Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2019:77
Faculty of Veterinary Medicine and Animal Science

Mastitis in dairy cows in Rwanda: Prevalence, aetiology, antimicrobial resistance, molecular epidemiology and effects on milk quality

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2019

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Cover: Ankole cow in Rwanda
(photo: Y. Persson)

ISSN 1652-6880
ISBN (print version) 978-91-7760-472-3
ISBN (electronic version) 978-91-7760- 473-0

Print: SLU Service/Repro, Uppsala 2019

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Abstract

The milk sector in Rwanda can be made competitive through improved udder health resulting in higher milk yields. This thesis investigated prevalence and aetiology of subclinical mastitis (SCM) in dairy cows, antimicrobial resistance and molecular epidemiology of udder pathogens. Screening for SCM with California Mastitis Test (CMT) was done in 828 cows in 429 herds from five regions in Rwanda. Milk was sampled from udder quarters with CMT score ≥3. Herd bulk milk quality and safety was investigated to generate knowledge for quality control. Overall SCM prevalence was 70.4% on herd level, 66.3% on cow level and 39% on quarter level. Overall 73.9% of all cultured milk samples were bacteriologically positive. Non-aureus staphylococci (NAS) followed by *Staphylococcus* (*S.*) *aureus* were the predominant pathogens. *Staphylococcus chromogenes*, *epidermidis* and *sciuri* were the most prevalent NAS. There was a high diversity of *S. aureus* sequence types, with both humans and cows as possible sources. Penicillin resistance exceeded 60% in all staphylococci. Among *S. aureus* isolates, 83.3% were resistant to penicillin, 100% to clindamycin and 20% to tetracycline. Main risk factors for SCM with implications on management routines included housing of cows in individual cattle kraal and on earthen floor, poor hygiene (hands, cows and milking area), absence of foremilk stripping, increasing stage of lactation, Holstein breed, lack of calf suckling and of feeding after milking. Total bacterial count and somatic cell count was high in milk from farms and milk collection centers, which indicate poor udder health and hygiene and contamination along the transport chain. Presence of *Escherichia coli*, *Salmonella* spp. and brucella antibodies in milk was common. Antimicrobial residues in milk was uncommon. In conclusion, SCM is common in dairy herds in Rwanda and the majority of causative pathogens exhibited penicillin resistance. The high microbial load has implications for milk quality, processability and public health. The high genetic diversity of *S. aureus* should be considered in future studies of disease spread. A mastitis control plan is recommended.

Keywords: Antimicrobial susceptibility, MALDI-TOF, intramammary infection, whole genome sequencing

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Mastit hos mjölkkor i Rwanda: Prevalens, etiologi, antimikrobiell resistens, molekylär epidemiologi och effekt på mjölkkvalitet

Sammanfattning

Mjölksektorn i Rwanda kan göras mer konkurrenskraftig genom förbättrad juverhälsa som ger högre mjölkavkastning. Denna avhandling undersökte prevalensen och etiologin för subklinisk mastit hos mjölkkor, antimikrobiell resistens och molekylär epidemiologi hos isolerade juverpatogener samt effekt på mjölkkvaliteten. Totalt 828 mjölkkor undersöktes med california mastitis test (CMT) i 429 besättningar lokaliserade i Rwandas fem olika regioner. Mjölkprov togs från juverfjärdedelar med CMT ≥3. Tankmjölksprover togs för studier av hygienisk mjölkkvalitet, för att få kunskapsunderlag till kvalitetskontroll. Prevalensen av subklinisk mastit var 70,4 % på besättningsnivå, 66,3 % på ko- och 39 % på juverfjärdedelsnivå. Totalt 74 % av mjölkproverna var bakteriologiskt positiva. Koagulasnegativa bakterier (KNS) och *Staphylococcus (S.) aureus* var dominerande patogener. *Staphylococcus chromogenes*, *epidermidis* och *sciuri* var mest prevalenta bland KNS. Det var hög genetisk diversitet hos *S. aureus*-sekvenstyperna, vilket indikerar både bovin och human källa. Penicillinresistensen var över 60 % hos alla stafylokocker. Bland *S. aureus* var 83.3 % resistenta mot penicillin, 100% mot klindamycin och 20% mot tetracyklin. Skötselrelaterade riskfaktorer var signifikant associerade med höga odds för subklinisk mastit: inhysning i individuell kraal (inhägnad) och på jordgolv, dålig hygien (mjölkarens händer, kons juver och bakben och mjölkningsplatsen), avsaknad av förmjölkning, sent laktationsstadium, kor av holsteinras, avsaknad av diande kalv och ingen utfodring direkt efter mjölkning. Det var högt totalantal bakterier och somatiskt celltal i mjölkprover från besättningar och mjölksamlingsställen, vilket ger ytterligare indikation på dålig juverhälsa och hygien och möjlig bakteriell kontamination längs transportkedjan. Förekomst av *Escherichia coli*, *Salmonella* spp. och brucellaantikroppar var vanligt i mjölken. Det var däremot ovanligt med antimikrobiella restsubstanser. Sammanfattningsvis visar avhandlingen att subklinisk mastit är vanligt förekommande i mjölkbesättningar i Rwanda, och att majoriteten av juverpatogenerna är av smittsam typ med hög penicillinresistens. Den mikrobiella belastningen på mjölken är hög, med betydelse för mjölkkvalitet, förädling och folkhälsa. Molekylär karakterisering av *S. aureus* påvisar hög diversitet, vilket bör tas i beaktande i framtida studier av smittspridning. Ett kontrollprogram mot mastit rekommenderas.

Nyckelord: Antimikrobiell känslighet, maldi-tof, juverinfektion, juverinflammation, helgenomsekvensering

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Dedication

To my family, colleagues at work and friends

Patience, persistence, and perspiration make an unbeatable combination for success.

Napoleon Hill

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ndahetuye*, J. B., Persson, Y., Nyman, A.-K., Tukei, M., Ongol, M. P., & Båge, R (2019). Aetiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. *Tropical Animal Health and Production*, 51, 2019, pp. 2037–2044.

II Ndahetuye*, J. B., Twambazimana, J., Karege, C., Persson, Y., Nyman, A.-K., Tukei, M., Ongol, M. P., Båge, R. A cross sectional study of prevalence and risk factors associated with subclinical mastitis and intramammary infections, in dairy herds linked to milk collection centers in Rwanda (accepted for publication in *Preventive Veterinary Medicine*)

III Ndahetuye, J.B., Ingabire, A., Nyman, A.-K., Karege C., Artursson K., Ongol, M.,P.,Tukei, M., Båge, R., Persson, Y. Microbiological quality and safety of milk from farm to milk collection centers in Rwanda. (manuscript)

IV Ndahetuye, J.B., Leijon, M., Artursson K., Båge, R., Persson, Y. Genetic characterization of *Staphylococcus aureus* from subclinical mastitis cases in dairy cows in Rwanda (manuscript)

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*Corresponding author.

The contribution of Jean Baptiste Ndahetuye to the papers included in this thesis was as follows

I. Contributed to the planning the experiment, conducted the fieldwork to screen cows for subclinical mastitis, milk sample collection and analyses. Performed the statistical analyses under supervision and wrote manuscript with regular input from co-authors.

II. Contributed to the planning of the experiment, responsible for recruitment of herds, participated in screening cows for subclinical mastitis, milk sample collection and analyses with research team. Performed the statistical analyses under supervision and wrote manuscript with regular input from co-authors.

III. Contributed to planning the study, was responsible for sample collection and analyses with the research team, responsible for statistical analysis under supervision with one of the co-authors and wrote the manuscript with regular input from co-authors.

IV. Participated in design of research project, responsible for isolates and DNA preparations; responsible for writing the manuscript with regular input from the co-authors.

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Abbreviations

AMR	Antimicrobial resistance
BMSCC	Bulk milk somatic cell count
CFU	Colony forming unit
cg-MLST	Core genome multilocus sequence typing
CM	Clinical mastitis
CMT	California mastitis test
DCC	DeLaval cell counter
EAC	East African Community
FAO	Food and Agriculture Organization
IFAD	International Fund for Agriculture Development
IMI	Intramammary infection
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCC	Milk collection centre
MIC	Minimum inhibitory concentration
MINAGRI	Ministry of Agriculture
MRSA	Methicillin resistant staphylococci
NAS	Non-aureus staphylococci
NMC	National Mastitis Council
PCR	Polymerase chain reaction
PVL	Panton valentine leucocidin
SCC	Somatic cell count
SCM	Subclinical mastitis
SNV	Netherlands Development Organisation
SOP	Standard operating procedure
SVL	Single locus variant
TBC	Total bacterial count

1 Introduction

Dairy cows have a great cultural and economic importance in Rwanda. The cattle population and milk production have increased recently, partly due to national programs such as Girinka Munyarwanda family (One cow per poor family) that distribute dairy cows to Rwandans in order to enhance nutritional status and food security (Ezeanya, 2014). Challenges in achieving the vision of quality milk production include the lack of updated research on prevention practices against major dairy cow pathogens, those causing mastitis being at forefront. The Rwandan Ministry of Agriculture has put appropriate research as the number one priority, aimed at disease control for increasing dairy production and productivity, mastitis being a disease at the forefront. Mastitis not only decreases milk yield but also causes high veterinary costs, increases culling rates, affects milk quality, causes animal suffering or impaired welfare and causes occasional animal fatalities. Moreover, mastitis leads to an increased use of antibiotics, which could, in case of imprudent use, lead to problems with antimicrobial resistance (AMR). Mastitis continues to be a costly problem in dairy cattle, not only in Rwanda but worldwide. The causative agents of bovine mastitis vary greatly among countries, regions and farms, and also between types of mastitis because of different management systems and local conditions.

Knowledge about the causative agents and risk factors is necessary in order to choose, dictate and recommend proper treatment regimens and preventive health care. Genotyping species of the causative pathogens is an important tool in understanding bovine mastitis dynamics. In many developing countries, milk is produced daily in small to large quantities and transported to cooling centers, sometimes using unrefrigerated equipment, raising concerns about bacterial proliferation in milk. Moreover, milk is at risk of contamination or infection with zoonotic pathogens, contaminants and chemical residuals when best practices in hygiene, withholding period for antibiotics etc. are not consistently followed. Furthermore, inconsistence monitoring of these contaminants through regular milk quality checks makes it difficult to discover and solve the problems early

enough. Such problems may be worse in informal dairy markets. Therefore, scientific studies on milk quality and safety aspects in developing countries are warranted. This thesis investigates prevalence, aetiology, molecular epidemiology of the most prevalent subclinical mastitis (SCM) pathogens in Rwanda, as well as quality and safety of milk in the milk chain from farm to milk collection centers (MCC) in Rwanda.

2 Background

2.1 Dairy sector in Rwanda

The dairy industry in Rwanda has experienced an important shift in cow breed composition intended for milk production. Since 2007, exotic breeds, mainly Holstein Friesian, have been imported to Rwanda (TechnoServe, 2008). Significant numbers of exotic cows are distributed to farmers through national programs such as "Girinka program" which aims to increase milk production and to assure nutritional and food security (Ezeanya, 2014). The transition from local breeds, such as Ankole, to exotic breeds of cow comes with increased requirements in udder health care as the prevalence of mastitis in the exotic breeds is higher than in local breeds in Rwanda (Iraguha et al., 2015).

Overall, the cattle population in Rwanda stands at 1,349,792, of which 615,631 (45%) are local breeds (mainly Ankole), 439,414 (33%) are dairy crossbreeds, and 294,747 (22%) are dairy improved pure breeds (IFAD, 2016). Daily milk production is estimated to be 2 L for the local Ankole breed, 8.6 L for the crossbreeds of Ankole and Holstein Friesian, and 14 L in purebred Holstein Friesian (Maximillian, 2018). Milk production from these cattle still does not meet the milk demand on the Rwandan market, as demonstrated in Figure 1, where projected milk demand is higher than projected milk supply in the coming 6 years.

Herd size and grazing system differ according to geographic locations. The majority of herds in the eastern province are large and practice open grazing as pasture is abundant. However, there are some herds with a small herd size, and zero grazing is practiced in the periphery of the province, such as in the Rwamagana region. Previously, dairy farmers in the northern and southern province practiced semi-grazing, but currently zero-grazing and smaller herd size (one to two lactating cows) with cows of mixed breeds prevail. In the western provinces the herds are small, with limited land area; semi-grazing

systems dominate except in the Gishwati where dairy farmers practice open range with large herd size and with pure, exotic breeds (TechnoServe, 2008). Herds located in peri-urban Kigali have a larger herd size than the national average, and producers are directly linked to milk consumers or milk processors in the capital city of Kigali (TechnoServe 2008; MINAGRI 2013).

In Rwanda, milk is typically produced by small holders and is transported in un-refrigerated cans by middlemen using bicycle or motorcycle to MCC (Figure 2). Individual large-scale farmers may supply milk directly to MCC. Hand milking is widely practiced. Small holders are characterized by low productivity, insufficient use of modern farm technologies and practices, which have challenges in accessing clean water and adequate training (IFAD, 2016; Doyle et al., 2015). Milk collection centers serve as centralized cooling and storage centers of milk from many producers before forwarding the milk to fresh milk selling kiosks or to factories for processing (Miklyaev, 2017). This system constitutes the formal milk chain in Rwanda. An informal milk chain also exists accounting for 500,000 L of milk per day traded, with direct sales to milk sellers such as restaurants or consumers without passing to the MCC where milk is quality-tested (SNV, undated).

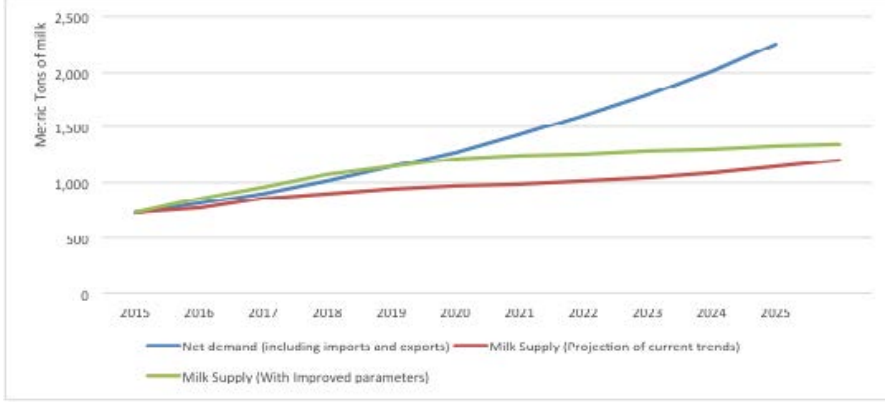

*Figure 1.*Projected milk supply and demand from 2015 to 2025 in Rwanda. (Rwanda Dairy Development Project) Source: MINAGRI livestock

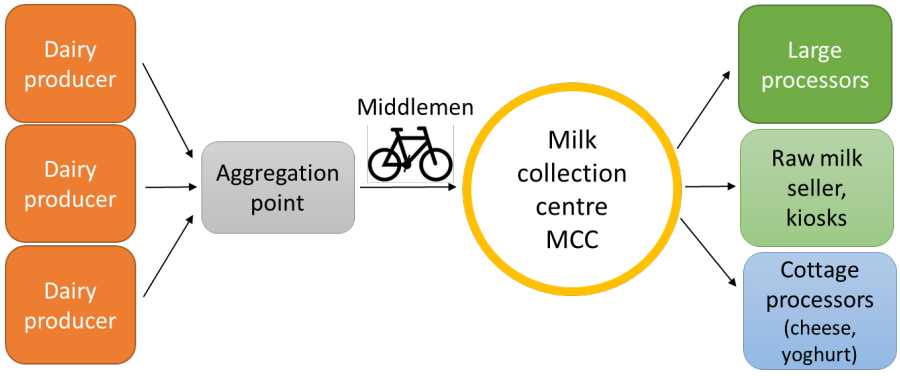

Figure 2. Dairy chain in Rwanda (modified after Miklyaev, 2017).

2.2 Mastitis

Mastitis is an inflammation of the mammary gland in both lactating and non-lactating cows due to an external stimulus, often caused by intramammary infections (IMI) with bacteria (Watts, 1988) or more rarely due to physical or chemical trauma on the udder. Mastitis can be clinical or subclinical based on signs, and acute or chronic based on duration. Clinical mastitis (CM) is characterized by local signs such as abnormal signs on the udder or its secretions, or general signs of disease such as fever, loss of appetite etc. In CM, the signs will include udder swelling, hardness of the affected quarter, pain, watery milk and reduced milk yield, and can occasionally cause fatalities of the cow (Gruet et al., 2001). Clinical mastitis can be further classified according to these signs into mild (changes in milk), moderate (changes in milk and visible signs of inflammation of the udder, or severe (changes in milk and udder, and systemic signs). On the other hand, SCM is a form of mastitis where there is inflammation without visible signs in the cow, udder or milk (Gruet et al., 2001), but there is a change in milk composition, change in pH and ion concentration, increase of somatic cells in milk, and reduced milk yield (Korhonen & Kaartinen, 1995).
Somatic cells such as leukocytes (white blood cells), mainly lymphocytes, macrophages, and polymorphonuclear neutrophils, and also small numbers of epithelial cells are present in milk during inflammation (Marta, 2006). A level of somatic cell count (SCC) of 200 x 10^3 cells/mL or less in composite milk from all quarters is often used in literature to indicate absence of mastitis in cows (Pitkälä et al., 2004; Deluyker et al., 2005), whereas the same cut-off level for a first lactation animal is 100 x 10^3 cells/mL or less (Marta, 2006). Non-infectious factors such as age, stage of lactation, season, stress, management, day-to-day variation, and diurnal variation could lead to fluctuations in SCC (Olde

Riekerink et al., 2017). Somatic cell count is also an indicator of bulk milk quality where different countries set limits required for milk producers to meet. For example, the EU sets an SCC limit in bulk milk of 400 x 10^3 cells/mL, whereas North America and Canada have an upper limit of 750 x 10^3 cells/mL and 500 x 10^3 cells/mL respectively (Schukken et al., 2003). The standard in East Africa is set at 300 x 10^3 cells/mL (EAS 67:2006). As SCC increases, milk yield, lactose and potassium content are reduced whereas sodium, chloride and whey N (protein) increase (Figure 3). This affects the suitability of the milk for processing, and also its organoleptic properties (Marta, 2006).

