767-71.

Calvinho, L. F., R. A. Almeida, and S. P. Oliver, 1998, Potential virulence factors of Streptococcus dysgalactiae associated with bovine mastitis: Vet Microbiol, v. 61, p. 93-110.

Capuco, A. V., S. A. Bright, J. W. Pankey, D. L. Wood, R. H. Miller, and J. Bitman, 1992, Increased susceptibility to intramammary infection following removal of teat canal keratin: J Dairy Sci, v. 75, p. 2126-30.

Carter, E. W., and D. E. Kerr, 2003, Optimization of DNA-based vaccination in cows using green fluorescent protein and protein A as a prelude to immunization against staphylococcal mastitis: J Dairy Sci, v. 86, p. 1177-86.

Cervinkova, D., H. Vlkova, I. Borodacova, J. Makovcova, V. Babak, A. Lorencova, I. Vrtkova, D. Marosevic, and Z. Jaglic, 2013, Prevalence of mastitis pathogens in milk from clinically healthy cows: Veterinarni Medicina, v. 58, p.567-575.

Diancourt, L., V. Passet, J. Verhoef, P. A. Grimont, and S. Brisse, 2005, Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates: J Clin Microbiol, v. 43, p. 4178-82.

Döpfer, D., H. W. Barkema, T. J. Lam, Y. H. Schukken, and W. Gaastra, 1999, Recurrent clinical mastitis caused by Escherichia coli in dairy cows: J Dairy Sci, v. 82, p. 80-5.

Fernandes, J. B., L. G. Zanardo, N. N. Galvão, I. A. Carvalho, L. A. Nero, and M. A. Moreira, 2011, Escherichia coli from clinical mastitis: serotypes and virulence factors: J Vet Diagn Invest, v. 23, p. 1146-52.

Filion, M., 2012, Quantitative real-time PCR in applied microbiology: Norfolk, UK, Caister Academic Press, vii, 242 p. p.

Garau, J., E. Bouza, J. Chastre, F. Gudiol, and S. Harbarth, 2009, Management of methicillin-resistant Staphylococcus aureus infections: Clin Microbiol Infect, v. 15, p. 125-36.

Gillespie, B. E., and S. P. Oliver, 2005, Simultaneous detection of mastitis pathogens, Staphylococcus aureus, Streptococcus uberis, and Streptococcus agalactiae by multiplex real-time polymerase chain reaction: J Dairy Sci, v. 88, p. 3510-8.

Graber, H. U., J. Naskova, E. Studer, T. Kaufmann, M. Kirchhofer, M. Brechbühl, W. Schaeren, A. Steiner, and C. Fournier, 2009, Mastitis-related subtypes of bovine Staphylococcus aureus are characterized by different clinical properties: J Dairy Sci, v. 92, p. 1442-51.

Hogan, J., 2005, Human Health Risks Associated with High Somatic Cell Count Milk 44th Annual Meeting, Orlando, FL, National Mastitis Council Inc.

Hogan, J. S., R. J. Gonzalez, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith, 1999, Laboratory Handbook on Bovine Mastitis: Madison, WI, National Mastitis Council.

Kanevsky-Mullarky, I., A. J. Nedrow, S. Garst, W. Wark, M. Dickenson, C. S. Petersson-Wolfe, and R. N. Zadoks, 2014, Short communication: comparison of virulence factors in Klebsiella pneumoniae strains associated with multiple or single cases of mastitis: J Dairy Sci, v. 97, p. 2213-8.

Keane, O. M., K. E. Budd, J. Flynn, and F. McCoy, 2013, Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture: Vet Rec, v. 173, p. 268.

Koskinen, M. T., J. Holopainen, S. Pyörälä, P. Bredbacka, A. Pitkälä, H. W. Barkema, R. Bexiga, J. Roberson, L. Sølverød, R. Piccinini, D. Kelton, H. Lehmusto, S. Niskala, and L. Salmikivi, 2009, Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens: J Dairy Sci, v. 92, p. 952-9.

Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W. A. van Haeringen, T. J. Lam, and S. Pyörälä, 2010, Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria: J Dairy Sci, v. 93, p. 5707-15.

Laberge, I., A. Ibrahim, J. R. Barta, and M. W. Griffiths, 1996, Detection of Cryptosporidium parvum in raw milk by PCR and oligonucleotide probe hybridization: Appl Environ Microbiol, v. 62, p. 3259-64.

Lau, H. W., and L. A. Archer, 2011, Gel electrophoresis of linear and star-branched DNA: Phys Rev E Stat Nonlin Soft Matter Phys, v. 84, p. 061916.

Leitner, G., E. Lubashevsky, A. Glickman, M. Winkler, A. Saran, and Z. Trainin, 2003, Development of a Staphylococcus aureus vaccine against mastitis in dairy cows. I. Challenge trials: Vet Immunol Immunopathol, v. 93, p. 31-8.

Makovec, J. A., and P. L. Ruegg, 2003, Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001: J Dairy Sci, v. 86, p. 3466-72.

Moxley, J. E., B. W. Kennedy, B. R. Downey, and J. S. T. Bowman, 1978, Survey of milking hygiene practices and their relationships to somatic cell counts and milk production: Journal of Dairy Science, v. 61, p. 1637-1644.

Muramatsu, Y., T. Yanase, T. Okabayashi, H. Ueno, and C. Morita, 1997, Detection of Coxiella burnetii in cow's milk by PCR-enzyme-linked immunosorbent assay combined with a novel sample preparation method: Appl Environ Microbiol, v. 63, p. 2142-6.

Norman, H. D., J. E. Lombard, J. R. Wright, C. A. Kopral, J. M. Rodriguez, and R. H. Miller, 2011, Consequence of alternative standards for bulk tank somatic cell count of dairy herds in the United States: J Dairy Sci, v. 94, p. 6243-56.

Oliveira, L., C. Hulland, and P. L. Ruegg, 2013, Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin: J Dairy Sci, v. 96, p. 7538-49.

Oliver, S. P., R. N. Gonzalez, J. S. Hogan, B. M. Jayarao, and W. E. Owens, 2004, Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality: Verona, Wisconsin, National Mastitis Council.

Over, K., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke, 2011, Current perspectives on Mycobacterium avium subsp.paratuberculosis, Johne's disease, and Crohn's disease: a Review: Critical Reviews in Microbiology, v. 37, p. 141-156.

Pereira, U. P., D. G. Oliveira, L. R. Mesquita, G. M. Costa, and L. J. Pereira, 2011, Efficacy of Staphylococcus aureus vaccines for bovine mastitis: a systematic review: Vet Microbiol, v. 148, p. 117-24.

Phuektes, P., G. F. Browning, G. Anderson, and P. D. Mansell, 2003, Multiplex polymerase chain reaction as a mastitis screening test for Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis in bulk milk samples: J Dairy Res, v. 70, p. 149-55.

Pitkälä, A., L. Salmikivi, P. Bredbacka, A. L. Myllyniemi, and M. T. Koskinen, 2007, Comparison of tests for detection of beta-lactamase-producing staphylococci: J Clin Microbiol, v. 45, p. 2031-3.

Rainard, P., and C. Riollet, 2006, Innate immunity of the bovine mammary gland: Vet Res, v. 37, p. 369-400.

Riffon, R., K. Sayasith, H. Khalil, P. Dubreuil, M. Drolet, and J. Lagacé, 2001, Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR: J Clin Microbiol, v. 39, p. 2584-9.

Romero, C., and I. Lopez-Goñi, 1999, Improved method for purification of bacterial DNA from bovine milk for detection of Brucella spp. by PCR: Appl Environ Microbiol, v. 65, p. 3735-7.

Rossen, L., P. Nørskov, K. Holmstrøm, and O. F. Rasmussen, 1992, Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions: Int J Food Microbiol, v. 17, p. 37-45.

Saah, A. J., and D. R. Hoover, 1997, "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings: Ann Intern Med, v. 126, p. 91-4.

Salyers, A. A., and D. D. Whitt, 2002, Bacterial Pathogenesis A Molecular Approach: Washington D.C. , ASM Press, 539 p.

Shpigel, N. Y., S. Elazar, and I. Rosenshine, 2008, Mammary pathogenic Escherichia coli: Curr Opin Microbiol, v. 11, p. 60-5.

Sreevatsan, S., J. B. Bookout, F. Ringpis, V. S. Perumaalla, T. A. Ficht, L. G. Adams, S. D. Hagius, P. H. Elzer, B. J. Bricker, G. K. Kumar, M. Rajasekhar, S. Isloor, and R. R. Barathur, 2000, A multiplex approach to molecular detection of Brucella abortus and/or Mycobacterium bovis infection in cattle: J Clin Microbiol, v. 38, p. 2602-10.

Srivastava, A. K., A. Kumaresan, A. Manimaran, and S. Prasad, 2015, Mastitis in Dairy Animals: An Update: Delhi, India, Satish Serial Publishing House.

Sukhnanand, S., B. Dogan, M. O. Ayodele, R. N. Zadoks, M. P. Craver, N. B. Dumas, Y. H. Schukken, K. J. Boor, and M. Wiedmann, 2005, Molecular subtyping and characterization of bovine and human Streptococcus agalactiae isolates: J Clin Microbiol, v. 43, p. 1177-86.

Sutra, L., and B. Poutrel, 1994, Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus: J Med Microbiol, v. 40, p. 79-89.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä, 2009, Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing: J Dairy Sci, v. 92, p. 2610-7.

Thompson, P. D., 1980, Milking Machines -- The Past Twenty-Five Years: Journal of Dairy Science, v. 64, p. 1344-1357.

Todhunter, D. A., K. L. Smith, and J. S. Hogan, 1995, Environmental streptococcal intramammary infections of the bovine mammary gland: J Dairy Sci, v. 78, p. 2366-74.

