Systematic review of diagnostic techniques for identification of pathogens in fresh milk from dairy herds with high bulk milk somatic cell count

by

Theo Kotzé

Submitted in partial fulfilment of the requirements for the degree of

Magister of Scientiae (Tropical Animal Health)

in the

Department of Veterinary Tropical Diseases.

Faculty of Veterinary Science University of Pretoria.

January 2021

DECLARATION I declare that this dissertation is my own work. It has been submitted for the Degree Magister of Scientiae (Tropical Animal Health) at the University of Pretoria. It has not been submitted before for any degree or examination in any other university. I further declare that the work presented was approved by the Animal Use and Care Committee for research of the University of Pretoria, South Africa. _____Signature of candidate

Day of 2021/ /

ABSTRACT

A private veterinary practitioner wants to identify diagnostic techniques that are practical, commercially available, reliable, rapid, and cost-effective to diagnose milk-borne pathogens in dairy herds with high Bulk Milk Somatic Cell Counts (BMSCC). To enable this, a systematic review was done to identify diagnostic techniques to diagnose pathogen(s) in dairy herds with high BMSCC. To enable this, a systematic review was done to identify diagnostic techniques for pathogen(s) identification in dairy herds with high BMSCC.The criteria to identify the diagnostic methods included commercial, rapid, and cost-effective methods with high diagnostic sensitivity and specific for aerobic mastitogenic, zoonotic, food-borne, antimicrobial resistant and state-controlled disease pathogens in fresh milk intended for human consumption. A model using practical methods to identify these pathogens included somatic cell count (SSC) screening, phenotypical culturing/isolation/biochemically identification, anti-microbial resistance evaluation together with conventional PCR and multiplex real-time quantitative PCR.

Results from the systematic literature identified the diagnostic sensitivity and specificity of SSC screening to be in the range of 49-94,5% and 57,7-86,8% respectively, while phenotypic identification of aerobic mastitogenic pathogens to be in the range of 9,1 -100% and 28,8- 100% respectively, Immuno-assay identification to be in the range of 75,5% - 100% respectively, conventional PCR 76,7-100% and 98,6-100% respectively and multiplex quantitative real-time PCR (Pathoproof -Thermofisher Scientific) with a 100 % analytic sensitivity and 99-100% analytic specificity as well as diagnostic sensitivity and specificity of 76,9-100% and 63,3-100% respectively , as accurate practical diagnostic

techniques. Based on the systematic review results, the high range of sensitivity and specificity using the combined diagnostic model makes it a suitable model that can successfully and with confidence be implemented by a veterinarian in private dairy practice. It will make an enormous contribution in diagnostic surveillance procedures for the milk industry - public health interface in a veterinary One Health orientated practice.

Diagnostic tests	**Sensitivity range**	**Specificity range**
SCC identification	49.0-94.5%	57,7-86.8%
Phenotypical identification	9.1-100%	28.8-100%
Immuno-assay identification	75,5-100%	98.8-100%
Conventional PCR	76.7%-100%	98.6-100%
Multiplex real- time qPCR	76.9-100%	63,3-100%

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following:

Dairy industry community.

Department Veterinary Tropical diseases. Faculty of Veterinary Science University of Pretoria;

Institute of Tropical Medicine. Antwerp, Belgium;

Supervisor: Professor Henriette van Heerden.

DEDICATION

This mini dissertation is dedicated to the returning Messiah King Jesus Christ. My effort is a humble contribution to One Health, while awaiting your Millennial reign on this earth.

(Mark 13:26; Revelation 20:4)

Contents

RESEARCH ETHIC APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Systematic literature review to design a diagnostic model of biotechnological techniques for surveillance and identification of pathogens influencing the Bulk Milk Somatic Cell Count (BMSCC) in large dairy herds.**
Project Number	**REC159-19**
Researcher / Principal Investigator	**Dr T Kotze**

Dissertation / Thesis submitted for	**Masters**

Supervisor	**Prof H van Heerden**

APPROVED	Date: **2019-09-26**
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadirulwa

INTRODUCTION:

Private dairy veterinarians are well trained to render a professional, profitable, unsubsidized, and affordable service to the farming communities, dairy industry, and public health administration. Therefore, a private One-Health orientated veterinary practitioner wants to identify diagnostic techniques that are practical, commercially available, reliable, rapid, and cost-effective to diagnose milk-borne pathogens in dairy herds with high bulk milk somatic cell counts (BMSCC). Private veterinary dairy consultancy laboratories are registered in South Africa under the South African Veterinary Council and comply to BSL-2 biohazard standards. Institutional SANAS accredited and Biohazard BSL-3 & 4 laboratories serve as backup laboratories for these private One-Health orientated practices. Veterinary practice laboratories do primary diagnostic isolations while subtyping is mostly performed in specialized laboratories (Flieger et al., 2013).

The purpose of this study is to investigate One-Health diagnostic opportunities on fresh milk samples in private veterinary dairy practice. At some points it overlaps with production animal mastitis studies and highly, sophisticated, accredited, and subsidized institution standards. In this research a diagnostic surveillance model is developed as the fundamental springboard from which a One -Health practice can be launched with emphasis on zoonotic diseases from fresh milk to implement biosecurity measures.

Bulk milk somatic cell count (BMSCC)

Bulk milk somatic cell count (BMSCC) is an indicator of herd immunological response (Baumert et al., 2009, Brunham et al., 1993, Smith, 2019, Sordillo, 2018). Somatic cells consist of inflammatory defense cells and are normally present in low levels in milk. These white blood cells as well as exfoliated old epithelial cells are normally present in healthy milk at low count numbers of <100,000 SCC (somatic cell count)/ml milk (Sumon et al., 2020). White blood cells consisting of macrophages, neutrophils and lymphocytes that fight infection

in the udder are reflected by high SCC levels that indicate intramammary infection (IMI; mastitis).

The basic explanation of a mammary immune response is when invading pathogens damage the mammary epithelial tissue and various chemical compounds are released into the mammary system (Stevens et al., 2012). The mammary immune system activates two components, namely the cellular leukocyte (Fonseca et al., 2015) and the humoral immunoglobulin components (complement proteins, antibodies, and antimicrobial peptides) (Waller, 2000, Bonizzi et al., 2003). The innate immunity is the first line of host defence and protects the host from the surrounding environment with nonspecific antibodies, leucocytes and macrophages that eliminate invading pathogens and toxins by neutralisation, opsonisation and phagocytosis (Fitzpatrick, 2001). Furthermore the innate immunity signal for recruitment and influx of more specialised immune cells, pro-inflammatory cytokines, interleukin-1, interleukin-8 (IL-8) and tumour necrosis factor-alpha, which induce chemotaxis of neutrophils from the surrounding blood vessels into the mammary gland (Alhussien and Dang, 2018). Cell-mediated immunity mechanisms by lymphocytes, plasma cells, and dendritic cells are then further activated as an inflammatory response and memory mechanism that causes a dramatic rise in SCC (Alhussien and Dang, 2018, Talbot and Lacasse, 2005, Tizard, 2017).

Intramammary infection (IMI) or mastitis is a syndromic disease in dairy practice. For the purposes of this study, the incidence of IMI can be defined as cow quarters with an increase in SCC from below to above 200,000 cells/ml milk in a quarter milk sample. IMI prevalence was historically defined as the proportion of cow quarters with a SCC above 200,000 cells/ml quarter milk (Ruegg and Pantoja, 2013). Well established private veterinary practices were in agreement with the general consensus that 200,000 somatic cells/ml are a more realistic threshold level for quarter and 150,000 somatic cells/ml for composite milk samples (Petzer et al., 2017a). BMSCC is the average SCC for all lactating cows contributing to the herds milk production bulk tank. SCC in milk inversely relates to dairy productivity and milk quality.

Schukken et al. (2003) stated: *"Herd and population somatic cell count are related to the inflammatory process in individual cows but much more reflect the udder health status of the herd and the quality of the raw milk in the herd and the population*." Low levels of BMSCC can be associated with a low prevalence of infection by mastitis pathogens and can be used to estimate milk quality and the level of mastitis and subclinical mastitis in a herd as suggested by Giesecke et al. (1994) already. BMSCC will also reflect immune depression effects caused by 'non-mastitis' pathogens, such as immunosuppressive pathogens e.g. bovine viral diarrhoea virus (BVDV) and bovine leukaemia virus (BLV) (Frie and Coussens, 2015); (Wellenberg et al., 2002).

Up to date evidence-based surveillance, diagnosis and preventative measures from reliable research are at the pivot point of decision making in veterinary preventative medicine, biosecurity and "One-Health". It is important to realize that it is a difficult, but manageable task according to Karzis et al. (2018) , to identify and understand the epidemiology of consortium pathogens in the quarters of a large dairy herd when a sudden rise of the BMSCC or a clinical mastitis outbreak occurs, which could have been mitigated by good management practices. (Karzis et al., 2018;Souza et al., 2020). Active surveillance to identify mastitis pathogens to understand the epidemiology of consortium of pathogens in variable seasonal, climatic, and farm management situations is the practitioner's primary challenge. The second challenge is to declare disease outbreaks or disease-free status on representative samples for instance in the case of zoonotic *Brucella abortus* or *Mycobacterium bovis* in the herd. In a competitive dairy practice environment, decisions must be made on recent scientifically based research and an accurate diagnostic strategy. To enable these clinical diagnostics, background data, laboratory diagnostics which include, cytological, bacteriological and phenotypical techniques need to be combined with molecular, mass-spectrometry and immunological techniques to create an accurate diagnostic surveillance in dairy practice.

As mentioned, it is common practice in the milk industry to use SCC along with microbiological test values as clinical diagnostic tools for subclinical and clinical mastitis prevention programs

.(Karzis and Petzer, 2012; Ruegg and Pantoja, 2013). In this study however SCC values will be seen from an immunological perspective as a primary screening diagnostic technique for prevalent intramammary infections as the source of One-Health calamities in private practice. Thus, IMI reflected by an elevated milk SCC due to an immune reaction from teat canal infections, Furstenberg rosette lymphoid stimulation, mucosal associated lymphoid tissue stimulation, subclinical mastitis, clinical mastitis, acute mastitis, per-acute mastitis, or chronic mastitis (Giovannini et al., 2000). A practical diagnostic surveillance model for the dairy was suggested using cytology, primary isolation and identification combined with molecular, mass -spectrometry and immunological techniques. The latter diagnostic techniques with high diagnostic sensitivity and specificity were selected using a systemic literature review to identify aerobic mastitis pathogens which influences the BMSCC.

There are > 150 known aerobic bacterial intramammary infection, apart from anaerobic bacterial pathogens as mentioned by Du Preez since 1989 (Du Preez, 1989) and teat canal pathogens(du Preez, 1986) plus other opportunistic organisms which effect the BMSCC (El-Sayed et al., 2017). This makes BMSCC an efficient indicator datatype and monitoring instrument that must be carefully interpreted for IMI and potential public health disease challenges in large dairy herds (Schwarz et al., 2020). Little or no information is available on the effect of “commensals” with low pathogenicity (Fitzpatrick, 2001) or other syndromic diseases on BMSCC such as the effect of zoonotic pathogen abortions (*Brucella abortus*), Q-fever (*Coxiella burnetii*), diarrhoea (*Escherichia coli, Enterococcus faecalis, Mycobacterium avium paratuberculosis*). These diseases were included in the systematic literature review due to their veterinary and public health importance. Recent research showed that as much as 37% *S. aureus* infections can be missed in low cell count milk investigations (Petzer et al., 2017b). It is important that these cases are thoroughly clinically examined and confirmed by a qualified veterinarian. A review of viral infections causing SCC elevations by Wellenberg et al. (2002) emphasised the influence of these infections on the milk somatic cell immunological response. These authors concluded that viral infections such as BVDV and BLV can also play

a direct or indirect role in the aetiology of bovine mastitis (Yang et al., 2016, Frie and Coussens, 2015) and were included in the systematic literature review.

The BMSCC count in milk for human consumption is acceptable between 150,000 somatic cells/ml milk to 500,000 somatic cells/ml milk. Bradley et al. (2007) stated that for every 100,000 cells/ml increase in BMSCC, there is likely to be a 10% increase in IMI prevalence in a herd. According to Petzer *et al*. (2016): *"The larger the herd the more critical the interpretation of BMSCC needs to be and the less valuable it becomes as a pro-active udder health monitoring tool because of the dilution effect of the milk masking high SCC of an individual cow."* In South African dairy veterinary practice, the ideal BMSCC range is commonly considered between practitioners to be 150,000-200,000 SCC/ml bulk milk target for healthy milk quality to feed the nation (Ruegg and Pantoja, 2013).

Individual cow pathogen(s) and collective pathogen consortiums in an individual dairy environment cause high SCC and different types of IMI in a herd. Virulence and anti-microbial resistance of the ruling pathogens also plays a role in somatic cell variations (Graves et al., 2010). This is reflected by the main epigenetic determinators of the SCC immune response to be (i) immune status of individual cows in the herd; (ii) virulence and resistance of individual pathogens; (iii) individual pathogens in the consortium that challenges the herd immunity and; (iv) environmental factors (Emam et al., 2019b). Epigenetics represents a vital balance between gene expression of the cow against the conflicting gene expression of the pathogen in the arms race for survival (Brunham et al., 1993). For the cow, as an immune responder, the challenge is disease resistance against infectious disease (Emam et al., 2019a, Fonseca et al., 2015). For the survival of pathogens on the other hand, resistance against antimicrobials/disinfectants and activation of virulence factors to overcome host immune defence mechanisms are important for co-existence with the host (Brunham et al., 1993). Mallard and co-workers hypothesised that high immune responding dairy cattle have half the disease occurrence of low responders and can pass their superior immune response genes on to future generations thereby accumulating health benefits within the dairy herd (Mallard et

al., 2015). Julie L. Fitzpatrick has a sobering thought on breeding for traits that may be associated with immune function such as SCC that may potentially and inadvertently lead to reduced udder immunity and mastitis susceptibility. The essence of selection should be to preserve the ability of the major histocompatibility complex (Sharif et al., 1998) that is involved in mastitis resistance by recognition of antigens, toll genes and molecular adhesion coding genes (Fitzpatrick, 2001). Employment of molecular genetic techniques can be used to investigate and incorporate animal resistance genotypes into a dairy herd (Mallard et al., 2011). Immunosuppressive pathogens endemic in a herd such as Enzootic bovine leukaemia (EBL), BVDV and Bovine herpes virus (BHV) play a similarly important role in herd immunological resistance (Herlekar et al., 2013). Virulence and antimicrobial resistance of ruling pathogens are determined by suspected epigenetic factors also influence BMSCC and should be considered after careful observations while interpreting BMSCC profiles when correlated to clinical udder examinations.

Physiological BMSCC fluctuations on a herd occurs during seasonal milking programs; heifers: mature cow lactation number ratios; herd size; genetic resistance against mastitis pathogens; culling rate of chronic cows; milking fractions (foremilk/after milk); lactation stage; number of lactations; age and breed; and yield dilution are all cow factors that play a role in daily BMSCC fluctuations (Harmon, 1994). Some researchers described distinct patterns of SCC based on analysis of consecutive sampling. Short and long periods of increased SCC, with or without recovery were used to identify pathogen profiles (de Haas et al., 2002). *Staphylococcus aureus* causes a fluctuating SCC over time in chronic mastitis cases whereas *Streptococcus uberis* causes a constantly high SCC observed in sub-clinical mastitis cases (Sorensen et al., 2009). Clinical practice observations showed that per- acute clinical endo-toxigenic *Escherichia coli* and exo-toxogenic *Staphylococcus aureus* strains of IMI on the other hand cause a sudden high SCC and/or often fatal toxic shock consequences without any elevated SCC warning alarm. The fact that some mycoplasmas have developed the capability of downregulating the synthesis of pro-inflammatory cytokines that was speculated by

(Chambaud et al., 1999) resulted in the re-evaluation of SCC profile interpretations by practitioners. Environment can also influence the BMSCC such as heat stress (Williams et al., 2016, Du Preez, 1995); pathogen carrier status of personnel in the parlour; milking machine maintenance regimes; traumatic factors; rainfall; season and climate; parlour hygiene; treatment and effectivity of prescribed antibiotics; exogenous melatonin/anti-inflammatory/cortisol treatments (Yang et al., 2017); current disinfectants used; thoroughness of milking; regularity of milking; feeding; treatment regimens; sample handling; sample age and counting methods are relevant in fluctuating BMSCC profiles (Nikodemusz et al., 1994). Therefore, BMSCC can be summarized as a collective multifactorial inflammatory immune response of a herd's endangered, threatened or compromised immune system that protects the mammary organ against invading pathogens after traumatic (teat injuries), parasitic (ticks/mites), chemical (teat dips), physical (milking machine dysfunction), toxicological (photosensitivity), metabolic (ketosis, hypocalcemia)), nutritional (milk urea nitrogen), neoplastic (squamous cell carcinoma), iatrogenic (vaccination), hormonal (adrenocorticoid, cortisone treatment), influences or exposures (heat stress).

Pathogens

Pathogens have specific antigenic factors that elicit antigen-antibody reactions and a resulting activated immune response causing a cell influx as part of the defense mechanism to the mammary glands of individual cows, which are responsible for the high collective BMSCC of the herd (Tizard, 2017). When elevated BMSCC indicates infection in a dairy, a collection of representative pathogen samples must be taken by the consulting veterinarian using a diagnostic surveillance protocol that will be proposed in this study. Accurate pathogen sampling and identification are crucial for pathogen specific therapy, selection of vaccine strategies and aids in limiting antimicrobial resistance. These pathogens can be classified into viruses, bacteria (aerobic, anaerobic, and facultative anaerobic), yeasts, algae, fungi, and

bacteriophages. According to their nature these pathogens can be further categorized in the milking parlor as infectious pathogens, environmental pathogens, zoonotic pathogens, food-borne pathogens, milking machine and utensils hygiene associated pathogens, water- borne pathogens, feed-borne pathogens, antimicrobial resistant pathogens, immune -suppressive pathogens, bio-security bypassed and new pathogens.

Milk is an essential food commodity and important as a component in the human diet (Delgado, 2003). Milk is also an excellent buffered growth and preservative medium for a variety of microorganisms (Oikonomou et al., 2012). The most important, common mastitogenic pathogens with the highest prevalence in South African herds were investigated and calculated from a dataset consisting of 62,230 over a 5 year period on pasture herds and total mixed ration herds (Blignaut et al., 2018; Petzer et al., 2009, Petzer et al., 2016a) . Blignaut et al. (2018) reported on the major mastitis pathogens namely Coagulase-Negative *Staphylococcus, Streptococcus uberis* and *Staphylococcus aureus*. Surveillance done in South African herds from 2000-2010 by Petzer et al. (2009), revealed a re-emergence of a beta hemolytic *Staphylococcus aureus* from human origin and *Streptococcus agalactiae* and *Enterococcus canis* causing several mastitis outbreaks. Furthermore the most prevalent IMI pathogens are Coagulase Negative *Staphylococcus* strains, although *Staphylococcus aureus* remains the most damaging pathogen in South African dairies (Petzer et al., 2009). *Streptococcus agalactiae*, *Strep. dysgalactiae*, *Strep. uberis* and *Enter. canis* were also commonly diagnosed, while *Enter. faecalis* was mainly found in dry cow samples. One-Health studies revealed that a much wider and more intensive approach on diagnostic protocol should be taken to include zoonotic pathogens than only looking at mastitis pathogens (Berge and Baars, 2020) Similarly Figure 1 illustrates the diversity of aerobic IMI pathogens identified during routine phenotypic investigation. Little information are available on *Mycoplasma* species prevalence (Azari et al., 2020), but is of global concern as a poorly diagnosed emerging disease in the dairy industry of Africa (Motaung et al., 2017). Angelopoulou et al.

(2019) identified some *Mycoplasma* species and these have been included in the systematic literature review.

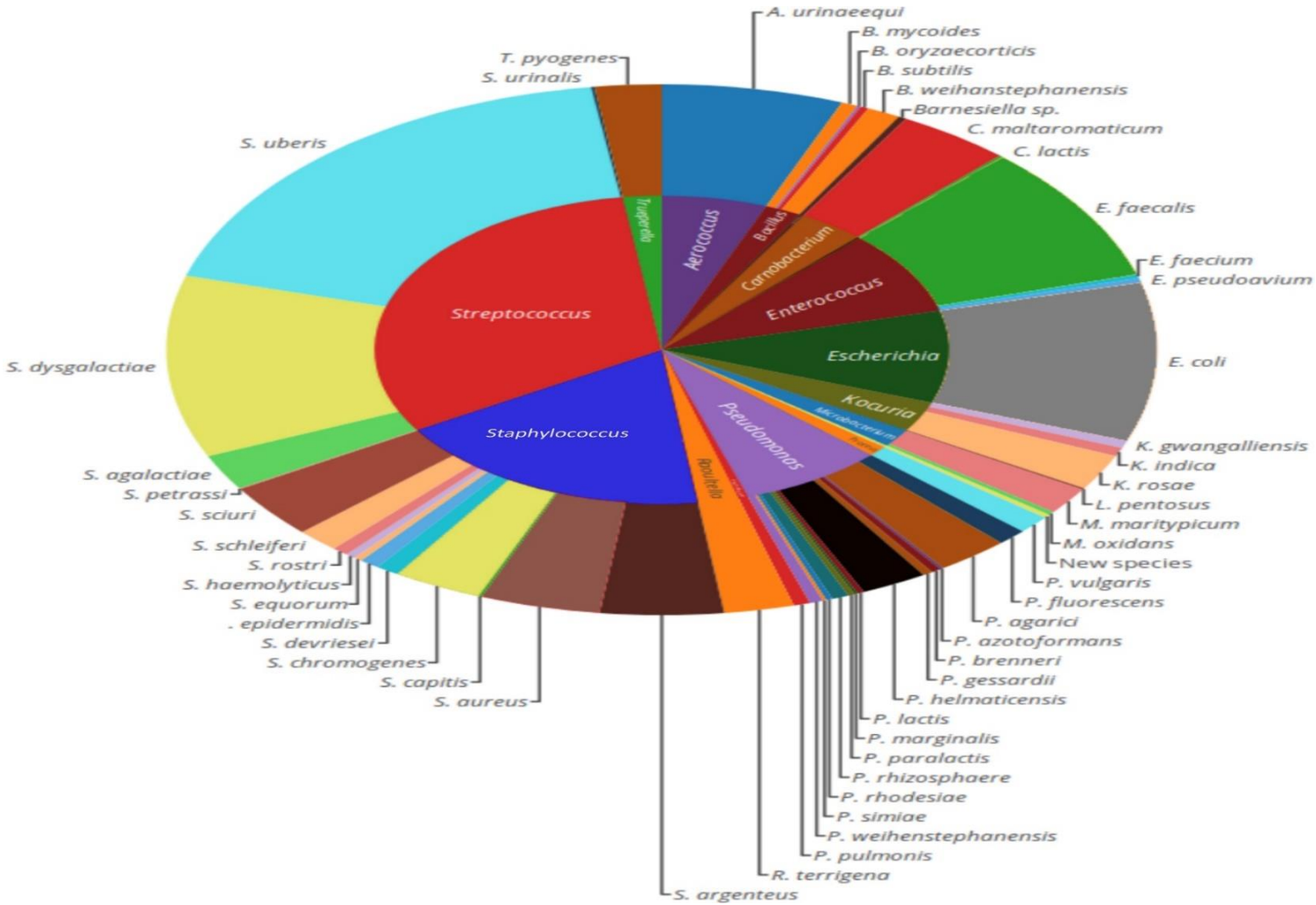

Figure 1. Average aerobic bacterial species identification on blood agar from fifty mastitis milk samples by Angelopoulou et al. (2019). Inner circle depicts genus and outer circle indicates species. Results depicting *Staphylococcus argenteus* was inconclusive as to whether it was *Staphylococcus argenteus* or *Staphylococcus aureus.*

Staphylococcus

Staphylococcus species were reported as a heterogenous group of gram-positive cocci, grape-like bacterial clusters, consists of 56 species and only 28 strains reliably categorized by phenotypical methods. Molecular methods added to the identification of the other 50% (Bes et al., 2000). This genus is categorized by coagulase positive staphylococci based on the ability to coagulate rabbit plasma (Swetha et al., 2017).