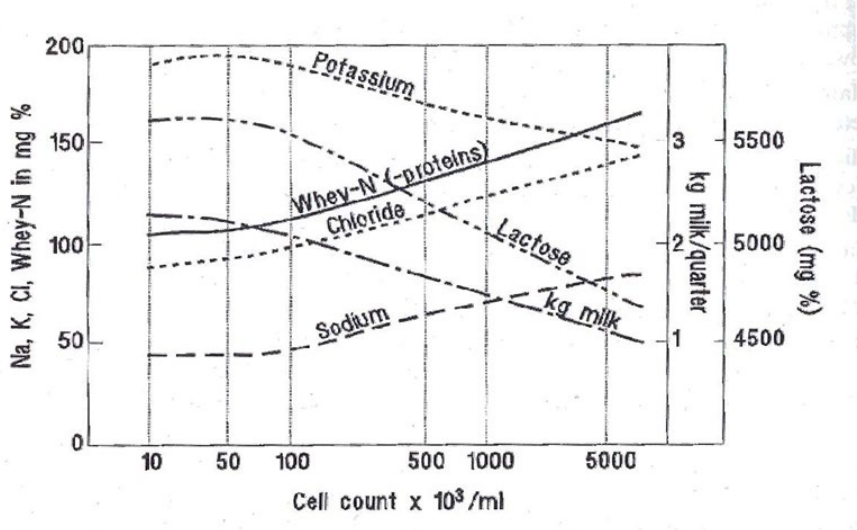

Figure 3. Changes in milk production and different milk components with increasing SCC (Korhonen & Kaartinen, 1995).

2.3 Mastitis microbiology

Several microorganisms cause mastitis infection in the cow's udder. The majority of mastitis cases are caused by bacteria, although fungal and algal infections have been reported (Watts 1988). Based on the source of bacteria, mastitis pathogens can be divided into environmental, which are transmitted from the cow´s environment (bedding, soil, manure etc.) to the teat canal, and contagious mastitis pathogens, which are spread from a cow with infected quarters to a healthy one mainly during milking (Ruegg, 2017). Contagious bacteria such as *Staphylococcus (S.) aureus* and *Streptococcus* (*Str.*) *agalactiae* cause mastitis in regions where mastitis control programs, including improved milking practices, post-milking teat disinfection, therapeutic and prophylactic

antimicrobial administration, as well as culling of persistently infected animals, have not been implemented (Gianneechini et al., 2002; Östensson et al., 2013). However, *S. aureus* can still be the major pathogen in countries where mastitis control programs have been well-implemented, such as in Sweden (Ericsson Unnerstad et al., 2009). Environmental pathogens include coliforms such as *Escherichia (E.) coli* or *Klebsiella (K.)* spp., streptococci (not *Str. agalactiae*), some species in the non-aureus staphylococcus (NAS) group, *Pseudomonas*, *Proteus*, *Serratia* species, Gram-positive bacilli, yeast and *Prototheca*. Their reservoirs include bedding, soil, walkways, and on pasture or any surface with which the cow or her manure comes in contact. The relative importance of environmental or contagious mastitis pathogens varies greatly according to the prevailing management practices of the specific countries or specific regions within the same country.

Some microorganisms are able to cause higher bulk milk SCC than others. Bradley (2002) reported that *S. aureus* and *Str. agalactiae* were isolated in milk samples from higher bulk milk somatic cell count (BMSCC) than, for example, other streptococci, whereas coliforms were isolated mainly from low BMSCC herds. Another characteristic of mastitis pathogens is that they have inherently evolved over time because of management practices, new breeds, changes in mastitis causative agents virulence etc. For examples, NAS which was once classified as minor pathogens, have re-emerged as important pathogens in many countries (Taponen et al., 2006). In addition, *S. aureus* which was the major contagious pathogen in U.K in the 60s has decreased over time, and environmental pathogens such as *E. coli* and *Str. uberis* have emerged as major pathogens in CM cases (Wilson & Kingwill, 1975, Wilesmith et al., 1986, Bradley & Green, 2001). *Staphylococcus aureus* is a particularly important pathogen to control in both CM and SCM, because of the recurrent, chronic type of mastitis it causes and its invasive nature(Oliveira et al., 2006). Its contagious nature means infected milk becomes a reservoir of bacteria which are transmitted to healthier animals in the herd mainly during milking (Capurro et al., 2010). In addition, the pathogen is hard to cure and eradicate in herds because of invasive nature in the udder and ability to persist in the cow´s environment and colonize skin or mucosal epithelia (Rainard et al., 2018). Reservoirs of *S. aureus* include the teat skin, the external orifices, housing, feedstuffs, humans, non-bovine animals, air, equipment, bedding, insects, and water (Roberson et al.,1994).

Staphylococcus aureus is common in mastitis cases in East African countries where implementation of the 10-point mastitis control plan is still lacking (Mekonnen et al. 2017). However, *S. aureus* is still a major cause of mastitis in many developed countries, which have been successfully implementing a mastitis control plan for decades (Tenhagen et al., 2006, Ericsson Unnerstad et

al., 2009). Thusthe need fornewer ways of studying *S. aureus* infection dynamics is highlighted.

Non-aureus staphylococci were previously known as coagulase-negative staphylococci. They were previously considered to be minor pathogens but now they have emerged as major pathogens causing mastitis in many countries (Tenhagen et al., 2006, Piepers 2007, et al., 2019). It is a heterogeneous group consisting of close to 50 staphylococcus species causing IMI, resulting in increased SCC and decrease in milk production and quality (Pyörälä et al., 2008). The same author indicated that *S. simulans* and *S. chromogenes* predominate in this group. Furthermore, the author revealed that multiparous cows generally become infected with NAS during later lactation whereas primiparous cows develop infection before or shortly after calving. Non-aureus staphylococci differ in their susceptibility pattern against antimicrobials with studies indicating, for example, that *S. epidermidis* is more resistant to ampicillin, erythromycin, methicillin and pirlimycin than other tested NAS species (Sawant et al., 2009), signifying that some NAS are more difficult to treat than others.

2.4 Detection and diagnosis of mastitis

Apart from clinical examination of the cow, udder and milk, there are several direct and indirect diagnostic tests available for detecting mastitis and IMI. These are especially important for the diagnosis of SCM where no clinical signs are visible. Measuring SCC is considered to be one of the most reliable udder heath indicators (Nyman et al. 2016).

2.4.1 Diagnosis of inflammation

California mastitis tests (CMT) can be used cow-side to evaluate SCC of quarter milk samples indirectly by estimating the DNA content of cells in the milk. It is based on an anionic detergent, Na-lauryl sulphate, which dissolves cell membranes and nuclei (Sandholm et al., 1995). Direct measurement of SCC could be achieved using equipment such as the portable Delaval Somatic Cell Counter (DCC) or the stationary Fossomatic somatic cell counter. N-acetyl-beta-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) are enzymes that are released during inflammation and their activity is positively correlated with severity of inflammation. Therefore, their measurement represents a diagnostic predictor of inflammation (Sandholm et al., 1995). Electrical conductivity is another method used to diagnose mastitis. This is made possible because ions such as sodium and chloride increase and fat decreases in mastitic

milk. Conductivity is thereby increased, allowing electrical current to flow more easily. The disadvantage of this method is that there is inter-cow variation, and conductivity may vary during milking (Sandholm et al., 1995). The choice of the one diagnostic method over the other depends on the purpose of the tests. It depends, for example, on whether the purpose is to study udder health on quarter basis, to study bulk milk quality of the herd, or for quality payments (Sandholm et al., 1995).

2.4.2 Diagnosis of intramammary infection

Detection of mastitis-causing microorganisms may require conventional culturing of milk samples on growth media, followed by biochemical tests to differentiate which agents are involved. The challenge with relying on culturing and biochemical tests is that they are time-consuming and need specific medium and reagents (Deb et al., 2013).

Recent advances in mastitis detection has led to the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for pathogen identification at species level. The method is more rapid, accurate and cost-effective than conventional biochemical methods and may lead to identification of previously unrecognized microorganisms (Barreiro et al., 2010). However, it does require conventional culturing of the sample as first step to identification. Since MALDI-TOF generates spectra of the microorganisms being analysed and matches them with spectra in a database, the bigger and more comprehensive the database is, the more microorganisms could be identified. The limitation on the use of MALDI-TOF in veterinary medicine is that most databases contain data on bacteria relevant for human medicine (Croxatto et al., 2011).

Polymerase chain reaction (PCR)-based methods are also increasingly gaining interest in identifying mastitis-causing organisms. The advantages of PCR methods are that they can detect lower numbers of organisms in milk samples than conventional culture and it is less time-consuming. The main disadvantage is that it detects both live and dead organisms and is thus unable to differentiate between active and non-active infections (Deb et al., 2013). Furthermore, genotyping mastitis bacteria is one of the methods used to understand variations within species (strains) and strain characteristics for a number of reasons. For example, cure rate of mastitis caused by bacteria such as *S. aureus* is very variable and may depend on prevalent genotypes (Lundberg et al., 2014). In addition, the same authors indicated that some strains may be more common than others. Haveri et al. (2005) indicated that persistence of IMI

depended on bacterial genotypes. The virulence and spread of *S. aureus* is also strain- dependent (Rainard et al., 2018).

2.5 Mastitis and antimicrobial resistance

Mastitis is the foremost disease that leads to the use of antimicrobials on dairy farms (Menéndez González et al. 2010). Imprudent use of antimicrobials can lead to a rise in AMR (Kapoor et al. 2017), and there is a risk of subsequent spread of resistant genes to other microbial populations (Xinglin et al. 2017). Resistance has been reported among mastitis pathogens where, for example, the frequency of resistance of *S. aureus* isolates to penicillin was 47% in Italy (Moronic et al. 2006), 52% in Finland (Pitkälä et al. 2004), and 88% in Tanzania (Suleiman et al. 2018). Björk et al. (2014) reported that 80% of the investigated NAS isolates in Uganda were resistant to penicillin through beta-lactamase production. The resistance mechanisms that *S. aureus* uses against antimicrobials include (i) enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes) (ii) alteration of the target with decreased affinity for the antibiotic (notable examples being penicillin-binding protein 2a of methicillin-resistant *S. aureus* and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains); (iii), trapping of the antibiotic (for vancomycin and possibly daptomycin); and (iv) efflux pumps (fluoroquinolones and tetracycline) (Pantosti et al., 2007). In addition, there are genetic determinants of AMR, including mecA and blaZ (penicillins), aacA-aphD (aminoglycosides), ermA/B/C (macrolides), tetK/M (tetracyclines), vanA (vancomycin), fusB (fusidic acid), ileS (mupirocin) and rpoB (rifampicin) (Jensen and Lyon 2009). High levels of AMR could lead to failure of bacteriological cure of mastitis infections (Barkema et al. 2006). Beside treatment failures, there is a risk that resistant strains may enter the food chain, thereby causing public health problems (White & McDermott, 2001). Microorganisms, such as *S. aureus,* have the ability to become resistant to antibiotics, such as in the case of methicillin resistant *S. aureus* (MRSA) which is currently a problem worldwide in both hospital and community settings (Rajan et al., 2015).

2.6 Epidemiology of mastitis

Minimizing both mastitis and IMI in certain regions requires information on animal and herd risks of these infections in these specific regions. Studies on risk factors associated with mastitis or IMI in Rwanda are limited. However, Iraguha et al. (2015) showed that an increase in teat-end damage, cow dirtiness,

and level of pure dairy breed genetics were associated with SCM. In Ethiopia, Abebe et al. (2016) indicated that cows from farms with larger herd size or farms with no milking order (vs. milking mastitic cows last) were more likely to contract mastitis than other cows. Furthermore, Mekonnen et al. (2016) and Tolosa et al. (2013) showed that an increasing stage of lactation was associated with a higher likelihood of mastitis, indicating continuous exposure of cows to mastitis pathogens throughout lactation. This is contradictory to the established pattern that early lactation is a more susceptible stage to new infections and CM due to periparturient immunosuppression or to infection acquired during the dry period. Chronic SCM is however common also in later lactation. In the dry period, absence of dry cow antibiotic therapy and/or teat sealants (Bradley and Green, 2001), or poor dry cow management leads to new infections in early lactation. Bihon et al. (2018) indicated that adult and older cows are more likely to contract mastitis than younger cows. The same author also indicated that cows in an intensive farming system were more likely to contract mastitis than cows kept in a semi-intensive farming system. A meta-analysis study, Getaneh and Gebremedhin (2017) indicated that cows with higher parity (above three) had significantly higher prevalence of mastitis than cows of lower parity (1-2). Furthermore, the same authors found factors including previous history of mastitis, floor type, milking hygiene, and udder injury had a significant effect on pooled prevalence of mastitis ($P < 0.05$).

Studies on risk factors associated with IMI are rare in Africa. However, Mekonnen et al. (2017) indicated that *S. aureus* was more often found in cows with a history of CM, and in larger herds. These authors reported that checking the udder for mastitis, feeding cows according to their requirements and allowing calves to suckle the cows, were negatively associated with SCM, culturing any bacteria and culturing CNS, respectively. In another study, Tolosa et al. (2015) concluded that quarters of cows in herds practicing bucket-fed calf-feeding (as opposed to suckling) had higher odds of IMI caused by *S. aureus*. The same authors indicated that the IMI caused by NAS was associated with absence of teat-drying before milking, increasing stage of lactation, right quarters (as opposed to a left quarter position) and quarters showing teat injury.

2.7 Mastitis prevention and control

It should be noted that mastitis cannot be completely to 100% eradicated. Therefore, targets are set for benchmarking producers: for example Bradley et al. (2012) indicated that producers should aim to achieve the following targets: lactational new IMI rate of less than 5%, the proportion of herds/cows in herds with SCC > 200 x 10^3 cells/ml should be less than 15%, fresh calver IMI rate

should be less than 10%, dry period new IMI rate should be less than 10%, dry period cure rate should be above 85%, incidence rate of CM (100 cow/year) should be less than 25%.

The prevention of mastitis relies on two principles: the first one is to identify and protect healthy cows, the second is to identify and minimize prevalence risk factors.

The five-point mastitis control plan is the foundation of controlling both SCM and CM. It was devised in developing countries in the 1960s and has evolved into the ten-point mastitis control plan. The pillars of the five-point plan are early detection and treatment of CM cases, application of dry cow therapy at the end of lactation, post- milking teat disinfection, culling chronically affected cows, and proper maintenance of milking machines (Bradley, 2002). This plan has helped to decrease the incidence of mastitis-causing contagious pathogens such as *S. aureus* and *Str. agalactiae*, the incidence of CM and SCM, and led to a reduction of BMSCC. Prevention of SCM relies on dry cow therapy, milking high-risk cows last, and culling chronic cases (Nyman, 2007). Other prevention measures include milking-time management, and reducing the reservoir of infection in the herd, use of milking gloves, as well as rigorous biosecurity protocols to prevent introduction of novel strains of contagious mastitis pathogens (Keefe, 2012). Mastitis vaccination is not yet providing a lasting solution for mastitis control. Available vaccines, such as *E. coli* J5 core antigen, are able to reduce the incidence and severity of clinical infections but are not able to prevent new coliform IMI (Hill, 1991; Hogan et al., 1992a; 1992b; Bradley, 2002). Similarly, Startvac (Hipra, Spain), a polyvalent vaccine, is able to decrease the prevalence of *S. aureus* and NAS significantly and increase milk yield. However, in some other studies, Startvac did not have any effect on udder health parameters (Ismail, 2017). In addition, a herd-specific auto-vaccine (Best Vac) is able to reduce the prevalence of *S. aureus* as much as Startvac. However, Ismail, (2017) concluded that vaccines alone will not be able to control mastitis effectively and economically, without other best practices such as hygiene, proper treatment of clinical cases etc. especially in herds where incidences are high . Although it is impossible to eradicate mastitis, it is possible to minimize its incidence using economic management routines (FAO, 1989).

2.8 Economic consequences of mastitis

The eventual consequences of mastitis are not only effects on animal health and welfare but also a significant economic loss for dairy farmers and processors. Several studies have associated an increase of SCC with a corresponding decrease in milk yield (Hagnestam-Nielsen et al., 2009) and others further

determined the monetary loss associated with mastitis (Van Soest et al., 2015). The economic loss depends on many factors including the type of mastitis-causing pathogen, (Cha et al., 2011; Sørensen et al., 2010), the parity and stage of lactation of the cow (Archer et al., 2013; Hortet et al., 1999; Huijps et al., 2009), and the breed (Heikkilä et al., 2012). Furthermore, processors have an economic burden since milk from cows that have mastitis is associated with lower product quality, more complex processing requirements, lower cheese and casein yield, shorter shelf life and flavour problems as significant factors (Hogeveen et al., 2010; Malcolm et al., 2005). Although it is difficult to harmonize methods used in evaluating losses due to both forms of mastitis, the total cost per cow per year was estimated to be 338 € in Sweden in 2010 (Nielsen et al. 2010), 240 € in the Netherlands between 2005 and 2009 (Van Soest et al. 2016), and 117.35 USD in the USA in 1979 (Blosser,1979). Economic estimations of losses associated with mastitis in Ethiopia indicate that quarters with SCM due to *S. aureus* lost an average of 34.5% of their potential milk production, and losses per cow were estimated to be 6.8% and the corresponding monetary loss per cow per lactation was estimated to be 78.6 USD (Tesfaye et al., 2010). Another study in the same country indicated that failure cost of having mastitis for a small-holder was 213.9 USD per farm per year, with SCM accounting for 54% of those costs (Mekonnen et al., 2019). Economic estimation of losses associated with mastitis in other African countries are rare and not well documented (Motaung et al. 2017). Thus, the overall motivation for its control is low.