Trun, N. J., and J. E. Trempy, 2004, Fundamental bacterial genetics: Malden, MA, Blackwell, xiii, 287 p. p.

United States. Animal and Plant Health Inspection Service. Veterinary Services., and National Animal Health Monitoring System (U.S.), 2008, Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations, 2007 Info Sheet, Fort

Collins, CO., U.S. Dept of Agriculture, Animal and Plant Health Inspection Service National Animal Health Monitoring System.

United States. Food and Drug Administration., 2014, Grade "A" pasteurized milk ordinance: Silver Spring, MD Washington, DC, U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration U.S. Government Printing Office, xvii, 412 pages p.

USDA-ERS, 2015, Milk Cows and Production by State and Region, in U. S. D. o. A. E. R. Service, ed., Washington DC, USDA ERS.

Watts, J. L., 1988, Etiological agents of bovine mastitis: Vet Microbiol, v. 16, p. 41-66.

Wellenberg, G. J., W. H. van der Poel, and J. T. Van Oirschot, 2002, Viral infections and bovine mastitis: a review: Vet Microbiol, v. 88, p. 27-45.

Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Ellis, and R. J. Magnuson, 2006, Escherichia coli isolates' serotypes, genotypes, and virulence genes and clinical coliform mastitis severity: J Dairy Sci, v. 89, p. 3408-12.

Wilson, I. G., 1997, Inhibition and facilitation of nucleic acid amplification: Appl Environ Microbiol, v. 63, p. 3741-51.

CHAPTER 3. QUANTITATIVE AND QUALITATIVE EVALUATION OF REAL-TIME PCR IN THE DETECTION OF BOVINE MASTITIS PATHOGENS

Abstract

Bovine mastitis is a common and costly disease of dairy cows and continues to be a persistent problem in the dairy industry. Bacterial infections are the most common cause of mastitis and can be detected through microbiological culture. Culture is the current gold standard diagnostic test to detect mastitis-associated bacteria although it does not favor the growth of fastidious organisms or bacteria in low concentrations. An alternative to culture is the use of PCR assays. PathoProof™ Mastitis PCR is a commercial multiplex real-time PCR assay capable of detecting 11 common bacteria associated with bovine mastitis. Analytical specificity was evaluated using the 11 target bacteria and 9 non-target isolates from a bovine mastitis teaching set and clinical isolates. Analytical sensitivity was evaluated using three bacterial isolates and serial dilutions in phosphate buffered saline (PBS) and milk to compare standard plate counts and PCR to determine a limit of detection. Data was analyzed using the manufacturer's Norden Lab Mastitis Studio software as well as the ABI 7500 Sequence Detection Software (SDS). PathoProof demonstrated 100% specificity detecting only the target bacteria. The limit of detection for PathoProof PCR was 10^4 CFU/mL which is lower than expected causing concern for false negatives in samples with concentrations below that limit. The Norden software analysis generated different results compared to the SDS software supplied by the ABI 7500. The Norden results were found to be false positives caused by non-sigmoidal curves that were not consistent with real-time PCR results.

PathoProof PCR has the potential to be an alternative diagnostic tool to bacterial culture when used with SDS software and careful observation of real-time PCR graphs.

Introduction

Mastitis or intramammary infection (IMI) causes decreased milk production and economic loss to dairy farmers affecting all dairy operations (Hogan et al., 1999). Bacteria are the most common cause of mastitis after invasion and colonization of the mammary gland. The bacteria most commonly detected in bovine mastitis include *Staphylococcus aureus*, coagulase-negative *Staphylococcus* species, environmental *Streptococcus* species and coliform bacteria such as *Escherichia coli* (Makovec and Ruegg, 2003; Watts, 1988). Bacterial infections of the mammary gland can manifest as clinical or subclinical mastitis although both result in decreased milk production (Watts, 1988). Preventing mastitis relies on adequate sanitation of both the lactating cow and the milking environment. Successful treatment requires rapid identification of causative agents and subsequent segregation and treatment of affected animals (Wilson et al., 1999). Multiple tests are available to diagnose mastitis in affected cows and can be used to help evaluate success of treatment (Viguier et al., 2009). However, diagnosing mastitis is a time consuming process and requires sensitive, specific and rapid diagnostic tests.

Bacterial culture of aseptically collected milk samples is considered the gold standard method for diagnosing mastitis (Hogan et al., 1999). Bacterial cultures are relatively simple to conduct, economical and have been well validated. With the proper media and incubation conditions, most bacterial causes of bovine mastitis can be cultivated in the laboratory with subsequent antibiotic sensitivity to help guide treatment decisions. However, limitations of

culture may include the length of incubation prior to detection, difficulty isolating fastidious organisms or lack of bacterial growth due to antibiotic treatment or other unknown reasons. Alternative methods that may help detect or diagnose mastitis are needed. One option is the use of real-time, multiplex PCR (qPCR).

In 2008 a commercial multiplex qPCR kit known as PathoProof Mastitis PCR™ (ThermoFisher Scientific, Waltham, MA) was approved for use to detect several common bacterial causes of bovine mastitis. The qPCR kit provides the reagents necessary to extract bacterial DNA directly from milk and conduct qPCR. The Complete 12 kit offers primer/probe sets for the following organisms: *Staphylococcus aureus*, coagulase negative *Staphylococcus* species, *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Enterococcus faecalis/faecium, Escherichia coli, Klebsiella pneumoniae/oxytoca, Corynebacterium bovis, Trueperella (Arcanobacterium*) *pyogenes/ Peptoniphilus indolicus* and *Serratia marcescens*. In addition, PathoProof PCR is able to detect the *Staphylococcus* spp. beta lactamase gene *blaZ* responsible for beta-lactam antimicrobial resistance.

Real-time PCR may be used as an alternative to culture due to several advantages. One difference between culture and PCR is the time it takes from processing samples to delivery of results. Within 4 hours, DNA extraction and real-time PCR with simultaneous detection of all 12 targets is completed. In contrast, bacterial culture requires at least 48 hours to allow enough time for fastidious mastitis pathogens, such as *Trueperella pyogenes*, to grow (Oliver et al., 2004). Furthermore, only viable bacteria are detected by culture. However, PathoProof PCR does not distinguish between viable and non-viable bacteria in a

sample and may complicate the ability to distinguish between infected and recovering animals.

One favorable aspect of culture has been the low cost to the dairy producer to perform the assay. Generally, PCR is more costly due to its requirement of more expensive equipment and trained technicians. Milk culture does not require extensive laboratory training or experience to perform the assay but additional training may be necessary for interpretation of the results. Bovine milk samples are easily contaminated during collection due to the presence of microbiota on the skin and environmental organisms, which must be considered when interpreting milk cultures. Populations of bacteria on culture plates must be evaluated for significance making the results highly subjective. PathoProof PCR delivers a list of detected pathogens with corresponding Cts.

Accompanying the PathoProof PCR kit is the interpretive software, Norden Lab Mastitis Studio (ThermoFisher Scientific, Waltham, MA). The PathoProof PCR kit and Norden software are compatible with 3 different real-time PCR systems including the Applied Biosystems™ 7500 Fast (ThermoFisher Scientific, Waltham, MA). Norden software uses the data collected from the real-time PCR instrument to produce a report containing cycle threshold values (Cts) for the detected targets. The software applies target-specific Ct ranges that correspond to a low (+), moderate (++) and high (+++) quantity of target. The Norden program issues results in a well-ordered format that can be easily understood by veterinarians and herd managers; Use of the Norden software for analysis of PathoProof data has been reported previously (Keane et al., 2013; Cervinkova et al., 2013) but its interpretation has not been addressed.

Several studies have analyzed the diagnostic specificity and sensitivity and analytical specificity of PathoProof PCR (Keane et al., 2013; Koskinen et al., 2009; Taponen et al., 2009;Wellenberg et al., 2010) however an established analytical sensitivity, or limit of detection (LOD) concentration, has not been determined. The objectives of this study were to a) determine the analytical specificity using an array of complete 12 target and non-target bacterial isolates and b) determine the limit of detection in colony forming units per milliliter (CFU/mL) using target bacterial dilutions spiked into phosphate buffered saline (PBS) and milk and c) compare cycle threshold values between Norden Lab Mastitis Studio software and the Applied Biosystems Sequence Detection System (SDS) software.

Materials and Methods

Specificity evaluation bacterial culture

Bacterial isolates used in the specificity analysis were obtained from the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) Clinical Microbiology Laboratory from a bovine mastitis teaching set. All 11 bacterial targets included in the PathoProof complete 12 kit were used for specificity analysis as well as 9 non-target bacteria. The eleven bacterial targets included *Staphylococcus aureus, Staphylococcus simulans* (coagulase-negative staphylococci), *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Corynebacterium bovis, Trueperella pyogenes* and *Serratia marcescens*. Nine non-target bacterial and fungal species isolated from bovine mastitis cases at the ISU VDL used in this evaluation included *Citrobacter koseri, Lactobacillus gasseri, Pasteurella multocida,* yeast, *Pseudomonas aeruginosa, Enterobacter cloacae, Lactococcus garvieae, Enterococcus saccharolyticus* and *Streptococcus pluranimalium*. Identity of bacteria were confirmed by

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) which has been used previously to identify bovine mastitis pathogens (Barreiro et al., 2010; Schabauer et al., 2014). Results from the MALDI-TOF MS were reported on a log scoring system with values between 0 and 3. Scores of 1.7 or higher are considered reliable for genus-level identification and scores higher than 2 are considered reliable for species identification (Barreiro et al., 2010).