Staphylococcus aureus and coagulase negative *Staphylococcus*

Udder infection with *Staph. aureus* as well as *Strep. dysgalactiae* and *Strep. uberis* are common causes of bovine mastitis (Figure 1) (Lundberg et al., 2016). *Staphylococcus aureus* is the major pathogenic bacterium found in milk (Schmidt et al., 2017) with public health concerns (Ismail, 2017). BinaxNOW (immuno chromatographic strip test) *Staphylococcus aureus* isolation was reported with 97.6% and 100% sensitive and specific on blood agar, respectively and the PCR was 93.8-100% sensitivity and 98.6-100% specificity (Qian et al., 2014). Bes et al. (2000) isolated 56 *Staphylococcus* strains from IMI that were identified using both phenotypic and genotypic techniques and 28/56 strains were identified by phenotypical characteristics while 28/56 rendered unreliable and atypical profiles. Combined phenotypic and genotypic techniques were used to classify the different strains by 16S-23S rDNA spacer region polymorphism with rapid and reliable results. The RAPIDEC *Staphylococcus* kit is an efficient, rapid, and cost-effective diagnostic method of *Staph. aureus* identification from cultures on bovine mastitis samples (Boerlin et al., 2003). It proved to be unpractical for private practice, but reliable and rapid technique for the classification of staphylococcal isolates down to subspecies level in specialized laboratories (Bes et al., 2000). *Staphylococcus aureus* were detected in clinical milk samples using a qualitative real-time PCR (qPCR) targeting the *nuc* (thermonuclease) gene and the analytical sensitivity of clinical samples were 50.7 times higher than conventional bacteriology (Graber et al., 2007). CHROMagar® introduced a chromogenic medium for the detection (isolation and differentiation) of MRSA (methicillin/oxacillin resistant *Staph. aureus*) with 100% sensitivity/specificity (Virgin et al.,

2009). Duplex PCRs are used to detect *nuc* gene and methicillin resistance gene, *mec*A, in bulk tank milk samples with confirmed methicillin resistant *Staph. aureus* (Virgin et al., 2009).

(Clothier et al., 2010) studied *Staph. aureus* strains, which are normally coagulase positive, although some strains are coagulase negative. The studies described coagulase positive *Staph. aureus* (CPS) causing a wide variety of diseases in animals and humans, like wound complications, pneumonia, septicemia, endocarditis, osteomyelitis, and pyoderma. In dairy animals it causes severe mastitis losses with accompanied systemic symptoms and even acute exo-toxic mastitis death. Coagulase Negative *Staphylococcus* (CNS) are associated with nosocomial infections in intensive care units (ICU) and neonatal hospitals and enterotoxin food poisoning. In dairy animals it causes sub-clinical and less severe clinical mastitis (Taponen et al., 2006, 2009). There is a vast number of CNS species that cause subclinical and clinical mastitis that is persistent and contribute to increased milk somatic cell count. Ten of the thirty-nine non-*Staphylococcus aureus* are associated with mastitis ((Taponen and Pyörälä, 2009). Characterization and identification of CNS species are traditionally done phenotypically, but sometimes differences occur compared to genotypical identification (Taponen et al., 2006, Ajitkumar et al., 2013). Bes et al. (2000) reported that various tests can be used to successfully identify species among non-*Staph.* aureus strains when using all the phenotypic characteristics including cultural, morphological, and biochemical techniques. Karzis et al. (2020) reported maltose negative strains of *Staph. aureus* differed in their antimicrobial resistance patterns over time, in comparison to maltose-positive *Staph. aureus* strains. Minimum inhibitory concentration (MIC) testing reported more multidrug-resistant maltose negative *Staph. aureus* isolates than reported for the maltose positive strains (Karzis et al., 2020b).

Emerging anti-microbial resistance in pathogens of milk producing animals are a global concern. Coagulase Positive Staphylococci (CPS) and Coagulase Negative Staphylococci (CNS) strains and other opportunistic pathogens causing infections and food poisoning have been reported in humans and reside on surfaces as well as cow udders in the dairy parlour

(Schmidt et al., 2015). The presences of methicillin and vancomycin resistances among staphylococci isolated from milk necessitate the periodic surveillance for antimicrobial resistance patterns of staphylococci in order to control the spread of anti-microbial resistance (El-Sayed et al., 2017) thus mitigating the hazard of food chain or direct transmission of resistant pathogens to humans (Swetha et al., 2017). Phenotypic and genotypic diagnosis shows an indistinguishable resemblance between human and animal isolates according to Schmidt et al. (2015). These authors reported that AMRs are common in CNS due to indiscriminate use of antibiotics by lay persons on farms (Schmidt et al., 2015). CNS species easily develop multidrug antimicrobial resistance with the most common being penicillin G and aminopenicillins, also methicillin/oxacillin resistance in organisms carrying the *mecA* gene.(Phophi et al., 2019). These MRSA strains became a serious global epidemiological problem. These strains can be detected with PCR (gold standard test) (Srinivasan et al., 2002). Swetha et al. (2017) found MRSA infections are most probably due to vancomycin resistant *Staph. aureus.*

AMR is of great concern as most *Staph. aureus* isolates were generally susceptible to all or almost all antimicrobials tested, while >50% of the CNS isolates were resistant to one or more antimicrobials tested (Taponen and Pyörälä, 2009). The important virulence factors and biofilm associated proteins are also found among bovine CNS(also referred to as NAS (Non-aureus staphylococci) mastitis isolates, namely *Staph. epidermidis, Staph. chromogenes, Staph. hyicus,* and *Staph. xylosus* (Taponen and Pyörälä, 2009).

Karzis mentioned in 2020 that, "*On a molecular basis, it was found that some non-aureus Staphylococci species are in fact environmental and some are actually pathogenic* (Piessens et al., 2011)*. Over time there seemed to be a shift from the environmental to the pathogenic NAS organisms, which is why at present NAS is the principal cause of mastitis worldwide, far overtaking S. aureus in terms of prevalence of mastitis"*(Piessens et al., 2011, Piessens et al., 2012).

Streptococcus

The bacteria involved in bovine mastitis are mainly *Streptococcus* spp., *Staphylococcus* spp., and coliforms (Figure 1). *Streptococcus* agalactiae is an infectious pathogen associated with transmission from cow to cow, while *Strep. dysgalactiae* and *Strep. uberis* are environmental pathogens (Minst et al., 2012).

Streptococcus uberis

Wald et al. (2017) emphasized the identification, differentiation, and susceptibility testing of Streptococcal IMI in dairy cows results in more effective treatment regimens with shorter treatment duration in some cases resulting in more effective use of antibiotics and lowering antimicrobial resistance. Routine diagnostic laboratories make use of phenotypical and biochemical diagnostic tests for *Strep. uberis* (Wald et al., 2017, Petzer et al., 2009). Additional multiplex PCR assays add to the practicality and accuracy of pathogen diagnosis in milk when more than one pathogen can be identified in a time frame. A multiplex qPCR for the detection of *Staph. aureus* (species specific genetic marker), *Strep. uberis* (plasminogen activator gene) and *Strep. agalactiae* (*cfb* gene encoding the Christie-Atkins-Munch-Petersen factor (CAMP)) in milk was compared with conventional microbiological method. The qPCR technique using overnight enrichment correctly identified 91.7% of *Staph. aureus,* 98.2% of *Strep. agalactiae,* and 100% of *Strep. uberis.* The sensitivity and specificity of the multiplex qPCR that correctly identified *Staph. aureus, Strep. agalactiae,* and *Strep. uberis* from milk were 95.5% and 99.6% respectively (Gillespie and Oliver, 2005). Petzer et al. (2016) argued that *Strep. uberis* strain typing of individual cow samples on a herd basis are not cost effective.

Streptococus dysgalactiae

Streptococcus dysgalactiae is a major cause of acute clinical and subclinical bovine mastitis especially in Europe (Alves-Barroco et al., 2019). It is a Gram-positive cocci organism with a tendency to form chains as a facultative anaerobe cultured on Edwards media, which is non (alpha)- haemolytic on blood agar, catalase and oxidase negative biochemically (Watts, 1988).

Bacterial culturing has a low sensitivity and specificity 38.8% and 92.8%(Addis et al., 2016) while qPCR detection in milk in separate qPCR with 95.5% and 99.6% sensitivity and specificity, respectively (Gillespie and Oliver, 2005). Historically Lancefield grouping classifies the catalase-negative gram-positive cocci based on the carbohydrate comparison of antigens found in their cell walls with group C classifying *Strep. dysgalactiae, Strep. equisimilis, Strep. equi* and *Strep. zooepidemicus*. New more accurate diagnostic techniques are currently available for identification and anti-microbial resistance characterization which are out of scope for the practitioner. (Hsieh et al., 2019).

Streptococcus agalactiae

Streptococcus agalactiae is known for causing chronic mastitis in bovines. It belongs to Lancefield Group B organisms with distinct beta-hemolysis on Edwards media and test positive on CAMP test and negative for aesculin hydrolysis (Keefe, 1997). As a zoonosis, Lancefield group B streptococci has been associated with severe human neonatal infections such as septicemia and meningitis. Chromagar StrepB® is a highly sensitive (92.0%) and specific (Ganda et al., 2016) diagnostic technique, coloring the organism colony a Mauve color for identification (Poisson et al., 2011). As mentioned, the sensitivity and specificity were 95.5% and 99.6% respectively of the multiplex qPCR correctly identified *Staph. aureus, Strep. agalactiae,* and *Strep. uberis* from milk (Gillipie & Olivier, 2005). The qPCR from bulk milk tank samples detected *Strep. agalactiae* accurately compared to conventional microbiological culture methods (de Carvalho et al., 2015).

Enterobacteriaceae

Escherichia coli as well as other less common IMI infections such as *Klebsiella oxytoca* and *Klebsiella pneumoniae, Enterobacter aerogenes* and non-coliform such as *Serratia marcescence (Klaas and Zadoks, 2018)* are *Enterobacteriaceae*. Phenotypic tests are used worldwide to identify enterobacteria; however, they tend to misdiagnose the species despite the multiple tests. In a study the enterobacteria from a dairy cattle environment identified

Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Serratia marcescence accurately, but misidentified *Enterobacter* spp including *Enterococcus aerogenes* (Rodrigues et al., 2017).

Escherichia coli mastitis is often seen in freshly lactating cows as a per-acute endo-toxemic mastitis because of endotoxin release from bacterial cell walls. Sub-clinical, clinical, and chronic mastitis can also occur because of pathogenicity of the organism and cow immunological factors. *Escherichia coli* surface antigens are named according to capsular (K), cell wall/somatic (O), flagellar (H), fimbrial (F) . These O, H, and K antigens can be used to serotype strains. Each serotype is designated by the numbers of antigens that it bears e.g. O157:K85:H19 (Markey et al., 2013).

Escherichia coli is a gram-negative rod shape organism with non-mucoid, round pink (lactose-fermenter) colonies on McConkey agar. It is occasionally hemolytic on blood agar and oxidase negative (Abbasi et al., 2014). Contamination of milk in dairy farms with mastitis and unhygienic conditions transmit zoonotic bacteria to milk consumers (Liu et al., 2020). Shiga toxin-producing *E. coli* (STEC) cause human diseases, ranging from diarrhoea to haemorrhagic and life-threatening complications such as haemolytic uremic syndrome (Awadallah et al., 2016). To distinguish between Shiga-toxin *E. coli* and *E. coli* O157 serotypes, commercially available CHROMagar® chromogenic media plates are available. CHROMagar STEC® for non-O157 Shiga-toxin producing *E. coli is* important in food poisoning. Non-O157 Shiga-toxin producing *E.coli* mauve coloring colonies can be diagnosed with 89.1% sensitivity and 91.4% specificity from other colorless serotypes or blue *Enterobacteriae* (Hussein and Bollinger, 2008, Gouali et al., 2013). CHROMagar O157® for selective isolation and differentiation of *E.coli* serotype O157 in milk samples gives mauve colonies with 98.0% sensitivity (Rhee et al., 2002).

Klebsiella is gram-negative with mucoid non-motile growth on McConkey agar whereas *Serratia marcescence* produces a distinctive red pigment on McConkey agar (Markey et al., 2013). CHROMagar KPC® for detection of carbapenem-resistant *Klebsiella* bacteria with

100% sensitivity and 98.8% specificity (Samra et al., 2008) and differentiated *Enterobacter/Klebsiella/Citrobacter, Escherichia coli* and *Pseudomonas* (Panagea et al., 2011). Anti-microbial resistance determination are particularly important in this group of bacteria because of the possibility of multiple drug resistance (Srinivasan et al., 2007). CHROMagar ESBL® can be used for differentiation and detection of extended spectrum beta-lactamase producing bacteria such as *E.coli Klebsiella/Enterobacter*, and *Proteus* with 100% sensitivity and 93.3% specificity (Saito et al., 2010).

Enterococcus faecalis and *Enter. faecium* are round white and smooth colonies with greenish non (alpha)-hemolysis on blood agar. These species are Gram-positive cocci with red pinpoint colonies on McConkey agar and group into the Lancefield group D (Markey et al., 2013). CHROMagar VRE® allows for the detection of vancomycin resistant *Enter. faecalis* and *Enter. faecium* using colony color after 24 hours incubation with 95.5% sensitivity and 90.4% specificity (Peterson et al., 2010). CHROMagar VRE® differentiate *Enterococcus* species with 86.0-99.0% sensitivity and 95.0-100% specificity while multiplex PCR provided 98.0% sensitivity and 99.0% specificity (Archibald, 2011). Antimicrobial resistant genes in enterococci can be transferred to other species through plasmids and conjugative transposons (Clewell, 1990) such as vancomycin resistant transferred to other gram-positive bacteria (Burrus et al., 2002). The qPCR based commercial PathoProof mastitis PCR assay was used to detect mastitis pathogens in milk and identified *Escherichia coli and*, *Enterococcus* with the assumption that this kit provided 100% analytical sensitivity and specificity using isolates from bovine mastitis. *Streptococcus pyogenes*, S*treptocccus salivarius*, *Streptococcus, sanguis* from human origin were identified as *Streptococcus uberis* while *Shigella* spp were identified as *Escherichia coli* which decreased the specificity to 99.0% in S*treptococcus uberis* and 99.5% in *Escherichia coli* as determined by Koskinen et al., (2009). Hiitio et al. (2015) and Sørensen et al. (2009) compared the PathoProof PCR assay with conventional culture and reported positive *Staphylococcus aureus* and *Staphylococcus spp* with 97.0% and 86.7% sensitivity and 95.8% and 75.4% specificity, respectively. Spittel & Hoedemaker (2012) also

compared the PathoProof PCR assay with conventional bacterial culture. The authors reported bacterial culture with 76.9-100% sensitivity and 63.3-98.7% specificity for six out of seven pathogens. With *Enterococcus* spp the sensitivity was 9.1% for bacterial culture. With the PCR assay *Corynebacterium bovis, CNS, Staph. areus, Arcanobacterium* (*Trueprella*) *pyogenes* were detected from most to least frequency while with both method *Streptococcus uberis* was the most frequent detected.

Trueperella pyogenes

Trueperella pyogenes causes an acute suppurative mastitis. It was previously classified as *Arcanobacterium pyogenes*, *Actinomyces pyogenes* and formerly as *Corynebacterium pyogenes* (Markey et al., 2013). *Trueperella pyogenes* is a gram-positive, pleomorphic, facultatively anaerobic rod that is catalase negative. Its growth requirements are not excessive, but media enriched with blood or serum need to be used for the culture. In co-infection with *Peptoniphilus indolicus* it causes a foul-smelling udder secretion that can by carried by flies to infect other cows. The condition is commonly known as summer mastitis (Rzewuska et al., 2019).

Molecular diagnosis by PCR -mediated gene targets including 16S rRNA, *sod*A , *plo* and 16S-23S rRNA intergenic spacer regions (Markey et al., 2013). Enterococci *resistance isolates* were observed against tetracycline (Clothier et al., 2010) and gentamicin (86.9%), while less resistance was reported for chloramphenicol (Soltau et al., 2017) and nitrofurantoin (10.9%) (Momtaz et al., 2016).

Nocardia spp

*Nocardia spp are w*hite, powdery colonies that are imbedded in the agar media (Markey et al., 2013). *Nocardia* spp. are saprophytes that commonly grows in soil that can become pathogens when introduction takes place into the udder. Milk is plated on blood agar (Columbia agar base) with gentamicin (25 mg/L). The antibiotic is added to reduce or eliminate background bacteria and allows *Nocardia* spp. to be detected more easily.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen that is frequently isolated in high cell count herds. The microorganism is considered an environmental pathogen in mastitis outbreaks (Kotzé, 2017). *Pseudomonas aeruginosa* is gram-negative rods on blood agar with large, flat colonies usually hemolytic with a greenish pigment (pyocyanin) and oxidase positive (Markey et al., 2013). Pseudomonas resistance can become a substantial antimicrobial resistance zoonotic problem (Livermore, 2002). Real-time PCR assays for *P. aeruginosa* in milk and soil samples was done using various target regions but the culture-based approach has been reported to be more sensitive than qPCR (Neamah, 2017). Highly sensitive and specific identification were observed with qPCR methods on soil and dung samples (Colinon et al., 2013).

Listeria monocytogenes

Listeriosis has been recognized as an emerging foodborne bacterial infection and a public health hazard (Farber and Peterkin, 1991). It is not a common mastitogenic organism, however this zoonotic pathogen can cause bovine mastitis under certain conditions and should be considered and eliminated (Goudar and Prasad, 2020). Detection of *Listeria monocytogenes* involves conventional selective enrichment subcultures on selective agar plates, followed by secondary confirmatory identification phenotypical tests, and this takes several days to complete with some unreliable outcomes. A rapid PCR detection protocol to screen for the presence of *Listeria monocytogenes* in milk are available (Holko et al., 2002). (Osman et al., 2016) used PALCAM agar plates and Listeria spp. were confirmed using biochemical and CAMP reactions. Omiccioli et al. (2009) used real-time PCR detection of *Salmonella* spp, *Listeria monocytogenes* and *E. coli O157* in milk.

Corynebacterium bovis

Corynebacterium bovis is an important sub-clinical mastitis pathogen. On sheep blood agar colonies are small, dry, white, and non-hemolytic in nature. Microscopic appearance shows

these organisms as gram-positive pleomorphic rods. Watts et al. (2000) identified *Corynebacterium* spp. from 183/ 212 coryneform bacteria from dairy cows based on isolation and biochemical tests. The 29 strains were misidentified as a yeast, *Bacillus* spp. (2) , *Enterobacteriaceae* (11), staphylococci (18), and a *Streptococcus* spp.and *Enterococcus* spp. The authors also tested the Biolog and API Coryne systems which correctly identified 54.0% and 88.0% of these strains, respectively (Watts et al., 2000).

Mycoplasma

Mycoplasma are pleomorphic bacteria that lack a cell wall, are contagious, and can cause high SCC and chronic clinical mastitis and is increasing globally .(Nicholas et al., 2007). *Mycoplasma bovis* is the causative organism in > 50% of cases (Clothier et al., 2010). Eleven (11) other *Mycoplasma* and *Acholeplasma* species have been also isolated from milk namely *Mycoplasma alkalescens, Mycoplasma arginini, Mycoplasma bovigenitalium, Mycoplasma bovirhinis, Mycoplasma californicum, Mycoplasma canadense, Mycoplasma dispar, Mycoplasma* species group 7, *Mycoplasma* F-38*, Acholeplasma laidlawii, and Acholeplasma axanthum* (González and Wilson, 2003).

Mycoplasmas are fragile and thus milk samples must be kept refrigerated and delivered before 24-48 hours or in a transport medium of 5 mg/ml ampicillin at room temperature for *Mycoplasma* isolation. *Mycoplasmas* are fastidious organisms that require an isotonic distilled water medium with cholesterol growth factors derived from 20% horse serum. Penicillin and thallium acetate are added to the medium to prevent contamination growth of bacteria and fungi (Nicholas et al., 2007, Sachse et al., 2010, Markey et al., 2013). Commercial Hayflick's medium are used for isolation (Riekerink et al., 2006). Comparison of certain conserved gene sequences, 16S rRNA and genomic restriction patterns have been effectively used in species and strain identification (González and Wilson, 2003). Multiplex Real-time PCR seems to be the most cost effective and practical technique to apply in the routine diagnostic work done in

the dairy industry (Behera et al., 2018a). ELISA technologies can be used for milk immunodiagnostic purposes (Uhaa et al., 1990).