2.9 Milk quality and safety

Apart from mastitis-causing pathogens, raw milk from dairy cows may be contaminated by microorganisms originating from the environment. These environmental organisms could be transferred to the milk through poor hygiene of udder and teat surfaces and from uncleaned and unsanitized milking equipment (Elmoslemany et al., 2008), but also from personnel performing milking or handling the milk. Improper cooling of milk during transport can also influence bacterial count by increasing the rate of bacterial growth before the milk reaches the MCCs or processors. The total bacterial count (TBC) test is used to evaluate to which extent such processes have affected milk quality. However, Murphy and Boor (2000) indicated that the tests should be interpreted with caution since different types of bacteria could contaminate the milk from various sources, such as equipment, milk handlers and different environmental niches. These microorganisms proliferate in milk because it contains key nutrients, high water activity and ideal pH for their growth and development

(Hassan & Frank, 2011). There are numerous groups of bacteria that can grow in milk. *Escherichia coli* is particularly used as an indicator organism for fecal contamination of foodstuff. Presence of these bacteria is therefore an indicator of the degree of hygiene and it can be associated with foodborne disease outbreaks (Tryland and Fiksdal, 1998).

Milk is produced daily in large volumes and has to reach the consumer quickly – compared to many other food products. Therefore, it involves frequent contact with human beings (hand milking personnel, milk transporters and sellers), further increasing the risk of zoonotic pathogens contaminating the milk. For examples, *Salmonella* (also referred to as non-typhoidal *Salmonella enterica*) can contaminate raw milk and milk products through infected persons and contamination of the environment (Mhone et al., 2012). Cattle can also serve as reservoir for *Salmonella* spp., which are transmitted to human beings through the fecal-oral route by eating contaminated foods (Grimont and Weill, 2007). The consequences of contamination of milk with *Salmonella* spp. are foodborne illnesses in people consuming the milk (Grimont and Weill, 2007). Kamana et al. (2014) reported a *Salmonella* spp. prevalence of 5.2% in raw milk samples from dairy farms, MCC and from milk shops in Rwanda.

Brucellosis is another disease that can potentially be transmitted to human beings through milk, it is a globally widespread zoonotic disease caused by bacteria of the genus *Brucella (B)*. There are ten species in the genus, of which three are considered major zoonoses; *B. melitensis* is the most pathogenic species, whereas *B. suis* and *B. abortus* cause milder symptoms in humans (Galińska and Zagórski, 2013). *Brucella abortus* causes abortion in cattle and can be transmitted to humans by bodily fluids, including milk, or by contaminated food products (Rock et al., 2016). There are different forms of brucellosis in humans: acute infection is characterized by fever, headache, gastrointestinal symptoms and joint pain, while the subacute and chronic forms usually are present with milder and unspecific symptoms. Brucellosis can also cause stillbirth or abortion in pregnant women (Rujeni and Mbanzamihigo, 2014). Rock et al., (2016) reported brucella antibodies in milk in Uganda at a level of 11% and 40% in Gulu and Soroti, respectively. Therefore, consumption of unpasteurized milk represents a risk of transmission of these pathogens to human beings.

Antimicrobial residues may arise in milk when dairy cows are treated with antimicrobials without conforming to the withdrawal period. Antimicrobial use in human and veterinary medicine has saved numerous lives through treatment and control of infection. However, imprudent use of antimicrobials may lead to AMR (van Den Bogaard and Stobberingh, 2000). Consequences of antimicrobial residues in milk include the risk of causing allergic reactions in human beings,

increased development of AMR (van Den Bogaard and Stobberingh, 2000), and can impact the suitability of milk for processing of product such as cheese and yogurt because antimicrobials impair the growth of starter cultures (Brady and Katz, 1988). Together, total bacterial contamination, presence of zoonotic pathogens or antimicrobials in milk cause a decrease in quality, thus having consequences for human health, nutrition and food security.

3 Research justification

Stunting among children in Rwanda stands at high rate (36.7%; WFP, 2015), milk as animal source food is anticipated to play a key role in alleviating such stunting levels. Dairy development is constrained by the fact that farm size is small due to high population density in Rwanda. Despite of this, efforts to increase milk production are made through several strategies, one of them being importation of dairy exotic breeds since 2006 (TechnoServe, 2008). These breeds may be more susceptible to mastitis than local breeds under local conditions (Iraguha et al., 2015). These highly improved and sensitive dairy breeds are at high risk for any disease, including mastitis, when they are not optimally fed and managed according to their needs, and are kept in a climate that is suboptimal for them (due to heat stress for example). Furthermore, there may be suboptimal milking routines, lack of many biosecurity measurements, both within and between herds, that make cows of improved breeds more susceptible to contracting mastitis more often than cows of local breeds. Zero grazing systems are becoming common in different regions in Rwanda (IFAD, 2016), which has implications for infection pressure around the cows, especially when manure is not removed regularly. Historically, mastitis has not been comprehensively studied in Rwanda. In the light of these recent developments in the dairy sector, determination of the prevalence and identification of risks factors associated with mastitis and udder pathogens is important for prevention of transmission of pathogens to healthy cows. Therefore, it is important to generate knowledge of prevailing risk factors and adjust herd management accordingly in Rwanda.

Furthermore, more knowledge on the causative organisms at species and strain level is needed for an accurate understanding of the dynamics and possible effects of prevalent pathogens on udder health and milk production. With more knowledge about infection dynamics, such as spread and transmission, appropriate preventive measures can be developed. To the best of our

knowledge, no such studies have been done to evaluate IMI dynamics at strain level in Rwanda.

Accurate identification of prevalent udder pathogens will pave the way not only to determine the level of AMR among mastitis pathogens for monitoring purposes, but also to guide mastitis prevention and treatment strategies and to detect emerging AMR. There is a risk that such high levels of resistant mastitis pathogens in Africa could persist and be transmitted in a contagious manner among cows or transfer resistant genes in a bacterial population. Therefore, it is important to monitor the level of AMR and develop control measures to reduce the prevalence of resistant pathogens in Rwanda and other countries in the region.

Milk is produced daily in large volumes and must reach the consumer quickly, in comparison to many other food products. Therefore, it involves frequent contact between milk and human beings (hand milking personnel, milk transporters). It is important to generate knowledge on the risk of possible zoonotic and hygienic indicator microorganisms that may contaminate milk, in the milk production chain from farms to MCC in Rwanda. In this way, these microorganisms can be controlled successfully, thus safeguarding the public health of milk consumers.

4 Aims of the thesis

The general aim of the current study was to generate knowledge on prevalence and risk factors of SCM in Rwanda. In addition, the study aimed to characterize the mastitis causing pathogens involved, to evaluate their AMR, and to study molecular epidemiology of the most prevalent udder pathogens.

More specifically the aims were to:

- Evaluate prevalence and determine the aetiology of SCM in Rwanda
- Genotype the most prevalent causative udder pathogen, in order to gain understanding about its' characteristics, distribution and transmission
- Study AMR in the most prevalent SCM causative udder pathogens
- Determine SCM associated risk factors on herd and cow level, with special focus on the most prevalent pathogens
- Determine important milk quality attributes including TBC, SCC, *E.coli*, *Salmonella* spp. and brucella antibodies, as well as antimicrobial residues in the milk chain from farm to MCC. In addition, to determine associated risk factors

5 Materials and methods

This thesis is built upon four distinct studies, resulting in four scientific papers. For a more detailed description of the methods used, see Papers I-IV.

5.1 General aspects of design of the four studies

Cows screened for SCM and milk sampled in this thesis came from two distinct production systems and from the five geographically representative regions in Rwanda. In Study I, the recruited cows were from herds that are considered large on the national scale, from the peri-urban area of Kigali, Rwanda. In total, 256 lactating cows from 25 herds kept in the Kigali peri-urban areas were examined. Herds were visited once between May and September 2016. In Study II, animals were recruited from small scale holders linked to eight MCCs from the four main provinces in Rwanda. The provinces cover possible differences in agro-ecology conditions and milk production systems, commonly known as milk sheds, in Rwanda (TechnoServe, 2008, IFAD, 2016). These eight MCCs were located in the following sites (Figure 4):

- MCC 1 and 2 in Rwamagana and Nyagatare, in the eastern province
- MCC 3 and 4 in Nyankenke and Rubaya, in Gicumbi in the northern province
- MCC 5 and 6 in Mudende and Rubengera, in the western province
- MCC 7 and 8 in Rugobagoba and Muyira, in the southern province

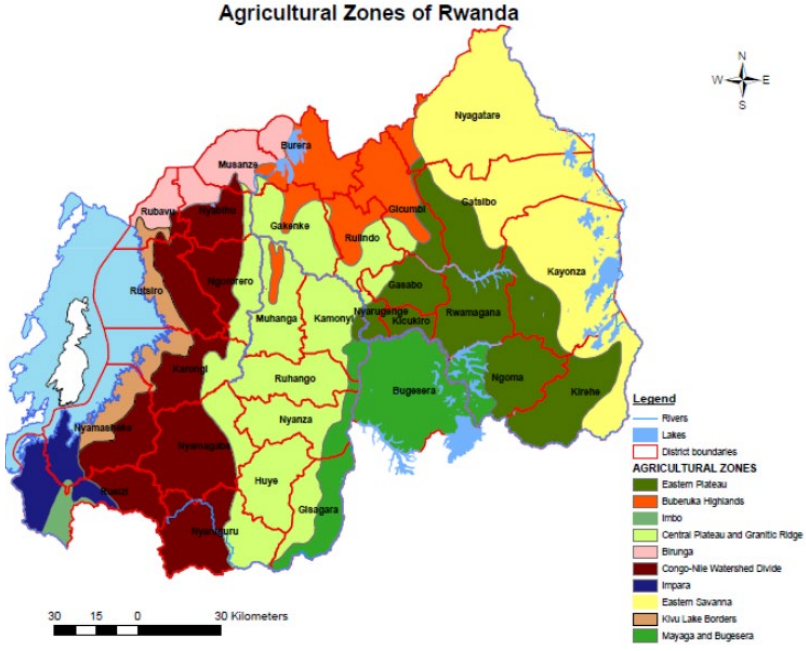

Figure 4. Agricultural zones of Rwanda (Delepierre, G. 1975).

Information on herd management practices was collected through a questionnaire, to be able to analyze risk factors associated with SCM or IMI. In total, 572 cows from 404 dairy herds were included in the study and the herds were visited once from April to September 2017.

In study III, bulk milk samples were collected from herds recruited in study II and from the respective MCC for analysis of milk quality attributes including TBC, SCC, *E. coli*, *Salmonella* spp. and brucella antibodies, as well as antimicrobial residues in the milk chain from farm to MCC. Information on herd management practices was collected through the same questionnaire as in study II, to be able to analyze factors associated with high or low TBC, SCC and *Salmonella* spp. presence in milk.

Study IV was built on study I and II, where the most dominant bacteria (*S. aureus*) isolated in SCM cases from herds in peri-urban area of Kigali and herds linked to MCCs in all provinces, underwent whole genome sequencing to gain understanding on bacterial transmission and AMR.

5.2 Detailed materials and methods

5.2.1 Screening for mastitis and milk sampling from dairy cows

Udder quarter milk samples were collected during ongoing milking by selected, trained personnel. For each udder half, the first two or three strips of milk were inspected for milk abnormality and discarded, followed by CMT testing. Subclinical mastitis prevalence was evaluated by CMT using the Scandinavian scoring system (grades 1–5), where 1 indicates a negative result (no gel formation, no indicative colour change), 2 is traceable (possible infection) and 3 or above indicates a positive result with 5 having the most gel formation and deep blue/violet colour change (Schalm et al. 1971; Saloniemi 1995). A cow was defined as positive for SCM if she had at least one positive quarter with CMT ≥ 3, with no signs of illness and/or visible inflammatory signs of the udder, and without visible abnormality in milk. Quarters with CMT ≥ 3 were recorded and sampled for bacteriological analyses according to the National Mastitis Council (NMC, 2017). After cleaning the teat ends with 70% alcohol, an aseptic milk sample was collected in a 10-mL sterile tube and samples were placed and transported on ice inside a cooler box to the microbiology laboratory of the University of Rwanda, College of Agriculture Animal Sciences and Veterinary Medicine, Busogo Campus for culture and identification of SCM causative agents (Study I, II).

5.2.2 Bulk milk somatic cell count measurements

Bulk milk samples were collected from each herd and transported in the same manner to the laboratory for SCC analysis with a DCC (DeLaval International AB, Tumba, Sweden). (Study I, III)

5.2.3 Bacteriological analyses

All milk samples were cultured on blood agar plates (5% bovine blood with 0.5% esculin) and incubated aerobically at 37 °C for 24 to 48 h before final examination. To be classified as a positive bacterial growth, at least one colony forming unit (CFU) was needed for the following major pathogens: *S. aureus*, *Str. uberis*, *Str. agalactiae*, and *Klebsiella* spp., and at least five CFUs for the other genera. Samples were classified as contaminated if two or more bacterial types were isolated from one milk sample and growth of the mentioned major pathogens was not identified. If growth of a major udder pathogen was found in combination with contaminating species and the CMT was high, the sample was

diagnosed as positive for growth of a major pathogen. Positive isolates were initially characterized based on colony morphology; α-, β-, or double hemolysis; and Gram reaction. Gram-positive isolates were further subjected to catalase and coagulase tests. Isolates were preserved in agar tubes and brought to the accredited laboratory at the National Veterinary Institute (SVA: accreditation number 1553 ISO/IEC 17025) in Uppsala, Sweden, for final identification of causative organisms at species level using MALDI-TOF MS. At SVA, each bacterial sample was first re-cultured on horse blood agar, and material from single pure colonies was spotted on a MALDI-plate without pre-treatment. The spots were covered with 1 µL matrix solution consisting of α-cyano-4-hydroxycinnamic acid (HCCA). Subsequently, isolates on MALDI-plate were analysed by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) to identify the species. Mass spectra were compared against 4613 spectra in the MALDI Biotyper database using the MALDI Biotyper 3.0 Real-time Classification (RTC) software (Bruker Daltonics, Bremen, Germany). Identification and classification of udder pathogens were done according to MALDI-TOF MS spectra score, where a score of ≥ 2.0 was considered reliable identification at species level, a score of ≥ 1.7 to < 2.0 was considered reliable identification to genus level, and a score of < 1.7 was considered as no identification.

5.2.4 Antimicrobial resistance testing

All staphylococcal isolates were examined individually for β-lactamase production by the clover leaf method as described by Bryan and Godfrey (1991). For quality control, the strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used. Identified isolates were stored in trypticase soy broth containing 15% glycerol at −80 °C. (Study I, II).

Sixty *S. aureus* isolates were selected and tested for antimicrobial susceptibility by determination of minimum inhibitory concentration (MIC) using a micro-dilution method according to recommendations from the Clinical and Laboratory Standards Institute using VetMIC™ panels (SVA, Uppsala, Sweden). Twelve isolates were selected from each of the five regions. Each isolate was selected randomly from individual herds within each region. Initially material from 3 to 5 fresh colonies of each isolate were suspended in 5 ml cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, MD, USA) and incubated for 3 to 5 hours at 37 °C to reach at least 108 CFU/ml. Subsequently, around 10 µl was further transferred into a broth of cation adjusted Mueller-Hinton broth to obtain a final inoculum density of approximately 5 x 10^5 CFU/ml. Finally, 50 µl of the inoculum from each isolate was dispensed in a

distinct well of VetMIC™ panels. The wells were sealed with transparent tape and panels were incubated for 16-18 hours at 37 °C. As quality control strains, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used. The MIC values were determined and defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate. The MIC distributions were studied, and isolates were reported as resistant or susceptible based on species-specific epidemiological cut-off (ECOFF) values issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org). (Study II)

For detection of antibiotic resistance genes, the Unicycler sequences assemblies were used by utilizing of the Resfinder 3.2 (Zankari et al., 2012) web server (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity and coverage threshold of 90 and 60%, respectively. In addition, the Unicycler assemblies were analysed with the resistance gene identifier service of the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/analyze/rgi) to detect antibiotic resistance genes with the search in 'Perfect' and 'strict' mode only (Jia B et al.,2017) . The results using the two databases were consistent except that aminoglycoside resistance were only found with Resfinder. In parallel, the 25 *S. aureus* isolates were tested for antimicrobial susceptibility by determination of MIC as described above.

5.2.5 Questionnaires

Questionnaires were used to collect cow and herd information on potential risk factors for SCM and the major pathogens, through interviewing herd owners or workers and by observations during the visit. Cow level information included parity (1, 2-3, 4-5, ≥6), if restraint measures were used during milking (yes versus no), average daily milk production per cow (litres), if the dam was suckled by the calf (yes versus no), lactation stage (≤ 3, 4-7, ≥ 8 months), age (≤ 5 versus > 5 years), udder and leg hygiene (clean, moderately dirty, very dirty) and breed. Herd level information included in the questionnaires are presented in Table 1. These factors were included in the questionnaire based on previous studies in Rwanda and in East Africa (Abrahmsén et al. 2014, Mekonnen et al. 2017) and their relevance for practices commonly used in the Rwandan dairy industry. Eight experts of various background assessed the questionnaire for relevance of each question to the mastitis outcomes studied. Trained research team members interviewed farmers in Kinyarwanda language on herd characteristics, management practices, milking routines and hygiene using closed-ended questions. The farmers responded freely without aid of the interviewer.

Table 1. *Herd variables related to subclinical mastitis prevalence in milk sheds in Rwanda included in the questionnaire. One questionnaire was completed for each of 404 farms across four regions in Rwanda. (Study I-III)*

Herd size	One/multiple lactating cow(s)
Type of cattle kraal[a]	Individual/grouped/no kraal
Type of floor of cow housing	Concrete/earthen/raised wood
Grazing type	Zero/semi/free grazing
Separate calving area; Separate milking area	Yes/no
Milking area hygiene	Clean/slightly dirty/very dirty
Cleaning milking area	Once/twice per day, Once/twice/thrice per week, Other
Type of milking	Hand/machine
Technique of milking	Stripping/full hand
Milking frequency	Once/twice
Who milks the cow	Owner/worker/child
Hand wash before milking	Water only/water and soap/no wash
Teat and udder wash before milking; Teat and udder drying; Clean towel for drying;	Yes/no
Pre-milking teat dipping; Post-milking teat dipping	Yes/no
Foremilk stripping; Performing CMT regularly; Milking mastitis cows last; Culling chronically infected cows	Yes/no
Feed cows after milking	Yes/no
Dry cow therapy	Yes/no
Knowledge of clinical/subclinical mastitis	Yes/no
Farm hygiene	Good/poor
Type of bedding materials	Sawdust/grass/none
Wet bedding	Yes/no
Bedding material replacement	Once/twice a week
Availability of veterinary service; Fly control; Data record of past diseases	Yes/no

5.2.6 Total aerobic bacterial count

To determine TBC, 1 ml of raw milk sample was mixed with 9 ml of diluent (sterilized peptone physiological saline solution) and vortexed thoroughly. Subsequently, serial dilutions (10-1 to 10-9) were prepared. From each dilution and starting from the highest dilution, 0.1 ml of test sample were inoculated on

to plate count agar (Titan Biotech Ltd, Rajasthan, India) culture medium plates in duplicate. The sample was spread evenly on the culture medium surface using a sterile spreading glass rod. Lastly, samples were incubated at 37 ºC for 24 hours. At the end of this incubation period, the number of colonies on plates with between 30 and 300 colonies on them was counted. The counted colony forming units were then converted (considering the dilution factor and the plated sample volume) into CFU per ml of raw milk. (Study III)

5.2.7 Escherichia coli

Enumeration of β-glucuronidase-positive *E. coli* in bulk milk samples from farm and MCC level was done according to ISO 16649-1:2001. Direct inoculation of 100 µl of milk sample was done on tryptone bile x-glucuronide TBX medium (bioMérieux, Marcy l'Etoile, France) plate in duplicate. Plates were incubated at 44 ºC for 24 hours. At the end of the incubation period, the number of colonies on plates with colonies between 30 and 300 were counted. (Study III)

5.2.8 *Salmonella* spp.

The ISO 6579:2002-A1 2007 method was followed to detect *Salmonella* spp. in bulk milk from farms and on MCC level. Initially, aseptic peptone water was prepared. Subsequently, 4.1 ml of milk sample was added into 9 ml of peptone water (BiolaZrt, Budapest, Hungary), and the mixture was incubated at 37 °C for 24 hours for pre-enrichment process. Subsequently 0.1 ml of the suspension was added to 10 ml modified semisolid Rappaport-Vassiliadis (Oxoid, Basingstonke, England) and the mixture was incubated at 41.5 °C for 48 h. Suspected *Salmonella* colonies were sub-cultured on xylose lysine deoxycholate (BiolaZrt, Budapest, Hungary).

Final identification of *Salmonella* spp. was done using the Oxoid *Salmonella* Latex Test (Hampshire, UK). (Study III)

5.2.9 Brucella antibody ELISA

I-enzyme-linked immunosorbent assay (ELISA) kits were used to detect antibodies to *B. abortus* and *B. melitensis* (SVANOVIR Brucella-Ab Boehringer Ingelheim, Uppsala, Sweden). The test kit specificity on milk samples is reported by the manufacturer to be 99–100%. Relative test kit sensitivity to the Rose Bengal test is 89.6% and 100% to the complement fixation test (Svanova 2009). All milk samples stored at −20 °C were thawed at room temperature, and I-ELISA was performed according to the manufacturer's protocol for milk samples. On each ELISA plate, positive and negative control sera were included

to ensure the accuracy of the test, and all samples and controls were run in duplicate. Skanlit Software for Thermo Scientific™ Multiskan™ FC (Ratastie, Finland) was used to read the ELISA plates and to calculate sample optical density (OD) values. A percent positivity (PP) value was then calculated as ¼ OD sample, or negative control OD and positive control 100. A milk sample with PP ≥ 10 was considered positive according to the manufacturer's instructions (Study III)

5.2.10 Antibiotic residues

Delvo SP NT kits were used as described by the manufacturer (DSM, Netherlands) to detect antibiotic residues in milk by incubating 100 µl of homogenized milk sample for 2 to 3 hours at 64 ˚C and observing for the colour change of 2/3 part of the test panel to yellow for negative test or positive when completely purple color. The kit sensitivity was penicillin G at 2 ppb (ng/g) and for sulfadiazine at 150 ppb (ng/g). (Study III)

5.2.11 Data analyses

The prevalence of SCM was calculated as the number of mastitis-positive cows (one or more quarters with SCM) divided by the total number of cows tested. The quarter SCM prevalence was calculated as number of quarters with SCM divided by the total number of quarters investigated. Herd level prevalence was calculated as number of positive herds (herds with at least one cow with SCM) over the total number of herds As almost half of the herds had only one lactating cow, we decided to divide the data set into single-cow herds and multiple-cow herds and to evaluate the data set separately, motivated by the fact that many of potential risk factors would not apply to single-cow herds (Study II). To evaluate cow and herd risk factors associated with SCM, as well as with NAS and *S. aureus* IMI, unconditional associations between each independent variable and the dependent variable, first with cow SCM status (0=negative and 1=positive), and subsequently in a separate analysis with NAS or *S. aureus* IMI (0=negative and 1=positive), were investigated using univariable logistic or univariable mixed-effect logistic regression analysis. Statistical significance in this step was assessed at P-value < 0.20. Factors that were significant in the univariable analyses were then investigated using Spearman's rank correlation in order to assess collinearity and if two variables showed collinearity ($r \geq 0.70$), the one with the lowest P-value was then offered to the multivariable regression models. A multivariable logistic regression model was used for one lactating cow herds, and multivariable mixed-effect logistic regression models, with herd as random

factor, was used for herds with more than one lactating cow. If herd as random factor was not significant ($P \geq 0.05$), an ordinary logistic regression model was used. The multivariable models were reduced using a manual, stepwise backward variable selection procedure where the initial model included all independent variables as main effects. Variables with a significant association ($P \leq 0.05$) with the dependent variable were kept in their respective final models. In each model, all variables with $P \leq 0.20$ for SCM, NAS or *S. aureus* IMI in the univariable analyses were then re-tested one at a time in their respective final model, and kept in the model if they were significantly associated with the dependent variable. In parallel, confounding was checked if removal of a variable in the final multivariable models changed the regression coefficients of the remaining variables (>25%). All plausible two-way interactions between the significant main effects were tested in all final models. Model fit was assessed by Hosmer-Lemeshow goodness-of-fit test. The statistical analyses were performed using Stata 15 (Stata Corp LLC, College Station, USA). (Study I, II). Data analysis in Study III was conducted in a similar manner using linear regression instead of logistic regression, since dependent variables such as TBC and SCC were continuous.

5.2.12 Genetic characterization of *Staphylococcus aureus*

Staphylococcus aureus isolates selection

Thirty *S. aureus* isolates from SCM cases were included. Six isolates were selected from each of the five provinces. Within each province, single isolates were selected from individual herds to simulate natural distribution.

DNA extraction

Prior to DNA extraction, isolates were cultured on horse blood agar plates to verify their purity. The EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extractions. Approximately 1 µL of each colony in pure culture from each of the 30 strains was suspended in 180 µL Digestion Buffer G2, plus 20 µL lysozyme (50 mg/ml; Sigma-Aldrich) and 10 µL lysostaphin (5 mg/ml; Sigma-Aldrich) and incubated at 37 °C for 90 minutes. Automated DNA extraction was then carried out using the EZ1 Advanced or Advanced XL robot (Qiagen) following the manufacturer's instructions, with a final elution volume of 50 µL. The extracted DNA was immediately stored at -20°C.

The DNA concentrations were adjusted to the range 5-15 ng/µL, suitable for sequencing using a Qubit® 2.0 fluorometric analysis double-stranded DNA high sensitivity kit (Thermo Fisher Science, Massachusetts, United States).

Sequencing of Staphylococcus aureus isolates

All library preparation and sequencing was carried out at Clinical Genomics Stockholm facility at Science for Life Laboratory (Stockholm, Sweden) using an Illumina Novaseq 6000 instrument with a S4 flow cell. Twenty-five of 30 samples produced sufficient sequence data for bioinformatic analysis. No further investigations were carried out on the five failed samples. The successfully sequenced samples had a mapping rate in the range 84.5-96.6% to the NCTC 8325 strain (GenBank accession NC_007795). The percentage of base pairs with a coverage better than 100 were in the range 89.6-93.8.

Bioinformatics analysis of sequences

Sequence assembly was carried using the UniCycler pipline (Wick et al., 2017). Unicycler employs read error correction and optimizes de novo assembly by SPAdes (Bankevich et al., 2012). In addition, UniCycler removes errors in the assembly by using pilon (walker et al., 2014). The UniCycler, SPAdes and pilon versions were v0.4.8-beta, 3.13.0 and 1.23, respectively. Minimum spanning trees were obtained by SeqSphere + version 5.1.0 (Kohl et al., 2014) using the assembled contigs obtained from UniCycler for the 25 isolates with the seed genome with GenBank accession NC_002951.2 and the *S. aureus* cgMLST version 1.3 containing 1861 loci (https://www.cgmlst.org/ncs/schema/141106/). The criteria for identification were 100% aligned length and 90% identity. For all 25 strains 1692 loci were found and used to create a minimum spanning tree. Strains with less than 200 different alleles were considered as members of a cluster. Sequence types (STs) are defined by alleles from the following standard set of *S. aureus* MLST genes: arcC, aroE, gpF, gmk, pta, tpi and yqiL (Enright et al., 2000).

6 Results and discussion

6.1 Prevalence of subclinical mastitis at herd, cow and quarter level (Paper I, II)

Overall prevalence of SCM in all five regions (peri-urban Kigali, eastern, northern, western and southern region) in Rwanda was 70.4% on herd level, 66.3% on cow level whereas quarter level prevalence was 39%. In Kigali, cow level prevalence of SCM was 76.2% (195 of 256 cows), whereas quarter level prevalence was 43.1% (433 out of 1004 udder quarters) (Paper I). Out of the 195 cows with SCM, 129 cows had more than one udder quarter affected. Subclinical mastitis was more common in rear (46.1%; 230 out of 499) than in front quarters (40.1%; 203 out of 505). The median score of quarter CMT was 2. The median and mean BMSCC for herds in the Kigali area was 1,108 × 10^3 cells/mL (central range = 760-1,531 cells/ml) and 1,179 × 10^3 cells/ml), respectively. Only two herds had BMSCC below the limit of 400 × 10^3 cells/ml, a level set according to EC regulation 853 (EC 2004) regarding milk intended for use as liquid milk for human consumption. The range of herd BMSCC was 352-2,196 × 10^3 cells/ml. The overall prevalence of SCM in small holders´ herds located in east, north, west and south regions of Rwanda was 62.0% at cow level and 37.3% at quarter level. These levels of prevalence are higher than the levels of less than 15% that can be achieved through appropriate udder health care (Ruegg and Pantoja 2013) or 52% (Iraguha et al. 2015) and 50.4% (Mpatswenumugabo et al. 2017) previously reported in the north-west and east of Rwanda, respectively.

The overall high SCM prevalence may be due to the lack of mastitis control practices, such as the five-point mastitis control plan, now evolved into a 10-point-plan, which has been shown to decrease the prevalence of mastitis and lower BMSCC in the last decades in developed countries (Hillerton et al. 1995). The zero-grazing system particularly prevalent in Kigali (MINAGRI, 2013)

favors increased infection pressure around the cow and higher chance of infection, especially when a mastitis control plan is not implemented, than in other regions where other systems than zero-grazing are prevalent. Furthermore, as the herd sizes in Kigali was greater than the generally smaller herds in the other regions, the risk was higher for disease transmission. The farmers lacked knowledge of which cows had SCM and did not apply a milking order based on udder health status. Low farmers' awareness of SCM (FAO 2014), lack of monitoring as demonstrated by lack of regular udder health screening using CMT and the lack of quality or payment standards for BMSCC in Rwanda could partly explain the high SCM prevalence and a low motivation to improve management practices known to prevent and control mastitis.

6.2 Udder pathogens in quarter milk samples from subclinical mastitis cases (Paper I, II)

A significant ($P < 0.05$) association was seen between parity and SCM as well as between breed and SCM in dairy cows in peri-urban areas of Kigali. Multiparous cows had significantly (OR=2.5, CI=1.32-4.71, P =0.005) higher odds to contract SCM compared to primiparous cow. The higher risk of SCM associated with increased parity in this study may be attributed to the fact that multiparous cows in the studied herds might have had cumulatively several exposures to mastitis pathogens from suboptimum hygiene during milking or from the environment. Poor integrity of the teat canal due to ageing leading to easy ingress of bacteria into the mammary gland after milking, decreased immunity, or a more pendulous udder prone to injury in older cows than younger cows might all have increased the susceptibility of the older animals to mastitis (Suleiman et al. 2018).

Holstein cows had significantly higher odds (OR=10.08, CI=1.54-66.12, P=0.016) for SCM than cows of other breeds (local breed, cross between Jersey and local breeds, cross between Sahiwal and local breeds). It has been reported that the Holstein breed is more susceptible to mastitis than other breeds (Bludau et al. 2014). The higher prevalence of SCM in the Holstein breed than in the Holstein-/local cross-breed or in other breeds reported in this study reflects recent trends in the cattle population in Rwanda, where mastitis-susceptible breeds, mainly Holstein, have been increasing since 2006 (TechnoServe 2008) without parallel improvement in udder health care practices (Paper I).

Both stage of lactation and udder and hind-leg hygiene were significantly associated with SCM in dairy cows in single cow herds in the multivariable model. Increased stage of lactation was associated with both SCM and NAS IMI in single cow herds. The odds for SCM increased significantly for cows ≥8

months in lactation compared to cows ≤3 months in lactation (OR=8.08, CI=2.29-28.61, *P*=0.001). No other significant differences were seen between other categories of month in lactation. Our data agree with Hortet et al. (1999), suggesting that the increased risk can be due to the accumulated exposure to mastitis pathogens cows undergo throughout lactation. Additionally, Abrahmsén et al. (2014) reported higher SCM prevalence in cows in late lactation than cows in early lactation stages in Uganda, and Tolosa et al. (2013) found a direct association between late stage of lactation and prevalence of SCM in Ethiopia.

In addition, the odds for SCM increased significantly for cows with moderately dirty udder and hind-legs (OR=2.22, CI= 1.25-3.99, *P*=0.007) compared with cows having a clean udder and hind-legs in single cow herds. Similarly, the odds for SCM increased significantly for cows in multiple cow herds with moderately dirty udder and hind-legs (OR=3.83, CI= 1.65-8.88, *P*=0.002) or a very dirty udder and hind-legs (OR=7.51, CI=1.81-31.07, *P*=0.005) compared with cows having a clean udder and hind-legs. Keeping cows under sub-optimal hygienic conditions increases the bacterial load and potential for transmission of both contagious and environmental mastitis pathogens. Our data corroborate those of Schreiner and Ruegg, (2003) and Abrahmsén et al. (2014) where cows with poor udder or poor hind-leg hygiene were found to be at increased risk of SCM. Sub-optimal hygiene conditions on udder and legs will facilitate udder pathogens gaining entry in the teat canal, for example during milking, and eventually causing SCM. Schreiner and Ruegg (2003) argue that a dirty udder will make teat dipping and sanitization ineffective.

The odds of SCM increased significantly for cows in multiple cow herds that did not have a calf suckling them (OR=3.30, CI= 1.88-17.75, *P*=0.002) compared with cows that had a calf suckling them. Calf suckling is generally practiced in Rwanda to stimulate milk ejection by the cow. Our study found that calf suckling can have a secondary benefit, as cows without this practice had an increased odds for SCM in multiple-cow herds. Other authors reported similar findings (Krohn, 2001, González-Sedano et al., 2010). The beneficial effect of calves suckling their dam might be due to the cow having a more complete milk ejection, a more complete udder emptying or removal of residual milk by the calf. Eventually, complete emptying of the udder will remove all residual milk which could otherwise function as a substrate for bacterial growth in the udder (González-Sedano et al., 2010). In addition, calf saliva might have possible antimicrobial elements that could inhibit growth of mastitis pathogens (González-Sedano et al., 2010).

Furthermore, the odds for SCM significantly decreased for cows not fed directly after milking (OR=0.39, CI=0.17-0.94, *P*=0.036) compared to cows fed

after milking. It is not clear why not feeding cows after milking resulted in lower odds for SCM than cows where feeding after milking is practiced, since feeding prevents the cow from lying down, thus preventing an open teat after milking becoming infected with udder pathogens. It is possible that prevalence of SCM is driven by other factors other than feeding cows after milking. This may include the fact that poor hygiene in the cow shed in a zero-grazing system is a disincentive for the cows to lie down, regardless of whether they are fed after milking or not.

Type of cattle kraal, hand washing between cows during milking, and type of flooring in cow housing were all significantly associated with NAS IMI in dairy cows in multiple cow herds. There were decreasing odds of NAS IMI in cows kept in grouped cattle kraal (OR=0.33, CI=0.11-0.95, *P*=0.041) and cows not kept in a cattle kraal (OR=0.17, CI=0.03-0.88, *P*=0.035) compared with cows kept in individual cattle kraal. In addition, there were increased odds of NAS IMI in cows from herds where there was an earthen floor in cow housing (OR=9.09, CI=1.77-46.72, *P*=0.008) compared to cows from herds kept in housing with concrete flooring. Individual cattle kraal, an enclosure where an individual cow is always kept, favors increased infection pressure compared to spacious grouping or no cattle kraal. It is possible that concrete floors and lack of cattle kraal maintain better cow hygiene because of better draining of manure on concrete flooring and lower infection pressure on cows kept on open ground without cattle kraal. Abrahmsén et al. (2014) reported a comparable situation in Uganda where he observed that cows in open grazing on pasture had lower odds for SCM than cows in zero grazing, because of better cow hygiene.

There were decreased odds of NAS IMI in cows from herds where washing the hands between cows during milking was not practiced (OR=0.24, CI=0.09-0.61, *P*=0.003) compared to cows from herds where such practice was present. It is, however, not clear why lack of hand washing between cows during milking resulted in less odds for NAS IMI. Since some NAS species such as *S. epidermidis* have been isolated from human skin (Thorberg et al., 2006), it can be postulated that hand washing between cows would minimize prevalence of NAS IMI. It is possible that during hand washing between cows using, for example, unsanitized water or without drying hands during hand milking, the water serves as a medium for proliferation and spread of NAS species. This theory might explain why cows in herds where hand washing between cows is practiced were more likely to contract NAS IMI.

The only significant variable in the multivariable analysis of variables associated with *S. aureus* IMI in single cow herds was hygiene of the milking area. There were increased odds ratio for *S. aureus* IMI in cows from single cow herds with a slightly dirty milking area (OR=2.52, CI=0.99-6.36, *P*=0.05) and a

very dirty milking area (OR=4.37, CI=1.66-11.55, *P*=0.003) compared with cows from herds where the milking area was cleaner. As mentioned above, poor hygiene will first facilitate, multiplication of udder pathogens in the cow environment which is a risk for SCM and IMI. Furthermore, poor hygiene will make control methods such as teat dipping and sanitization ineffective, which might be the main reason for increased odds of cows to contract SCM and *S. aureus* IMI.

Lack of foremilk stripping was the only variable significantly associated with *S. aureus* IMI in the multivariable analysis of risk factors for dairy cows in multiple cow herds. The odds of *S. aureus* IMI increased significantly if no foremilk stripping was performed (OR=3.16, CI=1.07-9.35, *P*=0.04). It has been stipulated that once mastitis pathogens have gained entry to the teat sinus, lack of foremilk stripping allows pathogens greater access to the mammary gland cistern where they cause infection (Phillips et al., 1969). In addition, foremilk stripping is also a good measure for checking the status of the milk before milking, making the milker aware of mastitis (Paper II)

6.3 Antimicrobial resistance in staphylococci (Paper I, II, IV)

6.3.1 β-lactamase production

Overall, β-lactamase production was common in staphylococci isolated from quarter milk samples from SCM cases in the Kigali area (77%, Paper I) and in the other four provinces (65.8%; Paper II). It is possible that the higher prevalence in Kigali is linked to antibiotic use since farm owners in Kigali have better financial means and access to antibiotics, which may lead to their overuse or misuse. This, in turn, can eventually have led to a higher selection for resistant clones compared to farms in the studied MCCs, which are located in more rural areas where access to antibiotics may be limited and smallholders do not have the financial means to purchase them. Overall, the levels in the present thesis are higher than the levels below 50% reported in developed countries (Persson Waller et al., 2010; Persson et al., 2011). The prevalence of β-lactamase production was higher in *S. aureus* (87.6% in the Kigali area and 78.8% in the other regions) than in NAS (62.2% in Kigali and 54.8% in the other regions). These findings are in agreement with Malinowski et al., (2002) who reported a higher prevalence of antimicrobial resistance in *S. aureus* than in NAS. It can be hypothesized that this is due to the blaZ gene that encodes β-lactamase

production being more common in *S. aureus* than in NAS. However, more research is needed.

The high prevalence of resistance in *S. aureus* is of concern because resistant clones of *S. aureus* survive in the deep inner tissue of the mammary gland, where it is more difficult for antibiotics to penetrate (Gruet et al., 2001). This has implications for the possible failure of a bacterial cure by widely used penicillin (Gruet et al., 2001), which might lead to chronic mastitis and failure to restore infected cells to full milk production capacity, even after treatment. Poor hygiene, mentioned earlier, purchase of cows without prior mastitis screening, and absence of culling of cows infected with resistant clones in Rwanda, may all be drivers in the spread and dominance of β-lactamase-producing staphylococci isolates. The reasons for differences in β-lactamase production in different staphylococci species are not known. Persson Waller et al. (2010) hypothesized that resistant isolates may belong to the same clonal group within each bacterial species, which inhabit specific virulence factors. However, further research at the molecular level would be worthwhile.

6.3.2 Antimicrobial sensitivity testing of *Staphylococcus aureus*

The majority of the 60 studied *S. aureus* isolates exhibited resistance to penicillin (83.3%) and clindamycin (100%), and fewer *S. aureus* isolates (20%) had high MIC to tetracycline, indicating a possible reduced clinical susceptibility to these drugs. Resistance to the other antimicrobials tested (cephalothin (Ct), cefoxitin (Fox), enrofloxacin (Ef), fusidic acid (Fu), erythromycin (Em), gentamicin (Gm), nitrofurantoin (Ni), and trimethroprim (T) was uncommon. Resistance to clindamycin may be due to the genetic potential of *S. aureus* isolates that carry erm genes or isolates acquiring that gene through horizontal gene transfer. The erm genes encode modification of the clindamycin-binding site on the ribosome, thus producing resistance in isolates (Lewis and Jorgensen, 2005). Although the results of antimicrobial susceptibility from different methods are to be interpreted with caution, our results are in line with those of Suleiman et al. (2018) who found high levels of *S. aureus* isolates from SCM resistant against penicillin (88%) and low levels of resistance against tetracycline (16.6%) in Tanzania. Similarly, Kasozi et al. (2014) reported 100% resistance to penicillin in *S. aureus* isolates from SCM cases in Uganda, and a different trend in tetracycline resistance (71.4%) than in current study. Ssajjakambwe et al. (2017) reported that tetracycline and penicillin are the most commonly used antibiotics in treating different infections including mastitis in Uganda, a country comparable with Rwanda. Antibiotics for animals can be procured without any veterinary prescription in Rwanda (Manishimwe et al., 2017). Therefore, it can

be hypothesized that the overuse or misuse of tetracycline and penicillin could have contributed to the selection pressure on resistant clones. Hence, the high resistance levels reported in this study.

Results of genotypic and phenotypic resistance of 25 out of 60 *S. aureus* isolates indicated that the BlaZ gene, which confers penicillin resistance, was prevalent at a level of 84% and was, in all cases, supported by MIC phenotypic resistance results. The prevalence of penicillin resistance found here (84%) for isolates from Rwanda is similar to the 86% that was recently observed for isolates derived from dairy cows in North-Western Ethiopia (Mekonnen et al., 2018). Four of the isolates (16%) were resistant to tetracycline, which is a significantly lower fraction than the 54% observed in the study from Ethiopia (Mekonnen et al., 2018). There is not full accordance between the presence of tet (M) or tetK genes and the observed resistance pattern. For two tetracycline-sensitive isolates, either the tetK or the tet(M) gene is found from the NGS-data, while one resistant isolate lacks both genes. The dfrG gene which encodes trimethoprim resistance was present for seven isolates, but only four of these actually displayed resistance. Thus the prevalence was 16%, which is considerably higher than what was observed by Mekkonen et al.,(2018) where only one of 79 isolates displayed trimethoprim resistance (Mekonnen et al., 2018). The str genes, which encode resistance among aminoglycosides including gentamicin, were found in three isolates but all were still susceptible to gentamicin. Since mechanisms of antimicrobial resistance is complex, it is possible to detect resistance genes in susceptible isolates or to find phenotypic resistance when there are no resistance genes (Yang et al., 2016). This may, for example, be due to lacking but crucial accessor genes.

6.4 Milk quality from farm to milk collection centers (Paper III)

6.4.1 Somatic cell counts in bulk milk from farms and milk collection centers

Average farm BMSCC varied between 180 x 10^3 and 920 x 10^3 cells/ml while the average SCC of bulk milk at MCC varied between 170 x 10^3 and 1,700 x 10^3 cells/ml in all MCCs. The farm bulk milk median SCC varied between 85 x 10^3 and 760 x 10^3 cells/ml whereas median BMSCC at MCC varied between 105 x 10^3 and 1, 091 x 10^3 cells/ml in all MCCs. The results of the final multivariable mixed-effect linear regression analysis showed that farms that offered cows concentrates had significantly higher BMSCC (CI:-0.38,-0.59 and P=0.007)

than farms which did not offer concentrates to cows. Moreover, farms that kept records of past diseases had significantly higher BMSCC (CI: -0.58,-0.05 and *P*=0.02) than those which did not keep such records, and farms with a more hygienic milking area had significantly lower SCC than farms with slightly dirtier milking area (CI:0.11,0.42 and *P*=0.001) and farms with a very dirty milking area (CI: 0.11-0.48 and *P*=0.001).

The MCCs included in the study did not screen bulk milk for SCC and were therefore unable to enforce the SCC standards for threshold limits, regardless of whether it concerns requirements for acceptance or rejection, or payment incentives with premium payment for a high quality product, or penalties. Milk samples from both farm and MCC level in seven out of eight MCC had average SCC above 300 x 10^3 cells/ml, an Easter African SCC standard (EAS 67:2006), implying udder health problems in the cows at the farms. There was considerable variation between minimum and maximum recorded BMSCC from farms, where the minimum recorded in MCC6 was 2 x 10^3 cells/ml and the maximum 7, 900 x 10^3 cells/ml in MCC4. This considerable variation validates the difficulty to set or comply to a relevant threshold for milk acceptance or rejection, or for quality compensation. Our results (35.9% of farm bulk SCC above 300 x 10^3 cells per ml) are lower than those of Kunda et al. (2016) who found 61.4% of milk samples from small holders' farms in Lusaka, Zambia, had SCC above recommended limit of 300 x 10^3 cells/ml. In order to give good advices on how to lower the BMSCC at farm level, knowledge of factors affecting the BMSCC is needed. Our results suggested that lack of feeding concentrates sometimes, not keeping records of diseases and having better milking area hygiene could improve in lowering bulk milk SCC. It is not clear why feeding concentrate was associated with high BMSCC, it could be that cows that are fed on concentrate are of Holstein breed, a breed that was found to be associated with mastitis in Rwanda (Ndahetuye et al., 2019). Similarly, it is not clear why keeping records was associated with higher SCC. It is possible that farmers who are keeping records are the ones who recently experience mastitis in their farms

Good hygienic conditions prevent or reduce transmission of mastitis bacteria from one cow to another (Philpot, 1979). The same author argues that if transmission of mastitis pathogens is prevented by good hygiene, there will be a parallel decrease in the incidence of IMI. This may explain why farms with a cleaner milking area in this study had a lower BMSCC. By applying best practices, several of the discussed issues can be mitigated or even completely overcome. Cattle owners are more prone to adopt innovations, management technologies and practices compared to farmers rearing other animal species (Amadou et al., 2012), and cattle are prioritized before other species in preventive health care and veterinary treatments (Amadou et al. 2012). Thus,

there is potential to increase and improve milk production and quality in Rwanda by cheap and simple means such as application of the 10-point mastitis control plan and other best practices.

6.4.2 Total bacteria count in bulk milk from farms and milk collection centers

Farm bulk milk average TBC varied between 1.1 x 10^6 and 1.6 x 10^7 CFU/ml, whereas average TBC of bulk milk at MCC varied between 5.3 x 10^5 and 2.4 x 10^8 CFU/ml. The farm bulk milk median TBC varied between 7 x 10^3 and 1.1 x 10^6 CFU/ml whereas median TBC of bulk milk at MCC varied between 2.5 x 10^5 and 1.423 x 10^8 CFU/ml in all MCCs.

Total bacterial count of bulk milk at MCCs was significantly ($P < 0.05$) higher than the average TBC of bulk milk at farms at MCC 4 (P=0.001), MCC5 (P= 0.000), MCC6 (P=0.000), MCC7 (P= 0.000) and at MCC8 (P=0.001)

Lack of a separate milking area was significantly ($P < 0.05$) associated with higher TBC levels at farm level. In milk samples from farms without a separate milking area, the TBC was 0.49 CFU/ml higher (C.I. = 0.15, 0.88, and P= 0.005) than in milk samples from farms with a separate milking area.

Except in two MCCs, there was an increase of TBC in milk samples from farms to MCCs. This increase suggests proliferation of bacteria in milk during transport using unrefrigerated equipment. This theory is in agreement with Doyle et al. (2015) who found that there is an increase in total microbial load in the milk chain from farm through milk transporters, MCCs and to end-consumers in Rwanda. The same trend was reported in Uganda where bacterial proliferation of milk from farm level through transportation to consumers reached 150-fold (Grimaud et al., 2007). Maximum-recorded TBC in milk samples from farms were very high suggesting that mixing such milk with milk of better quality at MCC level would raise overall TBC of the milk at MCC. Therefore, there is a need for infrastructure and equipment to discard low quality milk as early as possible in the milk chain, or preferably introduce economic incentives for farmers to produce and deliver milk with very low TBC. The highest recorded TBC (1.6 x 10^7 CFU/ml) was comparable with those reported in Zimbabwe (6.7 ± 5.8 log10 CFU/ml) by Mhone et al. (2011) in raw milk samples, and comparable to log 7.08 CFU/ml reported in milk samples from chilling centers in Sri Lanka (De Silva et al., 2016).

The lowest median TBC (7 x10^3 CFU/ml) was recorded in milk from MCC2, in Nyagatare, where farmers are known to have received more training in dairy husbandry and milk handling (TechnoServe, 2008). As farmers perform milking

in the same place where the cow is housed, they are thereby increasing the risk of milk being contaminated with environmental microorganisms.

6.4.3 *Escherichia coli* and *Salmonella* spp. in bulk milk from farms and milk collection centers

Escherichia coli was detected in 8.5% of on-farm bulk milk samples (range 5.00 to 11600 CFU/ml) and in 62.5% (20 out of 32 samples) from MCCs (range 5.00 to 2900 CFU/ml). Detection of *E. coli* was less frequent at farm level than at MCC level, suggesting better hygienic milk handling at the farm than at the MCC, and/or proliferation during transport and potential fecal contamination at these milk bulking sites. Potential routes of contamination at MCCs include personnel at MCCs, and equipment and tools used at MCCs level, whereas contamination at farm level may be due to animal faeces or poor hygienic level of animal husbandry practices (Kateřina et al., 2016). Our results are in agreement with Grimaud et al. (2007) who reported high *E. coli* counts in raw milk samples in Uganda, but even higher than levels indicated by Ppyz-lukazik et al. (2014) who detected *E. coli* in milk samples with levels ranging from 5.0 to 1.1×10^2 CFU/ml.

Overall *Salmonella* spp. prevalence in farm bulk milk samples was 14.0%. There were no *Salmonella* spp. detected in milk samples from MCCs. It is possible that due to dilution effect, *Salmonella* spp. concentrations at MCC were below the detection limit on the medium we used. The farm level results show a higher prevalence than previous results from Rwanda reported by Kamana et al. (2014) who found *Salmonella* spp. prevalence of 5.2% in raw milk samples from dairy farms, MCC and from milk shops. The results are also higher than a prevalence of 10.1% reported in raw milk in Tanzania (Schoder et al., 2013). The only on-farm factor remaining after the multivariable mixed-effect linear regression analysis was lack of teat washing before milking, resulting in significantly higher odds of also having a higher level of *Salmonella* spp. in bulk milk samples (O.R= 2.22, P=0.02, C.I.=1.13-4.36) than farms which do wash teats before milking. The farm environment is probably the place with the most interplay between various reservoirs and vehicles of the pathogens. It is possible that *Salmonella* spp. found in milk came from, for example, milker`s hands who have previously touched reservoirs of *Salmonella* spp., such as infected calves, salmonella-shedding cows or contaminated water supplies (Marth, 2006). Since shedding of *Salmonella* spp. is common in cattle (Wells, et al., 2001), poor hygiene through lack of teat washing will facilitate entry of the pathogen from the cow into the milk.

6.4.4 Brucella antibodies in bulk milk from farms and milk collection centers

No sample tested positive for brucella antibodies among farm bulk milk, but still brucella antibodies were detected twice in bulk milk from two MCCs. It is possible that these brucella antibodies came from farm bulk milk that was not sampled, since we did not visit all farmers associated with the MCCs. The prevalence of brucella antibodies in bulk milk of 22.7% at MCC level reported in this study was markedly higher than the level of 11% in Gulu in milk from milk delivery points, and markedly lower than 40% reported in milk samples from collections points in Soroti, both sites being in Uganda (Rock et al., 2016). The presence of brucella antibodies in milk can be attributed to infection burden and therefore it is only an estimation of the prevalence of brucellosis among milk-supplying cows (Godfroid et al. 2010).

6.4.5 Antimicrobial residues in bulk milk from farms and milk collection centers

Prevalence of antimicrobial residues in bulk milk was not common in the present study. Antimicrobial residues were detected only in bulk milk samples from farm level, connected to MCCs which had high SCC both in farms and MCC samples (unpublished data), suggesting that mastitis treatment without respecting withholding period could have been the origin of the antimicrobial residues in the milk samples. Despite being uncommon, consequences of antimicrobials residues exist, for example, antimicrobial residues can prevent optimum growth of starter cultures during processing, or if β-lactam antibiotics are present, they can cause allergic reactions in some people, and antimicrobial residues can facilitate selection of antimicrobial-resistant microorganisms when milk is consumed (Griffiths, 2010). Our results show a markedly lower rate of antimicrobial residues in milk than reported by others; 44.5% reported in Kenya (Teresiah et al., 2016), 30% in Zambia (Kunda et al., 2016) or 36% reported in Tanzania (Kurwijila et al., 2016). It is worth noting that the Delvo test had a high sensitivity for detection equal to 1-2 μg/L, so absence of antimicrobial residues could mean that any present are below the indicated detection limit.

6.5 Genetic characterization of *Staphylococcus aureus* (Paper IV)

Results from core genome multilocus sequence typing (cg-MLST) indicated that three main clusters could be discerned among the 25 isolates. The largest cluster

contains isolates of ST 152 and the closely related ST1633 (6+2 isolates). ST152 was first isolated from humans in Europe (Mueller-Premru et al., 2005) but has subsequently been shown to be an important and prevalent ST infecting humans in many African countries (Aiken et al., 2014; Shittu et al., 2012). These ST152 with a local predominance in Africa are typically of spa-type t355, Panton-Valentine leucocidin (PVL) positive and methicillin susceptible. In all eight ST152/ST1633 bovine isolates of the present study, the lukF-PV and lukS-PV genes are present signifying PVL positivity, and they are all of spa-type t355. Since hand milking is still prevalent in Rwanda it is possible that human ST152 strains have infected the cows from the person milking the cows. These strains were distributed over almost all provinces of Rwanda and apparently, bovine ST152 is prevalent all over the country. It can be hypothesized that since management of herds are similar and include hand milking and lack of post milking teat dipping, there will be opportunities for human contact with animals in the absence of consistent disinfection. This will facilitate transmission of human-adapted pathogens to dairy cows during milking. Similar management means that pattern of transmission is the same across regions, which is why positive identification of ST152/1633 was observed in all regions included in the study. Interestingly, when screening milk and dairy products in southern Italy for MRSA, Basanisi et al. (2017) found that PVL encoding ST152 (t355) accounted for 67.5% of all MRSA isolates (n=40). In fact, ST152 isolates have also been isolated sporadically from humans in Europe and are usually methicillin resistant (Dermota et al., 2015; Brauner et al., 2013). It has been suggested that ST152 is originally an African lineage which first acquired PVL and subsequently, after introduction to Europe, has also acquired methicillin resistance (Ruimy et al., 2008).

The second largest cluster (5 isolates) consists of the same single locus variant of ST3537 (SLV_ST3575) (Figure 5) geographically dispersed in Rwanda. ST3537 was isolated from mastitis cases in Kenya in 2009 (PubMLST, 2019). Very distantly related, but with an SLV_ST3575 isolate as its closest neighbor, among the isolates of the present study is a divergent strain whose closest match in the PubMLST database is ST3591, which was also isolated from a mastitis case in Kenya 2009 (PubMLST, 2019). However, the present strain has three different alleles compared to ST3591. A second singleton in the present data, also with a SLV_ST3575 isolate as the closest neighbor in the minimum spanning tree, albeit only distantly related, is a ST2430 (Figure 5). This ST was first discovered when isolated from inpatients in Thika, Kenya in 2011 (Aiken et al., 2014), but was later also found in an isolate from 1995 from a pyomyositis case in Uganda (PubMLST, 2019). Thus, the current evidence indicates that there exists a novel bovine clonal complex in the Eastern African region related

to ST3575. A third cluster contains four geographically dispersed isolates (Figure 5) that belong to the well-known bovine adapted CC97 clonal complex (Smith et al.,2005; Boss et al., 2016; Weinert et al., 2012). There are two isolates each of ST1 and SLV_ST20 and one isolate each of ST5 and SLV_ST101. All these STs have primarily been associated with globally dispersed human infections (Shepheard et al., 2013; WinertLA et al., 2012) although they are occasionally also found in bovine isolates (Boss et al., 2016; Shepheard et al., 2013; Hata et al., 2010). Overall, there is no geographic association linked to the ST of the isolates in the present study.

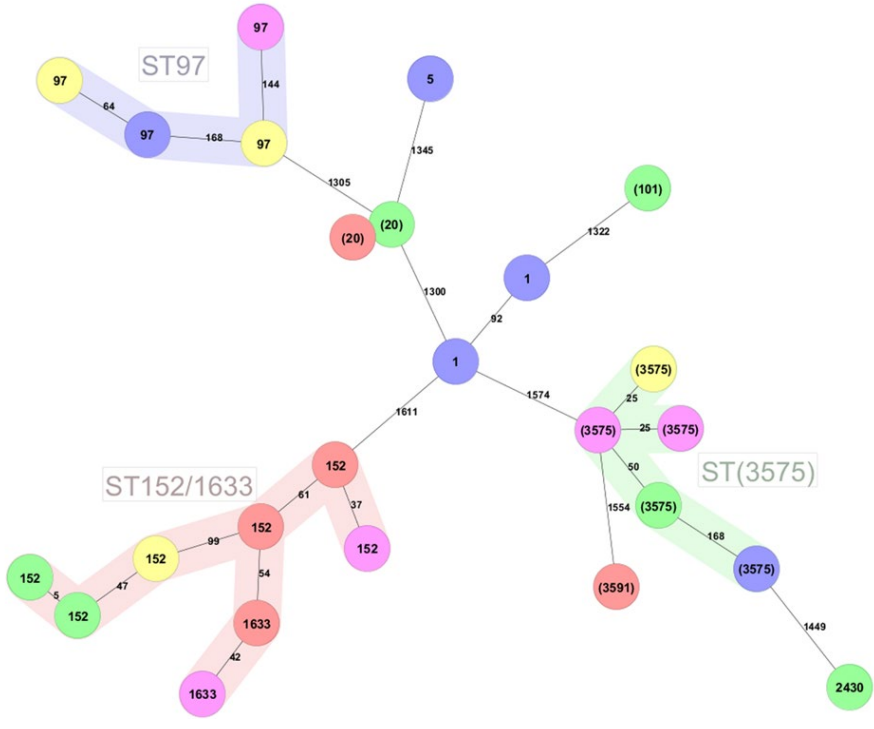

Figure 5. Figure 5. Minimum spanning tree created for the 25 *S. aureus* strains from the Kigali (yellow), eastern (red), western (pink), northern (green) and southern (blue) province of Rwanda. The tree was created using 1692 loci. Clusters connecting isolates with less the 200 different loci are indicated with background colors and named with the ST number in the cluster. The ST of the isolates are shown on the nodes. Novel STs are represented with the closed existing ST within parentheses.

6.6 Methodological considerations

In this thesis, SCM was defined as number of quarters with CMT ≥ 3 on a scale of 1 to 5, and positive quarters were milksampled for bacteriological culturing on blood agar plates and initial biochemical characterization of colonies, with final identification with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF). According to the NMC (1999), a threshold of 3 is equivalent to SCC of at least 400 x 10^3 cells per ml. Since mastitis is broadly defined as inflammation in udder quarters with at least 50 x 10^3 cells per ml in individual quarter or 200 x 10^3 cells per ml in cow level, and CMT is also subjective, it can be deduced that in this study, we might have missed many positive cases on quarter level. In addition, all milk samples from quarters with CMT ≥ 3 were sampled for bacteriological analyses. This was done in order to score as many cows as possible. Despite taking a higher threshold of SCC for mastitis, there were cases of negative bacteriological result on blood plates after incubation at the correct temperature and time. This may be due to low numbers of bacteria below the limit of detection in the sample, concurrent time of sampling when the immune system has successfully eliminated infection before decreasing SCC or with organisms being shed intermittently (Östensson et al. 2013). It could also be due to the fact that some pathogens, e.g. *Mycoplasma bovis* and others, do not grow on blood agar.

Bacteria growing on plates were initially characterized by biochemical tests and were transported to the SVA in Sweden for final identification with MALDI-TOF. This method is generally more sensitive and specific than the traditional culturing method (Raemy et al., 2013; Schabauer et al., 2014). MALDI-TOF is used routinely for identification of mastitis bacteria at SVA, thus ensuring a large database library for control. Bacteria were transported to Sweden in brain/heart infusion broth. It can be discussed whether transport had an effect on bacteria viability among different bacterial species. In addition, each sample was first re-cultured before being subjected to MALDI-TOF at SVA. All transported samples showed growth on horse blood agar plates.

In this thesis, ELISA was used to detect brucella antibodies in milk, instead of culturing the bacterium. It can be argued that we missed zoonotic aspects of the pathogens, and therefore culturing could have been included. The reason was lack of laboratory facilities at Biosafety Level 3 at the site where the analyses were being done.

The snowball sampling technique, as described by Faugier and Sargeant (1997), was used to locate dairy herds to be enlisted in the study to the north, east, west and south relative to the centre of the sampling site. It can be questioned why we did not use random sampling. It is because of the lack of a

registry of farmers at MCCs, and in addition, MCCs were not in contact directly with farmers.

6.7 Capacity building and research dissemination

In parallel to the actual research activities that were the basis for this thesis, capacity building and research communication were performed in Rwanda.

Given that mastitis prevalence in dairy cows was high compared to what can be achievable through good management and application of best practices, training and capacity building were conducted for farmers and other key stakeholders in the dairy value chain. Training occurred in two categories:

1 Best practices for udder health milk quality and safety training for farmers. Training materials for farmers were developed based on problems identified during this project and in the literature. Components of the training materials included: best practices for better mastitis prevention and control, cow shed management to maximize hygiene and cow comfort, and best practices for post-harvest milk handling (including sanitation and appropriate tools to store milk), milk safety (potential zoonosis contamination where best practices are not applied), and the role of milk in human nutrition. In total 226 farmers, comprising 155 males and 71 females, benefited from this training program.
2 Advanced training of trainers for veterinarians, students and extensionists, on udder health and milk quality.

In total, 45 participants (32 males and 13 females) underwent this training of trainers session. Participants came from MCCs, both private and public sectors, and final year students in the Veterinary Medicine, Animal Production and Food Science departments of the University of Rwanda. In addition to the concept of udder health and its diagnosis, and milk quality and its testing, but also preventive udder health and treatment strategies, and dairy herd reproductive management and milk productivity were communicated to participants.

7 General conclusions

- There was a high prevalence of SCM across regions of Rwanda, indicating udder health problems in dairy cows. Contagious udder pathogens, predominantly S. aureus and NAS, were isolated from SCM-positive cows in all Rwandan regions

- *Staphylococcus aureus* in particular revealed a high strain diversity, and its ST were distributed in all regions, with both a bovine and human possible source

- All staphylococci were resistant to penicillin to a high degree, and *S. aureus* was also resistant to clindamycin

- Main risk factors identified for SCM and its IMI with implications on management routines, included housing of cows in individual cattle kraal and on earthen floors, poor hygiene of cows and of the milking area, absence of foremilk stripping, increasing stage of lactation, Holstein breed, and lack of calf suckling.

- Milk quality indicators, such as TBC and SCC, were high in milk samples at farm and MCCs level, which indicate both poor udder health and hygiene in dairy cows and possible milk contamination in the chain from farm to MCC. Presence of *E. coli*, *Salmonella* spp. and brucella antibodies were common in the milk. Antimicrobial residues in milk were uncommon.

8 Practical implications and recommendations

- The predominance of contagious udder pathogens in SCM cases in all regions of Rwanda calls for prevention of spread among udder quarters and among cows. Therefore, it is recommended to raise awareness and apply the 10-point mastitis control plan among farmers in Rwanda. Notably, it is important to apply grouping of cows based on udder health status and apply a milking order with infected cows milked last, post-milking teat disinfectant, keep records of udder health status and infected cows, and apply selective dry cow therapy.

- The *S. aureus* causing SCM were diverse and included ST152 that has usually been found in human beings. This may indicating a recent transmission of these types from human to cows, for example, during hand milking. This implies that preventive measures such as wearing gloves during hand milking situations needs to be introduced in order to limit spread of *S. aureus* from personnel to cows.

- There were high levels of penicillin resistance, both phenotypic and genotypic, among the studied staphylococci. These results imply that treatment failures after antibiotic therapy therefore call for interventions for controlling mastitis based on a holistic approach that does not only include optimized treatment but also preventive measures and biosecurity to limit the spread of resistant clones.

- The identified herd and cow level risk factors also have implication on management routines of dairy cows to prevent SCM and IMI. This includes improving hygiene both on the cow and in the cattle shed. Zero grazing is becoming more common in Rwanda, and it is recommended that frequent cleaning and a comfortable cow lying area should be

established in order to minimize infection pressure around the cow. It is also recommended to frequently check the status of the cow whether it is with fore-milking practices or checking all udder quarters regularly with CMT. It is recommended to alert farmers to the fact that the Holstein breed can easily contract mastitis. Therefore, preventive measure should be practiced, and the choice of alternative, better adapted breeds can be discussed as a more sustainable option under the prevailing conditions.

- Establishment and dissemination of farm standard operating procedures (SOPs) is needed, and periodical benchmarking of farms of post-harvest milk handling at the farm and MCC level. Establishing SOPs will minimize negative effects on milk quality and decrease safety risks from dairy production to public health. This may include, for example, regular checking of TBC and SCC levels of milk in the MCC tank but also of milk samples from middlemen or farms delivering milk to the MCC. Such quality control would provide necessary feedback to middlemen and farmers to improve the practices that affect TBC and SCC. This may call for initiating training on best practices for milk handling or mastitis prevention and control. Another example may be to ensure good personal hygiene practices among personnel and to properly sanitize the equipment used to receive and cool milk. This will minimize fecal contamination of milk at MCC. It is equally recommended to transport milk using a proper cooling system that will minimize bacterial proliferation.

- Incentivize farmers to reduce SCC at cow level since its decrease correlates directly to improved milk yield and quality. This could be through, but not limited to, milk quality payments according to lower SCC, in addition to incorporating SCC measurements in evaluating MCC performance. Somatic cell count could serve as a rapid test for MCC cooperatives in order to initiate mastitis prevention and control activities in their affiliated farmers.

9 Future perspectives

- Are udder pathogen profiles the same for CM? This thesis focused on SCM but, so far, no studies have been done on CM in Rwanda. Therefore, it would add value to know the etiology of CM in Rwanda; how do the profiles of causative pathogens differ from SCM, how are they treated, are they more resistant to antibiotics, what are the risk factors for CM in Rwanda?

- Are there differences in penicillin resistance among NAS species? Non-aureus staphylococci species were predominant in SCM cases, and there were differences among them in penicillin resistance. Further research is needed to explain differences in antimicrobial susceptibility among NAS species.

- Are *S. aureus* from mastitis cases related to those found in extramammary sites, such as teat skin, and are they related to skin microflora of the milkers' hands? We found some isolated *S. aureus* strains to be of human origin: It would be worth sampling milker´s hands and extra-mammary sites to evaluate the genetic relatedness to isolates from milk samples. This will help understand major reservoirs of *S. aureus* infecting cows.

- What drives AMR in mastitis cases for small dairy holders? Our results indicated high levels of penicillin resistance among small dairy holders in Rwandan rural regions as well as in large dairy holders located in peri-urban areas of Kigali. Small holders may not have enough finances to procure antibiotics, which can potentially lead to AMR if over/misused. However, this thesis indicated that penicillin resistance exceeds 60% in these small holders, and it is worth exploring drivers of AMR in mastitis cases among them.

- What antibiotics are used on dairy farms in Rwanda and what are the perceptions of famers on antibiotics use and AMR? We found high levels of AMR; however, further information on antibiotic use and perception of antibiotic use and AMR among dairy farmers in Rwanda is still needed.

- Finally, improved udder health practices should be included in an overall herd health approach in order to reach sustainable improvements, for which, multidisciplinary research projects need to be formulated.

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Popular science summary

Thousands and thousands of people consume milk every day. Cows producing such milk can contract different diseases that threaten the supply of milk. One of the most common diseases in dairy cows is mastitis, which is an inflammation of the mammary gland that is caused mainly by different bacteria. Mastitis negatively affects milk yield and milk quality in dairy cows. Bacteria causing mastitis could be from environment around the cow, from other cows with infection in the udder or from human during milking. If precautions are not taken, dairy cows could suffer from mastitis for a long time and this will compromise their welfare and milk yield.

This thesis was undertaken to better understand mastitis and milk quality in Rwanda. Dairy cows have a great cultural and economic importance in Rwanda, where most of the milk is produced by small holder farmers. Milk has been recognized to be important for the nutritional status of young children and for their growth and cognitive development. The cattle population and milk production is increasing, partly due to national programs striving for enhanced human nutritional status and food security and safety.

Mastitis can be either clinical with obvious changes in the udder and milk or subclinical without visible signs. In this thesis I have focused on subclinical mastitis as it is more prevalent and it often goes undetected in the herd and affects milk yield and quality.

I have screened 828 cows in 429 herds from five regions in Rwanda with a cow-side test named California mastitis tests and taken milk samples from affected udder quarters for analysis of which bacteria cause the mastitis. In addition, I have studied antibiotic resistance in the isolated mastitis-causing bacteria. Furthermore, I have studied how milk quality and safety is affected by different practices at farm. Results showed that subclinical mastitis prevalence was around 70% on herd level, 65% on cow level and 40% on quarter level. That means around 6 cows out of 10 have inflammation in at least one of the udder quarters. About 75% of cultured milk samples in all regions were bacteriologically positive and 25% were bacteriologically negative. In all regions, staphylococci (non-aureus staphylococci (NAS) followed by *Staphylococcus (S.) aureus*) were the predominant bacterial pathogens causing

subclinical mastitis. *Staphylococcus chromogenes*, *S. epidermidis* and *S. sciuri* were the most prevalent NAS species in all regions. Tests for genetic diversity showed that the *S. aureus* were of many different types, implying several sources of infections, both from other cows and humans. Results also showed that more than half of all staphylococci were resistant to penicillin. A majority (83%) of the *S. aureus* isolates were resistant to penicillin, and all of them were resistant to clindamycin and one fifth to tetracycline.

Risk factor analysis indicated that cows were more likely to contract subclinical mastitis either caused by *S. aureus* or NAS if they were: housed in individual cattle kraal (pen) and on earthen floors, in late lactation stage, of Holstein breed , having dirty udder and legs, not having calf that suckle them, not being fed after milking, being milked without hand washing and with poor hygiene during milking, and at absence of foremilk stripping.

This study also indicated high bacterial load (total bacterial count) and high levels of inflammatory cells in the milk (somatic cell count) at farm and at milk collection center level, which further indicate possible milk contamination along the transport chain from farm to milk collection centre and poor udder health in dairy cows. It was common that the milk contained *Escherichia coli*, *Salmonella* spp. and brucella antibodies, which all are zoonotic pathogens with implications for public health. Finding antimicrobial residues in milk was uncommon.

In conclusion, subclinical mastitis is common in dairy herds in Rwanda and the majority of causative udder pathogens are resistant to penicillin. Contamination of milk in the production chain lead to a high microbial load in milk which is of importance for milk quality, processability and public health. Molecular characterization of *S. aureus* showed that this pathogen is highly diverse in subclinical mastitis cases in Rwanda, which provide knowledge on biosecurity, how mastitis is spread and how to best implement preventive measures. An overall mastitis control plan is recommended in order to optimize milk production in Rwanda.

Populärvetenskaplig sammanfattning

Tusentals människor konsumerar mjölkprodukter dagligen. Korna som producerar mjölken kan drabbas av sjukdomar som sänker produktionen. En av de allra vanligaste sjukdomarna hos mjölkkor är juverinflammation som oftast orsakas av bakterieinfektion. Mjölkavkastning och mjölkkvalitet sänks vid juverinflammation. Bakterierna som orsakar juverinflammationen kan komma från miljön runt kon, från andra smittsamma kor eller från människorna som mjölkar korna. Om inga åtgärder sätts in kan en ko bära på juverinflammationen under lång tid, och det kan äventyra kons hälsa och välmående och sänka mjölkproduktionen.

Denna avhandling vill öka kunskapen kring juverinflammation och mjölkkvalitet i Rwanda. Mjölkkor har stor kulturell och ekonomisk betydelse i Rwanda, där den mesta mjölken produceras av småbönder med enstaka kor. Mjölken har stor betydelse för de små barnens näringsstatus och deras tillväxt och kognitiva utveckling. Koantalet och mjölkproduktionen ökar i Rwanda, till stor del beroende på åtgärder inom nationella program som strävar efter förbättrad human näringsförsörjning och säkrad tillgång på livsmedel av god kvalitet.

Juverinflammation kan antingen vara klinisk med synliga förändringar i juver och mjölk, eller subklinisk, ”osynlig”, utan synliga förändringar. I avhandlingen har jag fokuserat på juverinflammationen utan synliga förändringar eftersom den drabbar fler kor, den kan finnas oupptäckt och pågå länge i en besättning, och den påverkar nivån på mjölkavkastningen och kvaliteten på mjölken.

Jag har undersökt 828 kor från 429 besättningar i Rwandas fem regioner. Med ett snabbtest, california mastitis test, har jag undersökt mjölken och tagit mjölkprov från de juverfjärdelar som har visat tecken på subklinisk juverinflammation. Mjölkproverna har odlats på laboratorium för att få reda på vilka bakterier som orsakat juverinflammationen. Jag har dessutom studerat antibiotikaresistensen hos dessa bakterier. Slutligen har jag studerat hur mjölkens kvalitet och livsmedelshygien påverkas av olika skötselrutiner i

besättningarna. Resultaten visade att subklinisk juverinflammation förekom hos 70 % av de studerade besättningarna, 65 % av korna och 40 % av juverfjärdedelarna. Det innebär att ungefär sex av tio kor har inflammation i minst en juverfjärdedel. Ungefär 75 % av de odlade mjölkproverna från alla regionerna hade växt av bakterier och 25 % av odlingarna var utan växt. I alla regionerna var stafylokocker (främst koagulasnegativa stafylokocker, följt av *Staphylococcus (S.) aureus*) de vanligaste bakterierna som orsakade juverinflammation. *Staphylococcus chromogenes*, *S. epidermidis* och *S. sciuri* var de vanligaste arterna bland de koagulasnegativa stafylokockerna. Tester för genetisk diversitet visade att *S. aureus*-bakterierna i denna studie var av många olika typer, vilket antyder att infektionen kan komma från flera olika källor, både från kor och människor. Resultaten visade också att mer än hälften av alla stafylokocker var penicillinresistenta. En majoritet, 83 %, av *S. aureus* var resistenta mot penicillin, och de var alla resistenta mot klindamycin, medan en femtedel var resistenta mot tetracyklin.

Enligt en analys av riskfaktorer hade korna hög risk att få subklinisk juverinflammation om de var inhysta i en individuell fålla (sk kraal) och på jordgolv, om de var i senare delen av sin mjölkproduktion, om de var av holsteinras, om de hade smutsiga juver och ben, om de inte hade en kalv som diade dem, om de inte fick foder direkt efter mjölkningen, om mjölkaren inte tvättade händerna och det var dålig hygien under mjölkningen, och om korna inte förmjölkades.

Vidare var det hög total bakterieförekomst och höga celltalsnivåer i mjölk både i besättningarna och på mjölksamlingsställena, vilket är ytterligare tecken på dålig juverhälsa och hygien, och att det sker kontamination eller tillväxt av bakterier längs transportvägen från besättning till mjölksamlingsställe. Bakterierna *Escherichia coli*, *Salmonella* spp. och brucellaantikroppar var vanligt förekommande i mjölken, och de är zoonotiska bakterier som kan innebära risker för folkhälsan. Det var ovanligt med antibiotikarester i mjölken.

Slutsatsen blir att subklinisk juverinflammation är vanligt förekommande i mjölkproducerande besättningar i Rwanda, och majoriteten av de sjukdomsframkallande bakterierna är av smittsam typ och i hög grad resistenta mot penicillin. Det höga bakterietrycket längs mjölkkedjan har betydelse för mjölkkvaliteten, för möjligheten att förädla mjölkprodukter och för folkhälsan. Den molekylära kartläggningen av *S. aureus* påvisar hög genetisk diversitet, vilket bör tas i beaktande i framtida studier av smittspridning. Ett kontrollprogram mot juverinflammation rekommenderas för att optimera mjölkproduktionen i Rwanda.

Acknowledgements

The work of this thesis was carried out at the Division of Reproduction, Department of Clinical Sciences, Swedish University of Agricultural Sciences.

I thank the former and current Heads of Department Björn Ekesten and Nils Fall, and the Coordinator of postgraduate studies Ylva Sjunnesson for ensuring my education ran smoothly in the department.

Funding for the project was provided by the Swedish International Development Agency (SIDA). Further funding was also provided by Feed the future innovation lab for livestock systems, University of Florida.

This thesis would not have been possible without the help and support from many people. I would like to thank all of you and in particular:

Associate Professor Renee Båge, main supervisor, for unwavering support throughout my PhD journey, for encouraging and motivating me when I felt weak, and for giving me time to ask any questions I had. Many thanks indeed.

Associate State Veterinarian Ylva Persson, for excellent supervision regarding mastitis, for excellent support regarding the work and for making sure I felt prepared to do what I had to do, for connecting me to staff at SVA, for support in writing manuscripts.

Associate professor Mikael Tukei for encouraging and teaching me great things in microbiology

Senior lecturer Karege Callixte for excellent support in Rwanda making sure I have all that I needed to carry out experiment in Rwanda.

Dr. Martin Ongol Patrick for encouraging me and giving me tips on how to be a good PhD student.

I would like to thank the extended supervision team that was added along the PhD journey:

Ann Nyman for unwavering support throughout my PhD journey on questions regarding statistics and statistic software. Without you it could have been very hard. Thank you for your enthusiastic courage while answering statistical questions

Karin Artursson for your time and always sharing ideas about microbiology.

I would like to thank colleagues at the Division of Reproduction, I have enjoyed sharing Fika time with each and every, with special thanks to Jane Morrell for linguistic review of the thesis.

I would like also to acknowledge and thank colleagues at University of Rwanda, College of Agriculture, Animal Sciences and Veterinary Medicine, especially staff in the microbiology laboratory.

I would like to acknowledge and thank staff at the innovation lab for livestock systems, University of Florida, for their time and sharing knowledge on how best to carry out capacity-building activities during the course of my PhD.

I would like to acknowledge and thank staff at University of Rwanda –Sweden program for the tireless support in administering funding on Rwandan side.

I would like to acknowledge and thank administrative staff at the Department of Clinical Sciences Swedish University of Agricultural Sciences for their tireless support in administering funding on the Swedish side

I would like to also acknowledge my fellow PhD student, and former PhD students Kristina, Theo, Gunilla, Tiger, Ida, Ziyad, Essraa, Elisabeth, Pongpreecha for fantastic company throughout the journey of PhD.

I would like to acknowledge staff in the Division of Reproduction for great work fellowship - you were so welcoming to me.

Special thanks goes to Maja Persson for endless support during laboratory analysis at SVA and ordering necessary materials for the project. Your support and smile encouraged me to complete the analysis.

I would like to acknowledge staff at the Department of animal health and antimicrobial strategies, and at the Mastitis laboratory, SVA, for their warm welcome during my time at the department.

I would like to thank Mikael Leijon for support in the bioinformatics and genotyping experiment I did at SVA.

I am very grateful to famers whose cows I sampled. Many thanks, and I hope the training and going through sampling can be helpful for your herds.

I would like to thank the family of Johan and Aleksia for warm welcoming me in Sweden

Finally, I would like to thank my family Murebwayire Marie Leonce, Izihirwe Maya Karmel and all extended family member for your emotional support throughout the whole journey of my PhD.

I

Tropical Animal Health and Production
https://doi.org/10.1007/s11250-019-01905-2

REGULAR ARTICLES

Aetiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda

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Received: 5 November 2018 / Accepted: 16 April 2019

Abstract
The aim of this cross-sectional study was to evaluate the prevalence of subclinical mastitis (SCM) and associated risk factors in dairy cows in peri-urban areas of Kigali, Rwanda, and identify causative udder pathogens. A sample of 256 cows from 25 herds was screened with the California Mastitis Test (CMT), and udder quarters with CMT score ≥ 3 (scale 1–5) were milk sampled for culture and final bacteriological identification with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). All resultant staphylococci species were tested for beta-lactamase production with the clover leaf method. In parallel, herd bulk milk somatic cell count (SCC) of each herd was analysed using a portable device, the DeLaval cell counter. The prevalence of SCM was 43.1% at quarter level and 76.2% at cow level based on CMT test. Multiparous, Holstein cows were 2.50 (C.I = 1.32–4.71) and 10.08 (C.I = 1.54–66.13) times more likely to contract SCM infection than primiparous animals or cows of other breeds, respectively. The median and mean SCC of all herds were 1108×10^3 cells/mL and 1179×10^3 cells/mL, respectively. The most prevalent pathogens were non-aureus staphylococci (NAS; 40.2%) followed by *Staphylococcus aureus* (22%) and less prevalent pathogens (6%). Samples with no growth or contamination constituted 30.4% and 1.4% of the diagnoses, respectively. The most prevalent species within NAS were *S. epidermidis* (38.2%) followed by *S. sciuri* (19.5%), *S. chromogenes* (9.8%), and nine less prevalent NAS species (32.5%). Out of 209 staphylococci isolates, 77% exhibited beta-lactamase production. The study shows that there is high prevalence of SCM and high herd bulk milk SCC in herds in Kigali, indicating udder health problems in dairy cows. Additionally, beta-lactamase production among staphylococci species was common. Improved milking hygiene and application of biosecurity measures, or a complete mastitis control plan, is required to lower the prevalence of SCM and minimize the spread of pathogens among dairy cows.

Keywords Beta-lactamase production · Risk factors · California Mastitis Test · Staphylococci

Introduction

Bovine mastitis is a common disease of high economic importance in dairy herds worldwide. The disease can be clinical—with visible signs of illness and milk abnormalities—or subclinical—without visible clinical signs and is affected by several environmental, animal, and management factors (Gruet et al. 2001; Ruegg 2017). Subclinical mastitis (SCM) is 15 to 40 times

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Published online: 28 April 2019

more prevalent than clinical mastitis (Seegers et al. 2003), and infected cows become major source of infection for healthier cows (Ruegg 2017). This happens when there is lack of implementation of preventive measures such as not wearing gloves during milking (Plozza et al. 2011) or not milking mastitis-infected cows last (Abebe et al. 2016). Cows with SCM have a reduced milk yield and produce milk of lesser quality (Bobbo et al. 2017), which affects farm revenues. Although it is difficult to harmonize methods used in evaluating losses due to both forms of mastitis, the total cost per cow per year was estimated to be 338 € in Sweden in 2010 (Nielsen et al. 2010), 240 € in the Netherlands between 2005 and 2009 (Van Soest et al. 2016), and $ 117.35 in the USA in 1979 (Blosser 1979). Economic estimations of losses associated with mastitis in Africa are rare and not well documented (Motaung et al. 2017); thus, there is an overall low motivation for its control.

Mastitis is a problem for the dairy industry in East Africa where the prevalence of SCM was 86.2% in Uganda (Abrahmsén et al. 2014), 75.9% in Tanzania (Karimuribo et al. 2008), and 62% in Ethiopia (Mekonnen et al. 2017). Mastitis is particularly a concern in Rwanda where there has been importation of high milk yield breeds, which are susceptible to mastitis, without parallel establishment of a mastitis control program (TechnoServe 2008). Mastitis is probably one of the factors that hinder the increase in net milk production yield in East Africa region (Motaung et al. 2017).

Several microorganisms are implicated in mastitis infection; some are environmental pathogens, whereas others are contagious pathogens (Ruegg 2017). The relative importance of each mastitis pathogen varies greatly according to the prevailing management practices of specific countries, or specific regions within the same country, and can change over time (Myllys et al. 1998). Successful mastitis control programs rely on accurate knowledge of the prevalent pathogens. Recently, MALDI-TOF MS has provided speed and reliability in routine analysis to identify mastitis pathogens in developed countries (Nonnemann et al. 2019). The relative importance of mastitis pathogen profile in SCM has not been comprehensively estimated in Rwanda.

Mastitis is the foremost disease that leads to the use of antimicrobials on dairy farm (Menéndez González et al. 2010). Imprudent use of antimicrobials can lead to a rise in antimicrobial resistance (AMR) (Kapoor et al. 2017), and there is risk of subsequent spread of resistant genes to other microbial populations (Xinglin et al. 2017). Resistance has been reported among mastitis pathogens where, for example, the frequency of resistance of *Staphylococcus* (*S.*) *aureus* isolates to penicillin was 47% in Italy (Moronic et al. 2006), 52% in Finland (Pitkälä et al. 2004), and 88% in Tanzania (Suleiman et al. 2018). Björk et al. (2014) reported that 80% of the investigated non-aureus staphylococci (NAS) isolates in Uganda were resistant to penicillin through beta-lactamase production. These levels of resistance could lead to failure of bacteriological cure of mastitis infections (Barkema et al. 2006). Therefore, there is a need not only to determine the level of resistance among mastitis pathogens, for monitoring purposes, but also to guide mastitis prevention and treatment strategies and to detect emerging AMR.

Despite mastitis being a problem for the dairy industry in Rwanda, knowledge about mastitis that could allow a customized control program remains limited. The aim of this study was to evaluate the prevalence of SCM and associated risk factors on cow level in dairy herds located in peri-urban areas of Kigali, in Rwanda. Additionally, the study aimed to identify causative udder pathogens and to evaluate the ability to produce beta-lactamase among resultant staphylococci species.

Materials and methods

Description of study area

The study was conducted in peri-urban areas of Kigali located in the geographic centre of Rwanda (− 1° 56′ 22.79″ S 30° 03′ 20.40″ E), surrounding the capital city. Herds located in peri-urban Kigali have higher herd size than the national average, and producers are directly linked to milk consumers or milk processors in the city of Kigali (TechnoServe 2008; MINAGRI 2013). Rwanda has a temperate tropical climate characterized by two seasons. The dry season happens in two periods of the year: the first from June to September and the second from December to February. The other season is wet, which also appears in two separate periods of the year: from February to June and then from September to December. Typical daily temperatures in Kigali range between 15 and 28 °C over the year (David 2007).

Study design

The study was reviewed, approved and performed in accordance with the ethics operational guidelines and the policy of the University of Rwanda, College Research Screening and Ethics Clearance Committee (RSEC-C) of the College of Agriculture Animal Sciences and Veterinary Medicine, University of Rwanda (UR-CAVM). The ethical guidelines of UR-CAVM were designed in accordance with international standards. Sample size was determined according to Dohoo et al. (2009) as follows:

$$n = \frac{z^2 p(1-p)}{L2}$$

where:

n sample size, 1.96 = the value of Z at 95% confidence interval

P expected prevalence
L desired absolute precision

Therefore, the sample size was determined at 95% confidence level, 6% precision, and with an expected prevalence of 52% from a previous study in Rwanda (Iraguha et al. 2015), thus yielding a sample size of 266 dairy lactating cows. The trained research team and local veterinarian of each subregion used a snowball sampling technique, as described by Faugier and Sargeant (1997), to locate the dairy herd to be enlisted in the study to the north, east and west relative to the centre of Kigali city. A herd was recruited if it had a minimum of five lactating cows, and the herd owner agreed to participate in the study. If a herd had more than 20 lactating cows, only 50% of the cows were included in the study. In total, 256 lactating cows from 25 herds kept in Kigali peri-urban areas were successfully examined. Herds were visited once between May and September 2016.

Screening for mastitis, milk sampling and bulk somatic cell count measurements

Udder quarter milk samples were collected during ongoing milking by selected trained personnel. For each udder half, the first two or three strips of milk were inspected for milk abnormality and discarded, followed by CMT testing. Subclinical mastitis prevalence was evaluated by CMT using the Scandinavian scoring system (grades 1–5), where 1 indicates negative result (no gel formation, no indicative colour change), 2 is traceable (possible infection) and 3 or above indicates a positive result where 5 has the most gel formation and deep blue/violet colour change (Schalm et al. 1971; Saloniemi 1995). A cow was defined as positive for SCM if she had at least one positive quarter (≥ 3), with no signs of illness and/or visible inflammatory signs of the udder and without visible abnormality in milk. Quarters with CMT ≥ 3 were recorded and sampled for bacteriological analyses according to the National Mastitis Council (NMC) (2017). After cleaning the teat ends with 70% alcohol, an aseptic milk sample was collected in a 10-mL sterile tube and samples were placed and transported on ice inside a cooler box to the microbiology laboratory of the University of Rwanda, College of Science and Technology, Nyarugenge Campus for culture and identification of SCM causative agents (NMC 2017). In parallel, bulk milk samples were collected from each herd and transported in the same manner to the laboratory for SCC analysis with the DeLaval Cell Counter (DCC; DeLaval International AB, Tumba, Sweden). Potential animal factors related to mastitis including breed (three categories: Holstein, Holstein local cross–breed, and other breeds which in turn included local breed, cross-breeds between Jersey and local breeds, and cross between Sahiwal and local breeds), parity (primiparous versus multiparous), stage of lactation (three categories: < 3 months in days in milk (DIM); 4 to 7 months in DIM; > 7 months in milk), calf suckling (yes versus no) and milk production (as continuous variable) were recorded at the time of herd visit through an interview with the herdsman and by observation.