DNA extraction

Bacteria were cultured on 6% sheep blood agar and incubated for 24 hours at 37° C. Using a sterile plastic loop, 5 bacterial colonies were selected and suspended directly in the lysis solution provided by the PathoProof DNA extraction kit. Subsequent steps for extracting bacterial DNA were performed with the KingFisher Flex Purification System (ThermoFisher Scientific, Waltham MA) for DNA purification by magnetic particle processing according to the kit's protocol.

Real-time PCR

PathoProof qPCR was conducted according to the manufacturer's instructions. Four separate PCR reactions with primer/probes for three of the 12 targets as well as an internal amplification control (IAC) for each set are used in this assay. Each PCR run used a negative control to monitor cross-contamination. PathoProof PCR targets are listed in Table 1.

The PathoProof real-time PCR profile for use with the Applied Biosystems 7500 Fast real-time PCR system consists of 10 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and one minute at 60°C and a final stage of 5 seconds at 25°C. Ct data was collected using Applied Biosystems Sequence Detection Software and uploaded into Norden Lab Mastitis Studio.

Sensitivity evaluation

To translate analytical sensitivity into CFU/mL, unpasteurized bovine milk and PBS were inoculated with pure culture of three different organisms and serially diluted for standard plate count. The organisms included were *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Escherichia coli*. The turbidity standard, 0.5 McFarland (10^8 CFU/mL), was created separately for each organism in sterile saline using a nephelometer. One milliliter of the 0.5 McFarland, acting as the 10^0 sample, was transferred to 9mL of PBS followed by 8 consecutive 10-fold dilutions. One hundred microliters of each dilution were dispensed and spread with a sterile plastic loop on a 20mL plate of 6% sheep blood agar. A 0.5 McFarland turbidity standard was also created in PBS in which 1mL was transferred to 9mL of unpasteurized, PCR-negative bovine milk followed by 8 consecutive 10-fold dilutions. Similar to the PBS dilutions, one hundred microliters of each milk dilution were dispensed and spread with a sterile plastic loop on a 20mL plate of 6% sheep blood agar. Cultures were incubated at 37° C for 48 hours and colonies counted. Serial dilutions and standard plate counts were performed in triplicate for each organism/diluent combination. An aliquot of 400µL of each dilution was used for DNA extraction and subsequent qPCR according to the manufacturer's protocol.

Results

Analytical specificity of PathoProof qPCR

The PathoProof qPCR correctly detected the eleven bacterial target isolates included in the assay, *S. aureus, S. simulans* (coagulase-negative staphylococci), *S. agalactiae, S. uberis, S. dysgalactiae, E. faecalis, E. coli, K. pneumoniae, C. bovis, T. pyogenes* and *S.*

marcescens. The bacteria used for the specificity analysis were also identified by MALDI-TOF MS with scores from each isolate above 2.0 (Table 2). The range of Ct values across all organisms detected by PathoProof and SDS analysis was 17.89 to 33.93 with an average of 21.77. The 9 non-target isolates, *C. koseri, L. gasseri, P. multocida, T. montevideense* (yeast), *P. aeruginosa, E. cloacae, L. garvieae, E. saccharolyticus* and *S. pluranimalium*, were also supported by MALDI-TOF MS analysis scores above 2.0 excluding *S. pluranimalium* and *T. montevideense* (Table 3). The *S. pluranimalium* and *T. montevideense* both received scores above 1.7 confirming identification to the genus level and *T. montevideense* was confirmed as a yeast organism with a Gram stain. All 9 non-target isolates were undetected by the PathoProof qPCR assay under the conditions of this study.

PathoProof qPCR data analysis

PCR data collected by SDS was transferred to the Norden software program. Subsequent Norden analysis correctly detected the bacterial target in the sample but also incorrectly reported bacteria not included in this analysis with a wide range of Ct values (Table 2). Upon further investigation of the raw qPCR data, extraneous targets were detected when the software interpreted atypical fluorescence curves as positive results. This error was not observed using the SDS software for qPCR data analysis.

Analytical sensitivity of PathoProof qPCR

Three organisms, *S. aureus, E. coli* and *S. dysgalactiae*, spiked into PBS and unpasteurized milk were diluted, tested by PathoProof qPCR in triplicate, and subjected to standard plate counting. Plates with colony counts in the reliable range of 25 to 250 (FDA, 2011) were used to calculate total CFU/mL for each dilution. The highest dilution detected

by PCR (the highest Ct) and its corresponding plate count were considered the analytical sensitivity or limit of detection (LOD) for that organism/diluent combination.

The overall mean LOD for all three bacteria was 10^4 CFU/mL ranging from 10^3 to 10^4 CFU/mL (Table 4). There was no noticeable trend in LOD CFU/mL between PBS and milk. The average LOD Ct values ranging from 30.05 to 39.5 demonstrated no discernable patterns between organisms and diluent used.

Discussion

The specificity analysis in this study demonstrated similar results to Koskinen et al. 2009 where 98% (634 of 643) of target isolates (n=525) and non-target isolates (n=118) were correctly detected or undetected, respectively, using PathoProof qPCR. In the current study, PathoProof correctly detected the 11 bacterial target isolates included in the assay. Conversely, the 9 non-target isolates were not detected by qPCR indicating 100% specificity under conditions of this study (Table 2). The staphylococcal *blaZ*, beta lactamase gene was not tested in this study. Beta lactamase-producing staphylococci were studied by Pitkälä et al. in which PathoProof PCR was able to correctly identify *blaZ* gene-positive isolates compared to a reference PCR method with 100% sensitivity. The results of this study and others (Keane et al., 2013; Koskinen et al., 2009; Taponen et al., 2009; Wellenberg et al., 2010) suggest the complete 12 version of the PathoProof qPCR assay is appropriate for accurately detecting 11 common pathogens isolated from bovine mastitis. However, dairy cows are exposed to a variable number of microorganisms in their environment that could cross-contaminate milk samples during collection. Therefore, it is necessary PCR assays for detecting mastitis-associated pathogens are highly specific. Nearly 140 microbial species have been isolated

from bovine mastitis cases (Watts, 1988) with variable prevalence in dairy herds. *S. agalactiae* has reduced in prevalence in recent years (USDA-APHIS, 2008) from a more devastating pathogen due to interventions by veterinarians and herd managers such as sanitation. *S. agalactiae* is an example of the way mastitis pathogens change in prevalence after intervention making the inclusion of primer/probes for *S. agalactiae* in a diagnostic PCR assay less appropriate due to the decreased likelihood of *S. agalactiae* infections. Typical culture media is capable of growing *S. agalactiae* without the addition of selective ingredients which is more efficient when *S. agalactiae* are infrequently isolated. Bacterial culture's lack of specificity can be advantageous as it allows many of the nearly 140 microorganisms isolated from bovine mastitis to propagate (Watts, 1988) as well as limiting because cross-contaminating organisms in the sample will grow as well making interpretation difficult.

To the author's knowledge this is the first study to report the analytical sensitivity of PathoProof PCR in CFU/mL. Analytical sensitivity reflects the concentration at which the target organism can be detected (Saah and Hoover, 1997). Analytical sensitivity is determined empirically to achieve a quantifiable value based on the LOD. The method used in this study revealed a relatively high LOD of 10^4 CFU/mL. An assay lacking in sensitivity is particularly concerning when working with pooled samples. Sampling all four quarters into one container, called composite milk samples, creates the risk of diluting the bacteria to a level where they would not be detectable. Composite samples are often used when testing cows. The same risk applies to bulk tank milk samples. Bulk tank milk samples are often tested for mastitis pathogens using standard plate count to gain an understanding of their prevalence within the herd. PathoProof has been evaluated for use on bulk tank milk

(Katholm et al., 2012) where PathoProof was able to detect all 12 targets in bulk tank milk from 4,258 herds. The study compared PCR results to culture which was also able to detect all 12 targets however the researchers did not perform quantitative analysis by culture. A comparison of the quantitative abilities of culture and PathoProof PCR would be instrumental in helping herd managers understand the presence as well as prevalence of mastitis pathogens in bulk tank milk.

Clinical milk samples have been used to compare conventional culture methods to PathoProof PCR in previous studies (Cederlöf et al., 2012; Hiitiö et al., 2015; Keane et al., 2013; Koskinen et al., 2010; Zadoks et al., 2014). With the exception of Hiitiö et al., PathoProof PCR detected bacteria in more clinical samples than culture. Two of these studies found significant numbers of samples that were culture negative/PCR positive for *Staphylococcus* spp. and *C. bovis* (Koskinen et al., 2010; Zadoks et al., 2014). *Staphylococcus* spp. and *C. bovis* are both present as microbiota on the skin of the udder making it possible that the high proportion of culture negative/PCR positive is due to the contamination of these organisms in low enough numbers that they would be undetected by culture. Contamination is taken into consideration when interpreting milk cultures. Limits developed by the National Mastitis Council assist in identifying significant growth on a culture plate (Hogan et al., 1999). Because PathoProof is semiquantitative, the significance of results is not as easily determined.

Nearly 30% of clinical milk samples are negative or no-growth in culture for several reasons, not all of them known (Makovec and Ruegg, 2003; Oliveira et al., 2013). PathoProof PCR has shown success in detecting bacteria in previously culture negative samples (Bexiga et al., 2011; Taponen et al., 2009). Raw milk has some natural components

bacteriostatic or bactericidal components such as fatty acids, lysozyme, lactoferrin and lactoperoxidase (Ekstrand, 1989). Although bacteria can overcome these compounds to cause infection in the mammary gland, bacteria may not be able to survive the effects of these compounds in a collected milk sample, decreasing the numbers of viable bacteria to an undetectable amount. Negative culture results are also observed in cows being treated with intramammary antibiotics (Makovec and Ruegg, 2003; Oliveira et al., 2013). PCR is able to detect bacteria in the presence of antibiotics or natural inhibitory compounds because it does not rely on viable bacteria for detection. This makes PathoProof PCR a suitable test for culture negative milk or when cows have been treated with antibiotics.