Coxiella burnetii (Q fever)

Coxiella burnetii is zoonotic and milk-borne occupational disease of farmers, abattoir workers and veterinarians. It is an obligate intracellular pleomorphic bacterium with a gram- negative type cell wall and grown in cell lines (Marrie and Raoult, 2000). Isolation can be achieved in a variety of methods, but is hazardous for laboratory staff and should be performed in a laboratory with biosafety level 3 (Dadimi and Nishanth), 2020). Identification of the organism can be achieved with Modified Ziehl-Neelson or Gimenez stains but is not normally detected by Gram stain. Immuno-histochemistry can also confirm bacterial identity PCR techniques are also available in some laboratories. Accurate diagnosis in milk can be made by commercial serological method to detected antibodies. (Markey et al., 2013). Several serological tests are also available (i.e., immunofluorescence (IFA), enzyme-linked immunosorbent assays (ELISA) and complement fixation test (CFT) as shown in Appendix 1.

Mycobacterium avium paratuberculosis (MAP)

Mycobacterium avium paratuberculosis affects domestic and wild animals by causing chronic enteritis, diarrhea, weight loss, progressive emaciation and eventually death (Szteyn et al., 2020). MAP has been linked as a zoonosis to Crohn's disease and is a controlled animal disease. This pathogen is occasionally capable of surviving commercial pasteurization (Paolicchi et al., 2012). It needs to be cultured in Biosafety level 3 laboratory and is slow growing. Antibodies to this pathogen can be detected using serology method (commercially IDEXX MAP ELISA antibody test-Appendix 1) as well as qPCR methods (Rodríguez-Lázaro et al., 2005, Szteyn et al., 2020).

Brucella abortus / melitensis.

Brucella abortus or B. melitensis (Dadar et al., 2020) is *a* state-controlled animal diseases (communicable disease) and needs to be cultured in laboratories with appropriate biosafety

level laboratories (Dadar et al., 2020). Due to strict legislation on veterinary diagnosticians, these important zoonotic diseases cannot be investigated, surveyed, or diagnosed by private professionals while they themselves are daily exposed by some of these pathogens that is endemic in certain districts of South Africa. Serological test can be used to detect antibodies of smooth *Brucella* spp (*B. abortus* and *B. melitensis*) in SANAS accredited laboratories. The detection of viable *Brucella* organisms in potentially contaminated milk can be done by a qPCR assay that is a reliable and sensitive method to identify infection risks without bacterial isolation and lower laboratory biosafety risks (Projahn et al., 2020, Dadar et al., 2020). The Milk Ring Test (MRT) was compared with ELISA on bulk milk and found that the sensitivity of the ELISA (98.1%) was higher than the MRT (72.2%) when using bulk milk samples with no statistically difference (*P*=1.0) between the specificity of MRT (90.5%) and the ELISA (88.1%) (Vanzini et al., 2001).

Other organisms

Other exotic organisms, species, sub-species, and strains could also be clinically diagnosed using culture and identified by the proposed diagnostic surveillance model e.g. teat sphincter *Fusobacterium necrophorum/ Staph. aureus* necrosis, secondary *Phithomyces chartarum* mycotoxicosis, *Staph. aureus* udder impetigo on the base of teats or between quarter cleavage, and *Criptosporidium* as zoonosis (Ursini et al., 2020).

Alge: *Prototheca spp*

Prototheca spp were associated with serious outbreaks of mastitis in dairy herds. *Prototheca* is an algae common in soil and aqueous habitats like dairy herd environments. Diagnosis is confirmed by real-time PCR (Pal et al., 2014) and therapy is mostly unsuccessful, and elimination of infected cows are indicated (Ranjan, 2015). Ricchi et al. (2010) developed qPCR assay for *P. zopfii* genotype 2*, P. wickerhamii and P. blaschkeae* targeting18S rDNA for the diagnosis of animal or human protothecosis.

Viruses

Bovine Herpes Virus (BHV)1 BHV 4, Foot-and-mouth disease virus and para -influenza virus 3 virus were isolated from mastitis cows (Tignon et al., 2017). Intramammary inoculations of Bovine Herpes Virus 1 and 4 or Para-influenza virus 3 induced high cell counts with sub-clinical or clinical mastitis (Herlekar et al., 2013). BHV, Cowpox virus, Vaccinia virus, Vesicular stomatitis virus, Pseudo cowpox virus and Bovine Pappiloma virus plays an indirect role in BMSCC as primary invaders for secondary bacterial IMI as commonly seen in practice. The immune-competence of the herd is also affected by primary viral disease which causes a fluctuation in the BMSCC. BHV1, Bovine Immunodeficiency Virus, BVDV and BLV have immunosuppressive properties (Wellenberg et al., 2002).

Bovine Viral Diarrhea Virus

The chronic, endemic form of BVDV in a dairy herd is associated with high somatic cell counts and mastitis (Berends et al., 2008a). BVDV genotype 1 is traditionally used in diagnostic techniques in milk. Persistently infected animals can constantly shed virus in the milk. As 80% of cows in infected herds will show antibodies in the milk, the milk antigen test is a more practical approach in identifying positive animals. Sero-conversion for diagnosing new infections, diagnostic demonstration of persistently infected animals by ELISA and identifying antigen positive animals by RT-PCR are accepted to confirming and monitoring BVDV infection in a herd (Waage, 2000, Berends et al., 2008b). Diagnosis in the milk can be confirmed by commercially available IDEXX BVDV Total Elisa antibody/antigen test as shown in Appendix 1.

Bovine Leukemia Virus/Enzootic Bovine Leucosis

Bovine leukemia virus is a retroviral disease of cattle characterized by persistent lymphocytosis in 30% of serologically positive cases and development of B-cell lymphosarcoma in a much smaller percentage of cases. Important transmission of BLV occurs via the colostrum and milk containing infected B- lymphocytes. High culling rates and

susceptibility to other diseases with infectious aetiology, with symptoms of mastitis, diarrhoea and/or pneumonia were demonstrated and reduced protective immunity following vaccination in BLV infected cattle has been reported (Frie et al., 2016; Yang et al., 2016). Diagnosis in the milk can be confirmed by the IDEXX BLV/EBL Leucosis milk verification ELISA antibody test, as shown in Appendix 1.

Other viruses

Other viruses which are indirectly responsible for an altered BMSCC because of secondary bacterial invasion of viral lesions ((Herlekar et al., 2013). Viral conditions commonly seen in South African dairy herds are:

- Bovine parapox virus (Pseudo cow pox)

  Clinically diagnosed inflammatory papules on the teats that develops in a ring-shaped scab forming lesion with secondary bacterial infection as a source of IMI. High-Resolution Melting (HRM) analysis assay can be used to detect the PCPV genome (Ziba et al., 2020).

  Clinically diagnosed Cauliflower- like cutaneous fibropapilomas types 1-10 on the skin or teats with secondary bacterial infections between growth (Prameela and Veena), 2020).

- Bovine Herpes Virus -2 (Ulcerative mammilitis)

  Severe painful clinically diagnosed oedema and erythema with a raw ulcerated wound on the teat skin. Secondary *Staphylococcus aureus* infection of healing viral wound .(Lelli et al., 2018).

- Lumpy skin disease can be successfully diagnosed by a qPCR assays (Mashamba, 2020).

Diagnostic techniques

According to the National Mastitis Council 1996 (Friendship et al., 2010), reliable identification of pathogens is a key factor to prevention of IMI. Diagnosis of IMI cannot be solely based on laboratory results alone and should be evaluated and analyzed in conjunction with history, data diagnostics, total herd diagnostic and biosecurity approach, clinical signs of inflammation and symptoms, teat end pathology, udder scar tissues, abscesses, fibrotic tissue, and changes in the milk (Ashraf and Imran, 2018). It is not realistic to launch a biosecurity program based on only on laboratory results, when prescribing antimicrobials, vaccines and other preventive measures (Hiitiö, 2018). To mitigate such unprofessional situations that can directly lead to anti-microbial resistance in South African dairy herds, the SANAS accredited Milk laboratory of the University of Pretoria set an example of excellence where a pro-active herd program is based upon and implemented successfully on strong working relationships between milk producers , consulting veterinarians, and well-trained samplers.

Somatic Cell Count Techniques:

The current reference method for determining the BMSCC/ individual quarter SCC in raw milk is the direct microscopic somatic cell counting analysis (DMSCC) by the Breed Prescot smear technique. Well trained staff are necessary, while it is time consuming to maintain accuracy and reproducibility(Moon et al., 2007).

COW-SIDE Techniques:

Cow-side tests, although convenient, fast, and cost effective for the practitioner, are not nearly as effective and accurate as SANAS accredited laboratory tests. On farm techniques should always be backed up by SANAS accredited techniques for accurate diagnosis.

DeLaval cell counter (DCC): The DCC is part of the consulting veterinarian's standard equipment for BMSCC determination on farm as a comfortable and quick monitoring apparatus. (https://www.delaval.com/en-za/our-solutions/milking/udder-health--hygiene/milk-

testing/delaval-cell-counter-dcc/). This apparatus counts somatic cells optically and automatically within 1 minute with some coefficient variation.

California Mastitis Test (CMT) and/or hand-held electrical conductivity (Draminski apparatus) applied individually or in parallel : Both CMT test and the Draminski apparatus are cost-effective and indicative methods to detect IMI in the dairy herd. (Karzis and Petzer, 2012, Petzer et al., 2013)

Milk conductivity: Electrical conductivity is measured during the first few seconds by a sensor for each quarter or per cow mounted in the milk line. Conductivity values and ratios can be determined between quarters and consecutive readings which are then used as an indicator of somatic cell count status. Sensor results are compared with visual inspection of in-line mastitis filters fitted to a milking unit. Filters are inspected for clots immediately after every cow's milking.

SANAS accredited high accuracy Somatic Cell Count techniques:

Far more accurate methods are the electronic particle counting method (Coulter counter-Coulter Electronics Ltd Bedfordshire, UK), fluoro-optic electronic cell counting method by disc (Fossomatic 90) and flow-cytometry method (Fossomatic 4000/ 5000- Foss Electric, Hillerod, Denmark), Somacount 150- (Bentley Instruments Inc, Chasca, MN), Somascope (Delta Instruments, Drachten, Netherlands) and C-reader system (Digital Bio Technology co. Seoul, Korea.) These instruments are calibrated by the microscope manual counting golden standard and are currently in use at state subsidized institutions like Universities, NOSA and Agricultural Research Council (Moon et al., 2007).

Classical microbial culture: Different selected techniques are commercially available, validated, rapid, user friendly, cost effective, practice applicable in the surveillance and will each be briefly mentioned (Adkins and Middleton, 2018; OIE Terrestrial Manual 2012).

Primary aerobic culturing which include the following commercial culture-based tests that are done consecutively on media namely Blood Triptose Agar (BTA-6% sheep blood agar or

Columbia equine blood agar), Chromagar Mastitis media (Griffioen et al., 2018b), McConkey agar (Ganda et al., 2016). The CHROMagar mastitis medium utilize chromogenic medium substrates for gram-positive bacteria with chromogenic mix, peptone, and yeast extract in one half of the plate and for gram-negative bacteria in the other half of the plate (Griffioen et al., 2018a).

Bacteriological culturing of quarter milk samples is performed in the veterinary practice laboratory where 0.01 ml milk is inoculated onto each media plate. Presumptive growth of mastitis-causing pathogens is examined after 24-48 hours incubation at 37°C under aerobic conditions. Growth of one colony of pure culture is considered for further characterization while contamination is defined as more than one phenotypically different colony types. Basic identification is done using microscopy staining from primary cultures which include Gram staining to differentiate between gram positive and gram-negative pathogens. “No- growth” samples should be investigated with complementary / alternative methods.

Isolation for many pathogens causing IMI is the gold standard technique for diagnosis and used as the standard diagnostic method in mastitis prevention schemes historically. Isolation is laborious for fastidious pathogens to be identified and characterized (Salina et al., 2020). Zoonotic pathogens have biosafety risks associated for isolation especially for practice laboratory staff. Between 30-50% clinical mastitis samples harbor ’’no-growth’’ as some are intracellular microorganisms, fastidious or due to the natural bacteriostatic and bactericidal components such as lactoferrin, lysozyme, fatty acids, and lactoperoxidase present in raw milk (Chakraborty et al., 2019). Negative cultures could also result from recent intramammary antibiotic treatments. The overall rate of false positive results to identify bacteria observed according to a European proficiency evaluation ranged between 9-37% across different mastitis culture laboratories (Pitkälä et al., 2005). Due to the low sensitivity of primary isolation complementary techniques are required for accurate identification. Ajitkumar (2011) and Barkema (2006) stated that it is critical to use improved techniques for accurate and rapid detection of causative organisms, as phenotypical methods fail to make distinctions between

isolates belonging to the same species because of variable gene expression of the phenotypical characteristics. Accurate pathogen identification is crucial for pathogen specific therapy, selection of vaccine strategies and aids in limiting AMR. Complementary methods such as qPCR that can detect DNA of the pathogens or immunological methods that detect the antibodies to a specific antigen or Mass- Spectrometry that can detect specific protein molecules.

A critical factor in mastitis studies was stated by Nicholas et al. (2007) concerning the exclusion of *Mycoplasmas* as possible etiological agents in a survey of the incidence and aetiology of mastitis on dairy farms in England and Wales (Bradley et al., 2007) as these organisms are not detected with standard culturing methods and not recorded. *Mycoplasma* require special growth medium (Riekerink et al., 2006) and will thus have to be added additionally to the current standard growth media or molecular technologies should be investigated and validated for IMI diagnosis (Rossetti et al., 2010; Ledger et al., 2020).

Different selective media and biochemical techniques: Different selective media are applicable and feasible in private practice laboratory such as mannitol salt agar (Merrill et al., 2017) for *Staphylococcus* characterization and Edward's medium selective for milk streptococci (Hsieh et al., 2019). Commercial selective media are also available for identification of specific virulent organisms for example Chromagar *Staphylococcus aureus* with 95.5% sensitivity and 99.4 % specificity (Gaillot et al., 2000, Bautista-Trujillo et al., 2013). Chromagar *Escherichia coli* STEC (Shigatoxin *Enterococcus*) and Chromagar *E.coli* 0157 media for specific pathogen selection.

Biochemical identification of specific genus and or bacterial species, the Biochemical Biomerieux API test strips can be used such as API *Staph. aureus*; API *Streptococcus*; API Coryne; API 20 Enterobacteria (https://www.biomerieux-usa.com/sites/subsidiary_us/files/18_api-ref-guide_v7.pdf).

Techniques for anti-microbial resistance determinations: The antimicrobial susceptibility testing is conducted using quantitative methods for the calculation of the minimum inhibitory concentration (MIC) using dilutions of antimicrobial agent in a broth where the highest dilution of the antibiotic or disinfectant inhibit growth. The antibiogram-disc diffusion tests by the Kirby–Bauer disk diffusion technique can also be used (Ismail, 2017)CLSI 2008; (Hsieh et al., 2019).

Clinical AMR breakdown points The Kirby Bauer technique (Bauer, 1966) with published breakpoints are used to determine antimicrobial susceptibility. The diameter of the inhibition zones are used to classify results as sensitive, intermediate or resistant in accordance with the Clinical and Laboratory Standard Institute but differs between veterinary animal specific vs human breakpoints used (CLSI 2008, CLSI 2012).(Karzis et al., 2020a).

The "VetPath study shows that mastitis pathogens were susceptible to most antimicrobials with exceptions of staphylococci against penicillin and streptococci against erythromycin or tetracycline. For most antimicrobials, the percentage resistance and $MIC_{50/90}$ values among the major pathogens were comparable to that of the preceding VetPath surveys. This work highlights the high need to set additional clinical breakpoints for antimicrobials frequently used to treat mastitis'' (El Garch et al., 2020).

VETCAST is a different approach based on dairy animals in Europe to determine clinical breakpoints. The standards used, make a huge difference on results and the products tested for. There are certain pathogens which have intrinsic resistance to certain products. VETCAST's approach to determine a clinical break point (CBP) is based on an epidemiological cut-off value (highest MIC that defines the upper end of the wildtype MIC distribution); Pharmacokinetic break points based on PK/PD breakpoints and clinical cure cut- off in relation to MIC (Silley, 2012).

Chromagar antimicrobial resistance selective media are economical and practically feasible in private practice (Griffioen et al., 2018b) such as :

- Chromagar MRSA for isolation and differentiation of methicillin resistant *Staphylococcus aureus* (MRSA) with 100% sensitivity and specificity according to Taguchi et al. (2004).

- Chromagar VRE with proof of 95.5% sensitivity and 90.4% specificity for vancomycin resistance in E*nterococcus faecalis* and *Enterococcus faecium* after 24 hours of incubation (Merquior et al., 2012).

- Chromagar KPC for the detection of carbapenem-resistant *Escherichia coli, Klebsiella, Enterobacter, Citrobacter* and *Pseudomonas* with 100.0% sensitivity and 98.8% specificity according to Panagea et al. (2010).

- Chromagar ESBL (B-lactamase) for the detection of extended spectrum beta-lactamase producing *E.coli, Klebsiella, Enterobacter* and *Proteus* with 100.0% sensitivity and 93.3% specificity according to Siato et al. (2010).

Molecular methods for detection of antimicrobial resistance in milk pathogens are used for the detection of resistance genes using real-time PCR (qPCR) assays, especially for slow growing organisms or organisms difficult to isolate (Wang et al., 2017). The disadvantage of molecular detection is that the presence of the resistant gene cannot ascertain that the gene will be phenotypically expressed under all epigenetic conditions (Fluit et al., 2001).

MALDI-TOF (matrix-assisted laser desorption/ionization time of flight spectroscopy)

This technique is a protein pattern-based diagnostic method directly from isolated, intact bacterial cells, mycobacteria, anaerobic bacteria, and certain fungal pathogens, to identify down to species and subspecies-level in the clinical microbiology laboratory. The pattern is a unique mass-spectral cellular “protein fingerprint” for individual isolates when compared to a spectral data base for accurate identification. The ability of this laser technology has been found to provide accurate and highly reproducible results. Analysis is technically, a quick and

simple procedure to perform. Relatively cost-effective compared with molecular identification techniques (Schmidt et al., 2018).

The ability of MALDI-TOF Mass Spectrometry to characterize large biomolecules led directly to obvious applications involving the analysis of isolated bacterial proteins, that could be applied directly to crude cellular fractions and suspensions, and that the resulting data from such complex mixtures could provide evidence for chemotaxonomic classification. "*The analysis of bacterial RNA and DNA, the detection of recombinant proteins, the characterization of targeted or unknown proteins, bacterial proteomics, the detection of virulence markers, and the very rapid characterization of bacteria at the genus, species, and strain level"* (Lay Jr, 2001) are outstanding capabilities of this technique.

MALDI-TOF MS can be used for rapid diagnosis of bacterial species in milk samples with minimum CFU /ml organisms for individual pathogens. and in bacterial isolates from sub-clinical mastitis milk samples. Detection of ribosomal-protein-fingerprints from intact pathogens and database searching with precise reproducible identification. This methodology is faster than conventional microbiological culture and DNA techniques (Barreiro et al., 2017).

According to literature in 2019 the shortcoming of *"MALDI-TOF MS using bacterial or fungal colony material; 93.5% could be identified to the species level, and 6.5% were identified only to the genus level. Isolates identified to the genus level required further identification to the species level by conventional methods or 16S rDNA sequencing. Mass spectra from verified species were used to expand the MALDI-TOF MS database to improve future identification ability in bovine mastitis, and the database can be continuously expanded and improved with additional species"(Nonnemann et al., 2019).* Therefore, database expansion needs further research and verification before it can be accepted as the new standard even for SANAS accredited milk laboratories. A further short coming according to Ngassam Tchamba et al. (2019) *"is the multiplex qPCR assay only targets the most important mammary gland pathogens and can detect DNA of bacteria both alive and dead. Conversely, bacteria only*

grow when alive and the MALDI-TOF MS databases do not include all bovine milk-associated bacterial species yet".

No sensitivity and specificity data on Bovine milk samples could be found in literature searches to determine accuracy for inclusion in the One-Health model although, MALDI-TOF MS and Biotyper data processing is a good alternative technique for routine identification of subclinical mastitis pathogens in large scale milk samples as it showed to be faster and an accurate alternate method as conventional culturing methods (Braga et al., 2018).

Enzyme linked immune sorbent assay (ELISA): ELISA is complementary/alternative method to identifying antibodies to antigen which has low sensitivity or cannot be cultured. ELISA is thus commonly used serological testing for milk pathogens. The assay use antigen such as proteins, polysaccharide and lipoprotein polysaccharides to bind antibodies in the sample using a color reaction the antibody concentration is determined by optical density. Different techniques can be applied:

- Direct ELISA test the antigen is coated to a multi-well plate and detector antibody has been conjugated to an enzyme is used to confirm the identification of the pathogen.
- Indirect ELISA with primary antibody incubated with the antigen, followed by binding with enzyme- conjugated labeled secondary antibody to indirectly confirm the identification of the pathogen.
- Sandwich ELISA quantifies antigen between 2 antigenic epitope combinations of a capture antibody layer and a detection antibody layer.
- Competitive or blocking ELISA with inhibitor antigen to measure the concentration of antibody or antigen. Labelled antigen and sample antigen compete for binding to the primary antibody. Lower concentration of antigen in the well, causes a stronger signal of labelled antigen.

IDEXX Milk Immunodiagnostic assay kits that are commercially available and commonly used to diagnose zoonotic, immunodepression, and state-controlled diseases in milk samples are the Q Fever antibody test (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-q-fever-ab-test/), BVD antibody test (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-bvdv-agserum-plus-test/), BLV/EBL antibody test (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-leukosis-milk-verification-ab-test/), MAP antibody test (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-map-ab-test), and the *Brucella* antibody test (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/pourquier-cft-brucellosis-ag/). These tests are not economically feasible for private practice diagnostic applications of these techniques due to the cost of the kit and request as diagnostics test (Appendix 1).

Molecular diagnostic techniques: Molecular diagnostic techniques enable the detection of the DNA of a specific genus, species and/or sub-species mastitis causing pathogens or zoonotic pathogens in the milk. The most commonly and rapid used molecular method is the polymerase chain reaction (PCR) which allows amplification of target region in a microtube which need to be visualized using agarose electrophoresis taking about 2-4 hours. More advance and faster PCR technology include qPCR where the application of the target region can be 'seen' in real time within an hour. Furthermore qPCR can be more sensitive as reported for the *Mycobacterium bovis uvr*C gene consisting of 40 copies that was 10^3 times more sensitive in spiked milk samples compared to traditional gel-based PCR (Behera et al., 2018b).