Bacteriological analyses

All milk samples were cultured on blood agar plates (5% bovine blood with 0.5% esculin) and incubated aerobically at 37 °C for 24 to 48 h before final examination. To be classified as a positive bacterial growth, at least one colony-forming unit (CFU) was needed for the following major pathogens: *S. aureus*, *Streptococcus* (*Str.*) *uberis*, *Str. agalactiae*, and *Klebsiella* spp., and at least five CFUs for the other genera. Samples were classified as contaminated if two or more bacterial types were isolated from one milk sample and growth of the mentioned major pathogens was not identified. If growth of a major udder pathogen was found in combination with contaminating species and the CMT was high, the sample was diagnosed as positive for growth of a major pathogen. Positive isolates were initially characterized based on colony morphology; α-, β-, or double hemolysis; and Gram reaction. Gram-positive isolates were further subjected to catalase and coagulase tests. All isolates, except 45 NAS (out of 169 NAS), were preserved in agar tubes and brought to an accredited laboratory, which is the National Veterinary Institute (SVA: accreditation number 1553 ISO/IEC 17025) in Uppsala, Sweden, for final identification of causative organisms at species level using MALDI-TOF MS. At SVA, each bacterial sample was first re-cultured on horse blood agar, and material from single pure colonies was spotted on a MALDI-plate without pre-treatment. The spots were covered with 1 μL matrix solution consisting of α-cyano-4-hydroxycinnamic acid (HCCA). Subsequently, isolates on MALDI-plate were analysed by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) to identify the species. Mass spectra were compared against 4613 spectra in the MALDI Biotyper database using the MALDI Biotyper 3.0 Real-time Classification (RTC) software (Bruker Daltonics, Bremen, Germany). The identification and classification of udder pathogens were done according to MALDI-TOF MS spectra score where a score of ≥ 2.0 was considered reliable identification at species level, a score of ≥ 1.7 to < 2.0 was considered reliable identification to genus level and a score of < 1.7 was considered as no identification. All Staphylococcal isolates were examined individually for beta-lactamase production by the "clover leaf" method as described by Bryan and Godfrey (1991). For quality control, the strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used. Identified isolates were stored in trypticase soy broth containing 15% glycerol at − 80 °C.

Data analysis

The data were recorded in a Microsoft Excel spreadsheet before statistical analysis. The prevalence of SCM was calculated as the number of mastitis-positive cows (one or more quarters with SCM) divided by the total number of cows tested. The quarter SCM prevalence was calculated as number of quarters with SCM divided by the total number of quarters investigated.

To evaluate risk factors associated with SCM, unconditional associations between each independent variable (breed, parity, stage of lactation, calf suckling and milk production) and the dependent variable, cow SCM status (0 = negative and 1 = positive) were investigated using univariable logistic regression analysis. Statistical significance in this step was assessed at P value < 0.2. Factors that were significant were then investigated using Spearman rank correlation to assess collinearity, and if two variables showed collinearity ($r \geq 0.70$), the one with the lowest P value was then offered to the multivariable logistic regression model. Initially, the multivariable mixed effect logistic regression model, with herd included as random variable, was used. However, herd as random effect was not significant; thus, the ordinary logistic regression model was used. The multivariable logistic regression model was reduced using a manual, stepwise backward variable selection procedure, where the initial model included all independent variables as main effects. Variables with a significant association ($P \leq 0.05$) with the dependent variable were kept in the model. All plausible two-way interactions between the significant main effects were tested in the final models. Model fit was assessed by Hosmer–Lemeshow goodness-of-fit test. The statistical analyses were performed using SPSS, version 20.0 for Windows (IBM Corp, Released 2011, IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Descriptive data

The studied cows had an average milk production of 9.5 L (range 2–22 L) per cow per day. One herd used milking machine; the remaining herds were milked by hand. All cows were milked twice a day. None of the visited herds screened cows regularly for SCM with CMT or included dry cow therapy in their management practices. Approximately 107, 138 and 11 cows were kept in zero-grazing, semi zero-grazing and grazing systems, respectively. The mean herd size was 35 (range 6–200), whereas the median was 22 head (range 5–40) of cattle (lactating cows, dry cows, heifers and weaned calves, inclusive) and the median of lactating cows was 10. There were 2 cows of local breed, 209 cows of cross-breeds between Holstein and local breeds, 3 cows of cross-breeds between Jersey and local breeds, 41 cows of Holstein (pure breed) and 1 cow that was a cross between Sahiwal and local breeds, among the sampled cows.

In total, 256 cows in 25 herds (with 4–20 screened cows per herd) were studied, and 1024 udder quarters were examined. However, there were 20 blind quarters, resulting in 1004 quarters being properly screened for SCM. At the time of quarter sampling, one herd had only four lactating cows. Additionally, 12 quarters from another herd were screened with CMT but sampling was not possible due to cows' behaviour. In total, 418 quarter milk samples were cultured for bacteriological analyses.

Prevalence of subclinical mastitis and herd bulk milk somatic cell count distributions

Cow level prevalence of SCM was 76.2% (195 of 256), whereas quarter level prevalence was 43.1% (433 out of 1004). Out of 195 cows with SCM, 129 cows had more than one udder quarter affected. Subclinical mastitis was more common in rear quarters (46.1%; 230 out of 499) than in front quarters (40.1%; 203 out of 505). Median of quarter CMT was 2 (range 1–5). The median and mean bulk-milk SCC for all herds visited was 1108×10^3 cells/mL (central range = 760–1531 cells/mL) and 1179×10^3 cells/mL (± 534 cells/mL), respectively. Only two herds had bulk-milk SCC below the SCC limit of 400×10^3 cells/mL, a level set according to EC regulation 853 (EC 2004) regarding herd milk intended for use as liquid milk and human consumption. The herd with the lowest bulk-milk SCC had 352×10^3 cells/mL, and the herd with the highest bulk-milk SCC had 2196×10^3 cells/mL.

Distribution of udder pathogens

Of the 418 cultured quarter milk samples, 127 (30.4%) were bacteriologically negative; the rest (69.6%) of the quarter milk samples were bacteriologically positive. The most common bacteriological finding was NAS (40.2%) followed by *S. aureus* (22%). Less common were *Acinetobacter iwoffi* (1%), *Enterococcus faecium* (0.7%), *Str. uberis* (0.7%), *Str. agalactiae* (0.7%), *Klebsiella* (*K.*) *oxytoca* (0.5%), *K. variicola* (0.5%), *Pseudomonas rhodesiae* (0.2%) and lastly *P. fluorescence* (0.2%). All above-mentioned species had MALDI-ToF M.S. spectra ≥ 2.0. *Staphylococcus* spp. that were identified at genus level were 1.2% (MALDI-ToF M.S. spectra: 1.7 ≤ score < 2), whereas contaminated samples were 1.4%. Only 0.2% of isolates were not identified by MALDI-ToF MS (score < 1.7). Of the 168 NAS, 123 were subtyped, and the most prevalent NAS species was *S. epidermidis* (38.2%), followed by *S. sciuri* (19.5%). Less common species were *S. chromogenes* (9.8%), *S. xylosus* (8.9%), *S. haemolyticus* (7.3%) and *S. capitis* (6.5%). Other less prevalent NAS species included *S. saprophyticus* (3.3%), *S. pasteuri* (2.4%), *S. devriesei* (1.6%), *S. kloosii* (0.8%), *S. lugdunensis* (0.8%) and *S. warneri* (0.8%). Out of 129 cows

that had more than one udder quarter with SCM, 44.9% (58 out of 129) were also bacteriologically positive in more than one quarter. Furthermore, 65.5% (38 out 58) had at least the same bacteriological finding in two of the infected quarters.

Animal factors associated with subclinical mastitis

Data on prevalence of SCM in each category of selected potential risk factors are presented in Table 1. Initially, mixed effects logistic regression model was used, but as the random effect was not significant, the ordinary logistic regression model was used. Individual factors associated with SCM in the univariable logistic regression analysis ($P \leq 0.20$) were breed, parity, stage of lactation and calf suckling. There was no statistical evidence of any high correlations among these variables. A significant ($P < 0.05$) association was seen between parity and SCM as well as between breed and SCM. Multiparous cows had significantly ($P < 0.05$) higher odds to contract SCM compared to primiparous cow; Holstein cows and Holstein cross with local breeds had higher odds for SCM than cows of other breeds (Table 2). Hosmer–Lemeshow goodness-of-fit test suggested that the model fit the data ($P = 0.98$).

Beta-lactamase production among staphylococci species

In total, 209 staphylococci isolates were individually tested for beta-lactamase production. Based on the clover leaf method, 77% (161of 209) of tested isolates exhibited phenotypic resistance to penicillin and thus were reported as positive. Beta-lactamase production was most prevalent in *S. xylosus*, *S. aureus* and *S. pasteuri* isolates (Table 3).

Discussion

The findings of this study showed that the prevalence of SCM (76.2%) and bulk-milk SCC was high in herds in the Kigali region. This is higher than the prevalence levels of below 15% that can be achieved through appropriate udder health care (Ruegg and Pantoja 2013) or 52% (Iraguha et al. 2015) and 50.4% (Mpatswenumugabo et al. 2017) previously reported in north-west and east of Rwanda, respectively. The overall high SCM prevalence may be due to the lack of mastitis control practices, such as the five-point mastitis control plan, which has proved to decrease the prevalence of mastitis and lower bulk milk SCC in the last decades in developed countries (Hillerton et al. 1995). The zero-grazing system particularly prevalent in Kigali (MINAGRI 2013) favours increased infection pressure around the cow, especially when a mastitis control plan is not implemented, resulting in a higher chance of infection in cows in Kigali than in other regions where "non-zero" grazing systems are prevalent. Low farmers' awareness of SCM (FAO 2014) and the lack of quality or payment standards for bulk milk SCC in Rwanda could explain a low motivation to improve management practices known to prevent and control mastitis, which probably explain the high level of SCM prevalence found in this study.

Our study found that NAS were the most prevalent pathogens causing intramammary infections in SCM cases, followed by *S. aureus*. MALDI-TOF MS used in this study is used routinely

Table 1 Descriptive statistics and results from univariable logistic regression analysis of associations between animal-level factors and the prevalence of subclinical mastitis defined as a score of ≥ 3 in the California Mastitis Tests in 256 dairy cows in peri-urban areas of Kigali, Rwanda

Factors	CMT negative	CMT positive	SCM prevalence (%)	*P* value
Breed				0.077
Holstein	5	36	87.8	
Holstein local cross breed	49	160	76.6	
Others	3	3	50.0	
Parity				0.012
Primiparous	24	48	66.7	
Multiparous	33	151	82.1	
Stage of lactation				0.106
Early lactation (1 to 3 months)	21	51	70.8	
Mid (lactation four to seven)	29	103	78.0	
Late lactation (equal and above eight)	7	45	86.5	
Milk production (mean in L per day)	9.1	9.6		0.464
Calf suckling				0.039
No	18	93	83.8	
Yes	39	106	73.1	

Others = Jersey local crossed breed, Sahiwal breed and local breed

CMT California Mastitis Test

Table 2 Final multivariable logistic regression model describing the association between risk factors and subclinical mastitis defined as a score of ≥ 3 in the California Mastitis Tests in 256 dairy cows in peri-urban areas of Kigali in Rwanda

Factor	B	S.E	O.R	95% C.I.	*P* value
Parity					
Primiparous	Ref				
Multiparous	0.92	0.32	2.50	1.32–4.71	0.005
Breed					0.048
Other	Ref				
Holstein cross with local breed	1.49	0.84	4.44	0.85–23.15	0.077
Holstein	2.31	0.96	10.08	1.54–66.12	0.016

Other = local breed, cross breeds between Jersey and local breeds, cross between Sahiwal and local breeds
OR odds ratio, *C.I.* confidence interval

for identification of mastitis bacteria at SVA, thus ensuring large library in database for control. This study identified, for the first time in Rwanda, the profile of NAS pathogens involved in SCM at species level. Recent studies conducted in Uganda (Abrahmsén et al. 2014), Ethiopia (Mekonnen et al. 2017) and Rwanda (Mpatswenumugabo et al. 2017) also found that both NAS and *S. aureus* were prevalent in intramammary infections. The increasing importance of NAS in intramammary infections is also reflected in their high prevalence in other geographic regions, such as Germany (Tenhagen et al. 2006), Sweden (Persson et al. 2011), Belgium (Piessens et al. 2011) and the USA (Schukken et al. 2009). The spread and reservoirs of each NAS species depend on several factors including housing systems, management factors, herd size and climate (Nyman et al. 2018). For example, *S. epidermidis*, which is an udder-adapted pathogen, has been isolated on human skin and can spread from cow to cow (Thorberg et al. 2006; Sawant et al. 2009; Piessens et al. 2011). These conditions, together with suboptimal hygiene at farm level in Rwanda (TechnoServe 2008), suggest that *S. epidermidis*, found as the most prevalent NAS species in this study, could have originated from milkers' hands and spread from cow to cow. Similarly, *S. chromogenes* could have originated from either the udder or the milker's hands, since these sites are known to be reservoirs of the pathogen and, rarely, the environment. On the other hand, *S. haemolyticus* may have originated from the environment since it is principally an environmental mastitis pathogen (Piessens et al. 2011).

Table 3 Prevalence in beta-lactamase production (β±) among staphylococci (S.) isolates from cases of subclinical mastitis in dairy cows in peri-urban areas of Kigali in Rwanda

	Total isolates	Positive	β+ (%)	Negative	β− (%)
S. aureus	89	78	87.6	11	12.4
S. epidermis	46	34	73.9	12	26.1
S. sciuri	23	12	52.2	11	47.8
S. chromogenes	9	6	66.7	3	33.3
S. xylosus	9	9	100	0	0.0
S. captis	8	5	62.5	3	37.5
S. haemolyticus	8	6	75.0	2	25.0
S. pasteuri	8	7	87.5	1	12.5
S. saprophyticus	4	2	50.0	2	50.0
S. devriesei	2	1	50.0	1	50.0
S. kloosii	1	0	0.0	1	100
S. lugdunensis	1	0	0.0	1	100
S. warneri	1	1	100	0	0.0
Total	209	161	77.0	48	23.0

The cause of the high prevalence of *S. aureus* reported in this study could be multifactorial. Beside unhygienic hand milking and lack of a mastitis-control plan mentioned earlier, contagious spread of *S. aureus* in the studied herds could also have been facilitated by not milking mastitis-infected cows last. This practice was found to be associated with mastitis in Ethiopia (Abebe et al. 2016), where conditions are likely similar to Rwanda. Since culling of *S. aureus*–infected cows is not practiced in Rwanda, this implies that the pathogen found in SCM cases in studied herds might have originated from chronic or persistent infections. Major environmental pathogens were less frequent in SCM cases in the studied herds; thus, measures need to focus on combating contagious pathogens. It is worth to note that SCM may be present despite negative bacteriological culture. This may be due to low numbers of bacteria below the limit of detection in the sample, concurrently with a time of sampling when the immune system has successfully eliminated infection before decreasing SCC or with organisms being shed intermittently (Östensson et al. 2013).

The higher risk of SCM associated with increased parity in this study may be attributed to the fact that multiparous cows in the studied herds might have had cumulatively several exposures to mastitis pathogens from suboptimum hygiene during milking or from the environment. Poor integrity of the teat canal due to ageing that leads to easy access of bacterial infection to the mammary gland after milking, decreased immunity or a more pendulous udder prone to injury in older cows than younger cows might all have increased the susceptibility of the older animals to mastitis (Suleiman et al. 2018). It has been reported that the Holstein breed is susceptible to mastitis (Bludau et al. 2014). The higher prevalence of SCM in the Holstein breed than in the Holstein-/local cross-breed or in other breeds reported in this

study reflects recent trends in the cattle population in Rwanda, where mastitis susceptible breeds, mainly Holstein, have been increasing since 2006 (TechnoServe 2008) without parallel improvement in udder health care practices.

Beta-lactamase production is the most common resistance mechanism in staphylococci against widely used beta-lactam antibiotics (Livermore and Brown 2001). Beta-lactamase production was common in this study with varying levels of prevalence within staphylococci species from SCM cases. Beta-lactamase prevalence was higher is *S. aureus* and *S. xylosus* than in other isolates. The results are markedly higher than levels of beta-lactamase production in selected mastitis isolates in Sweden (38%), in Finland (32%) and in the Netherlands (37%) (Pitkälä et al. 2004; Sampimon et al. 2009; Persson et al. 2011). The high prevalence of beta-lactamase producing udder pathogens might be due to low application of best practices for infectious disease control, such as culling cows infected with beta-lactamase-producing *S. aureus* strains, mastitis testing before animal trade, proper disinfection during hand milking or other biosecurity measures. Active monitoring for beta-lactamase production among mastitis pathogens, with subsequent control of infected cows, is required to counteract spread of beta-lactamase producing isolates within and among herds in Rwanda.

In conclusion, the high prevalence of SCM and dominance of contagious pathogens indicates that infected cows could be the major source of the infection in the studied herds. Some herds had low bulk-milk SCC, which implies that it is possible to have cows with good udder health in Rwanda. The study also reported that most identified staphylococci species pathogens exhibited beta-lactamase production. Application of biosecurity measures, such as grouping and milking mastitis high-risk cows last, good hygiene and raising awareness of a mastitis control plan among farmers, should be incorporated into herd management practices to effectively improve udder health in Rwanda.

Acknowledgments The authors would like to acknowledge funding from the Swedish International Development Agency (SIDA), within the "UR-Sweden programme for research, higher education and institutional advancement, subprogram agricultural sciences", project no. 20290000. The authors also thank Maria Persson for her assistance in MALDI-TOF MS and during the experiment at SVA and Charlotta Fasth for her assistance in mastitis bacteriology. Authors also thank all staff at the mastitis laboratory at SVA and all staff at the microbiology laboratory at the University of Rwanda. Finally, authors thank Jane Morell for language proofread.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved and performed in accordance with the ethics operational guidelines and procedures of the policy of the review to the College Research Screening and Ethics Clearance Committee (RSEC-C) of the College of Agriculture Animal Sciences and Veterinary Medicine, University of Rwanda (UR-CAVM). The ethical guidelines of U.R-CAVM were designed in accordance with international standards and guidelines for the care of animals; all participating herd owners gave their informed consent prior to inclusion in the study.

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Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2019:77

This thesis investigated prevalence and aetiology of subclinical mastitis (SCM) in dairy cows, antimicrobial resistance and molecular epidemiology of udder pathogens, and effects on milk quality. The results indicated that mastitis is common in dairy cows in Rwanda and it is associated with factors including poor hygiene, absence of foremilk stripping, increasing stage of lactation, Holstein breed, lack of calf suckling. Resultant udder pathogens were resistant to penicillin Establishing a mastitis control plan is recommended.

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ISSN 1652-6880
ISBN (print version) 978-91-7760-472-3
ISBN (electronic version) 978-91-7760- 473-0