The false positive results observed in the Norden software (Table 2) are concerning as its use does not appear to require much real-time PCR experience by the technician. These results confirm the importance of evaluating raw PCR data prior to interpreting the results. In the study reported here, some of the incorrectly reported Ct values were due to aberrant curves that crossed the PCR threshold at Ct values as low as 17.4. The analysis parameters used by the Norden software are proprietary and not provided by the manufacturer. The aberrant curves were not observed using the SDS software for analysis. SDS was chosen to analyze data for all PCR performed for this study.

Conclusion

PathoProof PCR has performed highly in analytical specificity, achieving 100% specificity in this study as well as others. The analytical sensitivity of the assay, 10^4 CFU/mL, is concerning and should be further investigated using clinical samples. Real-time PCR's rapid results and semi-quantitative ability are distinct advantages in diagnostic

microbiology, the latter of which deserves additional research for this assay. While Norden software did not accurately report the results, SDS was able to analyze the PCR data with high specificity. PathoProof PCR has the potential to replace bacterial culture however further investigation into its capabilities is warranted.

References

Barreiro, J. R., C. R. Ferreira, G. B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M. N. Eberlin, and M. V. dos Santos, 2010, Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: J Dairy Sci, v. 93, p. 5661-7.

Bexiga, R., M. T. Koskinen, J. Holopainen, C. Carneiro, H. Pereira, K. A. Ellis, and C. L. Vilela, 2011, Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing: J Dairy Res, v. 78, p. 49-55.

Cederlöf, S. E., N. Toft, B. Aalbaek, and I. C. Klaas, 2012, Latent class analysis of the diagnostic characteristics of PCR and conventional bacteriological culture in diagnosing intramammary infections caused by Staphylococcus aureus in dairy cows at dry off: Acta Vet Scand, v. 54, p. 65.

Cervinkova, D., H. Vlkova, I. Borodacova, J. Makovcova, V. Babak, A. Lorencova, I. Vrtkova, D. Marosevic, and Z. Jaglic, 2013, Prevalence of mastitis pathogens in milk from clinically healthy cows: Veterinarni Medicina, v. 58, p.567-575.

Ekstrand, B., 1989, Antimicrobial Factors in Milk - A review: Food Biotechnology, v. 3, p. 105-126.

Hiitiö, H., R. Riva, T. Autio, T. Pohjanvirta, J. Holopainen, S. Pyörälä, and S. Pelkonen, 2015, Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture: J Dairy Res, v. 82, p. 200-8.

Hogan, J. S., R. J. Gonzalez, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith, 1999, Laboratory Handbook on Bovine Mastitis: Madison, WI, National Mastitis Council.

Katholm, J., T. W. Bennedsgaard, M. T. Koskinen, and E. Rattenborg, 2012, Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens: J Dairy Sci, v. 95, p. 5702-8.

Keane, O. M., K. E. Budd, J. Flynn, and F. McCoy, 2013, Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture: Vet Rec, v. 173, p. 268.

Koskinen, M. T., J. Holopainen, S. Pyörälä, P. Bredbacka, A. Pitkälä, H. W. Barkema, R. Bexiga, J. Roberson, L. Sølverød, R. Piccinini, D. Kelton, H. Lehmusto, S. Niskala, and L. Salmikivi, 2009, Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens: J Dairy Sci, v. 92, p. 952-9.

Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W. A. van Haeringen, T. J. Lam, and S. Pyörälä, 2010, Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria: J Dairy Sci, v. 93, p. 5707-15.

Makovec, J. A., and P. L. Ruegg, 2003, Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001: J Dairy Sci, v. 86, p. 3466-72.

Oliveira, L., C. Hulland, and P. L. Ruegg, 2013, Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin: J Dairy Sci, v. 96, p. 7538-49.

Oliver, S. P., R. N. Gonzalez, J. S. Hogan, B. M. Jayarao, and W. E. Owens, 2004, Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality: Verona, Wisconsin, National Mastitis Council.

Orlandini, S., 2011, Reference system for somatic cell counting in milk 3rd International Symposium on Mastitis and Milk Quality, St. Louis, MO, National Mastitis Council.

Ott, S. L., 1999, Costs of Herd-Level Production Losses Associated with Subclinical Mastitis in U.S. Dairy Cows: National Mastitis Council 38th Annual Meeting.

Over, K., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke, 2011, Current perspectives on Mycobacterium avium subsp. paratuberculosis, Johne's disease, and Crohn's disease: a Review: Critical Reviews in Microbiology, v. 37, p. 141-156.

Saah, A. J., and D. R. Hoover, 1997, "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings: Ann Intern Med, v. 126, p. 91-4.

Schabauer, L., M. Wenning, I. Huber, and M. Ehling-Schulz, 2014, Novel physico-chemical diagnostic tools for high throughput identification of bovine mastitis associated gram-positive, catalase-negative cocci: BMC Vet Res, v. 10, p. 156.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä, 2009, Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing: J Dairy Sci, v. 92, p. 2610-7.

United States. Animal and Plant Health Inspection Service. Veterinary Services., and National Animal Health Monitoring System (U.S.), 2008, Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations, 2007 Info Sheet, Fort

Collins, CO., U.S. Dept of Agriculture, Animal and Plant Health Inspection Service National Animal Health Monitoring System.

United States. Food and Drug Administration., 2014, Grade "A" pasteurized milk ordinance: Silver Spring, MD Washington, DC, U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration U.S. Government Printing Office, xvii, 412 pages p.

Viguier, C., S. Arora, N. Gilmartin, K. Welbeck, and R. O'Kennedy, 2009, Mastitis detection: current trends and future perspectives: Trends Biotechnol, v. 27, p. 486-93.

Watts, J. L., 1988, Etiological agents of bovine mastitis: Vet Microbiol, v. 16, p. 41-66.

Wellenberg, G., O. Sampimon, A. Rothkamp, W. van Haeringen, and T. Lam, 2010, Detection of mastitis pathogens by real-time PCR in clinical and subclinical mastitis samples: Fifth International Dairy Federation Mastitis Conference.

Wellenberg, G. J., W. H. van der Poel, and J. T. Van Oirschot, 2002, Viral infections and bovine mastitis: a review: Vet Microbiol, v. 88, p. 27-45.

Wellnitz, O., and R. M. Bruckmaier, 2012, The innate immune response of the bovine mammary gland to bacterial infection: Vet J, v. 192, p. 148-52.

Wilson, D. J., R. N. Gonzalez, K. L. Case, L. L. Garrison, and Y. T. Gröhn, 1999, Comparison of seven antibiotic treatments with no treatment for bacteriological efficacy against bovine mastitis pathogens: J Dairy Sci, v. 82, p. 1664-70.

Zadoks, R. N., R. Tassi, E. Martin, J. Holopainen, S. McCallum, J. Gibbons, and K. T. Ballingall, 2014, Comparison of bacteriological culture and PCR for detection of bacteria in ovine milk--sheep are not small cows: J Dairy Sci, v. 97, p. 6326-33.

Tables

Table 1. PathoProof Mastitis PCR targets included in each PCR reaction. (IAC-internal amplification control, *blaZ*-staphylococcal gene for beta-lactamase)

PCR Targets Reaction 1	PCR Targets Reaction 2	PCR Targets Reaction 3	PCR Targets Reaction 4

• *Staphylococcus aureus*
• *Enterococcus* species
  • (including *E. faecalis* and *E. faecium)*
• *Corynebacterium bovis*
• IAC
	• *blaZ*
	• *Escherichia coli*
	• *Streptococcus dysgalactiae*
	• IAC
		• *Staphylococcus* species
		  • (including coagulase-negative *Staphylococcus* species)
		• *Streptococcus agalactiae*
		• *Streptococcus uberis*
		• IAC
			• *Klebsiella* species
			  • (including *K. oxytoca* and *K. pneumoniae*)
			• *Serratia marcescens*
			• *Trueperella pyogenes* and *Peptoniphilus indolicus*
			• IAC

Table 2. Specificity analysis of bacterial isolates, MALDI-TOF MS scores and PathoProof qPCR data analysis using System Detection Software and Norden Mastitis Studio software

Bacterial Isolate	**MALDI Score**	**SDS Analysis Result**	**SDS Ct Value**	**Norden Analysis Result**	**Norden Ct Value**
Staphylococcus aureus	2.321	*S. aureus* *Staphylococcus* spp.	21.1 19.9	*S. aureus* *Staphylococcus* spp. *blaZ* gene *T. pyogenes*	19.2 18.7 30.8 36.0
Staphylococcus simulans	2.316	*Staphylococcus* spp.	20.9	*Staphylococcus* spp. *S. aureus*	19.9 30.1
Streptococcus agalactiae	2.492	*S. agalactiae*	20.4	*S. agalactiae* *S. uberis* *T. pyogenes*	18.2 21.2 38.7
Streptococcus uberis	2.627	*S. uberis*	21.5	*S. uberis*	19.2
Streptococcus dysgalactiae	2.266	*S. dysgalactiae*	18.6	*S. dysgalactiae* *E.coli*	17.4 32.5
Enterococcus faecalis	2.418	*Enterococcus* spp.	23.0	*Enterococcus* spp.	21.4
Escherichia coli	2.500	*E. coli*	23.5	*E. coli* *blaZ* gene	20.6 37.6
Klebsiella pneumoniae	2.396	*Klebsiella* spp.	23.3	*Klebsiella spp.*	22.0
Corynebacterium bovis	2.239	*C. bovis*	18.5	*C. bovis*	19.9
Serratia marcescens	2.398	*S. marcescens*	33.9	*S. marcescens*	29.1
Trueperella pyogenes	2.364	*T. pyogenes/P. indolicus*	20.4	*T. pyogenes*	18.5

Table 3. Specificity analysis of non-target bacterial isolates, MALDI-TOF MS scores and PathoProof qPCR data analysis using System Detection Software and Norden Mastitis Studio software

Bacterial Isolate	MALDI Score	SDS Analysis	SDS Ct Value	Norden Analysis	Norden Ct Value
Citrobacter koseri	2.462	Undetected	N/A	Undetected	N/A
Lactobacillus gasseri	2.363	Undetected	N/A	Undetected	N/A
Pasteurella multocida	2.342	Undetected	N/A	Undetected	N/A
Trichosporon montevideense	1.888	Undetected	N/A	*E.coli*	29.1
Pseudomonas aeruginosa	2.468	Undetected	N/A	Undetected	N/A
Enterobacter cloacae	2.173	Undetected	N/A	Undetected	N/A
Lactococcus garvieae	2.334	Undetected	N/A	*Klebsiella* spp. *S. marcescens*	36.4 25.8
Enterococcus saccharolyticus	2.243	Undetected	N/A	Undetected	N/A
Streptococcus pluranimalium	1.886	Undetected	N/A	Undetected	N/A

Table 4. Analytical sensitivity analysis using *S. aureus, S. dysgalactiae* and *E. coli* diluted in PBS and milk.