Molecular diagnostic tools can be used as the gold standard for specific pathogens (El-Sayed et al., 2017). The applications of PCR in diagnoses and epidemiology include the rapid identification of pathogens on large scale up to sub-species level for epidemiological IMI studies to track the source, determine the focus of infection with transmission routes and calculate contagiousness of certain strains (Zadoks et al., 2011). Monitoring of emerging virulent or AMR strains (Fluit et al., 2001) aid diagnosis as well as allow a high accuracy in

resistant pathogen strain characterization for therapeutic antimicrobial treatment regimens and virulence factor determinations (Wang et al., 2017).

Molecular techniques can also be used in epidemiological studies to investigate the genetic diversity of the pathogen population(s) but these techniques are complex, time-consuming, not commercially available and/or cost effective. PCR was used to differentiate between bovine and human isolates in epidemiology studies (Sukhnanand et al., 2005, Ajitkumar et al., 2012a). Phenotypical identification and molecular genotyping based techniques can be combined and implemented in epidemiological outbreak investigation studies. Figure 2 shows dentification and genotyping techniques used by Werner (2016).

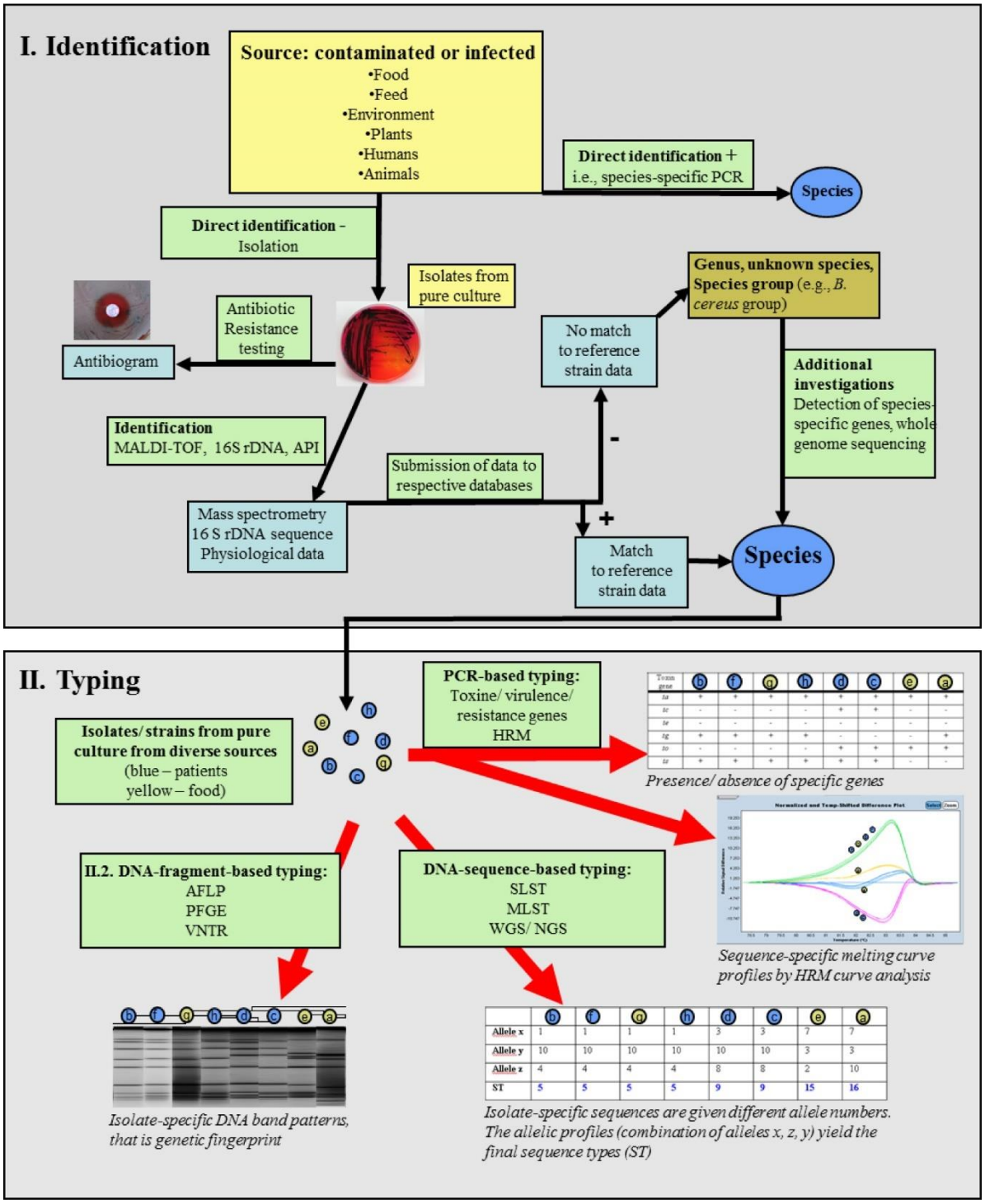

Figure 2: Identification and genotyping techniques for bacterial epidemiological outbreak used by Werner (2016) using first identification which is aided by molecular techniques such as MALDI-TOF (matrix-assisted laser desorption/ionization), 16 S ribosomal DNA sequencing or biochemical test using API followed by typing which include more complex molecular techniques such as AFLP (amplified fragment length polymorphism), PFGE (pulsed-field gel electrophoresis) and VNTR (variable number of tandem repeats) and various sequencing techniques (single locus sequence typing (SLST), multi-loci sequencing typing (MLST) and next generation sequencing (NGS) / whole genome sequencing (WGS).

With qPCR various chemistries varying in sensitivity and specificity are available and can be used for diagnosing IMI pathogens like as with conventional PCR. qPCR can also be multiplexed for detection of more than one pathogen. The advantages of PCR and multiplex PCR include detection of low concentration of pathogens in milk samples, identification of dead pathogens, automated with high throughput, quick turnaround times, high resolution (Kalin et al., 2017); (Soltau et al., 2017). Thus, qPCR can be used on high BMSCC samples to identify and differentiate pathogens. The following milk zoonotic targets amongst other, can be identified in the bulk milk tank namely *Mycobacterium tuberculosis, M. avium* (Bezerra et al., 2015); *M avium paratuberculosis, M. bovis* (Rodríguez-Lázaro et al., 2005), *Brucella abortus, B. melitensis* (Majid et al., 2016) and *Coxiella burnetii* (Muskens et al., 2011) using fluorescent signal detection known as TaqMan probes of specific target DNA regions with a probe which allows for high specificity (Kumar et al., 2018).

Thermo scientific PathoProof complete-16 multiplex qPCR kit is commercially available and used for the identification of the most common IMI pathogens in milk. Commercially available PathoProof qPCR assay has been used to detect the 16 pathogens causing mastitis under field conditions testing milk from animals suffering from clinical or subclinical mastitis, as well as spiked samples. This assay in the various studies was highly accurate (Ganda et al., 2016) at udder quarter or animal levels, as well as, analytical sensitive (100%) and specific (99–100%) (Koskinen et al.2009; Mahmmod 2013; Chakraborty et al., 2019). The PathoProof qPCR kit detects 15 mastitis causing pathogens and B-lactamase penicillin resistance gene in *Staph. aureus* as well as CNS species in 4 separate PCR reactions using TaqMan or dual labelled probes (probe with reporter and quencher on different ends). The mastitis causing pathogens can be detected from pooled or bulk milk tank samples within 4 hours without prior culturing steps. The 15 mastitis pathogens include *Staph. aureus, Staphylococcus* spp. (including all major coagulase-negative staphylococci), *Strept. agalactiae, Strept. dysgalactiae, Strept. uberis, E. coli, Enterococcus* spp. (incl. *Enter. faecalis* and *Enter.*

faecium), *Klebsiella oxytoca* and/or *K. pneumoniae, Serratia marcescens, Corynebacterium bovis, Trueperella pyogenes* and/or *Peptoniphilus indolicus,* Staphylococcal ß-lactamase gene (penicillin-resistance gene), *Mycoplasma bovis, Mycoplasma* spp., Yeast and *Prototheca* spp. As already discussed, Hiitio et al. (2015) reported positive *Staph. areus* and *Staphylococcus spp* with 97.0% and 86.7% sensitivity and 95.8% and 75.4% specificity, respectively compared with bacterial culture.

16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria.

Bacterial 16S r RNA genes contain nine hypervariable regions (V1-V9) with species specific sequences for useful diagnostic targets. With hypervariable species-specific sequence similarity dendrograms constructed and "MEGALIGN" / GENBANK DATABASE online data files, DNA probing, primer design and real-time PCR diagnostic identifications can be done with accurate outcomes (Chakravorty et al., 2007).

Other molecular techniques useful for diagnostic purposes in specialized and research laboratories but impractical in private practice is: AFLP (amplified fragment length polymorphism), PFGE (pulsed-field gel electrophoresis) and VNTR (variable number of tandem repeats) and various sequencing techniques (single locus sequence typing (SLST), multi-loci sequencing typing (MLST) and next generation sequencing (NGS) / whole genome sequencing (WGS) (Werner, 2016).

High resolution melting(HRM) Ajitkumar et al. (2012b) analysis is a qPCR-based technique which detect DNA polymorphism using a melting curve after the PCR which can distinguished amplicons on single base difference. HRM requires specific apparatus but can be used in diagnostics as low -cost, single -step, closed -tube, accurate and rapid method (Zhou and Wenjuan, 2017). This biotechnical technique can be used to identify different common mastitis pathogens such as *Escherichia coli, Streptococcus agalactiae /dysgalactiae, Klebsiella pneumoniae, Streptococcus uberis, Staphylocooccus aureus*, *Mycoplasma bovis* (Azari et al., 2020) and slow growing *Trueperella pyogenes* / *Corynebacterium bovis* pathogens in pooled

milk samples (Ajitkumar et al., 2012b, Athamanolap et al., 2017) (Figure 3). HRM multiplex qPCR technique targeting the nuclease (*nuc*) gene differentiated 13 *Staphylococcus* spp from milk with high SSC count and identified *Staph. Aureus, Staph. chromogenes, Staph. epidermidis, Staph. hyicus, Stap. hominis, Staph. lugdunensis, Staph. lentus, Staph. haemolyticus, Staph. schleiferi, Staph. saprophyticus, Staph. simulans Staph. warneri,* and *Staph. xylosus* (Ata and Buyukcangaz, 2019).

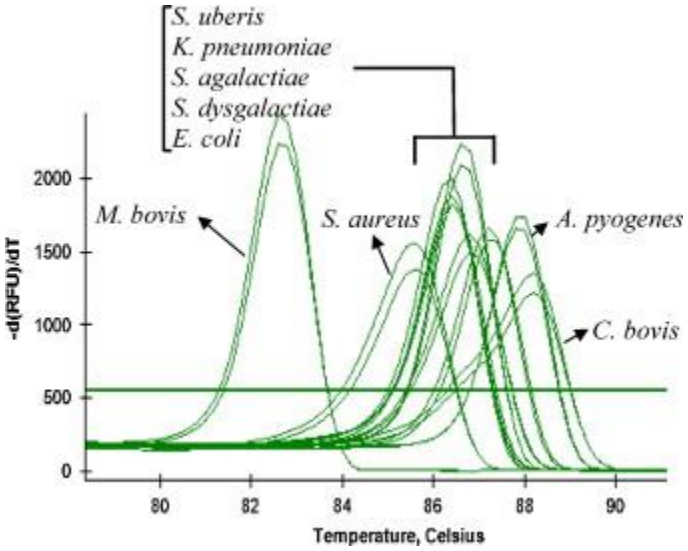

Figure 3. The real-time PCR data obtained with CFX Manager Software showing the DNA melting profile for 16S rRNA gene (V5–V6 region) for t 9 common mastitis pathogens. The image shows the derivative melt curve plots of the gene which differ in sequence and thus temperature which corresponds to the expected melting peaks based on the plotting of negative rate of change of fluorescence versus temperature (−d(RFU)/d*T*) (Ajitkumar et al., 2012a).

One-Health diagnostic surveillance model

From a One Health perspective, BMSCC management became a specialized field in veterinary science. As milk is a nutritious commodity for human consumption, pathogen identification and surveillance for zoonotic and reverse zoonotic /food-borne/AMR pathogens are crucial. Syndromic surveillance means a systematic collection, analysis and interpretation of biotechnical diagnosis or data results on a multi-factorial, multi pathogen disease complex

that influence milk quality and production or could affect the public health and contaminate the food chain. Mastitis is the most frequent and costly disease in the dairy industry.(Halasa et al., 2007; Hogeveen et al., 2010) In this research a diagnostic surveillance model is developed as the fundamental springboard from which a One-Health practice can be launched with emphasis on zoonotic diseases from milk.

Veterinary practice laboratories do primary diagnostic isolations while subtyping is mostly performed in specialized laboratories (Flieger et al., 2013). A One-Health practice orientated diagnostic surveillance model is proposed that combine primary cytology and isolation with biotechnological techniques for accurate diagnoses. This in- practice model is a procedure which investigate the causal pathogen(s) of an extraordinarily demanding, complex and dynamic system starting with BMSCC with infected quarter identification as the starting point in unravelling the influences of a fluctuating BMSCC. The diagnostic techniques to enable accurate determination of disease-causing pathogens in milk will be determined using a systematic literature review.

The diagnostic surveillance model combines cytological, phenotypical, immunoassays, molecular techniques, and mass-spectrometry to identify the pathogen members of the collective IMI consortium. This diagnostic surveillance model will be implemented on dairies in the milk producing areas of South Africa where the consulting veterinarian is contacted for high cell count challenges and mastitis outbreaks, problem solving and prevention of One-Health zoonotic disease and antimicrobial resistance. The chronological sequence where the model will be applied, is where the high BMSCC herd is identified by the milk procurement company, who tests procured milk daily at SANAS accredited somatic cell count facilities.

The alerted milk producer contacting the consulting veterinarian then to diagnose and prevent the biosecurity challenge, outbreak of mastitis or public health concern. The surveillance program will be on the individual herd, and the veterinarian will collect the following field data on the farm. Herd size and production group composition, breed and management practices,

milk machine ISO standard operating data, production analysis data, treatment, antibiotic, disinfectant history, feed flow program data, herd disease history data, history of individual high SCC cows and quarters, stage of lactation and parity. Data is maintained on an integrated milk management software program on farm as well as the SANAS accredited Agricultural Research Council, Lactodata system or Logix milk centralized data recording schemes. Milk recording schemes are managed by qualified and professional data processors, statisticians, and biometricians. These specialized milk recording data is available on request for indirect disease control and support.

The University of Pretoria also has implemented an udder health diagnostic data program to analyze milk samples on microbiological and cytological patterns to study pathogen specific udder health dynamics and assist veterinarians on informed practice decision making. Pathogen-specific group reports are issued as action lists for individual group therapy strategies according to pathogen epidemiology (Petzer et al., 2016a). A proactive udder health management programme, using the milk sample diagnostic computer programme (Abaci Systems, Aretsi SA, Pretoria) was developed and maintained over several years at the University of Pretoria (Petzer et al. 2016). The basic purpose of this management programme was to sample all lactating cows in a herd for both microbiological and cytological evaluations Farm management data and milk recording data are useful in epidemiological and prevention strategies for the private veterinarian. Milk buyer's SCC data are useful for monitoring of the prevention strategy successes. Milk recording data give a perspective of new, recurred, chronic, and cured cases along with other production data e.g. lactose, protein, milk urea nitrogen, individual SCC, and further milk quality and production criteria. Milk recording SCC data and Milk buyer SCC data can without enormous costs be implemented by private veterinarians as syndromic surveillance and early detection for public heath reporting when applying this model under investigation.

Currently average grazing herd sizes under center-pivot irrigation on commercial dairy farms could be more than 1000 cows per production unit. These herds are mostly along coastal and

river areas where cows graze on pastures for economical milk production for supplying milk to the dairy industry of South Africa as is shown in Figure 4. According to Lactodata (2019) the average herd size of South African dairies are 459 cows per farm. The expected outcome is to identify the most prevalent, most virulent, most resistant, and important zoonotic pathogens in a large dairy herd influencing the BMSCC by phenotypical, mass-spectrometry, immunological and molecular diagnostic techniques.

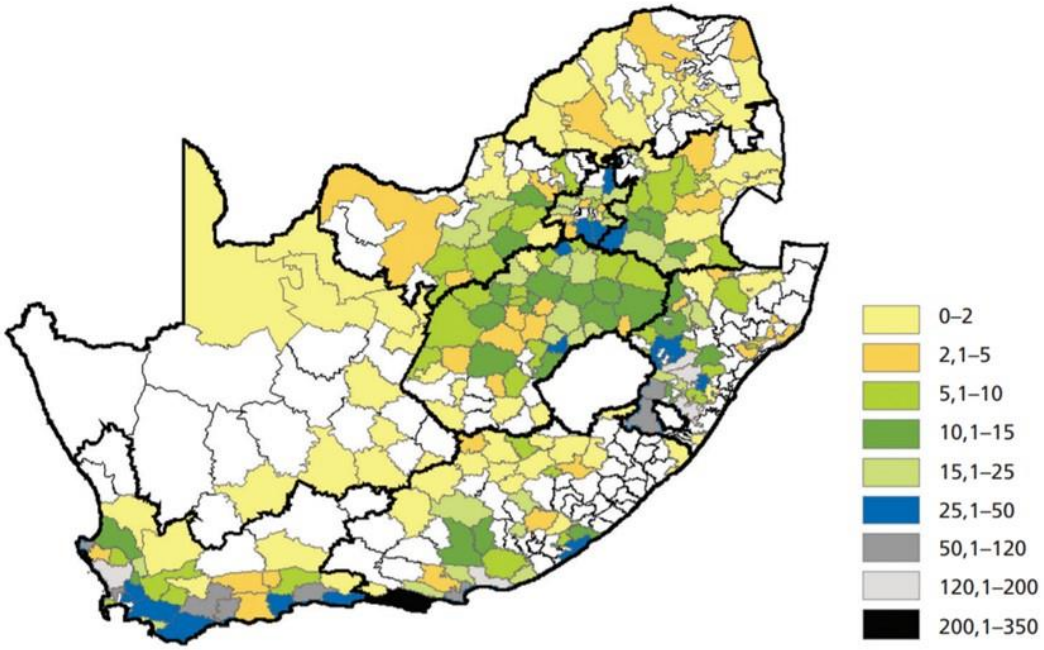

Figure 4: Milk production density per district in South Africa. (ℓ/km²) recorded in 2016 (source: MPO estimates from October 2016 statutory survey).

An important aspect of the model is the sampling strategy. This diagnostic surveillance model is designed to collect milk samples of high cell count cow udder quarters (>150,000 SCC/mL of quarter milk) in a large dairy herd to determine the pathogens that influence the SCC (Pantoja et al., 2009). After determining the BMSCC from a bulk tank sample with the Fossomatic 5000 cell counter, somatic cell values of quarters will be used as screening tests. The herds with lower BMSCC thresholds will identify more sensitively the overwhelming pathogens and herds with higher BMSCC thresholds will increase the specificity for these pathogens. The sensitivity and specificity of individual diagnostic techniques were extracted from a literature review. On arrival at a Milk Laboratory batch temperature of the milk samples is recorded. SCCs are performed using a Fossomatic 5000 (Petzer *et al*. 2012).

Schukken et al. (2003) showed that the most accurate relationship between SCC and IMI exists at quarter level sampling. This diagnostic surveillance model is designed to take samples as a snapshot in time. The ideal sample size is whole herd quarter milk samples. Microbiology (culture) results and SCCs are then available for each quarter milk sample. To perform a more economical but less reliable sample size determinations are based on Giesecke et al. (1994) calculations indicated in Table 1. In herds with an extreme high BMSCC, a higher threshold, e.g.,> 400,000 SCC/ml was set for sample size determination so that 15% final samples be enough to be cultured (Giesecke et al., 1994). Pure discretions of an experienced veterinary clinician indicated further selections based on clinical observations for sub-clinical, clinical, and physiological criteria while sampling. However, this method was deemed less accurate and of historic relevance, although definitely cost saving. The pooling of “no-growth” samples in this model is used to minimize testing costs but could influence the diagnostic sensitivity (Se) and specificity (Sp). Pooled samples are affected by analytic Se and Sp of the test, number of quarters combined in the sample pool, concentration and prevalence of cross- reacting agents and concentration and prevalence of analyte in individual samples (McKenna and Dohoo, 2006).

Table 1: One -Health method: The percentage (%) udder quarters positive in a herd using California mastitis test (CMT) relative to the bulk milk somatic cell counts (BMSCC) suggested by Giesecke et al. (1994) to use CMT as a cow side screening technique to estimate sampling numbers.

BMSCC per ml milk	**% Udder quarter CMT positive in the herd**
<125,000	<5%
125,000-250,000	5-10%
250,000-350,000	10-15%
>350,000	>15%

On the farm sampling procedure and examination of the diagnostic surveillance model (Figure 5) include sampling of only high cell count quarters. Samples will only be taken from herds where BMSCC is higher than 150,000 somatic cells per ml in bulk milk containers. Samples from cows with a high average conductivity variance on consecutive milking; samples taken from quarters identified by the consulting veterinarian by clinical diagnostics as clinical or sub-clinical cases after clinical udder examination of each high cell count cow in the herd, done immediately after milking. High SCC individuals will be grouped for further evaluation and sampling on CMT scores between 1 and 3 (400,000-5,000,000 somatic cells per ml quarter milk. All samples will be taken by the veterinarian personally to standardize the aseptic technique to make sure that appropriate samples are taken and that the sample quality is good and uncontaminated for One-Health diagnostic purposes and mastitis control.

According to the choice of the practitioner, whole herd samples can be taken by professional samplers that are trained and qualified according to SANAS requirements. Cold chain is maintained until the samples are taken to the practice laboratory or Specialized SANAS accredited milk laboratory for primary culturing within 24 hours. On the farm, infected cows are identified by observant “milk strippers” who stimulate the cows for let-down reflex and disinfecting teats during the pre-milk routine. This procedure will be an in-parlor organoleptic identification procedure of quarter milk mastitis cases to identify suspect cows with high cell counts (Claycomb et al., 2009). The veterinarian will follow up and conduct clinical udder examinations while collecting samples for One- Health pathogen Identification and grouping into a high cell count sub-clinical group for last milking and a mastitis group for immediate treatment (Figure 5).