Bacterial Isolate	Diluent	Mean LOD (CFU/mL)	Standard Deviation (CFU/mL)	Range (CFU/mL)	Mean LOD (CFU/per rxn)	Mean Ct	Standard Deviation Ct	Range Ct
S. aureus	PBS	$9.3x10^4$	$7.7x10^4$	$1.1x10^4$ - $1.6x10^5$	$1.2x10^3$	36	2.2	33.7-38.0
S. aureus	Milk	$2.0x10^4$	$7.3x10^3$	$1.2x10^4$ - $2.5x10^4$	$2.7x10^2$	33.5	1.8	32.4-35.5
S. dysgalactiae	PBS	$5.1x10^3$	$3.5x10^3$	$1.1x10^3$ - $7.7x10^3$	$6.8x10^1$	36.6	2.9	33.2-38.3
S. dysgalactiae	Milk	$9.2x10^3$	$2.1x10^3$	$7.4x10^3$ - $1.2x10^4$	$1.2x10^2$	30.1	0.2	29.8-30.2
E. coli	PBS	$1.0x10^4$	$7.7x10^3$	$1.4x10^3$ - $1.6x10^4$	$1.4x10^2$	37.6	1.8	36.3-39.6
E. coli	Milk	$9.9x10^3$	$1.4x10^3$	$8.5x10^3$ - $1.1x10^4$	$1.3x10^2$	39.5	0	39.7-39.8

CHAPTER 4. EVALUATION OF CONVENTIONAL BACTERIOLOGICAL CULTURE AND REAL-TIME PCR FOR DETECTION OF BACTERIA IN MILK FROM COWS TREATED FOR MASTITIS

Abstract

One of the most economically important diseases in the dairy industry is mastitis or intramammary infection. Inflammation of the mammary gland is often caused by opportunistic bacteria present in the environment that access the mammary gland. Production loss and treatment costs are the major sources of financial loss due to bovine mastitis making rapid diagnosis and treatment of high importance. Diagnosis is often done using microbiological culture, the industry standard for identifying mastitis-causing bacteria. Once the causative bacteria are identified proper treatment can be administered. Cows treated with intramammary antibiotics often produce negative culture results after subsequent culture due to a reduction in magnitude of bacteria remaining in the mammary gland. This can lead to false negative results and producers may prematurely consider the cow no longer infected. An alternative to culture is PathoProof™ Mastitis PCR (ThermoFisher Scientific, Waltham, MA), a real-time, multiplex PCR assay designed to detect 11 common mastitis-associated pathogens directly from milk. As PCR detection does not rely on organism viability, it may be a more sensitive diagnostic test on milk from cows treated for mastitis. The objective of this study was to collect milk samples from cows treated for mastitis and compare conventional culture with PathoProof PCR. Culture and PCR results agreed for day 0 in all 25 cows that were enrolled in the study. The subsequent collections revealed more culture negative/PCR positive samples on day 3 (19 cows), 7 (13 cows), 14 (13 cows) and 30 (6 cows) post treatment initiation. All samples positive on culture were also positive on PCR.

The number of culture positive cows declined after day 0 with 1 cow on day 3, 1 cow on day 7, 3 cows on day 14 and 1 cow on day 30. Although exact mechanisms remain unknown, PathoProof PCR may be useful for early mastitis detection or treatment performance in treated cows for mastitis.

Introduction

Mastitis is the inflammation of the mammary gland, and represents a common disease in the dairy industry. Bovine mastitis is most often caused by bacterial infections that occur through the teat canal colonizing the mammary gland (Hogan et al., 1999). Bovine mastitis has significant economic implications for the dairy industry due to cost of treatment, decline in milk production and quality and increased labor (Bar et al., 2008). Total costs are estimated from $100 to $300 per cow (Bar et al., 2008; Ott, 1999). Additional implications associated with mastitis include transmission of mastitis-causing bacteria to uninfected cows from milking equipment and the risk of antimicrobial resistant bacteria from the overuse of antibiotics (Wilson et al., 1999) that pose a public health threat if disseminated into the food supply (Barkema et al., 2006). Over 100 different microbial species have been implicated in bovine mastitis (Watts, 1988) and accurate detection is necessary for appropriate treatment.

The most common diagnostic method to detect bacteria from cows suspected of having mastitis is culture and identification with selective media and biochemical analysis. Bacterial culture is considered an accurate and reliable detection method for most common mastitis pathogens and remains a popular laboratory method because it is relatively inexpensive and simplistic. Culture also has limitations such as the length of time to obtain results which may take up to 72 hours. At a minimum, 48 hours are needed to allow bacteria

or fungi sufficient time to propagate in the presence of natural antimicrobial compounds in milk including immunoglobulins, complement, lysozyme, lactoferrin and lactoperoxidase (Ekstrand, 1989; Oliver et al., 2004) or bacteriostatic fatty acids (Hogan et al., 1988). Longer incubation times are also necessary to isolate potential fastidious organisms such as *Trueperella pyogenes* or *Nocardia* species (Oliver et al., 2004). *Mycoplasma* species is a significant and fastidious bovine mastitis pathogen that requires up to 7 days to be cultivated in the laboratory using special media and conditions (Oliver et al., 2004). These factors likely contribute to the approximate 30% of negative milk cultures reported by diagnostic laboratories in spite of the clinical signs observed in affected cows (Makovec and Ruegg, 2003; Oliveira et al., 2013). Restrictions of bacterial culture have provoked exploration and development of new technologies to more rapidly and accurately detect mastitis pathogens.

Several diagnostic methods have been investigated to replace or supplement conventional bacterial culture. Multiple immunoassays have been developed for mastitis and food safety pathogens such as *Staphylococcus aureus, Streptococcus agalactiae* and *Listeria monocytogenes* (Viguier et al., 2009). Immunoassays are not an ideal replacement for culture because most are only capable of detecting one target. Polymerase chain reaction (PCR) has been proposed as a replacement of milk culture especially for most common pathogens such as *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis,* and *Streptococcus dysgalactiae* (Phuektes et al., 2003; Riffon et al., 2001). Conventional PCR performed with gel electrophoresis is able to detect bacteria in milk samples at low concentrations but is not able to give quantitative information of the targets. Real-time or quantitative PCR (qPCR) provides semi-quantitative results by using fluorescent probes complimentary to the target sequence bound to a fluorophore molecule that is released and

emits light after DNA replication. Probes are used in addition to primer sequences to ensure specificity to the target bacteria sequence. Results are reported as the cycle threshold (Ct) values reflecting the PCR cycle at which fluorescence reaches a predetermined threshold. Cycle threshold values are inversely proportional to the amount of bacterial DNA present in the sample. Real-time PCR is a sensitive and specific assay previously evaluated to detect mastitis pathogens and aid the diagnosis of mastitis with success (Gillespie and Oliver, 2005) although commercial assays were unavailable.

PathoProof Mastitis PCR™ (ThermoFisher Scientific, Waltham, MA) is the first commercial, real-time, multiplex PCR assay designed to detect common bovine mastitis pathogens directly from milk. The complete 12 version detects 11 bacterial targets and 1 *blaZ* gene encoding the *Staphylococcus* beta-lactamase enzyme. The 11 targets include *Staphylococcus aureus,* coagulase negative *Staphylococcus* species, *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Enterococcus faecalis/faecium, Escherichia coli, Klebsiella pneumoniae/oxytoca, Corynebacterium bovis, Trueperella (Arcanobacterium*) *pyogenes/Peptoniphilus indolicus* and *Serratia marcescens.*

PathoProof PCR has been validated in several studies that evaluate the assay's sensitivity and specificity with culture isolates (Koskinen et al., 2009; Pitkälä et al., 2007) and clinical milk samples (Cederlöf et al., 2012; Hiitiö et al., 2015; Keane et al., 2013; Koskinen et al., 2010; Zadoks et al., 2014). Nearly one third of clinical milk samples are culture negative for a variety of reasons, not all of them known. PathoProof PCR has detected bacterial DNA in 47% (Bexiga et al., 2011) and 43% (Taponen et al., 2009) of milk samples from affected quarters that were negative or no-growth results in conventional culture.

A disadvantage of any molecular test compared to bacterial culture is cost; in spite of faster turn-around times and increased analytical sensitivity. Some instances require a more sensitive test to make them worth the extra cost. One example may involve testing milk samples from cows treated with intramammary antimicrobial products for mastitis. A cow treated with antibiotics may not represent an accurate culture result due to a reduction in the quantity of post-treatment bacteria present in the sample. Using an assay such as PCR that delivers the sensitivity to detect low levels of bacteria after antibiotic treatment may help veterinarians and producers determine treatment efficacy and if a cow's milk may be included back into the bulk tank. Currently, there is a lack of literature evaluating PCR and bacterial culture in treated clinical cases of mastitis. The objective of this study was to compare PathoProof PCR to bacterial culture in samples collected from cows with clinical mastitis during the first thirty days post initiation of antibiotic treatment.

Materials and methods

Cow selection and milk sample collection

Cows enrolled in this study came from the Iowa State University Dairy Farm and one additional dairy farm in central Iowa. Cows were selected from February 2014 through June 2015. Mastitis was diagnosed using monthly somatic cell count (SCC), electrical conductivity of milk and preliminary culture results if available. Cows were selected for antimicrobial therapy by veterinarians and herd managers. All cows received 10mL of a commercial cephalosporin antimicrobial treatment delivered by the intramammary route in the affected quarter once daily for 5 days. Duplicate milk samples were collected from each

affected quarter on day 0, prior to the first dose of antibiotic. Subsequent milk samples were collected on days 3, 7, 14 and 30 post treatment initiation.

Bacterial Culture

Milk samples were maintained on ice until delivered to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) to ensure quality. After vortexing one of the duplicate samples, 100µL was cultured on 20mL of 6% sheep's blood agar and MacConkey agar. Blood agar plates were incubated at 37°C with 5 to 10% CO_2 for 48 hours. MacConkey agar plates were incubated for 48 hours at 37°C without CO_2. Culture plates containing 1 or 2 different populations of bacteria with at least 5 colonies were considered significant based on the National Mastitis Council's laboratory guidelines for bovine mastitis milk cultures and were selected for subculture (Hogan et al., 1999). Identification of the subcultured bacteria was confirmed using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Billerica, MA).

MALDI-TOF MS has been validated for use with isolates selected from clinical mastitis cases and is able to identify over 100 different genera (Keys et al., 2004) including bovine mastitis pathogens (Barreiro et al., 2010). The isolates are given a log score using MALDI-TOF MS that ranges from 0 to 3. Scores of 1.7 or greater are considered accurate to a genus level and scores of 2.0 or greater are considered accurate to a species level (Barreiro et al., 2010).

PathoProof mastitis PCR

DNA extraction of one of the duplicate milk samples was performed by magnetic particle processing according to the manufacturer's protocol in the complete-12 kit using the KingFisher™ Flex Purification System (ThermoFisher Scientific, Waltham, MA). Four

separate PCR reactions contain primers and probes for three of the 12 targets in the assay as well as an internal amplification control (IAC) (Table 1). The PathoProof qPCR was conducted using the Applied Biosystems 7500 Fast real-time PCR system (ThermoFisher Scientific, Waltham, MA) according to the following profile: 10 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and one minute at 60°C and a final stage of 5 seconds at 25°C. Each primer/probe set included an IAC to detect internal inhibitors of PCR and serving as a positive control for each reaction. A *S. aureus* positive control was also included each time PCR was performed.

Results

Twenty-five of thirty-one (81%) of the cows enrolled in the study tested qPCR positive for at least one pathogen targeted in the PathoProof assay from 0 to 30 days post treatment (DPT). Five cows were positive for non-target bacteria identified by MALDI-TOF MS and were not included in the final analysis study. These included *Lactococcus garvieae* (2), *Enterobacter kobei* (1), *Enterococcus saccharolyticus* (1), and *Pseudomonas aeruginosa* (1). One additional cow was not included in the study due to negative culture results on day 0 despite a positive preliminary culture that was performed days before treatment by farm personnel. This brought the number of cows used in the final analysis to 25. Bacteria detected by qPCR from the remaining 25 cows included *S. dysgalactiae* (18), *S. uberis* (3), *E. faecalis* (1), *E. coli* (1) and coagulase-negative *Staphylococcus* spp. (4) including *Staphylococcus simulans* (1), *Staphylococcus haemolyticus* (2), and *Staphylococcus chromogenes* (1). Three cows were coinfected with 2 different bacteria based on day 0 milk cultures. These co-infections included *S. dysgalactiae* and *S. haemolyticus, S. dysgalactiae*

and *E. coli* and *E. faecalis* and the algae *Prototheca sp.* (not a PathoProof target) confirmed by Gram stain. Over the course of 30 days, some samples were not collected due to cows ending their lactation cycle or removal from the herd. The distribution of cows and bacteria identified by MALDI-TOF MS is described in Table 2.

Bacterial Culture

Bacterial cultures with growth detected from any sample were included for identification by MALDI-TOF MS. Bacteria isolated from the 25 cows included in the study received MALDI-TOF MS scores greater than 2.0 with the exception of the *S. dysgalactiae* isolated from 14 DPT milk samples from cow 28. All 25 cows were positive by culture on day 0 with at least one bacterial species associated with bovine mastitis and included as a PathoProof target. Isolation of significant bacteria by culture markedly declined on days 3, 7, 14 and 30 in cows that remained in the study. Less than 4 cows became culture positive again after day 0 (Table 3). All culture positive milk samples were PCR positive for PathoProof targets.

Real-time PCR

Twenty-five cows enrolled in the study were qPCR positive at 0 DPT for at least one target. Nineteen cows remained qPCR positive on 3 DPT. The number of positive cows decreased to 13 on day 7 and 14 and 6 cows on 30 DPT (Table 4). PCR and MALDI-TOF MS consistently detected the same bacteria when both culture and PCR were positive. Targets were detected by PathoProof qPCR with Cts ranging from 17.75 to 39.08 regardless of target. The average Ct for days 0 to 30 ranged from 29.13 to 35.25 and remained relatively steady from day 0 to day 30 on average.

Two cows were consistently culture negative/qPCR positive for at least 1 PathoProof target on 3, 7, 14 and 30 DPT (Table 3). Four cows were culture negative/qPCR positive at 3 DPT and then tested culture negative/PCR negative for the remainder of the study. Three cows were positive by PCR and negative by culture on days 3 and 7, and negative by both methods on days 14 and 30. Two cows were PCR positive and negative in culture on days 3, 7, 14 and negative on day 30. Eight cows who were negative in culture for days 3, 7, 14 and 30 had positive and negative PCR results on those days' samples. Culture and PCR results throughout the 30 days of the study are depicted in Figure 1.

Discussion

Bacterial cultures from milk samples collected from cows with clinical mastitis are often negative for a variety of reasons although many unknown (Makovec and Ruegg, 2003; Oliveira et al., 2013). One explanation is the effect of antimicrobial therapy on the success of post-treatment bacterial cultures from milk samples. This study showed that PCR and bacterial culture outcomes are consistent prior to treatment and have the ability to detect similar pathogens. However, post-treatment, PCR detected at least one target bacteria in more milk samples compared to bacterial culture on days 3, 7, 14 and 30 post treatment (Figure 1). Only one of the 25 cows enrolled in the study was culture positive on day 3 while PCR detected bacterial targets included in the assay in 19 cows. The treatment regimens for all cows continued into day 5 making that result reasonable. Withdrawal times indicated by the antibiotic's manufacturer indicate that the antibiotic may be present in the cow's milk up to 72 hours after the last dose corresponding to day 6 in this study. With that, day 7 samples should be devoid of antibiotic, removing the added inhibitory elements preventing bacteria

from growing. Day 7 results showed 13 cows as culture negative/PCR positive and 1 cow positive for both culture and PCR indicating that there may be another force preventing bacteria from growing or the population is diminished to a level not detectable by culture. Days 14 and 30 had more culture negative/PCR positive cows, similar to days 3 and 7.

Culture negative/PCR positive may also be attributed to PathoProof's ability to detect bacterial DNA from dead and non-infectious bacteria. Cows may indeed have overcome the infection but still harbor DNA from the dead bacteria that once caused the infection. PathoProof PCR is not able to distinguish between live, infectious bacteria and dead, non-infectious bacteria. It is possible that the assay could detect injured, non-infectious bacteria capable of repairing themselves and infecting or reinfecting the host however it still remains that the assay cannot determine the difference.

Questions remain as to the analytical sensitivity of PathoProof PCR in samples with low bacteria count and how PCR positive results should be interpreted from a clinical perspective. A sensitive mastitis diagnostic assay may be useful in the detection of bacteria from culture negative cows with low concentrations of bacteria due to chronic mastitis. Several pathogens associated with mastitis have been implicated in chronic and/or transient mastitis, most commonly *S. aureus* (Sears et al., 1990) but also *E. coli* (Döpfer et al., 1999), coagulase-negative staphylococci (Supré et al., 2011), *S. uberis* and *S. dysgalactiae* (Wyder et al., 2011). Chronic mastitis rotates through periods where clinical signs subside, SCC remains elevated, milk production remains low and pathogens are shed at a low rate (Döpfer et al., 1999). Low bacteria count has been overcome by some PCR assays reporting limit of detection (LOD) value of 10^2 CFU/mL (Riffon et al., 2001), and as low as 1 CFU/mL after including an enrichment step prior to PCR (Gillespie and Oliver, 2005). Previous studies

using PathoProof PCR have studied quantitative results and its correlation with clinical mastitis. A previous study used a standard curve to detect a Ct of 22.2 that represented $2.2x10^7$ copies per mL of milk and was associated with clinical mastitis (Taponen et al., 2009). Another study used clinical milk samples to understand how Ct related to target sequence copy number using *S. aureus* (Hiitiö et al., 2015). However, an LOD using genomic copies and organism count in cfu/mL has not been published for PathoProof PCR. In our laboratory, the analytical sensitivity using serial dilutions of 3 organisms averaged 10^4 cfu/mL as the limit of detection. This brings question about the sensitivity and its performance of the assay which is particularly problematic for pooled samples or bulk tank milk samples.