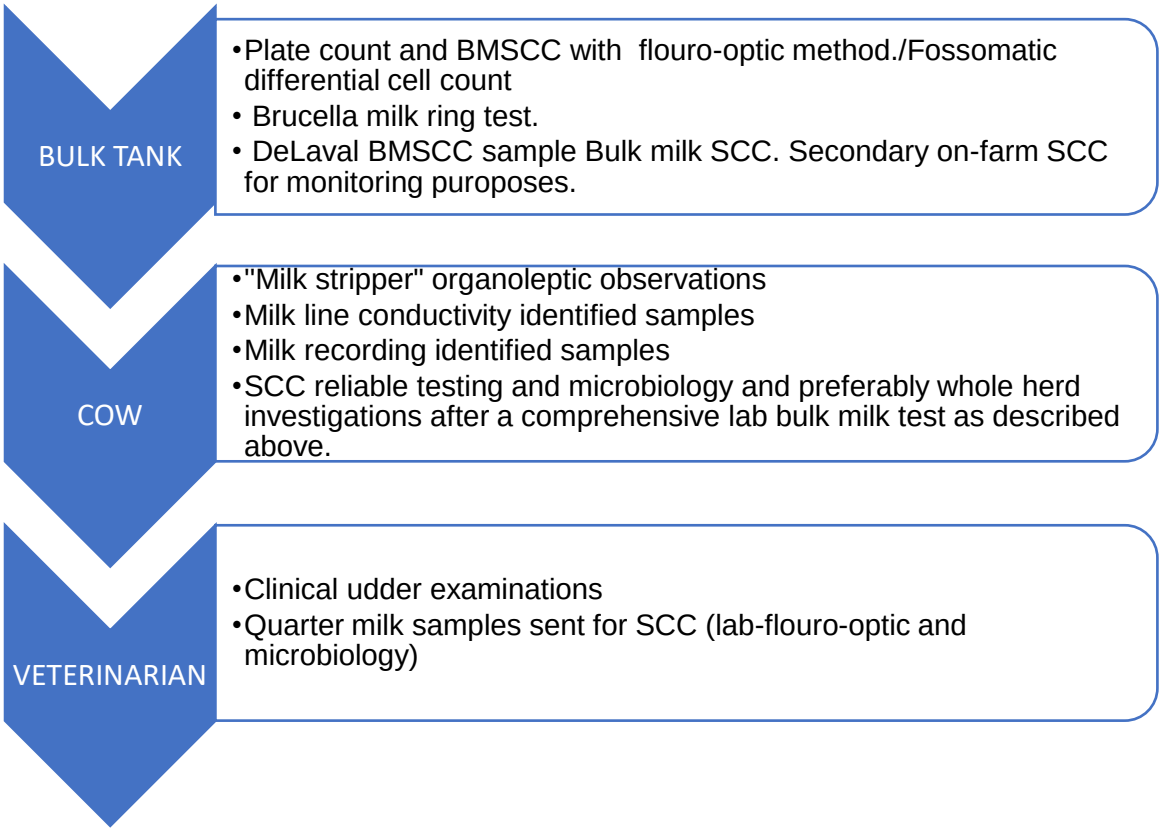

Figure 5: Sampling and observation on farm include samples from the bulk milk tank for somatic cell count (BMSCC)/Standard aerobic/anaerobic culturing as well as all quarters. The veterinarian will conduct clinical udder examinations.

Figure 6 indicates the laboratory phase of the diagnostic surveillance model consisting of the primary isolation and identification of pathogens from samples. On arrival of the 4°C CMT positive quarter milk samples primary isolation based on practice experience for optimal growth success will be done on BTA/ Chromagar mastitis/ McConkey agar plates, microscopic examination followed by selective media cultivation and further microbiological/biochemical tests. Anti-microbial/disinfectant resistance determination will be done on identified isolates. The final identification can be done on milk samples and/or primary cultures using multiplex real-time qPCR or high resolution melt curve analysis or MALDI-TOF (Vitek-MS) for pathogen, antimicrobial resistance(Vitek-2) and virulence region identification (Taponen et al., 2009). Samples can also be analyzed using ELISA Immuno-assay diagnosis (Figure 6). The final

identification can be done by specialized laboratories in South Africa or if the consulting private practice expands to conduct real time PCR, VITEK 2/ MS and/or serological tests.

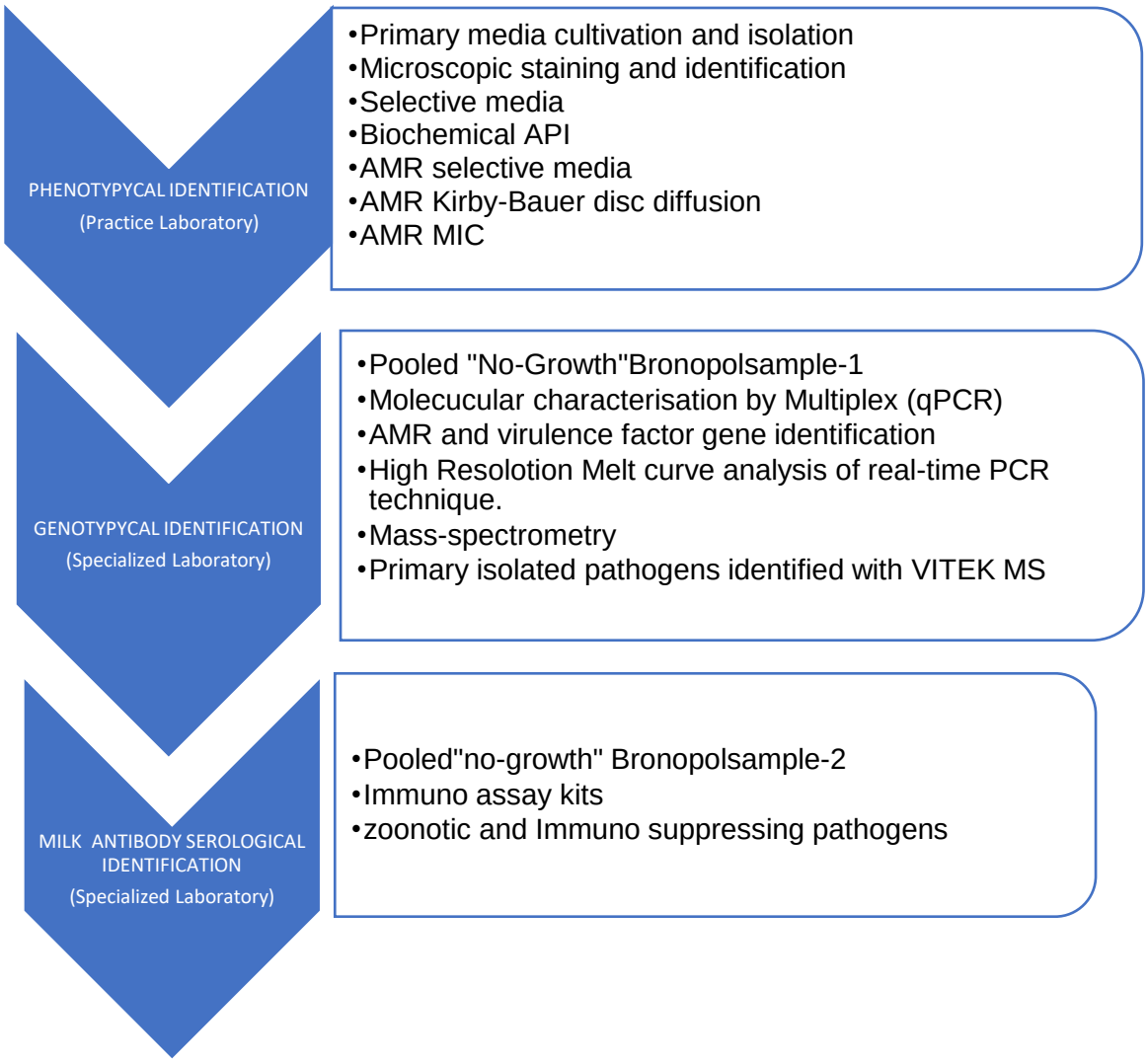

Figure 6: Illustrates a sample flow diagram for laboratory diagnostic purposes from clinical quarters and sub-clinical CMT (California mastitis test)/ Draminski positive quarters samples for phenotypical identification consisting of primary cultivation at the private practice laboratory followed by genotypical, and serological identification at specialized laboratories. The genotypical identification can be done on milk samples or on isolates obtained from primary isolation (Hijazin et al., 2011).

This diagnostic surveillance model is proposed for the consulting veterinary practice to identify pathogens in fresh milk from herds with high bulk milk SCC. Figure 7 illustrates the consulting veterinarian's equipped practice laboratory registered by the South African Veterinary Council that is equipped for primary phenotypical diagnostic procedures all done according to good laboratory practice.

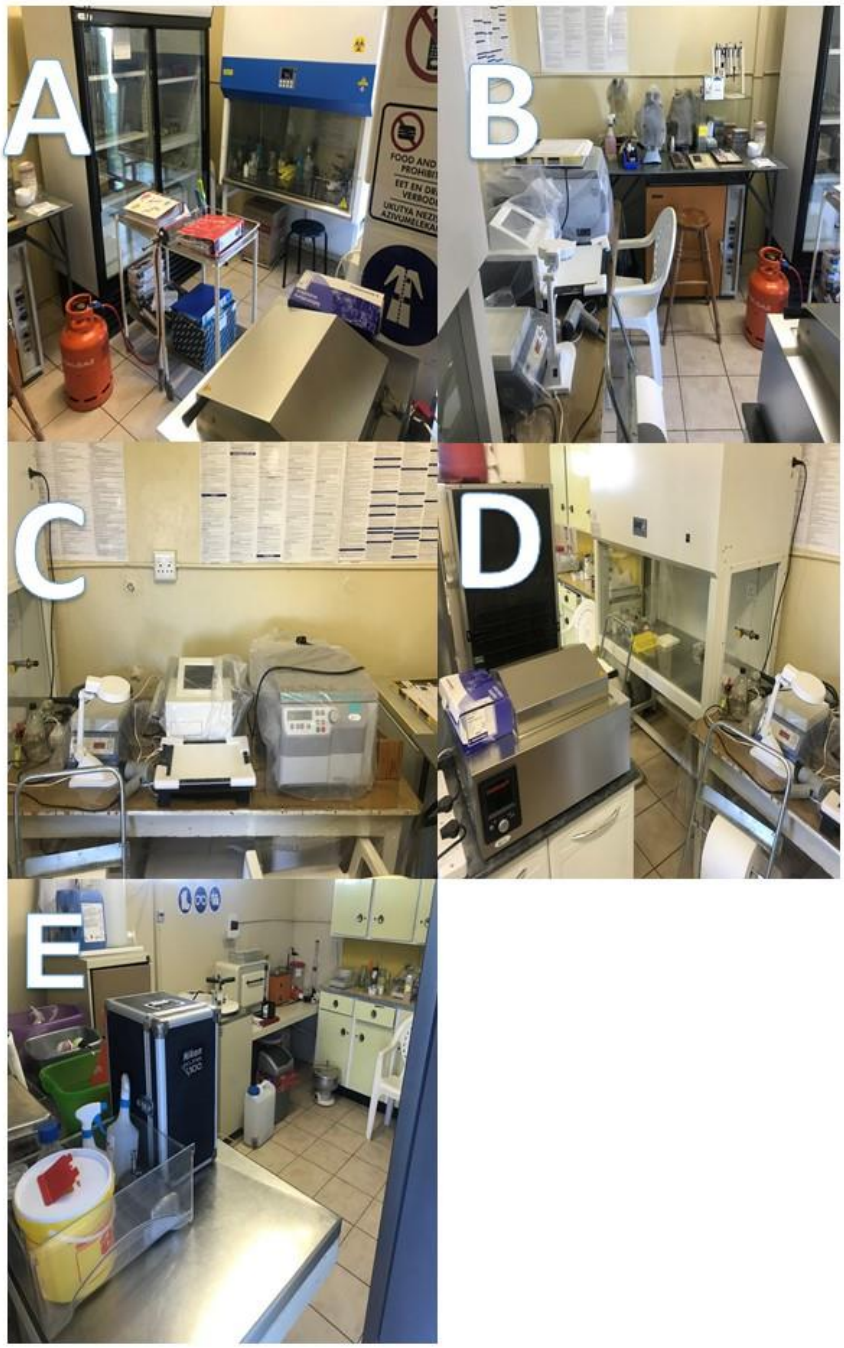

Figure 7: Shows the laboratory and instrumentation in a well maintained and validated practice laboratory consisting of (A) biosafety cabinet, water bath, bacterial isolation equipment; (B) microscopic examination table, incubator, micropipettes and centrifuge; (C) ELISA reader, (D) media preparation sterile flow cabinet, fridge/freezer and weighing scales and (E) sample preparation and examination stainless steel table, autoclave, antibiotic disc dispensers and reagent storage facilities.

METHOD:

In this study a private practice orientated diagnostic surveillance model was proposed. This model combined cytology, basic pathogen culturing/isolation/identification with genotypical and/or serological identification. Schukken, *et al.,* stated explicitly that:" *Monitoring tools are required to find the areas of risk in the herd. It is inevitable that more complete udder health programs and monitoring systems are to be developed and implemented. These programs are necessarily dynamic and complex. Implementation of complete udder health programs should be accompanied by research efforts to further fine-tune these complete udder health control and monitoring programs".* (Schukken et al., 2003)These diagnostic techniques will be selected with systematic literature review to allow for accurate surveillance and identification of pathogens from samples collected from large dairy farms. The criteria to select the diagnostic techniques included commercially available, practical, and cost-effective technologies for a private One-Health orientated veterinary dairy practice.

Systematic reviewing research and criteria

The literature was searched for diagnostic methods with high sensitivity and specificity allowing phenotypical, genotypical and/or serological identification of pathogens in fresh milk from herds with high bulk milk SCC. The systematic review was conducted according to a guideline protocol for systematic literature reviews described in Cochrane Handbook for Systematic Reviews of Interventions (www.training.cochrane.org/handbook). The literature was searched using diagnostic test accuracy (indicated by high diagnostic sensitivity and specificity) for pathogens of milk from herds with high bulk milk SCC consisting of books, including reference e-book collections; accredited journals, e-journals, world cat discovery; institutional repositories: UP Space, South African National Veterinary repository, controlled clinical trial registers; UP library databases e.g. PubMed, Google Scholar etc., books, patented techniques and advertised diagnostic kits for diagnosis of mastitis pathogens in dairy herds from 1990-2020. The search literature results were examined against the predefined inclusion

criteria primary focusing on diagnostic sensitivity and specificity, implemented, and used for analysis of the combined model. The criteria further included peer-reviewed papers written in the English language, diagnostic tests with focus on BMSCC, SCC, aerobic-, zoonotic/food-, antimicrobial resistant- mastitis pathogens and state-controlled disease pathogens (*Brucella abortus/ melitensis; Mycobacterium avium paratuberculosis; Coxiella burnetii*). The literature search results were first screened using diagnostic sensitivity and specificity followed by the ccommercially available, practical, and cost-effective technologies criteria.

Diagnostic test accuracy

Diagnosis means the positive identification of the specific pathogen identified, investigated, or tested for during the surveillance procedure. Accurate identification of the mastitis pathogen is crucial and thus diagnostic test accuracy was used as the main criteria to include biotechnological tests. Diagnostic test accuracy is a calculation and evaluation of sensitivity and specificity of individual tests against a reliable reference test according to a gold standard or parallel combination of tests. The combined diagnostic model's accuracy is tabulated (Table 2) and evaluated by the range of sensitivity (Se) and range of specificity (Sp) of all investigated techniques against a golden standard (McKenna and Dohoo, 2006).

Table 2 : Contingency tables are used in sensitivity (Se) / specificity (Sp) calculations where D+ represents infected quarters and D- non infective quarters, determined against the golden standard test. T+ represents a positive test result and T- a negative test result. TP= True Positive. FP= False Positive. FN=False Negative, TN=True Negative.

	D+	D-	Total
T+	TP	FP	TP+FP
T-	FN	TN	FN+TN
Total	TP+FN	FP+TN	TP+FN+FP+FN

True Positive is known infected reference quarters that test positive in the assay. False Negative is known Infected quarters that test negative in the assay are considered to have false-negative results. False Negative is known non-infected reference quarters that test positive in the assay. True Negative is non-infected reference quarters that test negative in the assay.

Sensitivity% (Se%) = TP/TP+FN*100/1 (Koskinen et al., 2009)

It is the probability that the evaluated screening technique or test gives a positive result in the sample in which the technique is applied or done.

Specificity% (Sp%) = TN/TN+FP*100/1 (Koskinen et al., 2009)

It is the probability that the evaluated screening technique or test gives a negative result among the quarter milk samples in which the technique is applied or done in absence of the tested factor. Se and Sp of a test should be considered relatively to each other and inter dependant for accuracy estimations.

It is important to distinguish between analytical- and diagnostic- sensitivity and specificity as high analytical sensitivity does not guarantee acceptable diagnostic sensitivity (Saah and Hoover, 1997). As explained above diagnostic sensitivity of a test is the percentage of infected samples that are found infected by the test, while the diagnostic specificity is the percentage of non-infected samples that are found negative by the test. Analytical sensitivity refers to the precision of the test, or the minimum amount detectable within a given system, whereas analytical specificity describes the degree of cross-reactivity in a test system.

RESULTS

Results from literature which of the diagnostic tests with diagnostic/analytic specificity and sensitivity are listed in Table 3.

Table 3: Systematic literature search results with diagnostic sensitivity and specificity for diagnostic techniques of milk pathogens

Pathogen	Techniques on fresh milk	Author	Sensitivity	Specificity
Somatic SCC				
Mastitis pathogens	Delaval Somatic cell counter	Kawai et al. (2013)	71.0%	81.0%
Sub-Clinical mastitis pathogens	Milk Electronic Conductivity Meter	Fosgate et al. (2013)	89.9%	86.8%
Sub-Clinical mastitis pathogens	Milk line conductivity test	Sheldrake and Hoare, *1981)	49.0%	79.0%
Sub-Clinical mastitis pathogens	California Mastitis Test CMT	Fosgate et al. (2013)	94.5%	77.7%
200,000 cells/ml Quarter milk samples of major pathogens	Fossomatic 5000	Petzer et al 2017	88.2%	57.7%
Phenotypical identification (primary isolation and biochemical tests)				
Staphylococcus aureus	Blood agar Blood culture broth	Bautista-Trujillo et al. (2013) Qiun et al. (2014)	68,8% 85.0%-100%	ND 100%
Staphylococcus aureus	CHROMagar *S. aureus*	Bautista-Trujillo et al. (2013)	95.5%	99.0%
Sterococcus dysgalactiae	Bacterial culturing	(Addis et al., 2016)	38,8%	92,8%
Streptococcus agalactiae	CHROMagar StrepB®	(Poisson et al., 2011).	92.0%	95.0%
Enterocoocus spp	Bacterial culturing	Spittel & Hoedemaker (2012)	9.1%	ND
Corynbacterium bovis, Arcanobacterium pyogenes, Peptoniphilus	Bacterial culturing	Spittel & Hoedemaker (2012)	76.9-100%	63.3-98.7%

indolicus, Streptococcus uberis, Staphylococcus spp, Staphulococcus areus				
Escherichia coli serotype O157	CHROMagar O157®	(Rhee et al., 2002)	98.0%	ND
Non-O157 Shiga-toxin producing *Escherichia colii*	CHROMagar STEC®	(Hussein and Bollinger (2008), Gouali et al. (2013)	89.1%	91.4%
Vancomycin resistant *Enterococcus*	CHROMagar VRE®	Archibald (2011) Peterson et al. (2010)	86.0%-99.0% 95.5%	95.0%-100% 90.4%
Carbapenem resistant *Klebsiella*	CHROMagar KPC	Samra et al. (2008)	100%	98.8%
Escherichia coli Klebsiella/ Enterobacter and Proteus beta lactamase	CHROMagar ESBL	Saito et al. (2010)	100%	93.3%
Mycoplasma	Modified Hayflick medium	Justice-Allen et al. (2011)	68.9%	100.0%
Staphylococcus aureus	Tube coagulase Test	(Sedky et al., 2020, Sundareshan et al., 2017) Qian et al., (2014)	65.0 -84.1%	98.7-100%
Staphylococcus aureus	Mannitol fermentation	Pumipuntu et al. (2017)	87.0%-100%	28.6%
Staphylococcus aureus	Deoxyribonuclease Test	(Pumipuntu et al., 2017)	53.1%	41.8%
Staphylococcus aureus	RAPIDEC Staph (Bio-Merieux) Enzyme based	 Boerlin, Patrick et al. (2003) Quin et al. (2014)	100% 90.5%-100%	100% 96.6%-100%

Immuno diagnostic assays				
Mycobacterium avium paratuberculosis	IDEXX Paratuberculosis ELISA Screening Ab Test for bovine milk samples	IDEXX Validation data report: Individual bovine milk samples	75,5%	98,8%
Coxiella burnetii	IDEXX Q-fever antibody test kit Coxiella burnetii Indirect Elisa Ab test for bovine milk samples Complement fixation test.	 IDEXX Validation data report. Serum, plasma, milk samples	100% 93%	100% 100%
Bovine viral diarrhoea virus (BVDV) and Mucosal disease (MD).	(ELISA) for the detection of antibodies directed against p80 proteins with maximum sensitivity and specificity are achieved within the 70.0%–85.0% interpretation threshold range for both applications	IDEXX Validation data report: Individual bovine serum, plasma, milk samples	For individual application, according to the ROC curve, maximum sensitivity and specificity are achieved within the 70%–85% interpretation threshold range.	For bulk tank milk application, according to the ROC curve, maximum sensitivity and specificity are achieved within the 70%–85% interpretation threshold range.
Bovine Leukosis Virus (BLV)	Indirect ELISA: Leukosis (BLV)milk anti-body test	IDEXX Validation data report: bovine bulk milk samples	No data available	99,6%
Molecular tests				
Staphylococcus aureus GTB (genotype B)	16S-23S r RNA intergenic spacer region (ISR)	(Syring et al., 2012)	100%	100%

Streptococcus agalactiae	16S-23S ribosomal RNA intergenic spacer region (ISR)	(Wu et al., 2008)	96.2%	98,6%
Staphylococcus aureus	PCR	Quin et al. (2014)	93.8%-100%	98.6%-100%
Vancomycin resistant Enterococcus	PCR	(Archibald, 2011)	98.0%	99.0%
Mycoplasma spp	PCR	(Justice-Allen et al., 2011)	76.7%	100%
Staphylococcus aureus, Streptococcus agalactiae and *Streptococcus uberis*	Multiplex real time PCR (qPCR)	Gillipie & Olivier (2005	95.5%	99.6%
Sterptococcus dysgalactiae	qPCR	Gillipie & Olivier (2005)	95.5%	99.6%
Staph. areus, Staphylococcus spp, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Enterococcus spp Klebsiella oxytoca, K. pneumoniae, Serratia marcescens, Corynebacterium bovis, Trueperella pyogenes, Peptoniphilus indolicus, beta-lactamase staphylococcal, *Mycoplasma* spp., yeast *Protiothecia* spp.	Multiplex PathoProof Mastitis PCR	Koskinen et al. (2009); Hiitio et al. (2015)	Analytical 100% 100% 86.0%	Analytica 100% Diagnostic 99.0% 75.4%

Corynbacterium bovis, Arcanobacterium pyogenes, Peptoniphilus indolicus, Streptococcus uberis, Staphylococcus spp, Staphulococcus areus, Beta-lactamase *staphylococcal*	Pathoproof	Spittel & Hoedemaker (2012)	76.9-100%	63.3-98.7%

The SCC diagnostic tests results have a sensitivity range of between 49.0-94.5% and specificity range of 79.0-86.8% for (Table 4), which includes the milk line conductivity test used in very large dairy herds. However, SCC diagnostic test using only CMT and Delaval SCC counter the sensitivity ranged from 71.0-94.5% (Table 4). As SCC are not absolute for pathogen species identification, it is followed up by primary isolation methods using phenotypical identification which gives a sensitivity and specificity range from 9.1-100% sensitivity and 28.8-100% specificity depending on the pathogens (Table 4). Spittel and Hoedemaker (2012) reported bacterial culture with 76.9-100% sensitivity and 63.3-98.7% specificity for six out of seven pathogens, however with *Enterococcus* spp the sensitivity was 9.1% for bacterial culture. *Mycoplasma* isolated on modified Hayflick medium with special growth requirements had low sensitivity of 68.9%. *Staphylococcus aureus* using blood agar has low sensitivity of 68.8% reported by Bautista-Trujillo et al. (2013) but improved to 85.0-100% when using blood broth as reported by Qian et al. (2014).