PathoProof PCR has been shown to detect *S. aureus* and coagulase negative staphylococci isolates originating from different mammalian species and geographic regions (Koskinen et al., 2009). However, when *S. aureus* is present in a sample the assay detects this as *S. aureus* and *Staphylococcus* species due to overlapping targets in the *Staphylococcus* genus and the *S. aureus* species. This presents a problem in the ability of detecting a non-*Staphylococcus aureus* species that may be present in a sample that also has *S. aureus*. The technician is not able to differentiate multiple *Staphylococcus* species in a sample if *S. aureus* is present whereas in culture two *Staphylococcus* species could be differentiated if they represent different phenotypes.

Conclusion

The results of this study suggest that PathoProof PCR may be useful to detect pathogens associated with bovine mastitis that are difficult to detect by culture. However,

qPCR results must be interpreted in the clinical context of individual cows. Real-time PCR has the capacity to be a quantitative assay although reference standards are necessary. If a similar experiment could be performed in a controlled fashion without some of the logistic issues a more accurate precedent could be made for use of the test. Additional research using a quantitative PCR assay and its correlation with clinical mastitis may indicate the future potential of real-time PCR as a diagnostic aid to diagnose bovine mastitis.

References

Bar, D., L. W. Tauer, G. Bennett, R. N. González, J. A. Hertl, Y. H. Schukken, H. F. Schulte, F. L. Welcome, and Y. T. Gröhn, 2008, The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming: J Dairy Sci, v. 91, p. 2205-14.

Barkema, H. W., Y. H. Schukken, and R. N. Zadoks, 2006, Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis: J Dairy Sci, v. 89, p. 1877-95.

Barreiro, J. R., C. R. Ferreira, G. B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M. N. Eberlin, and M. V. dos Santos, 2010, Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: J Dairy Sci, v. 93, p. 5661-7.

Bexiga, R., M. T. Koskinen, J. Holopainen, C. Carneiro, H. Pereira, K. A. Ellis, and C. L. Vilela, 2011, Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing: J Dairy Res, v. 78, p. 49-55.

Council, N. M., 1999, Laboratory and Field Handbook on Bovine Mastitis.

Döpfer, D., H. W. Barkema, T. J. Lam, Y. H. Schukken, and W. Gaastra, 1999, Recurrent clinical mastitis caused by Escherichia coli in dairy cows: J Dairy Sci, v. 82, p. 80-5.

Ekstrand, B., 1989, Antimicrobial Factors in Milk - A review: Food Biotechnology, v. 3, p. 105-126.

Gillespie, B. E., and S. P. Oliver, 2005, Simultaneous detection of mastitis pathogens, Staphylococcus aureus, Streptococcus uberis, and Streptococcus agalactiae by multiplex real-time polymerase chain reaction: J Dairy Sci, v. 88, p. 3510-8.

Hiitiö H, Riva R, Autio T, Pohjanvirta T, Holopainen J, Pyörälä S, Pelkonen S. 2015. Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture. J Dairy Res 82:200-208.

Hogan, J. S., K. L. Smith, D. A. Todhunter, and P. S. Schoenberger, 1988, Growth responses of environmental mastitis pathogens to long-chain fatty acids: J Dairy Sci, v. 71, p. 245-9.

Isaacs, C. E., R. E. Litov, and H. Thormar, 1995, Antimicrobial activity of lipids added to human milk, infant formula, and bovine milk: J Nutr Biochem, v. 6, p. 362-366.

Keys, C. J., D. J. Dare, H. Sutton, G. Wells, M. Lunt, T. McKenna, M. McDowall, and H. N. Shah, 2004, Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases: Infect Genet Evol, v. 4, p. 221-42.

Koskinen, M. T., J. Holopainen, S. Pyörälä, P. Bredbacka, A. Pitkälä, H. W. Barkema, R. Bexiga, J. Roberson, L. Sølverød, R. Piccinini, D. Kelton, H. Lehmusto, S. Niskala, and L. Salmikivi, 2009, Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens: J Dairy Sci, v. 92, p. 952-9.

Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W. A. van Haeringen, T. J. Lam, and S. Pyörälä, 2010, Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria: J Dairy Sci, v. 93, p. 5707-15.

Makovec, J. A., and P. L. Ruegg, 2003, Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001: J Dairy Sci, v. 86, p. 3466-72.

Oliveira, L., C. Hulland, and P. L. Ruegg, 2013, Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin: J Dairy Sci, v. 96, p. 7538-49.

Oliver, S. P., R. N. Gonzalez, J. S. Hogan, B. M. Jayarao, and W. E. Owens, 2004, Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality: Verona, Wisconsin, National Mastitis Council.

Ott, S. L., 1999, Costs of Herd-Level Production Losses Associated with Subclinical Mastitis in U.S. Dairy Cows: National Mastitis Council 38th Annual Meeting.

Phuektes P, Browning GF, Anderson G, Mansell PD. 2003. Multiplex polymerase chain reaction as a mastitis screening test for Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis in bulk milk samples. J Dairy Res 70:149-155.

Riffon, R., K. Sayasith, H. Khalil, P. Dubreuil, M. Drolet, and J. Lagacé, 2001, Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR: J Clin Microbiol, v. 39, p. 2584-9.

Sears, P. M., B. S. Smith, P. B. English, P. S. Herer, and R. N. Gonzalez, 1990, Shedding pattern of Staphylococcus aureus from bovine intramammary infections: J Dairy Sci, v. 73, p. 2785-9.

Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vaneechoutte, S. Piepers, and S. De Vliegher, 2011, Some coagulase-negative Staphylococcus species affect udder health more than others: J Dairy Sci, v. 94, p. 2329-40.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä, 2009, Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing: J Dairy Sci, v. 92, p. 2610-7.

Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R. 2009. Mastitis detection: current trends and future perspectives. Trends Biotechnol27:486-493.

Watts, J. L., 1988, Etiological agents of bovine mastitis: Vet Microbiol, v. 16, p. 41-66.

Wyder, A. B., R. Boss, J. Naskova, T. Kaufmann, A. Steiner, and H. U. Graber, 2011, Streptococcus spp. and related bacteria: their identification and their pathogenic potential for chronic mastitis - a molecular approach: Res Vet Sci, v. 91, p. 349-57.

Tables

Table 1. PathoProof Mastitis PCR targets included in each PCR reaction. (IAC-internal amplification control, *blaZ*-staphylocccal gene for beta-lactamase)

PCR Targets Reaction 1	PCR Targets Reaction 2	PCR Targets Reaction 3	PCR Targets Reaction 4

• *Staphylococcus aureus*
• *Enterococcus* species
  • (including *E. faecalis* and *E. faecium)*
• *Corynebacterium bovis*
• IAC
	• *blaZ*
	• *Escherichia coli*
	• *Streptococcus dysgalactiae*
	• IAC
		• *Staphylococcus* species
		  • (including coagulase-negative *Staphylococcus* species)
		• *Streptococcus agalactiae*
		• *Streptococcus uberis*
		• IAC
			• *Klebsiella* species
			  • (including *K. oxytoca* and *K. pneumoniae*)
			• *Serratia marcescens*
			• *Trueperella pyogenes* and *Peptoniphilus indolicus*
			• IAC

Table 2. Bacteria isolated from milk samples collected prior to antibiotic treatment and confirmed by MALDI-TOF MS

Cow Number	*S. dysgalactiae*	*S. uberis*	*Staph* sp. *(haemolyticus)*	*Staph* sp. (*chromogenes*)	*Staph* sp. (*simulans*)	*E. coli*	*E. faecalis*	*E. kobei*	*E. saccharolyticus*	*L. garvieae*	*P. aerugi nosa*	*Prototheca* sp.
1	x (2 quarters)											
2	x											
3		x										
4	x											
5					x							
6			x									
7	x		x									
8	x											
9												
10				x								
11		x										
12	x											
13									x			
14										x		
15										x		
16	x											
17	x											
18		x										
19											x	
20								x				
21	x											
22	x											
23	x											
24	x					x						
25	x											
26	x											
27	x											
28	x											
29	x											
30							x					x
31	x											

Table 3. Number of cows positive for bacterial culture on 0, 3, 7, 14 and 30 days post treatment initiation.

Pathogen	Day 0	Day 3	Day 7	Day 14	Day 30
S. dysgalactiae	18 of 18	0 of 18	1 of 16	2 of 16	1 of 10
S. uberis	3 of 3	1 of 3	0 of 3	0 of 3	0 of 2
***Staph* sp.**	4 of 4	0 of 4	0 of 3	0 of 4	0 of 3
E. coli	1 of 1	0 of 1	0 of 1	0 of 1	0 of 1
E. faecalis	1 of 1	0 of 1	0 of 1	1 of 1	0 of 0
Percent Positive	100.00%	4.00%	4.35%	13.04%	7.14%

Table 4. Number of cows PathoProof qPCR positive on 0, 3, 7, 14 and 30 days post treatment.

Pathogen	Day 0	Average Ct	Day 3	Average Ct	Day 7	Average Ct	Day 14	Average Ct	Day 30	Average Ct
S. dysgalactiae	18 of 18	25.16	14 of 18	28.9	11 of 16	31.46	11 of 16	34.24	3 of 10	31.21
S. uberis	3 of 3	31.34	2 of 3	32.25	1 of 3	32.23	0 of 3	N/A	0 of 2	N/A
***Staph* sp.**	4 of 4	29.7	3 of 4	36.73	1 of 3	36.65	2 of 4	34.48	3 of 3	37.09
E. coli	1 of 1	34.42	0 of 1	37.79	0 of 1	N/A	0 of 1	N/A	1 of 1	37.46
E. faecalis	1 of 1	25.02	0 of 1	N/A	0 of 1	N/A	1 of 1	28.24	0 of 0	N/A
Percent Positive	100.0%	29.13	76.00%	33.92	56.52%	33.45	56.52%	32.32	42.86%	35.25

Figures

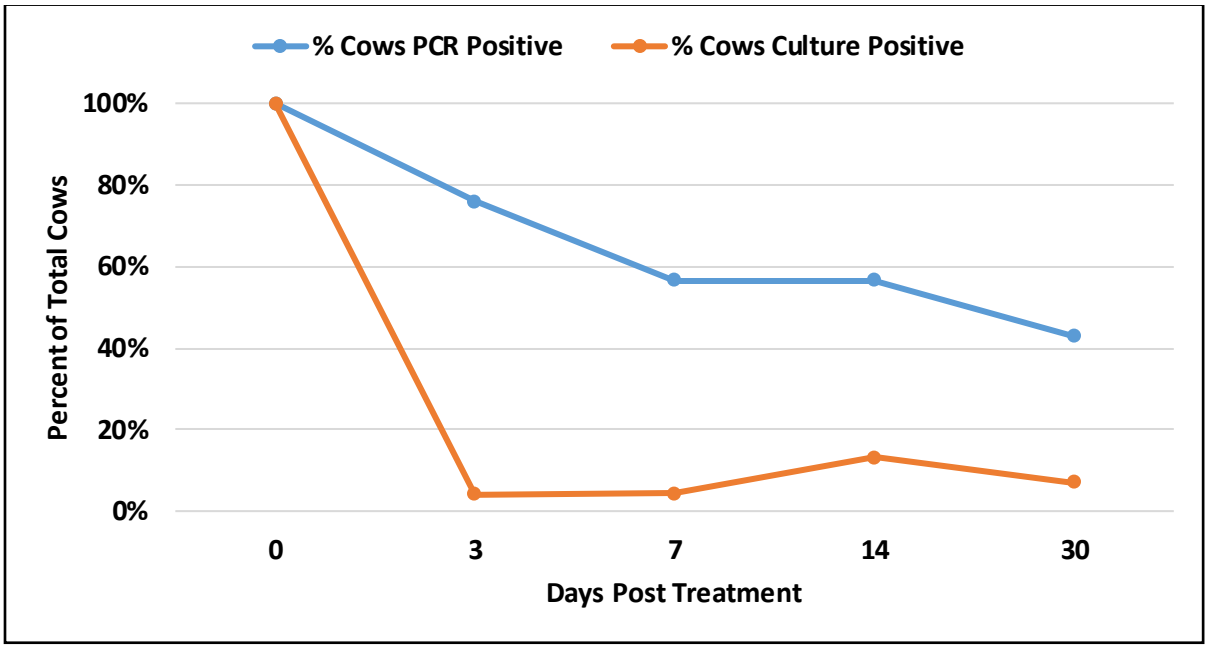

Figure 1. Percent qPCR and bacterial culture positive cows at 0, 3, 7, 14 and 30 days post treatment.

CHAPTER 5. GENERAL CONCLUSIONS

Microbiological culture has performed well for the detection of bovine mastitis pathogens for decades in veterinary diagnostic laboratories. However, culture tests are time consuming and may be unable to grow fastidious organisms warranting new technologies. No other methods of detecting bovine mastitis pathogens are as multiplexed and rapid as PathoProof PCR. The 11 target bacteria of the assay cover a wide range of common mastitis pathogens. The purpose of evaluating this assay was to determine its strengths and weaknesses in sensitivity and specificity and its use with milk from cows being treated with intramammary antibiotics.

Analysis with well-validated culture isolates from bovine mastitis revealed a high specificity for target and non-target organisms. Analytical sensitivity or limit of detection determined with serial dilutions and standard plate count of pure culture isolates revealed an average LOD of 10^4 cfu/mL. Although an infectious dose for all mastitis-causing bacteria in the mammary gland has not been established, PathoProof's LOD of 10^4 CFU/mL is relatively high with the potential of reporting false negatives.

PathoProof PCR was more successful detecting bacteria in milk from cows being treated for mastitis than culture. Cows sampled before treatment (day 0) and on days 3, 7, 14, and 30 post treatment initiation were negative for culture and positive for PCR on days 3, 7, 14 and 30. Results do not necessarily mean infectious bacteria are still in the mammary gland but could also indicate a cow's treatment has not been successful.

Diagnostic laboratories may want to choose PathoProof PCR for select cases when bacterial growth may be inhibited. Further investigation into PathoProof's sensitivity in

clinical samples and pooled samples such as bulk tank milk is necessary to better understand its capabilities.

APPENDIX: ADDITIONAL COW DATA

Table 1. Individual cow data including target, culture and PCR results for days 0, 3, 7, 14 and 30.

Cow Number	Target	Day 0 culture/PCR	Ct value	Day 3 culture/PCR	Ct value	Day 7 culture/PCR	Ct value	Day 14 culture/PCR	Ct value	Day 30 culture/PCR	Ct value	Comments
1	*Streptococcus dysgalactiae*	+/+	32.2675	-/+	26.9	-/+	33.74	N/A	N/A	N/A	N/A	Dried off before D14
2	*Streptococcus dysgalactiae*	+/+	23.33	-/+	24.85	-/+	28.66	-/+	33.45	-/+	35.26	Teat lesion seen on D14 believed to be positive for *S. aureus*
3	*Streptococcus uberis*	+/+	31.57	-/-	N/A	-/-	N/A	-/-	N/A	-/-	N/A	Mycobacterium spp. Found after prolonged incubation of D30 culture
4	*Streptococcus dysgalactiae*	+/+	24.19	-/+	31.55	-/-	N/A	-/-	N/A	-/-	N/A	
5	*Staphylococcus* spp.	+/+	25.19	-/+	36.42	-/+	36.65	-/-	N/A	-/+	36.85	
6	*Staphylococcus* spp.	+/+	34.74	-/+	36.65	-/-	N/A	-/+	37.07	-/+	38.08	
7	*Streptococcus dysgalactiae*	+/+	22.76	-/-	N/A	N/A	N/A	-/+	38.22	-/-	N/A	No D7-collected incorrect quarter
	Staphylococcus spp.	+/+	28.54	-/-	N/A	N/A	N/A	-/+	31.89	-/+	36.33	
8	*Streptococcus dysgalactiae*	+/+	17.75	-/+	26.77	-/+	31.52	-/-	N/A	-/-	N/A	
9	*Streptococcus dysgalactiae*	-/+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	D0 culture negative
10	*Staphylococcus* spp.	+/+	30.32	-/+	37.13	-/-	N/A	-/-	N/A	N/A	N/A	Dried off before D30
11	*Streptococcus uberis*	+/+	38.4	-/+	39.08	-/-	N/A	-/-	N/A	-/-	N/A	
12	*Streptococcus dysgalactiae*	+/+	33	-/+	39.19	-/-	N/A	-/-	N/A	-/-	N/A	
13	*Enterococcus saccharolyticus*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Non-target
14	*Lactococcus garvieae*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
15	*Lactococcus garvieae*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
16	*Streptococcus dysgalactiae*	+/+	27.75	-/+	28.56	-/+	N/A	-/+	36.69	-/-	N/A	
17	*Streptococcus dysgalactiae*	+/+	19.64	-/+	25.24	-/+	31.61	+/+	29.39	N/A	N/A	No D30 sample
18	*Streptococcus uberis*	+/+	24.05	+/+	25.42	-/+	32.23	-/-	N/A	N/A	N/A	No D30 sample, culture positive for Yeast on D14
19	*Pseudomonas aeruginosa*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Non-target
20	*Enterobacter kobei*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Non-target
21	*Streptococcus dysgalactiae*	+/+	20.9	-/+	25.42	-/+	36.06	-/+	35.59	-/-	N/A	
22	*Streptococcus dysgalactiae*	+/+	28.77	-/+	35	-/+	34.38	-/-	N/A	N/A	N/A	No D30 sample
23	*Streptococcus dysgalactiae*	+/+	28.8	-/-	N/A	-/-	N/A	-/+	37.33	N/A	N/A	No D30 sample
24	*Streptococcus dysgalactiae*	+/+	18.31	-/+	22.01	-/+	22.68	-/+	34.24	-/+	34.56	
	E. coli	+/+	34.42	-/+	37.79	-/-	N/A	-/-	N/A	-/+	37.46	
25	*Streptococcus dysgalactiae*	+/+	31.01	-/+	31.01	-/-	N/A	-/+	32.48	N/A	N/A	No D30
26	*Streptococcus dysgalactiae*	+/+	23.64	-/+	27.64	-/+	33.1	-/+	35.01	-/-	N/A	
27	*Streptococcus dysgalactiae*	+/+	26.54	-/+	27.22	-/+	31.19	N/A	N/A	+/+	23.82	No D14
28	*Streptococcus dysgalactiae*	+/+	27.24	-/-	N/A	+/+	26.21	+/+	26.9	N/A	N/A	No D30
29	*Streptococcus dysgalactiae*	+/+	18.56	-/-	N/A	-/+	36.94	-/-	N/A	N/A	N/A	No D30

30	*Enterococcus faecalis/faecium*	+/+	25.02	-/-	N/A	-/-	N/A	+/+	28.24	N/A	N/A	No D30 and positive for *Prototheca* spp.
31	*Streptococcus dysgalactiae*	+/+	21.39	-/+	33.24	N/A	N/A	-/+	37.35	N/A	N/A	No D7 and D30