Further immunodiagnostic techniques reported sensitivity range of 70-100% (Table 4). The molecular tests were evaluated according to conventional and real time PCR. The conventional PCR diagnostic sensitivity and specificity ranged from 76.7-100% and 98.0-100%, respectively, but conventional PCR tests are not always practical, more time consuming than real-time PCR and/or not commercially available. The real time PCR assays diagnostic sensitivity and specificity ranged from 86.7-100%% and 75.4-100%, respectively (Table 4). Patho-proof multiplex qPCR assay proved to be 100% analytical sensitive and specific, while at animal levels reported 100% diagnostic sensitive and 99-100% specific (Koskinen et al.2009). Hiitio et al. (2015) evaluate the diagnostic performance of the PathoProof qPCR assay on a total of 294 quarter milk samples from routine mastitis using bacterial culture and qPCR assay. The bacterial culture identified 85·7% (251/294) while the qPCR mastitis assay amplified DNA targets 83·0% (244/294) samples. The most common bacterial species detected in the samples was the CNS group followed by *Stap. aureus*. For *Stap. aureus* the sensitivity and specificity for the PCR assay was 97.0% and 95·8%, respectively compared

with blood culture. *Staphylococcus* spp. were 86·7 and 75·4% sensitivity and specificity, respectively. The other molecular assay literature search results listed in Table 3 were not commercially available and /or practical. No explicit sensitivity and specificity range determinations for high resolution melt analysis or mass spectrometry analysis on fresh milk samples could be found in the literature review.

Table 4: Summary of the ranges of sensitivity and specificity of each diagnostic tests from systematic literature search

Diagnostic tests	**Sensitivity range**	**Specificity range**
SCC identification	49.0-94.5%	57,7-86.8%
Phenotypical identification	9.1-100%	28.8-100%
Immuno-assay identification	75,5-100%	98.8-100%
Conventional PCR	76.7%-100%	98.6-100%
Multiplex real- time qPCR	76.9-100%	63,3-100%

DISCUSSION

In this project a diagnostic surveillance model applied in a One- Health orientated practice, was proposed that combine basic pathogen identification with genotypical and/or serological identification. The systematic literature review indicated that the basic pathogen identification from high BMSCC fresh milk will depend on the pathogen ability to grow on routinely used media. The only commercially available molecular techniques that had high sensitivity and specificity was the PathoProof mastitis multiple PCR assay. The PathoProof mastitis PCR assay can identify 15 mastitis pathogen and beta lactamase Staphylococci from milk which include bacteria that will not grow on routinely used media such as *Streptococcus dysgalactiae, Trueperella pyogenes* and *Mycoplasma spp*. Bacterial culture of *Enterococcus* was reported to have a low sensitivity of 9.1% (Spittel and Hoedemaker, 2012) but increase

dramatically using CHROMagar VRE® (Archibald, 2011; Peterson et al., 2010). Thus, combining the phenotypical and genotypical identification will allow for an improved diagnostic sensitivity and specificity in the private veterinary dairy practice. As mentioned, no sensitivity and specificity range determinations for high resolution melt analysis and mass-spectrometry on milk samples could be found in the literature review but this could change in future. The serological identification had high sensitivity and specificities (Table 3) but were expensive. To conclude the proposed diagnostic surveillance model can be implemented for the private practice laboratory using diagnostic techniques identified with the systematic literature search. The phenotypical identification can be done using routine media as well as specialized selection media which will allow for higher sensitivity and specificity of selected pathogens. As the private practice laboratory has an ELISA apparatus, serological ELISA identification can be done at the laboratory or outsourced to specialized laboratories. The mass-spectrometry and genotypical identification can be done by a specialized laboratory or if the practice obtains a Vitek MS and real-time PCR apparatus in future, it can be done economically in-practice.

California Mastitis Test (CMT) is a fast, easy and cost-effective cow side technique for estimating quarter milk SCC of individual cows (Patil et al., 2015) by the veterinarian. However, the CMT is too time-consuming for large herd processing where milking time is a critical factor; thus, not suitable for large dairy herd and replaced by rapid diagnostic techniques (Chakraborty et al., 2019). (Fosgate et al. (2013 reported the sensitivity and specificity of clinical mastitis pathogen using CMT to be 94 % and 77.7% compared to Delaval counter with sensitivity of 89.9% and increase specificity of 86.8%. SCC has been found to be an ideal method for herd immune response profiling and subclinical mastitis diagnosis. The DeLaval cell counter is a practical on-farm instrument for the veterinarian to evaluate BMSCC but too expensive for individual cow or quarter milk evaluations. The Fossomatic cell counter on the other hand is an accurate but expensive laboratory instrument for large numbers of

individual quarter samples that can be analysed with ease and automaticity for SANAS accredited milk recording programs and mastitis prevention schemes on whole herd basis. This instrument measures SCC in many samples together, with high capacity based on the principle of flow cytometry(www.fossanalytics.com).

It is essential to discuss the importance of culture negative or “no-growth” results when researching diagnostic techniques for SCC. Culture negative results can be caused by the raised SCC in the milk as part of the immune response that has the purpose to eliminate the pathogens, determined that at least 10-25% of quarters with > 200,000 SCC will be bacteriologically negative (Abdelmegid et al. 2017). It is important to have the proper knowledge of the microbial/bacteriological aetiology of subclinical mastitis and concurrently understand the immune responding mechanisms of the cow by a veterinarian to successfully interpret the individual quarter SCC as well as the BMSCC profile over a time frame where a graphic profile of the individual quarter milk SCC and the BMSCC is monitored and evaluated. Microbiological/bacteriological culture is ultimately important as is “no growth” results that could indicate normal advantageous immunological responses especially in the modern era of prevention by means of vaccines (Chakraborty et al., 2019). “No growth” is observed upon bacteriological examination of milk samples in 10–40% of cases in clinical mastitis at the quarter level. Various reasons may be responsible for such situation, viz., presence of very few organisms, or samples may contain fastidious pathogens and even intra-cellular pathogens, which require special technique and media for culturing, or the cultural conditions may simply not be feasible (Chakraborty et al., 2019). Furthermore anti-microbial treatment, disinfectants, lowered numbers of bacteria or the low inoculated number of bacteria, sampling time , transport times and conditions as well as media used, temperatures such as freezing of collected samples/ incubator settings and age of samples to name only the most important factors that causes ”no growth” on isolation plates (Dohoo and Leslie, 1991). Crucial culture negative or “no growth” roles may be played by latent infections or shedding cycles in case of pathogen specific subclinical mastitis or “commensal” low pathogenicity, non- specific

immunological responses which influences the most active, responsive defending SCC patho-physiology mechanisms of the cow. Further research and investigations should be done on this controversial topic (Bexiga et al., 2011).

Lam et al. (2009) stated that specific culturing techniques are essential for specific diagnosis. More effective isolation techniques of pathogens have increased isolation rates gradually by improving various diagnostic techniques such as specific culture media, incubation times, temperatures, culturing specificities and increase in volume of inoculation. Primary conventional blood agar media e.g. nutrient agar or broth for initial bacteria culturing like streptococci and staphylococci, and secondary, more specific media (e.g. MacConkey agar for Gram-negative bacteria initial growth. Further progression to highly selective media, like Mannitol salt agar for *Staphylococcus* spp., Eosin Methylene Blue agar for *E. coli* to Hayflicks medium for *Mycoplasma* spp. to eventually more advanced chromogenic diagnostic media. In case of clinical mastitis, it is pertinent from a timely diagnostic point of view to have bacteriological culture results at the earliest time available for effective treatment. This reduces the cost of treatment and helps proper and prudent use of antimicrobial treatments (Lam et al., 2009).

Over the last two decades the use of various molecular methods to detect pathogens have been increasingly implemented. Detection of a variety of mastitis causing pathogens and descriptions about the diagnostic techniques based on PCR were available (Lakshmi, 2016). PCR is rapid as it can be done in 1-2 days as well as sensitive 76.9–100% and specific 63.3–98.7% diagnostic technique for IMI or mastitis (Parker et al., 2017; Spittel and Hoedemaker 2012; Chakraborty et al., 2019). Bacterial culture methods for detection of mastitis pathogens are far less sensitive 32.2% than PCR assays 70.6% (Parker et al., 2017). According to literature novel PCR techniques became essential in modern day milk pathogen diagnostics especially in diagnosing slow growing microbes, e.g. *Mycoplasma* (Parker et al., 2017), and subclinical mastitis (El-Sayed et al., 2017). Molecular techniques are rapid and more sensitive than the laboratory culture techniques in detecting pathogen and thus more useful for the

clinician to plan the treatment regimen early (Cantekin et al., 2015). Short coming of conventional PCR include poor resolution, identification of organism based on amplification of DNA and it is not quantitative at end point (Duarte et al., 2015). The pPCR assay can detect bacteria that are either dead or with inhibited growth, thereby decreasing false negative results and has been applied as a diagnostic tool for detection of organisms causing mastitis from samples of milk (including composite samples) (Behera et al., 2018). The serological identification using immunodiagnostic techniques reported a sensitivity range of 70-100% (Table 4), but as indicated these techniques are expensive and can be used on request or if needed as the private practice laboratory has an ELISA apparatus.

The commercially available Patho-proof multiplex qPCR assay detects pathogens most frequently encountered in mastitis milk (15 mastitis pathogens) from fresh or preserved samples during a short throughput time. False negative results are minimised as this assay identifies dead or inhibited pathogens (Chakraborty et al., 2019). Hittio et al. (2015) showed the benefit of the Pathoproof qPCR assay to accurately identify the Coagulase Negative Staphylococcus group with a Se and Sp of *Staph. aureus* as 97.0% and 95·8%, respectively compared to bacterial culture as 86·7% and 75·4% Se and Sp respectively. As mentioned, high resolution melt analysis or MALDI-TOF MS technique is commercially available and can be applied to the proposed diagnostic surveillance model but is currently not included as the diagnostic sensitivity and specify for milk pathogens are unknown and it is not cost effective. No explicit sensitivity and specificity range determinations for high resolution melt analysis or MALDI-TOF MS on milk samples could be found in the literature review.

CONCLUSION & RECOMMENDATIONS

I can conclude by practical experience and this literature review that BMSCC diagnostic investigations, profiling and interpretations are extraordinarily demanding, complex and

dynamic. Diagnostic techniques with available sensitivity and specificity are not always available in literature for all possible pathogens that could influence the BMSCC. More research needs to focus on diagnostic techniques for milk samples to identify milk pathogens with negative culture results or minor pathogens in conventional bacterial culturing techniques. The systematic literature search with high diagnostic sensitivity and specificity as well as commercial and practical applications identified the Pathoproof multiplex qPCR assay as an essential technique for future accurate BMSCC investigations.

The diagnostic surveillance model is a suitable protocol that can be successfully implemented by a veterinarian in private dairy practice using the combined diagnostic techniques determined with the systematic literature search with high levels of sensitivity and specificity. This described model will make an enormous contribution in diagnostic surveillance procedures for the milk industry - public health interface in a veterinary One Health orientated veterinary practice.

BIBLIOGRAPHY

ABBASI, P., KARGAR, M., DOOSTI, A., MARDANEH, J., GHORBANI-DALINI, S. & DEHYADEGARI, M. A. 2014. Characterization of Shiga-toxin producing E.coli (STEC) and enteropathogenic E.coli (EPEC) using multiplex Real-Time PCR assays for stx1 , stx2 , eaeA. *Iran J Microbiol,* 6**,** 169-74.

ADDIS, M. F., TEDDE, V., PUGGIONI, G. M. G., PISANU, S., CASULA, A., LOCATELLI, C., ROTA, N., BRONZO, V., MORONI, P. & UZZAU, S. 2016. Evaluation of milk cathelicidin for detection of bovine mastitis. *Journal of Dairy Science,* 99**,** 8250-8258.

ADKINS, P. R. F. & MIDDLETON, J. R. 2018. Methods for Diagnosing Mastitis. *Vet Clin North Am Food Anim Pract,* 34**,** 479-491.

AJITKUMAR, P., BARKEMA, H. W. & DE BUCK, J. 2012a. Rapid identification of bovine mastitis pathogens by high-resolution melt analysis of 16S rDNA sequences. *Veterinary microbiology,* 155**,** 332-340.

AJITKUMAR, P., BARKEMA, H. W. & DE BUCK, J. 2012b. Rapid identification of bovine mastitis pathogens by high-resolution melt analysis of 16S rDNA sequences. *Veterinary Microbiology,* 155**,** 332-340.

AJITKUMAR, P., BARKEMA, H. W., ZADOKS, R. N., MORCK, D. W., VAN DER MEER, F. J. & DE BUCK, J. 2013. High-resolution melt analysis for species identification of coagulase-negative staphylococci derived from bovine milk. *Diagnostic microbiology and infectious disease,* 75**,** 227-234.

ALHUSSIEN, M. N. & DANG, A. K. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. *Veterinary world,* 11**,** 562-577.

ALVES-BARROCO, C., ROMA-RODRIGUES, C., RAPOSO, L. R., BRÁS, C., DINIZ, M., CAÇO, J., COSTA, P. M., SANTOS-SANCHES, I. & FERNANDES, A. R. 2019. Streptococcus dysgalactiae subsp. dysgalactiae isolated from milk of the bovine udder as emerging pathogens: In vitro and in vivo infection of human cells and zebrafish as biological models. *Microbiologyopen,* 8**,** e00623.

ARCHIBALD, L. K. 2011. Rapid Diagnostic Testing of Infectious Diseases. Medscape.

ASHRAF, A. & IMRAN, M. 2018. Diagnosis of bovine mastitis: from laboratory to farm. *Tropical animal health and production,* 50**,** 1193-1202.

ATA, Z. & BUYUKCANGAZ, E. 2019. A 13-Plex PCR for high-resolution melting-based screening of clinically important Staphylococcus species in animals. *Journal of microbiological methods,* 164**,** 105673.

ATHAMANOLAP, P., HSIEH, K., CHEN, L., YANG, S. & WANG, T.-H. 2017. Integrated Bacterial Identification and Antimicrobial Susceptibility Testing Using PCR and High-Resolution Melt. *Analytical Chemistry,* 89**,** 11529-11536.

AWADALLAH, M., AHMED, H., MERWAD, A. & SELIM, M. 2016. Occurrence, genotyping, shiga toxin genes and associated risk factors of E. coli isolated from dairy farms, handlers and milk consumers. *The Veterinary Journal,* 217**,** 83-88.

AZARI, A. A., AMANOLLAHI, R., JOZANI, R. J., TROTT, D. J. & HEMMATZADEH, F. 2020. High-resolution melting curve analysis: a novel method for identification of Mycoplasma species isolated from clinical cases of bovine and porcine respiratory disease. *Tropical animal health and production,* 52**,** 1043-1047.

BARREIRO, J. R., GONÇALVES, J. L., BRAGA, P. A. C., DIBBERN, A. G., EBERLIN, M. N. & VEIGA DOS SANTOS, M. 2017. Non-culture-based identification of mastitis-causing bacteria by MALDI-TOF mass spectrometry. *Journal of Dairy Science,* 100**,** 2928-2934.

BAUER, A. 1966. Kirby Bauer method antimicrobial susceptibility testing by a standardized single disk method. *American journal of clinical pathology,* 45**,** 493-496.

BAUMERT, A., BRUCKMAIER, R. M. & WELLNITZ, O. 2009. Cell population, viability, and some key immunomodulatory molecules in different milk somatic cell samples in dairy cows. *Journal of Dairy Research,* 76**,** 356-364.

BAUTISTA-TRUJILLO, G., SOLORIO-RIVERA, J., RENTERIA-SOLORZANO, I., CARRANZA-GERMAN, S., BUSTOS-MARTINEZ, J., ARTEAGA-GARIBAY, R., BAIZABAL-AGUIRRE, V., CAJERO-JUAREZ, M., BRAVO-PATINO, A. & VALDEZ-ALARCON, J. 2013. Performance of culture media for the isolation and identification of Staphylococcus aureus from bovine mastitis. *Journal of medical microbiology,* 62**,** 369-376.

BEHERA, S., RANA, R., GUPTA, P. K., KUMAR, D., SONAL, REKHA, V., ARUN, T. R. & JENA, D. 2018a. Development of real-time PCR assay for the detection

of Mycoplasma bovis. *Tropical Animal Health and Production,* 50**,** 875-882.
BEHERA, S., RANA, R., GUPTA, P. K., KUMAR, D., SONAL, REKHA, V., ARUN, T. R. & JENA, D. 2018b. Development of real-time PCR assay for the detection of Mycoplasma bovis. *Tropical Animal Health and Production,* 50**,** 875-882.
BERENDS, I., SWART, W., FRANKENA, K., MUSKENS, J., LAM, T. & VAN SCHAIK, G. 2008a. The effect of becoming BVDV-free on fertility and udder health in Dutch dairy herds. *Preventive veterinary medicine,* 84**,** 48-60.
BERENDS, I. M. G. A., SWART, W. A. J. M., FRANKENA, K., MUSKENS, J., LAM, T. J. G. M. & VAN SCHAIK, G. 2008b. The effect of becoming BVDV-free on fertility and udder health in Dutch dairy herds. *Preventive Veterinary Medicine,* 84**,** 48-60.
BERGE, A. & BAARS, T. 2020. Raw milk producers with high levels of hygiene and safety. *Epidemiology & Infection,* 148.
BES, M., GUÉRIN-FAUBLÉE, V., MEUGNIER, H., ETIENNE, J. & FRENEY, J. 2000. Improvement of the identification of staphylococci isolated from bovine mammary infections using molecular methods. *Veterinary Microbiology,* 71**,** 287-294.
BEXIGA, R., KOSKINEN, M. T., HOLOPAINEN, J., CARNEIRO, C., PEREIRA, H., ELLIS, K. A. & VILELA, C. L. 2011. Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing. *Journal of Dairy Research,* 78**,** 49-55.
BEZERRA, A. V. A., DOS REIS, E., RODRIGUES, R. O., CENCI, A., CERVA, C. & MAYER, F. Q. 2015. Detection of Mycobacterium tuberculosis and Mycobacterium avium Complexes by real-time PCR in bovine milk from Brazilian dairy farms. *Journal of food protection,* 78**,** 1037-1042.
BLIGNAUT, D., THOMPSON, P. & PETZER, I.-M. 2018. Prevalence of mastitis pathogens in South African pasture-based and total mixed ration-based dairies during 2008 and 2013. *Onderstepoort Journal of Veterinary Research,* 85**,** 1-7.
BOERLIN, P., KUHNERT, P., HÜSSY, D. & SCHAELLIBAUM, M. 2003. Methods for identification of Staphylococcus aureus isolates in cases of bovine mastitis. *Journal of clinical microbiology,* 41**,** 767-771.
BONIZZI, L., MENANDRO, M., PASOTTO, D. & LAUZI, S. 2003. Transition cow: non-specific immune response. *Veterinary research communications,* 27**,** 137.
BRAGA, P. A. C., GONÇALVES, J. L., BARREIRO, J. R., FERREIRA, C. R., TOMAZI, T., EBERLIN, M. N. & SANTOS, M. V. 2018. Rapid identification of bovine mastitis pathogens by MALDI-TOF Mass Spectrometry. *Pesquisa Veterinária Brasileira,* 38**,** 586-594.
BRUNHAM, R. C., PLUMMER, F. A. & STEPHENS, R. S. 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. *Infection and immunity,* 61**,** 2273-2276.
BURRUS, V., PAVLOVIC, G., DECARIS, B. & GUÉDON, G. 2002. Conjugative transposons: the tip of the iceberg. *Molecular microbiology,* 46**,** 601-610.
CANTEKIN, Z., ERGÜN, Y., DOĞRUER, G., SARıBAY, M. K. & SOLMAZ, H. 2015. Comparison of PCR and culture methods for diagnosis of subclinical mastitis in dairy cattle.
CHAKRABORTY, S., DHAMA, K., TIWARI, R., IQBAL YATOO, M., KHURANA, S. K., KHANDIA, R., MUNJAL, A., MUNUSWAMY, P., KUMAR, M. A. & SINGH, M. 2019. Technological interventions and advances in the diagnosis of

intramammary infections in animals with emphasis on bovine population—a review. *Veterinary Quarterly,* 39**,** 76-94.
CHAKRAVORTY, S., HELB, D., BURDAY, M., CONNELL, N. & ALLAND, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of microbiological methods,* 69**,** 330-339.
CHAMBAUD, I., WRÓBLEWSKI, H. & BLANCHARD, A. 1999. Interactions between mycoplasma lipoproteins and the host immune system. *Trends in microbiology,* 7**,** 493-499.
CLAYCOMB, R. W., JOHNSTONE, P. T., MEIN, G. A. & SHERLOCK, R. A. 2009. An automated in-line clinical mastitis detection system using measurement of conductivity from foremilk of individual udder quarters. *New Zealand Veterinary Journal,* 57**,** 208-214.
CLEWELL, D. B. 1990. Movable genetic elements and antibiotic resistance in enterococci. *European Journal of clinical microbiology and infectious diseases,* 9**,** 90-102.
CLOTHIER, K. A., JORDAN, D. M., THOMPSON, C. J., KINYON, J. M., FRANA, T. S. & STRAIT, E. L. 2010. Mycoplasma bovis real-time polymerase chain reaction assay validation and diagnostic performance. *J Vet Diagn Invest,* 22**,** 956-60.
COLINON, C., DEREDJIAN, A., HIEN, E., BROTHIER, E., BOUZIRI, L., COURNOYER, B., HARTMAN, A., HENRY, S., JOLIVET, C. & RANJARD, L. 2013. Detection and enumeration of P seudomonas aeruginosa in soil and manure assessed by an ecfX q PCR assay. *Journal of applied microbiology,* 114**,** 1734-1749.
DADAR, M., FAKHRI, Y., SHAHALI, Y. & KHANEGHAH, A. M. 2020. Contamination of milk and dairy products by Brucella species: A global systematic review and meta-analysis. *Food Research International,* 128**,** 108775.
DADIMI, B. & NISHANTH, M. A. D. Q-Fever: A Neglected Zoonosis.
DE CARVALHO, N. L., GONÇALVES, J. L., BOTARO, B. G., E SILVA, L. F. D. P. & DOS SANTOS, M. V. 2015. Detection and enumeration of Streptococcus agalactiae from bovine milk samples by real-time polymerase chain reaction. *Current microbiology,* 71**,** 363-372.
DE HAAS, Y., BARKEMA, H. W. & VEERKAMP, R. F. 2002. The Effect of Pathogen-Specific Clinical Mastitis on the Lactation Curve for Somatic Cell Count. *Journal of Dairy Science,* 85**,** 1314-1323.
DELGADO, C. L. 2003. Rising consumption of meat and milk in developing countries has created a new food revolution. *The Journal of nutrition,* 133**,** 3907S-3910S.
DOHOO, I. R. & LESLIE, K. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. *Preventive veterinary medicine,* 10**,** 225-237.
DU PREEZ, J. H. 1986. The prevalence of teat canal infections in lactating dairy cows as determined from foremilk and teat canal swab samples. *J S Afr Vet Assoc,* 57**,** 193-8.
DU PREEZ, J. H. 1989. The role of anaerobic bacteria in bovine mastitis: a review. *J S Afr Vet Assoc,* 60**,** 159-68.
DU PREEZ, J. H. 1995. The role of heat stress in the performance of dairy cattle in southern Africa.
DUARTE, C. M., FREITAS, P. P. & BEXIGA, R. 2015. Technological advances in bovine mastitis diagnosis: an overview. *J Vet Diagn Invest,* 27**,** 665-72.

EL-SAYED, A., AWAD, W., ABDOU, N.-E. & CASTAÑEDA VÁZQUEZ, H. 2017. Molecular biological tools applied for identification of mastitis causing pathogens. *International journal of veterinary science and medicine,* 5**,** 89-97.

EL GARCH, F., YOUALA, M., SIMJEE, S., MOYAERT, H., KLEE, R., TRUSZKOWSKA, B., ROSE, M., HOCQUET, D., VALOT, B. & MORRISSEY, I. 2020. Antimicrobial susceptibility of nine udder pathogens recovered from bovine clinical mastitis milk in Europe 2015–2016: VetPath results. *Veterinary Microbiology***,** 108644.

EMAM, M., LIVERNOIS, A., PAIBOMESAI, M., ATALLA, H. & MALLARD, B. 2019a. Genetic and epigenetic regulation of immune response and resistance to infectious diseases in domestic ruminants. *Veterinary Clinics: Food Animal Practice,* 35**,** 405-429.

EMAM, M., LIVERNOIS, A., PAIBOMESAI, M., ATALLA, H., MALLARD, B. O. M. O. A. & RURAL AFFAIRS, S. R. W. G. O. N. G. Y. C. 2019b. Genetic and Epigenetic Regulation of Immune Response and Resistance to Infectious Diseases in Domestic Ruminants. *Veterinary Clinics of North America: Food Animal Practice,* 35**,** 405-429.

FITZPATRICK, L. Milk somatic cells–what do they do. Proceedings of the British Mastitis Conference Garstang, 2001. 56-62.

FLIEGER, A., MIELKE, M. & TIETZE, E. 2013. [Role of pathogen surveillance and subtyping in outbreak recognition of food-borne bacterial infections. Microbiological perspective - Aims, methods and prospects of pathogen subtyping]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz,* 56**,** 42-6.

FLUIT, A. C., VISSER, M. R. & SCHMITZ, F. J. 2001. Molecular detection of antimicrobial resistance. *Clinical microbiology reviews,* 14**,** 836-871.

FONSECA, I., CARDOSO, F. F., HIGA, R. H., GIACHETTO, P. F., BRANDÃO, H. M., BRITO, M. A. V. P., FERREIRA, M. B. D., GUIMARÃES, S. E. F. & MARTINS, M. F. 2015. Gene expression profile in zebu dairy cows (Bos taurus indicus) with mastitis caused by Streptococcus agalactiae. *Livestock Science,* 180**,** 47-57.

FOSGATE, G. T., PETZER, I.-M. & KARZIS, J. 2013. Sensitivity and specificity of a hand-held milk electrical conductivity meter compared to the California mastitis test for mastitis in dairy cattle. *The Veterinary Journal,* 196**,** 98-102.

FRIE, M. C. & COUSSENS, P. M. 2015. Bovine leukemia virus: a major silent threat to proper immune responses in cattle. *Vet Immunol Immunopathol,* 163**,** 103-14.

FRIE, M. C., SPORER, K. R., WALLACE, J. C., MAES, R. K., SORDILLO, L. M., BARTLETT, P. C. & COUSSENS, P. M. 2016. Reduced humoral immunity and atypical cell-mediated immunity in response to vaccination in cows naturally infected with bovine leukemia virus. *Veterinary immunology and immunopathology,* 182**,** 125-135.

FRIENDSHIP, C., KELTON, D., VAN DE WATER, D., SLAVIC, D. & KOSKINEN, M. 2010. *Field Evaluation of the Pathoproof Mastitis PCR Assay for the Detection of Staphylococcus aureus Infected Cows Using DHI Samples*.

GAILLOT, O., WETSCH, M., FORTINEAU, N. & BERCHE, P. 2000. Evaluation of CHROMagar Staph. aureus, a new chromogenic medium, for isolation and presumptive identification of Staphylococcus aureus from human clinical specimens. *Journal of clinical microbiology,* 38**,** 1587-91.

GANDA, E. K., BISINOTTO, R. S., DECTER, D. H. & BICALHO, R. C. 2016. Evaluation of an On-Farm Culture System (Accumast) for Fast Identification of Milk Pathogens Associated with Clinical Mastitis in Dairy Cows. *PloS one,* 11**,** e0155314-e0155314.

GIESECKE, W., DU PREEZ, J. H. & PETZER, I.-M. 1994. *Practical mastitis control in dairy herds. A field guide on various aspects of udder health for the dairy practitioner*, Butterworth Publishers (Pty) Ltd.

GILLESPIE, B. E. & OLIVER, S. P. 2005. Simultaneous Detection of Mastitis Pathogens, Staphylococcus aureus, Streptococcus uberis, and Streptococcus agalactiae by Multiplex Real-Time Polymerase Chain Reaction. *Journal of Dairy Science,* 88**,** 3510-3518.

GIOVANNINI, G., PICCININI, R. & ZECCONI, A. Epidemiology of clinical mastitis in Italy. ANNUAL MEETING-NATIONAL MASTITIS COUNCIL INCORPORATED, 2000. National Mastitis Council; 1999, 176-178.

GONZÁLEZ, R. N. & WILSON, D. J. 2003. Mycoplasmal mastitis in dairy herds. *Veterinary Clinics: Food Animal Practice,* 19**,** 199-221.

GOUALI, M., RUCKLY, C., CARLE, I., LEJAY-COLLIN, M. & WEILL, F.-X. 2013. Evaluation of CHROMagar STEC and STEC O104 chromogenic agar media for detection of Shiga toxin-producing Escherichia coli in stool specimens. *Journal of Clinical Microbiology,* 51**,** 894-900.

GOUDAR, V. & PRASAD, N. 2020. A Critical Review on Listeria monocytogenes.

GRABER, H. U., CASEY, M. G., NASKOVA, J., STEINER, A. & SCHAEREN, W. 2007. Development of a Highly Sensitive and Specific Assay to Detect Staphylococcus aureus in Bovine Mastitic Milk. *Journal of Dairy Science,* 90**,** 4661-4669.

GRAVES, S. F., KOBAYASHI, S. D. & DELEO, F. R. 2010. Community-associated methicillin-resistant Staphylococcus aureus immune evasion and virulence. *Journal of Molecular Medicine,* 88**,** 109-114.

GRIFFIOEN, K., VELTHUIS, A. G., LAGERWERF, L. A., HEUVELINK, A. E. & LAM, T. J. 2018a. Agreement between four commercial diagnostic tests and routine bacteriological culture of milk to determine the udder infection status of dairy cows. *Preventive veterinary medicine,* 157**,** 162-173.

GRIFFIOEN, K., VELTHUIS, A. G. J., LAGERWERF, L. A., HEUVELINK, A. E. & LAM, T. J. G. M. 2018b. Agreement between four commercial diagnostic tests and routine bacteriological culture of milk to determine the udder infection status of dairy cows. *Preventive Veterinary Medicine,* 157**,** 162-173.

HALASA, T., HUIJPS, K., ØSTERÅS, O. & HOGEVEEN, H. 2007. Economic effects of bovine mastitis and mastitis management: A review. *Veterinary quarterly,* 29**,** 18-31.

HARMON, R. 1994. Physiology of mastitis and factors affecting somatic cell counts. *Journal of dairy science,* 77**,** 2103-2112.

HERLEKAR, D. A., SHASHIKANT, C. S., GURJAR, A. A. & JAYARAO, B. M. 2013. Presence of viral and bacterial organisms in milk and their association with somatic cell counts. *J Dairy Sci,* 96**,** 6336-46.

HIITIÖ, H. Multiplex Real-Time PCR in Bovine Mastitis Diagnostics. 2018.

HIJAZIN, M., ULBEGI-MOHYLA, H., ALBER, J., LAMMLER, C., HASSAN, A. A., ABDULMAWJOOD, A., PRENGER-BERNINGHOFF, E., WEISS, R. & ZSCHOCK, M. 2011. Molecular identification and further characterization of Arcanobacterium pyogenes isolated from bovine mastitis and from various other origins. *J Dairy Sci,* 94**,** 1813-9.

HOGEVEEN, H., KAMPHUIS, C., STEENEVELD, W. & MOLLENHORST, H. 2010. Sensors and clinical mastitis—The quest for the perfect alert. *Sensors,* 10**,** 7991-8009.

HOLKO, I., URBANOVA, J., KANTIKOVA, M., PASTOROVA, K. & KMEŤ, V. 2002. PCR detection of Listeria monocytogenes in milk and milk products and differentiation of suspect isolates. *Acta Veterinaria Brno,* 71**,** 125-131.

HSIEH, J.-C., YEN, Y.-S. & CHUANG, S.-T. 2019. Identification of Streptococcus spp. isolated from bovine milk and characterization of their antimicrobial susceptibility profiles in Taiwan. *The Thai Journal of Veterinary Medicine,* 49**,** 57-63.

HUSSEIN, H. S. & BOLLINGER, L. M. 2008. Influence of selective media on successful detection of Shiga toxin–producing Escherichia coli in food, fecal, and environmental samples. *Foodborne Pathogens and Disease,* 5**,** 227-244.

ISMAIL, Z. B. 2017. Molecular characteristics, antibiogram and prevalence of multi-drug resistant Staphylococcus aureus (MDRSA) isolated from milk obtained from culled dairy cows and from cows with acute clinical mastitis. *Asian Pacific Journal of Tropical Biomedicine,* 7**,** 694-697.

JUSTICE-ALLEN, A., TRUJILLO, J., GOODELL, G. & WILSON, D. 2011. Detection of multiple Mycoplasma species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. *Journal of Dairy science,* 94**,** 3411-3419.

KALIN, R., KARAHAN, M., AÇIK, M. N., TASDEMIR, B. & ÇETINKAYA, B. Development of A Multiplex PCR Method for Direct Detection of Common Mastitis Pathogens in Bovine Milk Samples. 2017.

KARZIS, J. & PETZER, I.-M. 2012. Curb mastitis with fast diagnosis.

KARZIS, J., PETZER, I.-M., DONKIN, E. F. & NAIDOO, V. 2018. Proactive udder health management in South Africa and monitoring of antibiotic resistance of Staphylococcus aureus in dairy herds from 2001 to 2010. *Journal of the South African Veterinary Association,* 89**,** 1-8.

KARZIS, J., PETZER, I.-M., DONKIN, E. F., NAIDOO, V. & ETTER, E. 2020a. Surveillance of antibiotic resistance of maltose-negative Staphylococcus aureus in South African dairy herds. *Antibiotics,* 9**,** 616.

KARZIS, J., PETZER, I.-M., DONKIN, E. F., NAIDOO, V. & ETTER, E. M. 2020b. Surveillance of antimicrobial resistance of maltose negative Staphylococcus aureus in South African dairy herds.

KEEFE, G. P. 1997. Streptococcus agalactiae mastitis: a review. *The Canadian veterinary journal,* 38**,** 429.

KLAAS, I. C. & ZADOKS, R. N. 2018. An update on environmental mastitis: Challenging perceptions. *Transboundary and Emerging Diseases,* 65**,** 166-185.

KOSKINEN, M., HOLOPAINEN, J., PYÖRÄLÄ, S., BREDBACKA, P., PITKÄLÄ, A., BARKEMA, H., BEXIGA, R., ROBERSON, J., SØLVERØD, L. & PICCININI, R. 2009. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *Journal of Dairy Science,* 92**,** 952-959.

KOTZÉ, T. 2017. Pasop vir pseudomonas. *The Dairy Mail,* 24**,** 87-90.

KUMAR, V., MAAN, S., KUMAR, A., BATRA, K., CHAUDHARY, D., DALAL, A., GUPTA, A. K., BANSAL, N., SHEORAN, N. & MAAN, N. 2018. Real time PCR assay for differentiation of Brucella abortus and Brucella melitensis. *Indian Journal of Animal Research,* 52**,** 1037-1042.

LAKSHMI, R. 2016. Bovine mastitis and its diagnosis. *Int J Appl Res,* 2**,** 213-216.
LAM, T., RIEKERINK, R. O., SAMPIMON, O. & SMITH, H. 2009. Mastitis diagnostics and performance monitoring: a practical approach. *Irish Veterinary Journal,* 62**,** 1-6.
LAY JR, J. O. 2001. MALDI-TOF mass spectrometry of bacteria. *Mass spectrometry reviews,* 20**,** 172-194.
LEDGER, L., EIDT, J. & CAI, H. Y. 2020. Identification of Antimicrobial Resistance-Associated Genes through Whole Genome Sequencing of Mycoplasma bovis Isolates with Different Antimicrobial Resistances. *Pathogens,* 9**,** 588.
LELLI, D., LUINI, M., GAZZOLA, A., BOCCARDO, A., SOZZI, E., SALA, G., TROGU, T., CANZIANI, S., MORENO, A. & LAVAZZA, A. Pseudo-lumpy skin disease (BoHV-2): a case study [Conference poster]. XVIII Congresso Nazionale SI Di. LV, Perugia (PG), Italia, 7-9 Novembre 2018, 2018. Società Italiana di Diagnostica di Laboratorio Veterinaria (SIDiLV), 152-153.
LIU, C., LIU, Y., FENG, C., WANG, P., YU, L., LIU, D., SUN, S. & WANG, F. 2020. Distribution characteristics and potential risks of heavy metals and antimicrobial resistant Escherichia coli in dairy farm wastewater in Tai'an, China. *Chemosphere,* 262**,** 127768.
LIVERMORE, D. M. 2002. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? *Clinical infectious diseases,* 34**,** 634-640.
LUNDBERG, Å., NYMAN, A. K., ASPÁN, A., BÖRJESSON, S., UNNERSTAD, H. E. & WALLER, K. P. 2016. Udder infections with Staphylococcus aureus, Streptococcus dysgalactiae, and Streptococcus uberis at calving in dairy herds with suboptimal udder health. *Journal of Dairy Science,* 99**,** 2102-2117.
MAJID, Y., SOMAYEH, N., PARISA, S., BEHROOZ, A., REZA, F. N., JAVAD, R., MEHDI, F., NASRIN, A. A. & MARZIEH, K. 2016. Prevalence of Brucella melitensis and Brucella abortus in raw milk and dairy product by real time PCR technique. *THE ULUTAS MEDICAL JOURNAL,* 2**,** 7-11.
MALLARD, B. A., ATALLA, H., CARTWRIGHT, S., HINE, B., HUSSEY, B., PAIBOMESAI, M., THOMPSON-CRISPI, K. & WAGTER-LESPERANCE, L. Genetic and epigenetic regulation of the bovine immune system: Practical implications of the high immune response technology. Proc. National Mastitis Council 50th Annual Meeting (1), 2011. 53-63.
MALLARD, B. A., EMAM, M., PAIBOMESAI, M., THOMPSON-CRISPI, K. & WAGTER-LESPERANCE, L. 2015. Genetic selection of cattle for improved immunity and health. *Japanese Journal of Veterinary Research,* 63**,** S37-S44.
MARKEY, B., LEONARD, F., ARCHAMBAULT, M., CULLINANE, A. & MAGUIRE, D. 2013. *Clinical veterinary microbiology e-book*, Elsevier Health Sciences.
MARRIE, T. J. & RAOULT, D. 2000. Coxiella burnetii (Q fever). *Mandell, Douglas and Bennett's, Principles and Practice of Infectious Diseases***,** 2043-50.
MASHAMBA, P. 2020. *Molecular characterisation of lumpy skin disease virus in Mahikeng local municipality.* North-West University (South Africa).
MCKENNA, S. L. & DOHOO, I. R. 2006. Using and interpreting diagnostic tests. *Veterinary Clinics: Food Animal Practice,* 22**,** 195-205.
MERQUIOR, V. L., FARIA, A. R., ROCHA, F. S., MORAIS, J. M., NEVES, F. P. & TEIXEIRA, L. M. 2012. Emergence and characterisation of vanB vancomycin-resistant Enterococcus faecalis in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz,* 107**,** 557-60.

MERRILL, R., ROGERS, K., WARD, S., OJO, O., KAKAĪ, C. G., AGBEKO, T. T., GARBA, H., MACGURN, A., OPPERT, M., KONE, I., BAMSA, O., SCHNEIDER, D. & BROWN, C. 2017. Responding to Communicable Diseases in Internationally Mobile Populations at Points of Entry and along Porous Borders, Nigeria, Benin, and Togo. *Emerging Infectious Disease journal,* 23.

MINST, K., MÄRTLBAUER, E., MILLER, T. & MEYER, C. 2012. Short communication: Streptococcus species isolated from mastitis milk samples in Germany and their resistance to antimicrobial agents. *Journal of Dairy Science,* 95**,** 6957-6962.

MOMTAZ, H., GHAFARI, A., SHEIKH-SAMANI, M. & JHAZAYERI, A. 2016. Detecting virulence factors and antibiotic resistance pattern of Trueperella Pyogenes isolated from bovine Mastitic Milk. *International Journal of Medical Laboratory,* 3**,** 134-141.

MOON, J., KOO, H., JOO, Y., JEON, S., HUR, D., CHUNG, C., JO, H. & PARK, Y. H. 2007. Application of a new portable microscopic somatic cell counter with disposable plastic chip for milk analysis. *Journal of dairy science,* 90**,** 2253-2259.

MOTAUNG, T. E., PETROVSKI, K. R., PETZER, I.-M., THEKISOE, O. & TSILO, T. J. 2017. Importance of bovine mastitis in Africa. *Animal Health Research Reviews,* 18**,** 58-69.

MUSKENS, J., VAN ENGELEN, E., VAN MAANEN, C., BARTELS, C. & LAM, T. 2011. Prevalence of Coxiella burnetii infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Veterinary Record*.

NEAMAH, A. A. 2017. Molecular Detection of virulence factor genes in pseudomonas aeruginosa isolated from human and animals in Diwaniya province. *Kufa Journal For Veterinary Medical Sciences,* 8**,** 218-230.

NGASSAM TCHAMBA, C., RAO, A.-S., BOYEN, F., HAESEBROUCK, F., DUPREZ, J. N., THÉRON, L., THIRY, D. & MAINIL, J. 2019. Comparison of quantitative PCR and MALDI-TOF mass spectrometry assays for identification of bacteria in milk samples from cows with subclinical mastitis. *Journal of applied microbiology,* 127**,** 683-692.

NICHOLAS, R., AYLING, R. & MCAULIFFE, L. 2007. Mycoplasma mastitis. *Veterinary record,* 160**,** 382-382.

NIKODEMUSZ, E., BEDÖ, S., PICKLER, A. & SZEP, P. 1994. Variations in milk somatic cell count and haematologic values of dairy cows during lactation. *Acta Veterinaria Hungarica,* 42**,** 131-139.

NONNEMANN, B., LYHS, U., SVENNESEN, L., KRISTENSEN, K. A., KLAAS, I. C. & PEDERSEN, K. 2019. Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. *Journal of Dairy Science,* 102**,** 2515-2524.

OIKONOMOU, G., MACHADO, V. S., SANTISTEBAN, C., SCHUKKEN, Y. H. & BICALHO, R. C. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. *PLoS One,* 7**,** e47671.

OSMAN, K. M., SAMIR, A., ABO-SHAMA, U. H., MOHAMED, E. H., ORABI, A. & ZOLNIKOV, T. 2016. Determination of virulence and antibiotic resistance pattern of biofilm producing Listeria species isolated from retail raw milk. *BMC Microbiology,* 16**,** 263.

PAL, M., ABRAHA, A., RAHMAN, M. T. & DAVE, P. 2014. Protothecosis: an emerging algal disease of humans and animals. *International Journal of Life Sciences Biotechnology and Pharma Research,* 3**,** 1.

PANAGEA, T., GALANI, I., SOULI, M., ADAMOU, P., ANTONIADOU, A. & GIAMARELLOU, H. 2011. Evaluation of CHROMagar™ KPC for the detection of carbapenemase-producing Enterobacteriaceae in rectal surveillance cultures. *International journal of antimicrobial agents,* 37**,** 124-128.

PANTOJA, J. C. F., HULLAND, C. & RUEGG, P. L. 2009. Dynamics of somatic cell counts and intramammary infections across the dry period. *Preventive Veterinary Medicine,* 90**,** 43-54.

PAOLICCHI, F., CIRONE, K., MORSELLA, C. & GIOFFRÉ, A. 2012. First isolation of Mycobacterium avium subsp paratuberculosis from commercial pasteurized milk in Argentina. *Brazilian journal of microbiology,* 43**,** 1034-1037.

PARKER, A. M., HOUSE, J. K., HAZELTON, M. S., BOSWARD, K. L. & SHEEHY, P. A. 2017. Comparison of culture and a multiplex probe PCR for identifying Mycoplasma species in bovine milk, semen and swab samples. *PLoS One,* 12**,** e0173422.

PATIL, M., NAGVEKAR, A., INGOLE, S., BHARUCHA, S. & PALVE, V. 2015. Somatic cell count and alkaline phosphatase activity in milk for evaluation of mastitis in buffalo. *Veterinary World,* 8**,** 363.

PETERSON, J. F., DOERN, C. D., KALLSTROM, G., RIEBE, K. M., SANDER, T., DUNNE, W. M. & LEDEBOER, N. A. 2010. Evaluation of Spectra VRE, a New Chromogenic Agar Medium Designed To Screen for Vancomycin-Resistant Enterococcus faecalis and Enterococcus faecium. *Journal of Clinical Microbiology,* 48**,** 4627-4629.

PETZER, I.-M., KARZIS, J., DONKIN, E. F. & WEBB, E. C. 2016a. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations. *Onderstepoort Journal of Veterinary Research,* 83**,** 1-12.

PETZER, I.-M., KARZIS, J., DONKIN, E. F. & WEBB, E. C. 2016b. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations. *Onderstepoort Journal of Veterinary Research,* 83**,** 1-12.

PETZER, I.-M., KARZIS, J., DONKIN, E. F., WEBB, E. C. & ETTER, E. M. C. 2017a. Somatic cell count thresholds in composite and quarter milk samples as indicator of bovine intramammary infection status. *Onderstepoort Journal of Veterinary Research,* 84**,** 1-10.

PETZER, I.-M., KARZIS, J., DONKIN, E. F., WEBB, E. C. & ETTER, E. M. C. 2017b. Validity of somatic cell count as indicator of pathogen-specific intramammary infections. *2017,* 88.

PETZER, I.-M., KARZIS, J., MEYER, I. A. & VAN DER SCHANS, T. J. 2013. A cost-benefit model comparing the California milk cell test and milk electrical resistance test. *Onderstepoort Journal of Veterinary Research,* 80**,** 00-00.

PETZER, I.-M., KARZIS, J., WATERMEYER, J. C., VAN DER SCHANS, T. J. & VAN REENEN, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association,* 80**,** 17-22.

PHOPHI, L., PETZER, I.-M. & QEKWANA, D. N. 2019. Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species

isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. *BMC Veterinary Research,* 15**,** 420.

PIESSENS, V., DE VLIEGHER, S., VERBIST, B., BRAEM, G., VAN NUFFEL, A., DE VUYST, L., HEYNDRICKX, M. & VAN COILLIE, E. 2012. Intra-species diversity and epidemiology varies among coagulase-negative Staphylococcus species causing bovine intramammary infections. *Veterinary microbiology,* 155**,** 62-71.

PIESSENS, V., VAN COILLIE, E., VERBIST, B., SUPRÉ, K., BRAEM, G., VAN NUFFEL, A., DE VUYST, L., HEYNDRICKX, M. & DE VLIEGHER, S. 2011. Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. *Journal of dairy science,* 94**,** 2933-2944.

PITKÄLÄ, A., GINDONIS, V., WALLIN, H. & HONKANEN-BUZALSKI, T. 2005. Interlaboratory Proficiency Testing as a Tool for Improving Performance in Laboratories Diagnosing Bovine Mastitis. *Journal of Dairy Science,* 88**,** 553-559.

POISSON, D.-M., EVRARD, M.-L., FRENEAUX, C., VIVÈS, M.-I. & MESNARD, L. 2011. Evaluation of CHROMagar™ StrepB agar, an aerobic chromogenic medium for prepartum vaginal/rectal Group B Streptococcus screening. *Journal of Microbiological Methods,* 84**,** 490-491.

PRAMEELA, D. R. & VEENA, P. Molecular diagnosis of bovine papillomatosis and a successful management with autogenous vaccine.

PROJAHN, M., HAMMERL, J. A., DIECKMANN, R. & DAHOUK, S. A. 2020. A Proof of Principle for the Detection of Viable Brucella spp. in Raw Milk by qPCR Targeting Bacteriophages. *Microorganisms,* 8**,** 1326.

PUMIPUNTU, N., KULPEANPRASIT, S., SANTAJIT, S., TUNYONG, W., KONG-NGOEN, T., HINTHONG, W. & INDRAWATTANA, N. 2017. Screening method for Staphylococcus aureus identification in subclinical bovine mastitis from dairy farms. *Veterinary world,* 10**,** 721.

QIAN, Q., EICHELBERGER, K. & KIRBY, J. E. 2014. Rapid Identification of Staphylococcus aureus Directly from Bactec Blood Culture Broth by the BinaxNOW S. aureus Test. *Journal of Clinical Microbiology,* 52**,** 319.

RANJAN, R. 2015. *Bovine protothecal mastitis: A review*.

RHEE, M.-S., GRAY, P. & KANG, D. 2002. Improvement of selectivity for isolation of Escherichia coli O157: H7 from generic Escherichia coli. *Journal of Rapid Methods & Automation in Microbiology,* 10**,** 27-35.

RIEKERINK, R. G. O., BARKEMA, H. W., VEENSTRA, S., POOLE, D. E., DINGWELL, R. T. & KEEFE, G. P. 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. *The Canadian Veterinary Journal,* 47**,** 567.

RODRIGUES, N. M. B., BRONZATO, G. F., SANTIAGO, G. S., BOTELHO, L. A. B., MOREIRA, B. M., COELHO, I. D. S., SOUZA, M. M. S. D. & COELHO, S. D. M. D. O. 2017. The Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) identification versus biochemical tests: a study with enterobacteria from a dairy cattle environment. *Brazilian Journal of Microbiology,* 48**,** 132-138.

RODRÍGUEZ-LÁZARO, D., D'AGOSTINO, M., HERREWEGH, A., PLA, M., COOK, N. & IKONOMOPOULOS, J. 2005. Real-time PCR-based methods for detection of Mycobacterium avium subsp. paratuberculosis in water and milk. *International Journal of Food Microbiology,* 101**,** 93-104.

ROSSETTI, B. C., FREY, J. & PILO, P. 2010. Direct detection of Mycoplasma bovis in milk and tissue samples by real-time PCR. *Molecular and cellular probes,* 24**,** 321-3.

RUEGG, P. & PANTOJA, J. 2013. Understanding and using somatic cell counts to improve milk quality. *Irish Journal of Agricultural and Food Research***,** 101-117.

RZEWUSKA, M., KWIECIEŃ, E., CHROBAK-CHMIEL, D., KIZERWETTER-ŚWIDA, M., STEFAŃSKA, I. & GIERYŃSKA, M. 2019. Pathogenicity and virulence of Trueperella pyogenes: A review. *International journal of molecular sciences,* 20**,** 2737.

SAAH, A. J. & HOOVER, D. R. 1997. "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Ann Intern Med,* 126**,** 91-4.

SACHSE, K., SALAM, H. S., DILLER, R., SCHUBERT, E., HOFFMANN, B. & HOTZEL, H. 2010. Use of a novel real-time PCR technique to monitor and quantitate Mycoplasma bovis infection in cattle herds with mastitis and respiratory disease. *Vet J,* 186**,** 299-303.

SAITO, R., KOYANO, S., NAGAI, R., OKAMURA, N., MORIYA, K. & KOIKE, K. 2010. Evaluation of a chromogenic agar medium for the detection of extended-spectrum β-lactamase-producing Enterobacteriaceae. *Letters in applied microbiology,* 51**,** 704-706.

SALINA, A., TIMENETSKY, J., BARBOSA, M. S., AZEVEDO, C. M. & LANGONI, H. 2020. Microbiological and molecular detection of Mycoplasma bovis in milk samples from bovine clinical mastitis. *Pesquisa Veterinária Brasileira,* 40**,** 82-87.

SAMRA, Z., BAHAR, J., MADAR-SHAPIRO, L., AZIZ, N., ISRAEL, S. & BISHARA, J. 2008. Evaluation of CHROMagar KPC for Rapid Detection of Carbapenem-Resistant Enterobacteriaceae. *Journal of Clinical Microbiology,* 46**,** 3110-3111.

SCHMIDT, T., KOCK, M. M. & EHLERS, M. M. 2015. Antimicrobial Resistance in Staphylococciatthe Human–-Animal Interface. *Antimicrobial Resistance: An Open Challenge***,** 85.

SCHMIDT, T., KOCK, M. M. & EHLERS, M. M. 2017. Molecular Characterization of Staphylococcus aureus Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission. *Front Microbiol,* 8**,** 511.

SCHMIDT, T., KOCK, M. M. & EHLERS, M. M. 2018. Identification and characterization of Staphylococcus devriesei isolates from bovine intramammary infections in KwaZulu-Natal, South Africa. *BMC veterinary research,* 14**,** 324.

SCHUKKEN, Y. H., WILSON, D. J., WELCOME, F., GARRISON-TIKOFSKY, L. & GONZALEZ, R. N. 2003. Monitoring udder health and milk quality using somatic cell counts. *Veterinary research,* 34**,** 579-596.

SCHWARZ, D., SANTSCHI, D. E., DUROCHER, J. & LEFEBVRE, D. M. 2020. Evaluation of the new differential somatic cell count parameter as a rapid and

inexpensive supplementary tool for udder health management through regular milk recording. *Preventive Veterinary Medicine,* 181**,** 105079.
SEDKY, D., GHAZY, A., SOLIMAN, N. & SHAAPAN, R. 2020. Comparative diagnosis of infectious bacteria in bovine milk. *J. Anim. Health Prod,* 8**,** 171-182.
SHARIF, S., MALLARD, B., WILKIE, B., SARGEANT, J., SCOTT, H., DEKKERS, J. & LESLIE, K. 1998. Associations of the bovine major histocompatibility complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. *Animal genetics,* 29**,** 185-193.
SILLEY, P. 2012. Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? *Revue Scientifique et Technique-OIE,* 31**,** 33.
SMITH, D. R. 2019. Herd Immunity. *Vet Clin North Am Food Anim Pract,* 35**,** 593-604.
SOLTAU, J. B., EINAX, E., KLENGEL, K., KATHOLM, J., FAILING, K., WEHREND, A. & DONAT, K. 2017. Within-herd prevalence thresholds for herd-level detection of mastitis pathogens using multiplex real-time PCR in bulk tank milk samples. *Journal of dairy science,* 100**,** 8287-8295.
SORDILLO, L. M. 2018. Mammary Gland Immunobiology and Resistance to Mastitis. *Vet Clin North Am Food Anim Pract,* 34**,** 507-523.
SØRENSEN, L. P., MARK, T., MADSEN, P. & LUND, M. S. 2009. Genetic correlations between pathogen-specific mastitis and somatic cell count in Danish Holsteins. *Journal of Dairy Science,* 92**,** 3457-3471.
SOUZA, F. N., BLAGITZ, M. G., BATISTA, C. F., TAKANO, P. V., GARGANO, R. G., DINIZ, S. A., SILVA, M. X., FERRONATTO, J. A., SANTOS, K. R., HEINEMANN, M. B., DE VLIEGHER, S. & DELLA LIBERA, A. 2020. Immune response in nonspecific mastitis: What can it tell us? *Journal of dairy science*.
SPITTEL, S. & HOEDEMAKER, M. 2012. Mastitis diagnosis in dairy cows using PathoProof real-time polymerase chain reaction assay in comparison with conventional bacterial culture in a Northern German field study. *Berliner und Munchener tierarztliche Wochenschrift,* 125**,** 494-502.
SRINIVASAN, A., DICK, J. D. & PERL, T. M. 2002. Vancomycin Resistance in Staphylococci. *Clinical Microbiology Reviews,* 15**,** 430.
SRINIVASAN, V., GILLESPIE, B. E., LEWIS, M. J., NGUYEN, L. T., HEADRICK, S. I., SCHUKKEN, Y. H. & OLIVER, S. P. 2007. Phenotypic and genotypic antimicrobial resistance patterns of Escherichia coli isolated from dairy cows with mastitis. *Veterinary microbiology,* 124**,** 319-328.
STEVENS, M. G., DE SPIEGELEER, B., PEELMAN, L., BOULOUGOURIS, X. J., CAPUCO, A. V. & BURVENICH, C. 2012. Compromised neutrophil function and bovine E. coli mastitis: is C5a the missing link? *Veterinary Immunology and Immunopathology,* 149**,** 151-156.
SUKHNANAND, S., DOGAN, B., AYODELE, M. O., ZADOKS, R. N., CRAVER, M. P. J., DUMAS, N. B., SCHUKKEN, Y. H., BOOR, K. J. & WIEDMANN, M. 2005. Molecular subtyping and characterization of bovine and human Streptococcus agalactiae isolates. *Journal of clinical microbiology,* 43**,** 1177-1186.
SUMON, S., PARVIN, M., EHSAN, M. & ISLAM, M. Relationship between somatic cell counts and subclinical mastitis in lactating dairy cows, Veterinary World, 13 (8): 1709-1713. 2020. Abstract.
SUNDARESHAN, S., ISLOOR, S., BABU, Y., SUNAGAR, R., SHEELA, P., TIWARI, J., WYRYAH, C., MUKKUR, T. & HEGDE, N. 2017. Isolation and molecular

identification of rare coagulase-negative Staphylococcus Aureus Variants Isolated from Bovine milk samples. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases,* 38**,** 66-73.

SWETHA, C. S., SUPRIYA, R. A., GOUD, S. S., BABU, A. J. & RAO, T. M. 2017. A study on the prevalence of zoonotic important methicillin resistant and vancomycin resistant Staphylococcus aureus (MRSA & VRSA) and coagulase negative Staphylococci (MR-CNS & VR-CNS) in raw milk samples of Tirupati, Andhra Pradesh. *The Pharma Innovation,* 6**,** 17.

SYRING, C., BOSS, R., REIST, M., BODMER, M., HUMMERJOHANN, J., GEHRIG, P. & GRABER, H. U. 2012. Bovine mastitis: The diagnostic properties of a PCR-based assay to monitor the Staphylococcus aureus genotype B status of a herd, using bulk tank milk. *Journal of dairy science,* 95**,** 3674-3682.

SZTEYN, J., WISZNIEWSKA-ŁASZCZYCH, A., WOJTACKA, J., WYSOK, B. & LIEDKE, K. 2020. Occurrence and differentiation of Mycobacterium avium ssp. paratuberculosis (MAP) strains from milk of cows from herd with low prevalence of MAP. *Journal of Dairy Science,* 103**,** 8526-8529.

TALBOT, B. G. & LACASSE, P. 2005. Progress in the development of mastitis vaccines. *Livestock production science,* 98**,** 101-113.

TAPONEN, S. & PYÖRÄLÄ, S. 2009. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from Staphylococcus aureus? *Veterinary Microbiology,* 134**,** 29-36.

TAPONEN, S., SALMIKIVI, L., SIMOJOKI, H., KOSKINEN, M. T. & PYÖRÄLÄ, S. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *Journal of dairy science,* 92**,** 2610-7.

TAPONEN, S., SIMOJOKI, H., HAVERI, M., LARSEN, H. D. & PYÖRÄLÄ, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary microbiology,* 115**,** 199-207.

TÉLLEZ-CASTILLO, C. J., ARMENGOL-PORTA, M., BANDT, D., COLEMAN, D. C., GAVIER-WIDEN, D., HOTZEL, H., KINNEVEY, P., LAZARIS, A., MATTSSON, R. & PETERS, M. Performance of CHROMagar MRSA II in identification and recovery of newly emerging methicillin-resistant Staphylococcus aureus carrying mecC gene.

TIGNON, M., DE BAERE, M., HANON, J. B., GOOLAERTS, A., HOUTAIN, J. Y., DELOOZ, L. & CAY, A. B. 2017. Characterization of three commercial ELISA kits for detection of BOHV-1 gE specific antibodies in serum and milk samples and applicability of bulk milk for determination of herd status. *J Virol Methods,* 245**,** 66-72.

TIZARD, I. R. 2017. *Veterinary Immunology-E-Book*, Elsevier Health Sciences.

UHAA, I. J., RIEMANN, H. P., THURMOND, M. C. & FRANTI, C. E. 1990. The use of the enzyme-linked immunosorbent assay (ELISA) in serological diagnosis of Mycoplasma bovis in dairy cattle. *Veterinary Research Communications,* 14**,** 279-285.

URSINI, T., MORO, L., REQUENA-MÉNDEZ, A., BERTOLI, G. & BUONFRATE, D. 2020. A review of outbreaks of cryptosporidiosis due to unpasteurized milk. *Infection*.

VANZINI, V., AGUIRRE, N., VALENTINI, B., DE ECHAIDE, S. T., LUGARESI, C., MARCHESINO, M. & NIELSEN, K. 2001. Comparison of an indirect ELISA

with the Brucella milk ring test for detection of antibodies to Brucella abortus in bulk milk samples. *Veterinary microbiology,* 82**,** 55-60.
VIRGIN, J., VAN SLYKE, T., LOMBARD, J. & ZADOKS, R. 2009. Methicillin-resistant Staphylococcus aureus detection in US bulk tank milk. *Journal of dairy science,* 92**,** 4988-4991.
WAAGE, S. 2000. Influence of new infection with bovine virus diarrhoea virus on udder health in Norwegian dairy cows. *Preventive Veterinary Medicine,* 43**,** 123-135.
WALD, R., BAUMGARTNER, M., URBANTKE, V., STESSL, B. & WITTEK, T. 2017. Diagnostic accuracy of a standardized scheme for identification of Streptococcus uberis in quarter milk samples: A comparison between conventional bacteriological examination, modified Rambach agar medium culturing, and 16S rRNA gene sequencing. *Journal of dairy science,* 100**,** 1459-1466.
WALLER, K. P. 2000. Host resistance to mastitis. *Ruminant Physiology. Digestion, Metabolism, Growth and Reproduction.* CABI Publishing.
WANG, W., ZIJLSTRA, R. T. & GÄNZLE, M. G. 2017. Identification and quantification of virulence factors of enterotoxigenic Escherichia coli by high-resolution melting curve quantitative PCR. *BMC microbiology,* 17**,** 114.
WATTS, J., LOWERY, D., TEEL, J. & ROSSBACH, S. 2000. Identification of Corynebacterium bovis and other coryneforms isolated from bovine mammary glands. *Journal of Dairy Science,* 83**,** 2373-2379.
WATTS, J. L. 1988. Characterization and identification of streptococci isolated from bovine mammary glands. *Journal of dairy science,* 71**,** 1616-1624.
WELLENBERG, G., VAN DER POEL, W. H. & VAN OIRSCHOT, J. 2002. Viral infections and bovine mastitis: a review. *Veterinary microbiology,* 88**,** 27-45.
WERNER, R. 2016. Molecular typing of bacteria for epidemiological surveillance and outbreak investigation / Molekulare Typisierung von Bakterien für die epidemiologische Überwachung und Ausbruchsabklärung. *Die Bodenkultur: Journal of Land Management, Food and Environment,* 67**,** 199-224.
WILLIAMS, R., SCHOLTZ, M. & NESER, F. 2016. Geographical influence of heat stress on milk production of Holstein dairy cattle on pasture in South Africa under current and future climatic conditions. *South African Journal of Animal Science,* 46**,** 441-447.
WU, J., LIU, Y., HU, S. & ZHOU, J. 2008. Development of a rapid PCR test for identification of Streptococcus agalactiae in milk samples collected on filter paper disks. *Asian-Australasian Journal of Animal Sciences,* 21**,** 124-130.
YANG, M., SHI, J., TIAN, J., TAO, J., CHAI, M., WANG, J., XU, Z., SONG, Y., ZHU, K. & JI, P. 2017. Exogenous melatonin reduces somatic cell count of milk in Holstein cows. *Scientific Reports,* 7**,** 43280.
YANG, Y., FAN, W., MAO, Y., YANG, Z., LU, G., ZHANG, R., ZHANG, H., SZETO, C. & WANG, C. 2016. Bovine leukemia virus infection in cattle of China: association with reduced milk production and increased somatic cell score. *Journal of Dairy Science,* 99**,** 3688-3697.
ZADOKS, R. N., MIDDLETON, J. R., MCDOUGALL, S., KATHOLM, J. & SCHUKKEN, Y. H. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. *Journal of mammary gland biology and neoplasia,* 16**,** 357-372.

ZHOU, A. & WENJUAN, W. 2017. Clinical application of high resolution melting curve assay in bacteria identification and antimicrobial susceptibility testing. *Chinese Journal of Laboratory Medicine,* 40**,** 84-87.

ZIBA, M. W., CHITALA, C., SETTYPALLI, T. B. K., MUMBA, M., CATTOLI, G., FANDAMU, P. & LAMIEN, C. E. 2020. First detection and molecular characterisation of pseudocowpox virus in a cattle herd in Zambia. *Virology Journal,* 17**,** 1-8.

APPENDIX 1

Appendix 1: Commercially available immune diagnostic test kits and quotation: