Pathotyping and antimicrobial resistance-characterization of *Staphylococcus aureus* in milk for human consumption in Marsabit and Isiolo Counties, Kenya

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DECLARATION

This is my original work and I declare that it has not been submitted for award of a degree in any other University.

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Dedicated to my lovely wife and friend, Elicah, daughter Briellah and son Omwenga Jnr

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LIST OF ABBREVIATIONS

Agr:	Accessory gene regulator
AMR:	Antimicrobial resistance
AMU:	Antimicrobial use
ASAL:	Arid and Semi-arid lands
AST:	Antimicrobial Sensitivity Test
ATCC:	American Type Culture Collection
BLAST:	Basic Local Alignment Search Tool
Ccr:	Cassette chromosome recombinase
CLSI:	Clinical & Laboratory Standards Institute
EU:	European Union
HA-MRSA:	Healthcare-Acquired Methicillin-resistant *Staphylococcus aureus*
CA-MRSA:	Community associated Methicillin-resistant *Staphylococcus aureus*
IWG-SCC:	International Working Group on the Classification of Staphylococcal Cassette Chromosome
MDRSA:	Multidrug-resistant and methicillin-resistant Staphylococcus
MSSA:	Methicillin-susceptible *S. aureus*

MRSA: Methicillin-resistant *Staphylococcus aureus*

PBP 2a': penicillin-binding protein 2a (peptidoglycan transpeptidase)

PBP2: penicillin-binding protein 2

PCR: Polymerase chain reaction

SCCmec: Staphylococcal Cassette Chromosome mec

SEA: Staphylococcal enterotoxin A

SEB: Staphylococcal enterotoxin B

SEC: Staphylococcal enterotoxin C

SED: Staphylococcal enterotoxin D

SEE: Staphylococcal enterotoxin E

SSSTI: serious skin and soft tissue infections

SEs: Staphylococcal enterotoxins

SFP: Staphylococcal food poisoning

ABSTRACT

Staphylococcus aureus (S. aureus) is a ubiquitous Gram-positive bacterium commonly encountered in the environment as well as mucus membranes of animals and humans. *S. aureus* food poisoning results from consumption of preformed *S. aureus* enterotoxins in food. The risk posed by contamination of milk intended for human consumption by pathogenic *S. aureus* in pastoral areas in Kenya is still not well documented, yet this information is critical for ensuring safety to consumers who sometimes may take unpasteurized milk. The prevalence of antimicrobial resistant (AMR)-*S. aureus* including methicillin-resistant *S. aureus* (MRSA) in livestock raw milk consumed by pastoralists in Kenya remains unclear. This study therefore determined the phenotypes, genetic determinants for virulence associated with *S. aureus* enterotoxins, the phenotypic resistance profiles and the genetic determinants responsible for antimicrobial resistance phenotypes of milk borne *S. aureus*. In addition, the relationship between antimicrobial usage (AMU) and emergence of multidrug-resistant (MDR) *S. aureus*, including MRSA in raw milk of livestock was determined by correlation analysis.

A cross-sectional study design involving 188 households in the two counties was conducted. In total, 603 milk samples from 57 zebu cattle, 346 Galla goats, 8 red Maasai and dorper sheep, 4 one-humped camels (*Camelus dromedaries*) and 188 pooled from all animals were collected from Isiolo and Marsabit counties of Kenya. *S. aureus* isolates were cultured from milk samples using a selective media, mannitol salt agar (MSA). Suspect colonies of *S. aureus* were further analysed using biochemical tests including Gram staining, catalase activity, mannitol fermentation, coagulase activity and β-hemolysis. The isolates were confirmed by amplification of *S. aureus* specific staphylococcal terminase gene (*satm*) and BLAST analysis.

The isolates confirmed to be potentially pathogenic *S. aureus* were further evaluated to determine whether they harbour enterotoxin genes responsible for milk-borne food poisoning. Oligonucleotide primers were designed and used for the amplification of *sea*, *seb*, *sec*, *sed* and *see* enterotoxin encoding genes. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards Institute Guidelines. Moreover, genetic determinants responsible for the resistance phenotypes of *S. aureus* were analyzed by PCR, sequencing, and Blast analysis. Genes encoding for resistance to oxacillin (*mec*A), tetracyclines (*tet*K, *tet*M), other beta-lactams (*bla*Z), aminoglysosides [*aac* (*6')/aph (2"), aph (3')-IIIa*], and macrolides (*msr*A*/erm*A) were used for the analysis. Data on the commonly used and/or sold antimicrobials in the study area were collected from wholesalers and veterinary pharmacies for a period of one year. Correlation between AMU and occurrence of resistance was determined by Pearson's correlation coefficient (r) method.

Overall, potentially pathogenic *S. aureus* harboring enterotoxic genes were detected in 85 (14.09%, 95% CI: 11.55–17.1%) of the total milk samples. Genes encoding enterotoxins were detected in the *S. aureus* bacteria isolated from the milk samples. At least one type of *S. aureus* enterotoxin gene (*SE*) was detected in 74.11% (95% CI: 63.91–82.24%) of the 85 isolates. The most frequently encountered gene in the two counties was *see* (51; 60%, 95% CI: 49.73–69.76%) followed by *sea* (22; 25.88%, 95% CI: 17.76–36.09%) and *sec* (19; 22.35%, 95% CI: 14.8–32.29%). None of the isolates tested positive for *sed*. Overall, 21 of the 85 (24.7%, 95% CI: 16.76–34.83%) strains harbored more than one enterotoxin gene. More than half of the *S. aureus* isolates harbored at least one of the enterotoxin coding genes, indicating milk samples contaminated by *S. aureus* could have a high chance of causing staphylococcal food intoxication.

The *S. aureus* isolates were mainly resistant to tetracycline (79%), ampicillin (58%), and oxacillin (33%), respectively. A few isolates (5–18%) were resistant to clindamycin, cephalexin, erythromycin, kanamycin, and ciprofloxacin. Most of the MDR-*S. aureus* isolates were MRSA (94%). The genetic determinants found in the AMR isolates included *tet*K/*tet*M (96.5%/19%) for tetracycline, *bla*Z (79%) for penicillin, *aac (6')/aph (2")/aph (3')-IIIa* (53%) for aminoglycosides, *mec*A (41%) for oxacillin, and *msr*A/*erm*A (24%/7%) for macrolides.

The antimicrobial classes used in the study site were as follows; 4,168 kg of oxytetracycline, 70 kg of sulfonamides, 49.7 kg of aminoglycosides, 46 kg of beta-lactams, 39.4 kg of macrolides, and 0.52 kg for trimethoprim. Oxytetracycline usage was correlated to *tet*K/*tet*M (r = 0.62/1) detection, penicillins to *mec*A/*bla*Z (r = 0.86/0.98), aminoglycoside to *aac (6')/aph (2")/aph (3')-IIIa* (r = 0.76/-13), and macrolide usages for detection of ermA/msrA (r = 0.94/0.77). There was association between AMU and the occurrence of MDRSA and the *tet*M detection.

In conclusion, milk consumed in Marsabit and Isiolo counties is contaminated with multidrug resistant *S. aureus* that harbour enterotoxigenic and antimicrobial resistance genes hence its consumption could increase the risk of staphylococcal food poisoning. AMU appeared to be associated with occurrence of MDRSA and the *tet*M detection. Judicious use of antimicrobials is recommended in the study areas to mitigate against development of AMR

Key Words: *Staphylococcus aureus*: raw milk: enterotoxins genes; Antimicrobial usage; Multidrug resistant-MRSA; Kenya

CHAPTER 1: INTRODUCTION

1.1 Background

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that is responsible for a wide range of infections in both humans and animals. In humans, *S. aureus* causes skin, soft tissue infections and bacteremia. *Staphylococcus aureus* is one of the most common causative agents of subclinical and clinical mastitis in dairy farms resulting in significant economic losses (Madzgalla *et al*., 2016; Thomer *et al*., 2016; Gussmann *et al*., 2019). The bacterium is widely implicated in food poisoning outbreaks in humans and has been associated with food products from various parts of the world, including milk (Chiang *et al*, 2008; Zouharova and Rysanek, 2008). A pH range of between 4.2 to 9.3 and temperature range of 7 ^{0}C to 48.5 ^{0}C provides optimum conditions for the growth of *S. aureus*. The bacterium can adapt to grow in various foods and causes food poisoning by secreting enterotoxins. Production of Staphylococcal enterotoxins (SEs) rapidly increases at suitable temperatures and pH ranges of 20–37 ^{0}C and pH 4–7.4 respectively (Tamarapu *et al*., 2001; Grispoldi *et al*., 2019). Rapid onset of nausea, vomiting, diarrhea and abdominal cramps are characteristic of food poisoning resulting from *S. aureus* (Suzuki, 2019). Enterotoxin production by some strains of *S. aureus* is the single most important virulence factor responsible for staphylococcal food poisoning (Suzuki, 2019). A wide variety of enterotoxins are produced by *S. aureus* strains. The ability of *S. aureus* to produce enterotoxins and occurrence of food poisoning of staphylococcal origin have shown a significant association in previous studies (Suzuki, 2019). A variety of foods can be contaminated by SEs,

especially foods high in moisture containing starch and protein, for example meat, poultry and egg products, milk as well as other dairy products.

Milk provides a good medium that supports the growth of *S. aureus* as well as enterotoxin production. Pasteurization of raw milk eliminates *S. aureus* from milk; however, once SEs have been produced, they remain stable after pasteurization (Yehia *et al*., 2019). Staphylococcal eneterotoxins are highly heat resistant, an example being staphylococcal enterotoxin A (*sea),* that was exposed to a temperature of 121°C for 28 minutes and still remained biologically active (Yehia *et al*., 2019).

A very low level of SEs, in the range of 20 ng to < 1 μg, is adequate to cause classical staphylococcal intoxication. For example, the LD_{50} of staphylococcal enterotoxin B (SEB) in monkeys is 0.02 μg/kg of body weight (Verreault, 2019). Consequently, SEB has been identified as a restricted agent. Therefore, SEs pose a threat to both food safety and food security especially when produced in a purified form that can be used as a deliberate adulterant. Consumption of milk and other dairy products is the main source of exposure of humans to enterotoxins (Owusu-Kwarteng *et al*., 2020). It is therefore necessary to investigate the capability of *S. aureus* to produce enterotoxins in order to prevent the public from the health risk associated with milk and dairy product consumption.

Antimicrobials are essential in the treatment of staphylococcal food poisoning. Food poisoning with milk-borne antimicrobial-resistant (AMR) *S. aureus* is currently gaining attention in both developed and developing world (Asiimwe *et al*., 2017). For example, AMR *S. aureus* has been isolated from milk value chain in various countries in Africa such as Uganda, Egypt, Tunisia, Algeria, Nigeria and other world regions, including Asia, Europe and the United States of

America, (Al-Ashmawy *et al*., 2015; Ahmed *et al*., 2020; Klibi *et al*., 2018; Titouche *et al*., 2019). Most of the antimicrobial agents used for the treatment of human infections are also used in animal health (Toutain *et al*., 2016). For instance, tetracyclines, beta-lactams, streptogramins, linconsamides, fluoroquinolones, sulfonamides, rifamycins, macrolides, and aminoglycosides have been used to control *S. aureus* illnesses in both animals and humans (Vestergaard *et al*., 2019). Consequently, there is an increase in the cases of AMR *S. aureus* resulting from either overuse and/or misuse of the drugs during livestock production (Kateete *et al*., 2013). This extensive use of antibiotics has resulted in selective pressure that is responsible for the appearance and spread of AMR *S. aureus*, including the multidrug-resistant (MDR) isolates (Gomes and Henriques, 2016). Previous studies have reported *S. aureus* be highly resistant to commonly used antimicrobial agents thereby presenting major public health challenges to humans (Kateete *et al*., 2013; Vestergaard *et al*., 2019). *Stapyloccocus aureus* manifests resistance phenotypes to beta-lactams such as penicillins and cephalosporins, as well as tetracyclines, fluoroquinolones, macrolides, and aminoglycosides (Faires *et al*., 2010). Consequently, cases of prolonged hospital admissions reduced therapeutic efficacy of the antimicrobial agents, increased virulence, high cost of treatment and even mortalities have been reported from staphylococcal infections (Li *et al*., 2018).

Staphyloccocus aureus can develop resistance to beta-lactams by degrading the antibiotics through the production of beta lactamases. The beta-lactamases that hydrolyze penicillins and cephalosporins are encoded by *bla*Z gene. Alternatively, some strains of *S. aureus* may acquire an altered penicillin binding protein 2a' (PBP 2a') encoded by *mec*A gene (Siiriken *et al*., 2016). The *S. aureus-Mec*A gene encodes resistance phenotypes to methicillin yielding the methicillin-

resistant *S. aureus* (MRSA). The MRSA are well known to be exhibit resistance to a range of antibiotics commonly used for the treatment of livestock and humans thereby complicating treatment outcomes (Lobanovska and Pilla, 2017). Indeed, the MDR phenotype is a particular characteristic of the MRSA strains (Herrmann *et al*., 2013; Egyir *et al*., 2014). The *mec*A gene encoding the MRSA phenotypes is usually present in the staphylococcal cassette chromosome *mec* (SCC*mec*) (Siiriken *et al*., 2018). The SCC*mec* subtypes I, II, III, IV, and V are known to harbor other AMR genetic determinants, such as *blaZ*, *tet*K, *tet*M, *aac (6')/aph (2'')*, *aph (3')-IIIa*, *msr*A, and *erm*A (Katayama *et al*., 2000). These genetic determinants are believed to be responsible for the emergence of MDR MRSA. The *tet*K and *tet*M gene are known to confer resistance phenotypes to tetracyclines, whereas *aac (6')/aph (2'')* and *aph (3')-IIIa* genes confer resistance to the aminoglycosides. The presence of AMR genes in mobile genetic elements, such as SCC*mec*, may lead to horizontal transfer of the molecular genetic determinants among *S. aureus* strains in clinical set up and along various food chains, including the dairy value chain (Igbinosa *et al*., 2016).

Many studies show that continuous antimicrobial usage (AMU)) resulting from misuse and overuse in both animal and human selects for AMR, and that reducing AMU often results in reduced AMR (Valsangiacomo *et al*., 2000). Furthermore, with the increasing disease burdens and extensive livestock production, the global antimicrobial use is on the rise (Van Boeckel *et al*., 2017). In addition, antibiotics used in the prophylaxis and treatment of infectious diseases in livestock and human health, including the critically important antibiotics, are similar by pharmacological classification (Feikin *et al*., 2011). Therefore, resistant zoonotic strains in

animals may lead to infections of humans that are difficult to treat as is the case with MDR-MRSA.

Recently, there has been an increase in reported cases of MRSA contamination in food. For example, MRSA is known to contaminate foods of animal origin, like food products that were recently sampled from passengers aboard flights originating from 45 non-European states at various international airports in EU (Rodrı´guez-La´zaro *et al*., 2017). The contamination has also been reported in various foods sampled from markets near the EU land borders. Furthermore, increase in MRSA contamination in milk has been reported in many countries, including China, Italy, and Uganda (Kasozi *et al*., 2014; Wang *et al*., 2018). Previous studies indicate that AMU by livestock farmers and animal health professionals is rampant in pastoral areas of northern Kenya (Lamuka *et al*., 2017). Consequently, it is possible that the overuse and/or misuse could be associated with high prevalence of AMR *S. aureus*, including the MDR-MRSA phenotypes in unpasteurized raw milk consumed regularly by these communities.

Therefore, this study investigated the level of contamination of raw milk of cows, goats, sheep and camels with potentially pathogenic *S. aureus*. The study determined phenotypic antimicrobial resistance profiles of *S. aureus* isolates including multidrug resistant *S. aureus* (MDR-*S. aureus*) and MRSA contaminating raw milk of sheep, cattle, goats and camels in households from the two counties. In addition, the study investigated whether the resistant *S. aureus* phenotypes harbour genetic determinants responsible for the antimicrobial resistance phenotypes including the multidrug-MRSA.

1.2 Problem statement

Globally, it is estimated that *S. aureus* causes approximately 185,000 food poisoning cases in humans annually (Xu *et al*., 2016). Food poisoning can be caused by enterotoxigenic *S. aureus* which is resistant to commonly used antimicrobials. Studies around the world show increasing staphylococcal food poisoning cases as well as increase in antimicrobial resistance in *S. aureus*, including MRSA (Xu *et al*., 2016; Asiimwe *et al*., 2017; da Silva *et al*., 2020). For the last 30 years, a few studies have investigated the potential of milk borne *S. aureus* to cause staphylococcal food poisoning (SFP) in Kenya (Ombui *et al*., 1992; Ombui *et al*., 2001; Mathenge *et al*., 2015). Moreover, in cases where these studies were conducted, the focus was not on the *S. aureus* resistance profile to commonly used antimicrobials. In spite of these reports, the actual risk of staphylococcal food poisoning caused by antimicrobial resistant *S. aureus* in Kenya and particularly in pastoral areas generally remains unknown yet this information is crucial for the management of foodborne illnesses. Currently, the extent of antimicrobial usage by pastoralist communities remains unknown. It is hypothesized that the widespread use of antimicrobials by the pastoralists and animal health professionals could lead to emergence of antimicrobial resistant *S. aureus* which may be difficult to treat in case of *S. aureus* infections, including SFP.

Therefore, this study determined the pathotypes and characterized antimicrobial resistance patterns of *S. aureus* in raw milk for human consumption in Marsabit and Isiolo counties, Kenya.

1.3 Justification

Staphylococcal food poisoning and infections due to enterotoxigenic *S. aureus* including antimicrobial resistant strains of *S. aureus* are linked to higher mortality rates as compared to infections caused by antimicrobial-susceptible strains. Therefore, there is need to conduct surveillance and generate data on SFP and MDR- *S. aureus* in the milk value chain. This study provides novel findings on SFP and MDR-*S. aureus* including MRSA along milk value chain in pastoral areas of Northern Kenya. In addition, information generated in this study will be used to develop mitigation strategies against SFP outbreaks and contamination of raw milk by antimicrobial resistant *S. aureus* hence safeguard human health. Knowledge generated from this study will be used to advise policy makers on interventions to reduce milk contamination and SFP cases and spread of MRSA as well as supporting rational therapy in clinical set up. Also, the information generated will help promote food safety and food security in the study area.

1.4 Objectives:

1.4.1 Overall objective: To evaluate the pathotypes and antimicrobial resistance patterns of *S. aureus* obtained from milk meant for human consumption in Marsabit and Isiolo Counties, Kenya

1.4.2 Specific Objectives:

a) To characterize the phenotypes of *S. aureus* isolated from milk intended for human consumption in Marsabit and Isiolo Counties

b) To determine the genetic determinants for virulence associated with *S. aureus* enterotoxins isolated from milk in Marsabit and Isiolo Counties

c) To evaluate phenotypic antimicrobial resistance profiles of *S. aureus* isolated from milk in Marsabit and Isiolo Counties

d) To determine the genetic determinants responsible for antimicrobial resistance phenotypes of *S. aureus* isolated from Marsabit and Isiolo Counties.

1.5 Hypotheses

- Raw livestock milk for human consumption in Marsabit and Isiolo is not contaminated with enterotoxigenic S. *aureus*
- The enterotoxigenic *S. aureus* resistant to commonly used antimicrobials do not harbour genetic determinants responsible for the antimicrobial resistance phenotypes.

CHAPTER 2: LITERATURE REVIEW

2.1.1 History of *Staphylococcus aureus*

Staphylococcus aureus was first identified in 1884 by a German surgeon, Anton Rosenbach (Lakhundi *et al*., 2018; Turner *et al*., 2019). In addition, two strains of staphylococci were identified, *S. aureus* and *S. epidermidis*, named according to the pigmented appearance of their colonies on growth media (Lakhundi *et al*., 2018). During the period of discovery, most of the *S. aureus* infected patients died, with mortality rate as high as 82 % for patients with *S. aureus* bacteremia (Oliveira *et al*., 2018).

Staphylococcus aureus is a ubiquitous nonmotile, coagulase-positive, coccoid bacterium of the Firmicutes phylum. The *Staphylococcu*s genus consists of 52 species and 28 subspecies. In *Staphylococcus* genus, *S. aureus* is the most clinically relevant bacterium. The bacterium is found in the environment including air, water and the skin surfaces including mucus membranes of animals and humans (Tong *et al*, 2015; Bradley *et al*., 2017). *Staphylococcus aureus* is normally found in the skin and nasal cavities of humans and animals. About 20-40 % of humans are thought to be intermittent carriers while the other 20% are known to be permanent carriers (Van Belkum *et al*., 2009; Sakr *et al*., 2018; Becker, *et al*., 2018). The varied prevalence is attributed to differences in the quality of sampling, culture techniques utilized, demographics and size of the study populations (Dilnessa and Bitew, 2016).

2.1.2 *Staphylococcus aureus* classification

Taxonomically, *S. aureus* belongs to the genus *Staphylococcus* in the family of *Staphylococcaceae*, together with other five less popular *genera*. Currently, there are 53

recognised species of staphylococci and 28 subspecies most of which are found only in lower mammals. The staphylococci most frequently associated with human infection are *S. aureus, S. epidermidis* and *S. saprophyticus*. Other Staphylococcus species may also be associated with human infection (Euzeby, 2019). Staphylococcus species are Gram positive, non-sporing cocci, non-motile of varying size occurring singly, in pairs and in irregular clusters. Colonies are opaque and may be white or cream and occasionally yellow or orange. Staphylococci are tolerant to high concentrations of salt (Jeong *et al*., 2017) and show resistance to heat (Kennedy *et al*., 2005). Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood (Kennedy *et al*., 2005). This distinguishes the coagulase positive strains, *S. aureus* (a human pathogen), and *S. intermedius* and *S. hyicus* (two animal pathogens), from the other staphylococcal species that are coagulase-negative (CoNS). *Staphylococcus aureus* grows well at an optimum temperature range of 30°C-37°C. The bacterium is a facultative anaerobe with a fermentative metabolism mechanism (Jorgensen *et al*., 2005). Usually, staphylococcus species are catalase positive. In addition, this species is oxidase negative with the exception of *S. sciuri* group as well as macrococcus group (Vernozy-Rozand *et al*., 2000). On the other hand, the genus streptococci are catalase negative in addition to having different cell wall composition to staphylococci, hence are easily distinguished. Furthermore, the production of a thermostable deoxyribonuclease (DNase) can be used to identify staphylococci (Tang *et al*., 2008).

The *aureus* species derive their name from the golden color of their colonies when grown on solid media, in contrast to CoNS that form translucent, pale white colonies (Melter and Radojevič, 2010).

2.1.3 The cell structure of *Staphylococcus aureus*

The *S. aureus* cell wall has a single lipid membrane layer, sandwiched between a thick peptidoglycan film and lipoteichoic acid attached to diacylglycerol (Rajagopal *et al*., 2017). Furthermore, peptidoglycan forms the main constituent of the staphylococcal cell wall (up to 50%) and is made up of two interchanging polysaccharide subunits namely N--acetylmuramic acid and N-acetylglucosamine, together with 1, 4-linkages (Misra *et al*., 2014). The peptidoglycan chains determine the shape of the bacterium in addition to offering protection from osmotic lysis since it is the main determinant of the cell rigidity (Misra *et al*., 2014). Teichoic acids, a group of phosphate containing polymers make up to 40% of the total cell wall mass (Brown *et al*., 2013). These polymers provide a negative charge to the bacterium cell surface that is key in retention of metal ions. The two cell wall components account for a total of 90% cell wall mass, whilst the 10% remaining is composed of surface proteins, exoproteins, and autolysins (Oliveira *et al*., 2018)

2.1.4 Detection of *Staphylococcus aureus*

Various methods have been applied to detect *S. aureus* or MRSA from screening or clinical samples. Phenotypic methods include obtaining *S. aureus* cultures by plating specimens on various species specific and other relevant culture media followed by biochemical methods such as Gram staining, catalase activity, mannitol fermentation, coagulase activity and β-hemolysis. According to Kateete *et al*. (2010), none of the available *S. aureus* phenotypic identification tests (including the coagulase test) can guarantee reliable results. Consequently, new methods such as PCR have been introduced for the identification of *S. aureus* nucleic acids in both screening and clinical specimens to supplement the cultural methods. PCR technique has been regarded as a

"gold standard" method for identification of various pathogens including *S. aureus* (Yamamoto, 2002).

Various molecular targets have employed for detection of *S. aureus* isolates using PCR. For example, *nuc* gene responsible for production of a thermostable nuclease exoenzyme (thermonuclease [TNase]), with a similar frequency similar to of coagulase production have been used successfully to identify *S. aureus* (Nemeghaire *et al*., 2014). The TNase is an endonuclease with capabilities to degrade both RNA and DNA, and the kinetics of this enzyme can withstand a temperature of 100^0C for an hour or even more (Bonnin and Bouloc, 2015). The TNase protein has been well characterized (Aydin *et al*., 2011), and the gene responsible for its production, the nuc gene, has previously been cloned and sequenced (Kennedy *et al*., 2008). According to Brakstad *et al*. (1992) the detection of the nuc gene permits up to 100% identification of the isolates of *S. aureus,* utilizing only 0.69 pg. or even less of chromosomal DNA equivalent to about 10 bacterial CFU cells.

2.1.5 *Staphylococcal aureus* infection and emergence of antimicrobial resistance

Although some strains are non-pathogenic, others can cause fatal infections in both animals and human (Tong *et al.*, 2015). When normal primary barriers such as skin and mucous membranes are compromised, for example, as a result of wounds or surgical intervention or chronic skin conditions, the bacterium can invade the exposed tissues or enter the bloodstream leading to severe infections. In addition, immunocompromised patients or persons with invasive medical devices are particularly vulnerable to *S. aureus* infection. Other infections may include food poisoning, bacteremia, necrotizing pneumonia and toxic shock syndrome (Tong *et al.*, 2015). In animals the bacterium causes mastitis, dermatitis, urinary tract infections and abscesses (Peton and Le Loir, 2014). *Staphylococcus aureus* can evade the host natural defenses by expressing various antimicrobial resistance mechanisms as well as virulence factors (Honeyman *et al*., 2006). For example, multiple virulence factors are produced by *S. aureus* including various toxins, enzymes and cell surface-associated antigens (Honeyman *et al*., 2006). Staphylococcal toxins act by various mechanisms of action of including weakening of host response through degradation of some host cells, manipulation of host adaptive and innate immune responses, and degradation of inter-cellular junctions leading to increased proliferation of *S. aureus* (Grumann *et al*., 2014). As a result, a strong link between *Staphylococca*l virulence genetic determinants and the occurrence of certain diseases has been established. For example, there is a strong correlation between toxins and various diseases including necrotizing pneumonia, Staphylococcal food poisoning (SFP), staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS), or deep-seated infections (Jarraud *et al*., 2002; Ladhani, 2003). Therefore, the correlation between toxins and the various *S. aureus* diseases has clearly demonstrates the *S. aureus* as the causative

agent as well as the pathogenesis, epidemiology and the highly regulated mechanism of toxin production.

In the past, *S. aureus* infected patients exhibited high mortality rates, especially those with *S. aureus* bacteremia where up to 82 % mortality rate was reported (Oliveira *et al*., 2018). However, this rate decreased swiftly following the discovery and introduction of penicillin into clinical practice (Ladhani and Garbash, 2005). Unfortunately, the first penicillin-resistant *S. aureus* strains emerged soon after in the early 1940s, and by close of the decade, up to 25% of hospital-associated strains had developed resistance to penicillin (Rammelkamp and Maxon, 1942; Kirby, 1944; Chambers, 2001). Consequently, oxacillin and methicillin were introduced to overcome this resistance challenge in the 1960s. Unfortunately, resistant strains emerged again towards the end of the first year of their use, and the new resistant strains were classified as MRSA (Jevons, 1961). Consequently, infections due to hospital-acquired MRSA become more common in the period between 1960s and the 1980, with infections related to MRSA reaching 29 % in the 1990s (David and Daum, 2010). Since then, contact with patients with MRSA and the receipt of antimicrobials have been shown to increase the probability of being infected with MRSA (Chambers, 2001; Thompson, 1982). However, in the late 1990s, MRSA infections in young, patients with no history of visits to hospital settings appeared (Herold *et al*., 1998). Currently hospitalized patients with reduced immunity, those under prolonged treatment with antimicrobials, and those using unsterilized indwelling devices such as catheters or ventilators are still at high risk of acquiring these infections (Millar *et al*., 2007; Chatterjee and Otto, 2013).

2.1.6 *Staphylococcus aureus* enterotoxins

Staphylococcal enterotoxins (SEs) are generally classified in a family of 20 different exotoxins that share a common phylogenetic relationship, structure, function, and sequence homology produced by staphylococcal and streptococcal exotoxins. Currently, 23 enterotoxins have been identified as distinct serological entities (Schlievert and Case, 2007) and these includes *Staphylococcal enterotoxin* A (SEA), *Staphylococcal enterotoxin* B (SEB), *Staphylococcal enterotoxin* C (SEC), *Staphylococcal enterotoxin* D (SED), and *Staphylococcal enterotoxin* E (SEE).

These toxins are basic proteins comprising of about 220–240 amino acids and molecular weights of about 25–30 kDa. Staphylococcal enterotoxins SEA and SEB are the most common. SEA is the most frequent cause of food poisoning caused by staphylococcus (Pinchuk *et al.*, 2010). SEB has also been identified biological weapon of war and bioterrorism in addition to causing food poisoning and has therefore been restricted (Greenfield *et al*., 2002). Common among the foods that can be contaminated by SEs are dairy products, including milk.

Production of SEs rapidly increases at optimum temperatures (20–37^{o}C) and pH (4–7.4) (Greenfield *et al.*, 2002). Children suffer SFP by ingesting as little as 100 ng of SEs, and vulnerable populations may develop staphylococcal food poisoning with a few micrograms of toxin (Larkin *et al.*, 2009).

In Kenya, *S. aureus* is responsible for up to 38% of reported milk borne disease outbreaks (Ombui, *et al*., 2001). In another similar study, Ombui *et al*., (1992) reported a prevalence of 74.2% for enterotoxins-producing *S. aureus* in raw milk from Nairobi and its environs. Out of these isolates, three (4.17%) were found to produce SEA on screening using latex agglutination

test. Mathenge *et al*., (2015) also identified enterotoxin producing *S. aureus* strains in dairy products as well as meat in Nairobi County and its surroundings.

2.1.7 Pathophysiology of *Staphylococcus aureus* enterotoxins

The mechanism of action and pathophysiology of SE-induced food poisoning has not been fully elucidated. However, it is hypothesized that the stimulation of the vagus nerve in the abdominal viscera by the SEs followed by transmission of the signals to the vomiting center in the brain, is responsible for vomiting in cases of SFP. Furthermore, the cholinergic receptors located on the afferent vagal neurons are key in SEA-induced emesis (Hu *et al*., 2007), and that capsaicin from chili peppers has been found to deplete peptidergic sensory nerve fibers, leading to diminished SE effects in mammals (Larkin *et al.,* 2009). Moreover, SEs can penetrate the gut epithelium and activate both local and systemic immune systems (Shupp *et al*., 2002). Consequently, mediators of inflammation released such as neuroenteric peptide substance P, histamine and leukotrienes, leads to vomiting. The resulting emesis can be diminished by H_2- and calcium channel-blockers, through blockade of histamine release. In addition, the observed gastrointestinal damage may as well be caused by local immune system activation as a result of SE ingestion. Inflammatory lesions are commonly found in various regions of the GI tract with the jejunum and ileum showing the most severe lesions (Banwell and Sherr, 1973). The diarrhea associated with SEs induced SFP results from diminished reabsorption of water and electrolyte in the small intestine.

Some studies have shown that SEs do not directly act on the gastrointestinal tract, but indirectly affect the expression of cytokines and metabolites produced by T cells, macrophages, monocytes and mastocytes (Marrack and Kappler, 1990; Kotzin *et al*., 1992).

It has been postulated that superantigenicity and enterotoxicity is a result of enterotoxin activity facilitating transcytosis, leading to entrance of SE toxin into the bloodstream, hence facilitate the interaction with antigen presenting- and T-cells ultimately leading to superantigen activity (Balaban and Rasooly, 2000). Therefore, systemic invasion of SEs following ingestion and their spread from a localized *S. aureus* infection site, could have more severe effects upon the host than when the toxin remains localized (Larkin *et al*., 2009).

2.1.8 *Staphylococcus aureus* food poisoning

Staphylococcal food poisoning (SFP) is an intoxication caused by exposure to sufficient amounts of one (or more) preformed enterotoxin in food (Le *et al.*, 2003). Staphylococcal food poisoning is characterizes by rapid onset (2–8 h), and include nausea, abdominal cramping, violent vomiting, with (or without) diarrhea (Murray, 2005). The disease is self-limiting most of the time, and typically resolves within 1-2 days following onset. Once in a while, SFP can result in severe illness that warrant hospitalization, especially when infants, immunocompromised or elderly or people are affected (Murray, 2005).

Staphylococcus aureus is a normally found on the mucosal membranes and skin of humans and animals, with an estimation of about 20-30% persistent carriers and about 60% for intermittently colonized (Kluytmans, 2010; Sakr *et al*., 2018). Food handlers with enterotoxigenic *S. aureus* on their hands or noses are the main source of food contamination, mainly through manual contact or respiratory secretions. Indigenous microbiota found in raw foods outcompete *S. aureus*, hence contamination is mainly associated with poor handling of processed or cooked foods, followed by storage under conditions that support the growth of *S. aureus* and enterotoxin production.

However, food animal such as sheep and goats, dairy cattle particularly those with mastitis act as the source of *S. aureus* in milk (Stewart, 2005). In addition, dust, air and food contact surfaces may also serve as vehicles through which *S. aureus* get to foods. Staphylococcal intoxication is mainly caused by milk and dairy products contaminated by preformed SEs (Tamarapu *et al*., 2001).

2.2 Antibiotics used against *Staphylococcus aureus*

2.2.1 β-Lactam antibiotics

The capacity of penicillin to impede the growth of *S. aureus in vitro* was first reported in 1929 by Alexander Fleming. The classification of penicillins is based on chemical substitutions of the residue anchored to the beta-lactam ring, which in turn confer varied activities (Fig. 2.1). For instance, benzylpenicillins are first generation penicillins effective against Gram-positive bacteria especially the cocci group, such as pneumococci, staphylococci, other streptococci and bacilli, including *Bacillus anthracis, Corynebacterium diphtheriae* and *Clostridium perfringens*, but less effective against Gram-negative bacteria. Methicillin was developed and introduced to clinical use in 1959, being the first semisynthetic penicillin resistant to β-lactamase. The need to improve on the narrow spectrum of activity of these early antibiotics and the desire for broader coverage to also include Gram-negative organisms necessitated the expansion of the second generation penicillins. Consequently, the broad spectrum third generation penicillins also referred to as aminopenicillins, were introduced in the 1960s. Amoxicillin and ampicillin represent this group. In contrast to their predecessors, these broad spectrum penicillins have proved to be efficacious against the wide group of Gram-negative bacteria including *Haemophilus influenzae, Salmonella*

spp., Escherichia coli, and Shigella spp. due to their high stability to penicillinases (Birch and Wright, 1969). The final generation of penicillins (fourth generation such as ureidopenicillins and carboxypenicillins) further increased the spectrum of penicillin coverage against most Gram-negative bacteria, and most importantly exhibited potency against *Pseudomonas aeruginosa* (Wu, 1994).

2.2.2 Mechanism of action of penicillins

β-Lactam antibiotics act by binding to the serine active site of the transpeptidase (TP) located in the PBP2 by mimicking the structurally similar molecule, D-Ala4-D-Ala5. Consequently, the β-lactam bond is severed, resulting to penicilloyl-O-serine intermediate. Penicilloyl-O-serine intermediate differs from peptidoglycan acyl enzyme intermediate in that the penicilloyl-O-serine is quite stable, hence takes a longer period (1–4 h) for the addition of a water molecule to restore the active site Ser and liberate the penicilloic acid product. In contrast, it takes an extremely short period, milliseconds scale, for the natural uninhibited reaction to be accomplished. Basically, the active site of the TP enzyme active site is blocked and the biosynthesis of peptidoglycan ceases. Therefore, penicillins induce malfunctioning of the biosynthetic apparatus of the cell wall, with cyclic synthesis and autolysis. This indicates that the bactericidal effect observed in penicillin use is complex than simple inhibition of PBP activity (Cho *et al*., 2014).

2.2.3 New β-lactams with activity against MRSA-Cephalosporins

Just like penicillins, cephalosporins have a beta lactam ring in their structure (Fig. 2.2). Cephalosporins are bactericidal antibiotics with the same mechanisms of action and resistance as penicillins. Cephalosporin C was discovered as the first family member of the Cephalosporins (a

sub-class of β-lactams) originally discovered by Giuseppe Brotsu in 1945 from *Cephalosporium acremonium* (now renamed *Acremonium chrysogenum*) (Bo, 2000**)**. The antibiotic was rediscovered in 1955 and its structure revealed using X-ray crystallographic analysis as well as chemical degradation studies in 1961 (Abraham and Newton, 1961). However, cephalosporin C failed clinical trials, and its structure only served as a design template for the design of analogs leading to emergence highly effective antibacterial drugs, including cefalotin as the first cephalosporin clinically approved. Unfortunately, it is the fifth generation of semisynthetic cephalosporins, ceftaroline, ceftobiprole, and fosamil demonstrated adequate pharmacological activity against the PBP2a containing MRSA. Consequently, only the fifth generation cephalosporins have been approved to treat *S. aureus* infections (SSSTIs), as well as treatment of community acquired streptococcal pneumonia (Saravolatz *et al*., 2011). Ceftaroline however has a unique mechanism of action (Fisher and Mobashery, 2016). In addition, the functional and structural properties of ceftaroline components have been described (Laudano, 2011) (Fig. 2.3). Previous studies conducted demonstrate that two moles of ceftaroline bind to PBP2a binding sites of MRSA. One inactivates the serine active site while the other targets the allosteric binding site. Moreover, the attachment to the second site establishes a conformational change resulting from binding to the second site leading to the opening up the deep active site pocket permeating drug access. Therefore, the clinical use of ceftaroline has been restricted to resistant strains with mutations causing amino acid substitutions near the active site and as well as the allosteric binding site of PBP2a presumed to alter the drug binding (Lahiri and Alm 2016; Schaumburg *et al*. 2014).

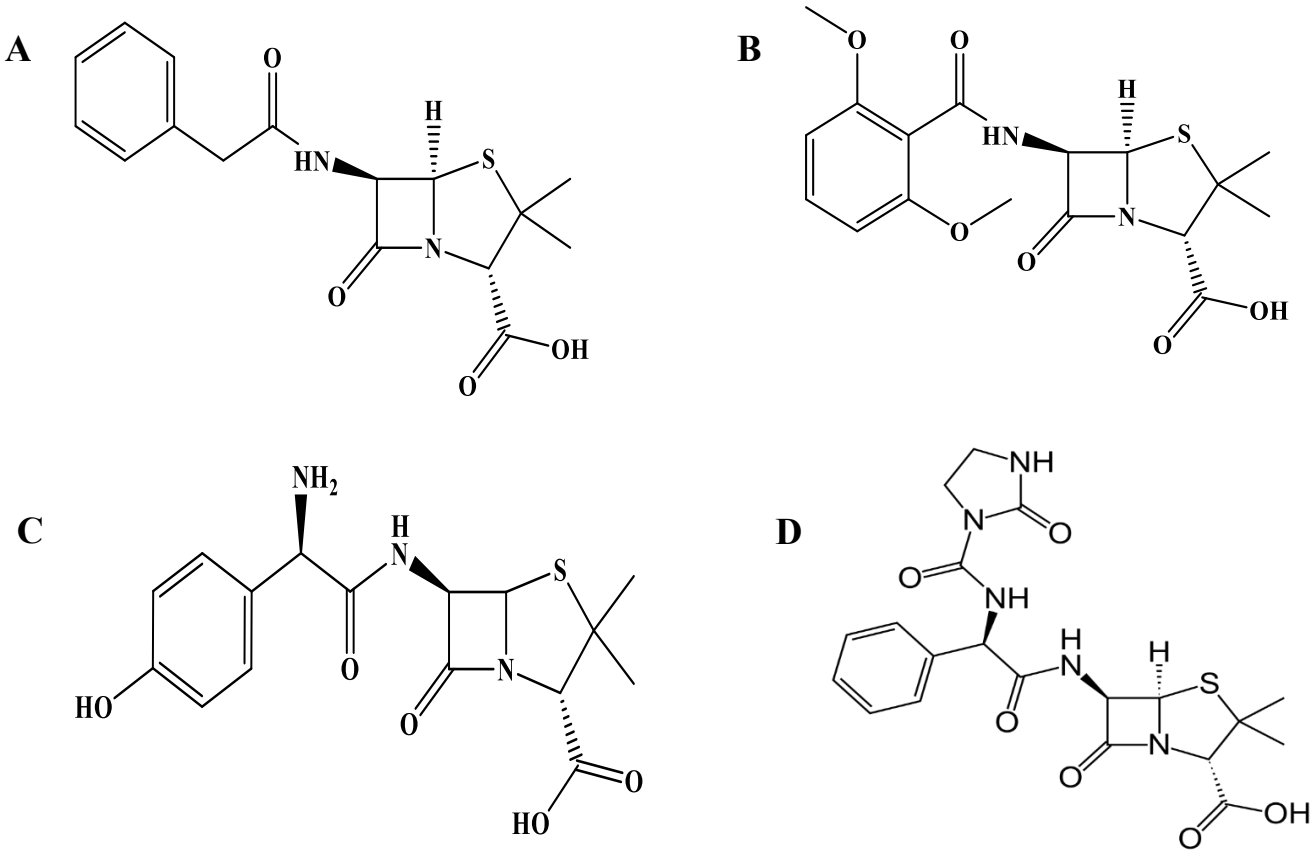

Figure 2.1. Various classes of penicillins used against *S. aureus.* A-Benzylpenicillin (first generation penicillin), B-Methicillin (Second generation penicillin), C- Amoxycillin (Third generation penicillin) and D – Azlocillin (fourth generation penicillin)

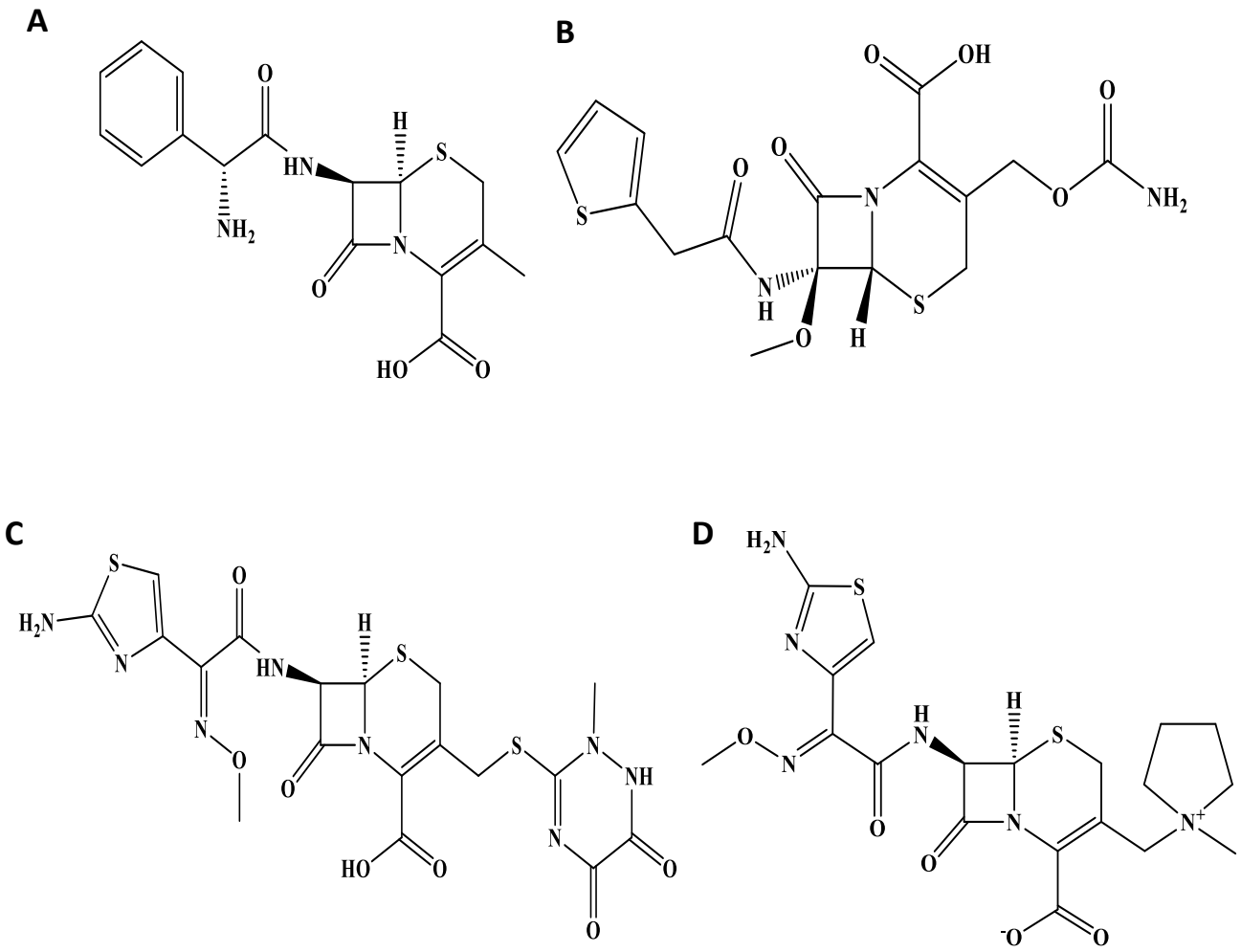

Figure 2.2. Cephalosporin generations used for treatment of *S. aureus* infections. A- Cephalexin (first generation cephalosporin), B-Cefoxitin (Second generation cephalosporin), C- Ceftriaxone (Third generation cephalosporin) and D – Cefepime (fouth generation cephalosporin).

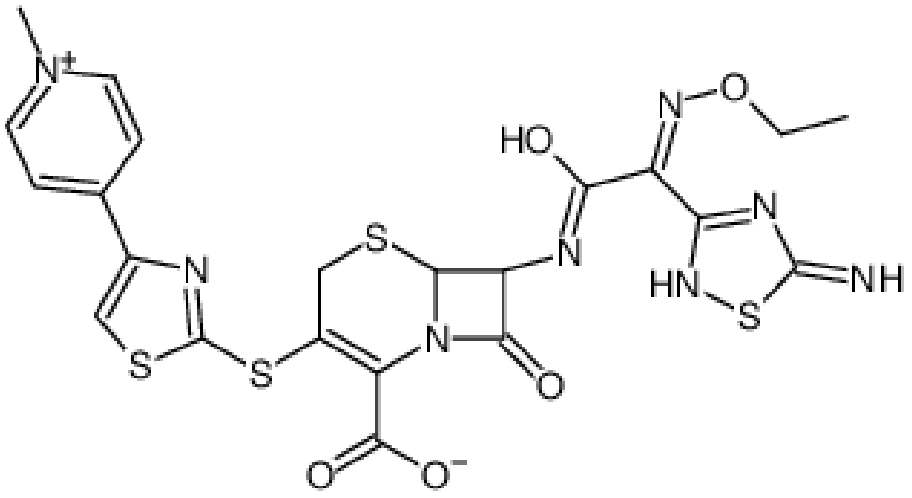

Figure 2.3. Fifth generation cephalosporin used for treatment of *S. aureus* infections.

2.2.4 Vancomycin and other glycopeptides

The first member of glycopeptide family of antibiotics was isolated from *Streptomyces orientalis* (now *Amycolatopsis orientalis*), actinobacterium species found in a soil sample from Borneo in 1953 (Yushchuk et al., 2020). Later, the antibiotic was designated vancomycin (due to its ability to vanish bacteria). This important antibiotic was found to be particularly effective against Gram-positive bacteria, and especially, against penicillin-resistant Staphylococci and was approved for clinical application as an antibiotic in 1958. However, due to toxicity reasons, its use was later diminished, although it continues to be used in severe drug-resistant bacterial infections (Filippone et al., 2017). Vancomycin has a similar mechanism of action to that of the β-lactams in that it alters the bacterial cell wall synthesis. The drug works by binding to the dipeptide D-Ala4-D-Ala5 of lipid II and hinders transpeptidation and transglycosylation catalysed by PBP2a hence counteracts peptidoglycan remodeling (Maya-Martinez et al., 2019). Vancomycin is used widely to treat serious MRSA infections in hospital patients.

Several semisynthetic lipoglycopeptides related to vancomycin are currently approved for treating severe acute bacterial SSSTIs following successful therapy using vancomycin. For instance, televancin and oritavancin have a bactericidal action against MSSA, MRSA and VISA strains by eliciting membrane damage as well as inhibition of peptidoglycan biosynthesis even in cells that are not actively growing (Crotty et al. 2016; Maya-Martinez et al., 2019) hence they are effective against persisters. The high potency is associated with the lipophilic substitution and increased formation of drug dimers.

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2.2.5 Quinolones

The first member of the group, nalidixic acid was synthesized in 1962 and reported to be highly effective both in vitro and in vivo (Lesher *et al*., 1962), with high potency pronounced against Gram-negative bacteria, while exhibiting low toxicity (Lesher *et al*., 1962). It is a 1-ethyl-1, 4-dihydro-7-methyl-4-oxo-1, 8-naphthyridine-3-carboxylic acid (Fig 2.4). Depending on the concentration, Nalidixic acid can act as a bacteriostatic or bactericidal agent (Hamatake *et al*., 1981). This antibiotic, first approved for clinical use in 1967 (Emmerson and Jones, 2003) was later replaced by more potent fluoroquinolones like ciprofloxacin. The biological target of quinolones is DNA Gyrase and topoisomerase IV (D'Atanasio *et al*., 2020), a specific enzyme responsible for the ATP-dependent negative supercoiling of double stranded closed-circular DNA, (D'Atanasio *et al*., 2020) thus causing interruption of bacterial DNA biosynthesis (Fournier *et al*., 2000).

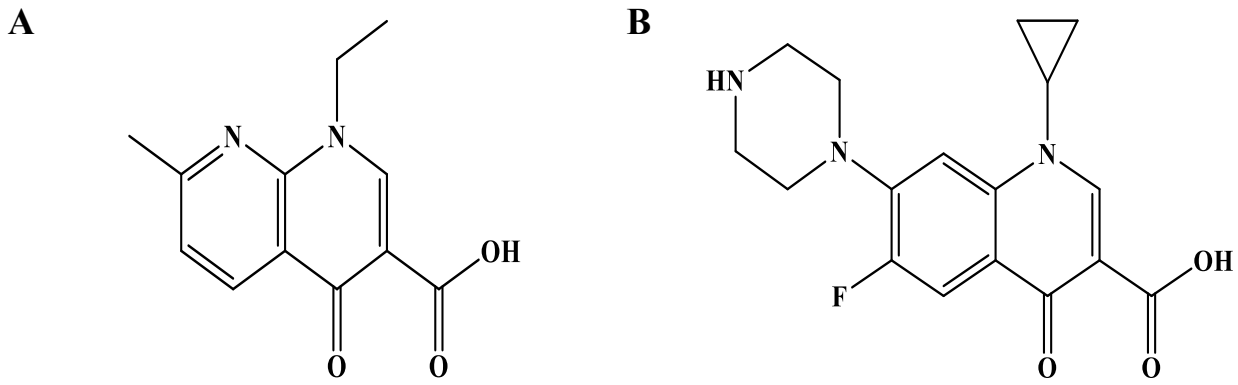

Figure 2.4. The chemical structure of quinolones (A-Nalidixic acid and B-ciprofloxacin) used for treatment of *S. aureus* infections.

2.2.6 Daptomycin

Daptomycin (Dap) a cyclic lipopeptide is produced by *Streptomyces roseosporus* (Fig 2.5). It has activity against MDR Gram positives, including MRSA and *S. aureus* with low susceptibility to vancomycin (Rybak *et al*., 2000). Currently, daptomycin is the antibiotic of choice against MRSA (John, 2020). The antibiotic is 4 to 8-fold as active as vancomycin, and about 30-fold as active as linezolid against MSSA and MRSA (Tally and de Bruin, 2000). Previous studies have demonstrated that, in cases of bacteremia caused by *S. aureus* with MIC of vancomycin >1mg/L, the administration of daptomycin early enough leads to a significantly better clinical outcome (Moore *et al*., 2012; Murray *et al*., 2013), though some authors report no significant differences (Kalil *et al*., 2014).

The mechanism of action of daptomycin is unique and is currently not fully understood. Daptomycin acts by binding to the bacterial cytoplasmic membrane in the presence of physiological concentrations of calcium ions (50 μg/ml), both in actively growing or cells in stationary phase, causing depolarization as a result transpeptidation of potassium ions loss from the cytoplasm. As a consequence, there is the interruption of multiple physiological factors in the bacterial cell membrane without necessarily penetrating the cytoplasm. This change in cellular homeostasis leads to inhibited bacterial key processes leading to cell death (Zhang *et al*., 2013). *Staphylococcus aureus* not susceptible to daptomycin have been identified from either patient treated with daptomycin or those patients treated with other antibiotics and even those who have not been treated at all (Pfaller *et al*., 2007; Jones *et al*., 2008; Howden *et al*., 2011). However, daptomycin resistance is not common in the clinical setting.

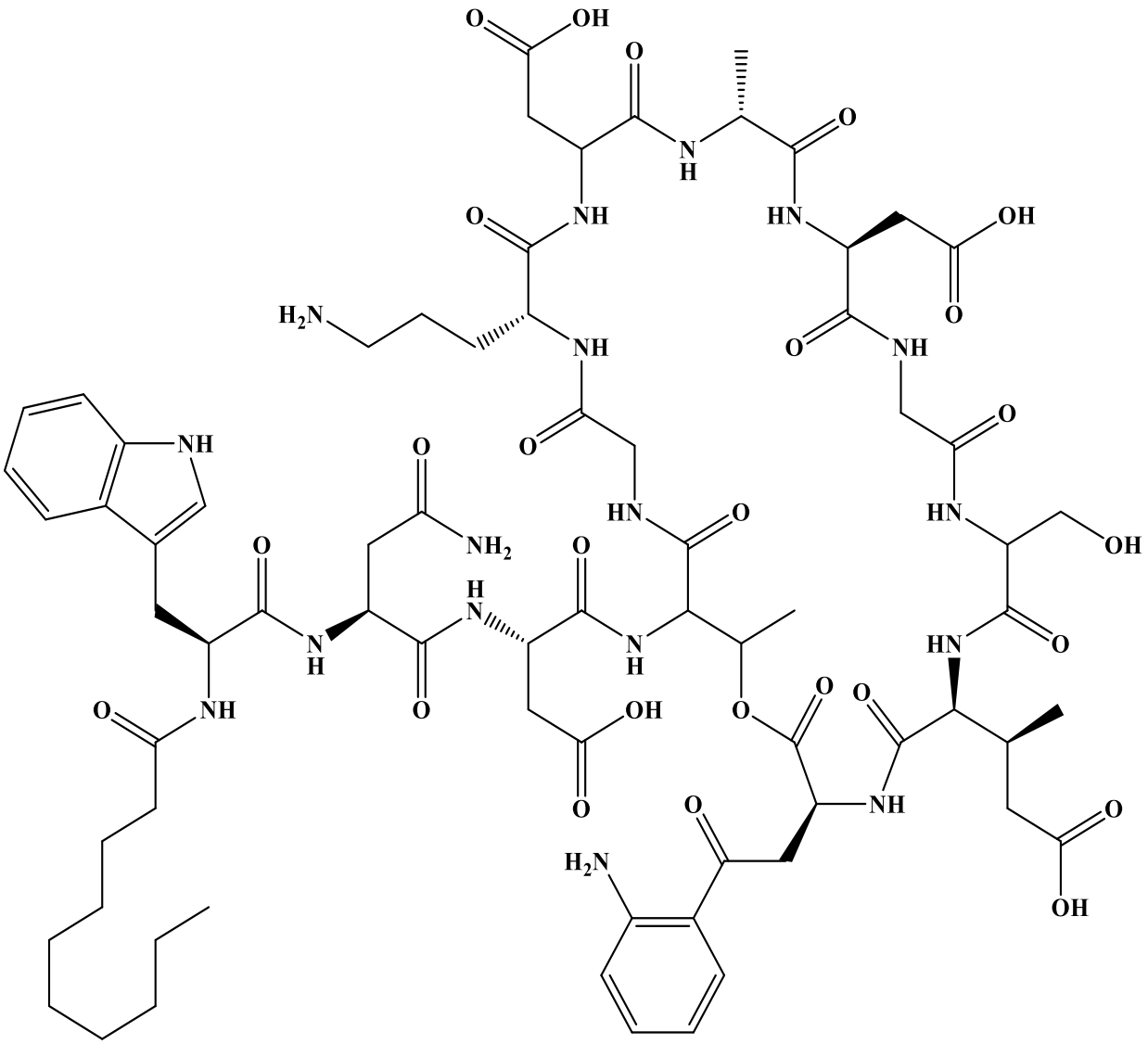

Figure 2.5. The chemical structure of daptomycin used for the treatment of multi-drug resistant *S. aureus*, including MRSA *S. aureus* infections.

2.2.7 Tetracyclines

Tetracyclines were first isolated from *Strep. aureofaciens* in 1945 by an American plant physiologist Benjamin M. Duggar. Structurally, tetracycline molecules contain a linear fused tetracyclic nucleus to which a variety of functional groups are attached (Fig. 2.6). The first member of this class of antibiotics was chlortetracycline (originally named aureomycin due to its golden color) (Grossman, 2016), and was approved for clinical use as a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria in 1948 (Chopra and Roberts, 2001). Soon, thereafter, several other members of the tetracycline family were isolated including oxytetracycline (from *Strep. rimosus*) and tetracycline (from *Strep. aureofaciens*). Tetracyclines exert their bacteriostatic properties inhibition of bacterial protein biosynthesis by binding to the 30 S subunit of rRNA (Grossman, 2016). In order to overcome the emergence of resistance, numerous tetracycline derivatives have been synthesized, including tigecycline which was approved by the US Food and Drug Administration in 2005 (Rose and Rybak, 2006). Tigecycline has a glycylamido moiety linked to the 9-position of minocycline in addition to the central four-ring carbocyclic skeleton responsible for antimicronial activity other tetracyclines (Fig.2.6). This modification creates the ability to overcome most tetracycline resistance mechanisms.

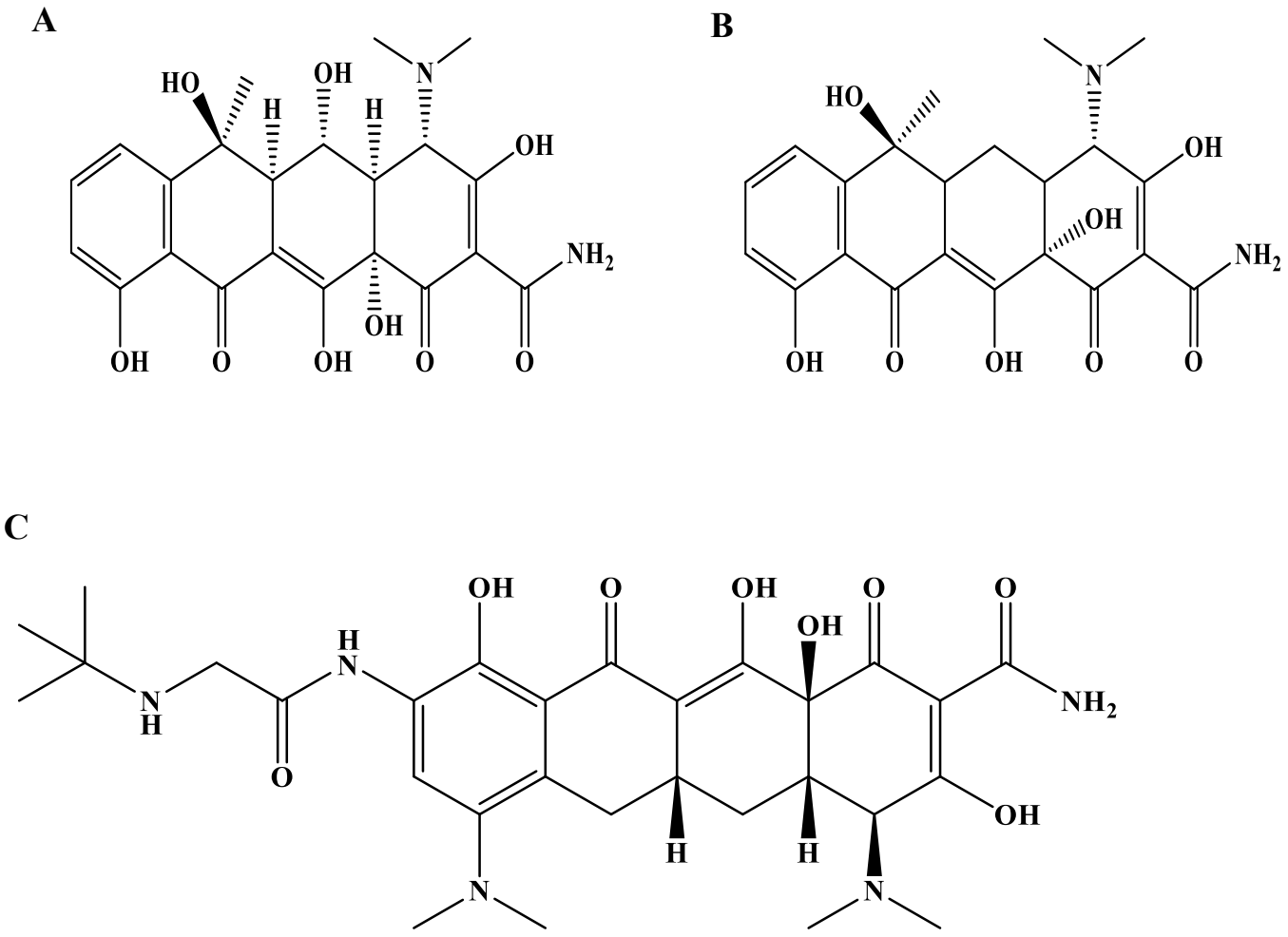

Figure 2.6. Tetracyclines (A- oxytetracycline, B- tetracycline and C-Tigecycline) used for treatment of *S. aureus* infections.

2.2.8 Aminoglycosides

Aminoglycosides comprise a core structure of amino sugars joined via glycosidic linkages to a dibasic aminocyclitol, in most cases 2-deoxystreptamine (Fig 2.7). These antibiotics were first discovered in 1943, with the first member, streptomycin being isolated from *Actinomyces griseus* (now *Streptomyces griseus*) by Albert Schatz and Selman Waksman (Krause *et al*., 2016). Gentamicin was introduced in the 1970s to combat serious nosocomial infections caused by *S. aureus*. However, its usage declined due to emergence of high-level resistance mediated by mobile genetic elements (Jensen and Lyon 2009). Aminoglycosides are the only bactericidal agents that target the ribosome. They inhibit protein synthesis by binding, tightly to the A-site of the 30S subunit of the 16S rRNA of the 30S ribosome leading to misreading of codons during translation. Consequently, the error rate in translation is increased from <1 in 1000 to around 1 in 100 so that every average sized protein has several incorrect amino acids (Walsh and Wencewicz, 2016). Previous studies propose that the lethal event is mediated by faulty membrane proteins causing lethal membrane damage (Davis *et al*., 1986). Other members of the aminoglycoside class of antibiotics include amikacin, neomycin, kanamycin, netilmicin and tobramycin, which is a derivative of kanamycin.

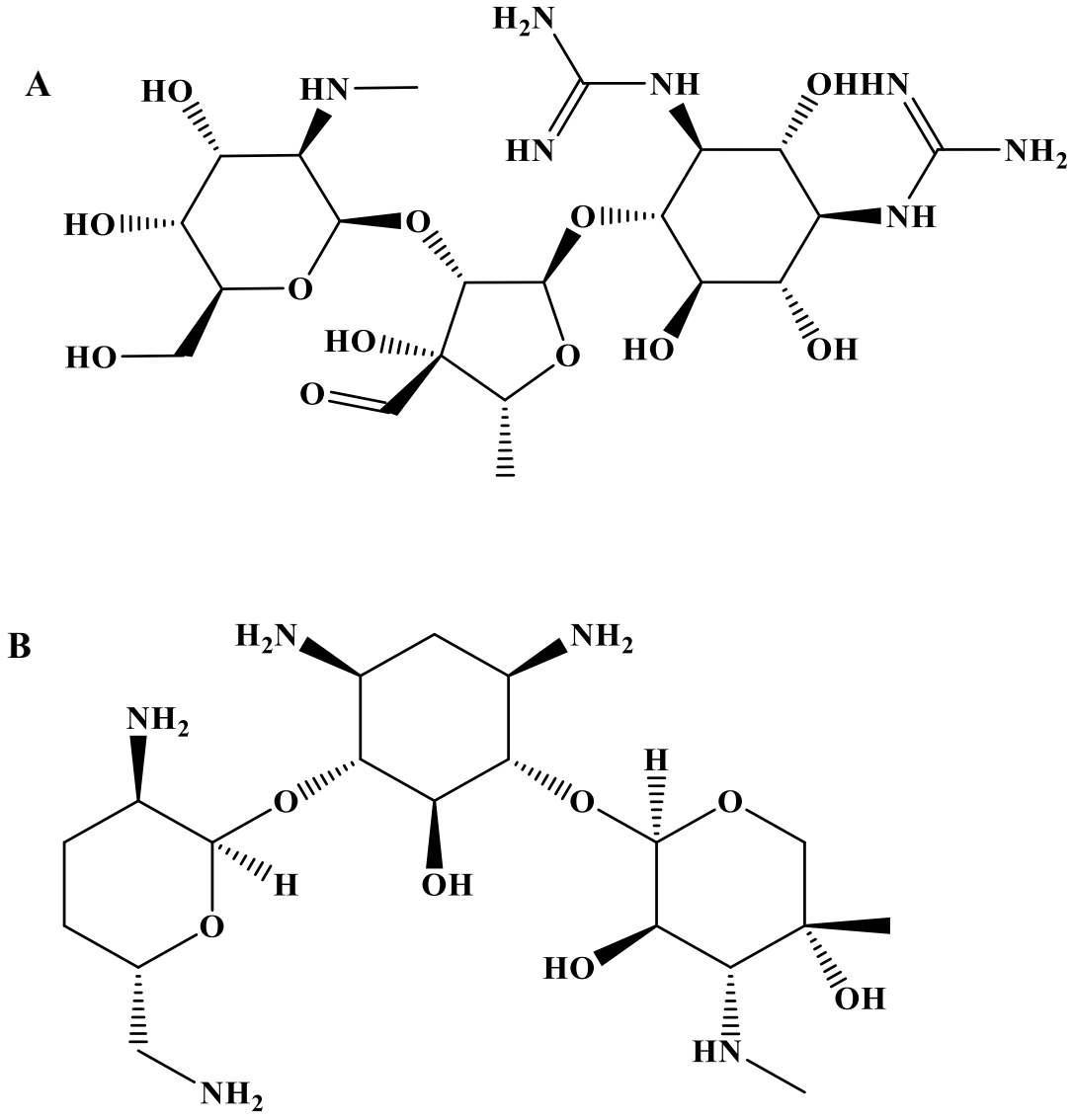

Figure 2.7. Aminoglycosides used for treatment of *S. aureus* infections (A-Streptomycin, B-Gentamicin).

2.2.9 Folic acid synthesis inhibitors (Sulphonamides and Trimethoprim)

Sulfonamide antimicrobials consists of a sulfur atom with two sets of double bonds to two oxygen atoms, a carbon-based side group, and a nitrogen atom linked to the sulfur (Fig. 2.8). The sulfonamides are bacteriostatic agents, acting through a mechanism that involves inhibition of folic acid biosynthesis in bacteria (Fernández-Villa *et al*., 2019). These antibiotics inhibit dihydropteroate synthase (DHPS) which converts p-aminobenzoic acid (pABA) to dihydropteroate, a precursor of folic acid (Babaoglu *et al*. 2004; Walsh and Wencewicz, 2016). This enzyme is essential in prokaryotes which, unlike mammals, synthesize folic acid *de novo*. Sulfonamides compete with pABA at the active site of the enzyme, at the same time acts as an alternative substrate forming a dead-end pteroate-sulfonamide product (Walsh and Wencewicz, 2016). Consequently, this shuts off a precursor of folic acid resulting in a slow acting, bacteriostatic affect (Skold, 2000).

Trimethoprim, an aminopyrimidine antibiotic consists of pyrimidine 2, 4-diamine and 1, 2, 3-trimethoxybenzene moieties linked by a methylene bridge (Fig. 2.8). It is an early synthetic antibiotic found to be effective against both Gram-positive and Gram-negative strains. Trimethoprim acts bacteriostatically through the inhibition of dihydrofolate reductase, an enzyme involved in the bacterial folic acid biosynthesis (Brogden *et al*., 1982; Estrada *et al*., 2016). The drug was synthesized in 1962 and its synergistic effects with sulfonamides were discovered in the same period. The first combined drug consisted of one-part trimethoprim to five parts sulfamethoxazole, and later also as a single molecular entity drug (Estrada *et al*., 2016). Currently, only Sulfadiazine and sulfamethoxazole are used clinically, the later in combination with trimethoprim in a formulation called co-trimoxazole (Wormser *et al*., 1982; Estrada *et al*.,

2016) and the former as the silver-sulfadiazine combination used prophylactically in wound and burns dressings.

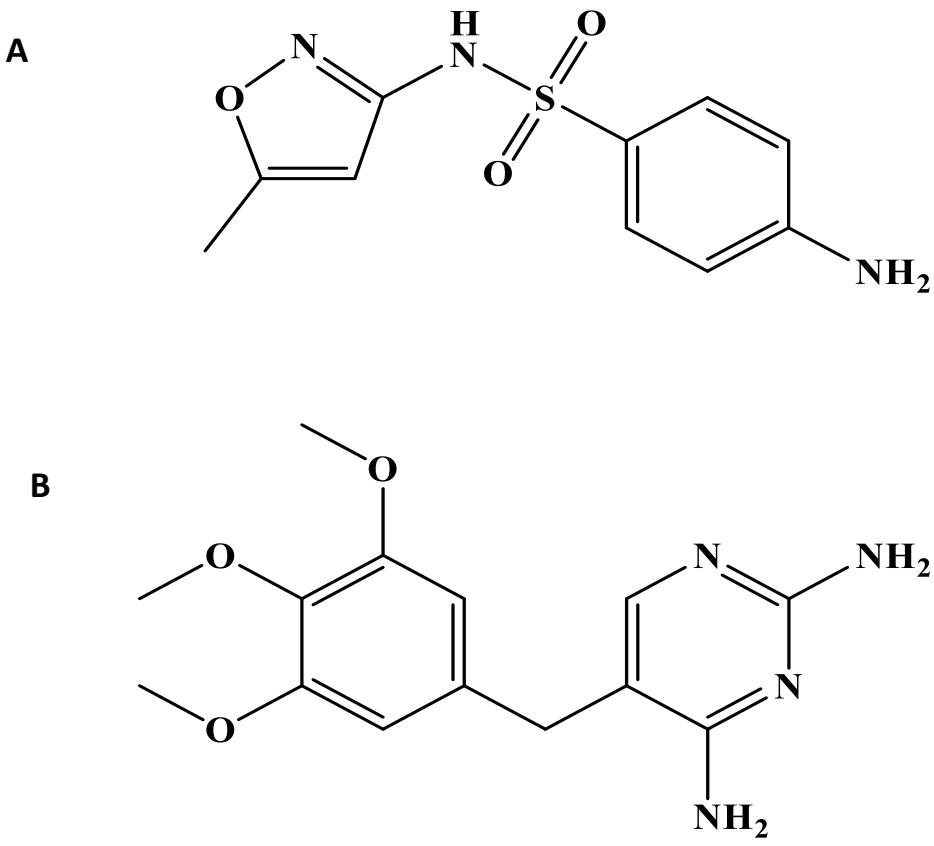

Figure 2.8. Folic acid synthesis inhibitors (A– Sulfamethoxazole and B- Trimethoprim) used for treatment of *S. aureus* infections.

2.3 New targets and drugs against *Staphylococcus aureus*

2.3.1 Fatty acid biosynthesis

Staphylococcus aureus fatty acid elongation is a cyclical pathway that is enzyme mediated and involves the fatty acid biosynthesis Fab proteins (FabI, FabG, FabF and FabZ). Therefore, the fatty acid biosynthetic pathway is vital and is currently an important target for development of novel antimicrobial agents (Yao and Rock 2017). Already, a compound originally discovered by Glaxo Smith Kline targets FabI and is now known as Debio1452 (Foster, 2017). The compound has been commercially developed and has entered phase 2a clinical trial for SSSTIs in 2015 (Flamm *et al*. 2015).

The mechanism of action of Debio1452 is specific inhibition of the enoyl-acyl carrier protein reductase FabI in the elongation cycle of fatty acid biosynthesis (Flamm *et al*. 2015). To improve solubility and bioavailability including potential for oral administration, a prodrug of Debio1452 called Debio1450, previously AFN1720, is being developed. Debio1452 has a very narrow spectrum of activity, specifically targeting staphylococci and has limited activity on other Gram-positive cocci including enterococci and streptococci

2.3.2 FtsZ and cell division

The FtsZ protein has been identified as a potential target for antibiotic action due to its essential role in cell division. The monomeric protein has GTPase activity utilised during polymerization at early initiation stage of cell division and serves as a scaffold for recruitment and organization of the septum (Pinho *et al*., 2013). Currently, PC190723 which is an inhibitor prevents cell division by disrupting FtsZ function and causes displacement of the Z ring (Andreu *et al*. 2010).

Another inhibitor, ClpP protease, kills persisters. A small proportion of a susceptible population of *S. aureus* growing in exponential phase survives exposure to bactericidal antibiotics such as quinolones, aminoglycosides, rifampicin and β-lactams. These cells are only temporarily resistant because they regain sensitivity when re-cultured. Upon a second exposure to the drug, the population shows the same killing phenotype as the original. Thus, in any population of bacterial cells a small proportion is in a transiently insensitive state.

2.4 *Staphylococcus aureus* and antimicrobial resistance

Staphylococcus aureus is a versatile organism that best exemplifies the adaptive evolution of bacteria in the antibiotic era (Pantosti *et al*., 2007). For instance, up to 28% of *S. aureus* isolates in Boston City Hospital developed resistance within the first decade of penicillin use (Maranan *et al*., 1997). Currently, a large proportion of Hospital acquired *S. aureus* are resistant to penicillin. Furthermore, *S. aureus* has demonstrated a unique ability to quickly respond to each new antibiotic through development of a new resistance mechanism.

Staphylococcus aureus resistance to the commonly used antibiotics is almost exclusively mediated by genetic determinants acquired through horizontal gene transfer. Through this acquisition, the bacterium gains a pre-assembled all-inclusive packet encoding resistance to multiple antibiotics, hence becomes MDR (Aslam *et al*., 2018). For example, horizontal gene transfer is responsible for *S. aureus* resistance against methicillin and vancomycin. In addition, endogenous resistance, resistance acquired through the random process of mutation and selective pressure in presence of antibiotics is responsible for development of resistance in the clinical setting, providing an important route for resistance to antibiotics such as vancomycin (for

intermediate resistance), fluoroquinolones, linezolid, daptomycin among others (Chen *et al*., 2020).

2.4.1 Mechanism of resistance to penicillins

Penicillin-resistant strains of *S. aureus* emerged soon after the introduction of the antibiotic in the early 1940s (Lowy, 2003; Walsh, 2016). The resistant strains expressed a β-lactamase enzyme that hydrolyzed the key β-lactam bond resulting in destruction of the drug's antibacterial activity. The *S. aureus* resistance to penicillins is mediated by β-lactamase, which is a typical serine β-lactamase (BlaZ) that forms an acyl enzyme intermediate similar to TP as well as PBP2 (Lowy, 2003). The kinetics of deacylation forms the main difference between TP and *Bla*Z. In presence of *bla*Z, the addition of water molecules is fast leading to the rejuvenation of the active site serine and immediate release of the ring-opened penicilloic acid, a hydrolytic degradation metabolite with no inhibitory activity.

Structurally, the β-lactamase gene *bla*Z gene is located in the transposon Tn552 or Tn552-like components (Jensen and Lyon, 2009). The transposon is integrated into the bacterial chromosome or situated on a large plasmid which is the prototype of pI524. β-lactamase expression is inducible and is under the control of the *Bla*R sensor and the *Bla*I repressor (Zhang *et al*. 2001; Lowy, 2003). The lipoprotein enzyme is located partly on the extra cytoplasmic surface of the cytoplasmic membrane, located strategically to protect PBP2s. Some amount of the β-lactamase is secreted into the surrounding medium (Bush, 2018).

2.4.2 Mechanism of resistance to methicillin and oxacillin

The *S. aureus* resistance to oxacillin and methicillin results from acquisition of PBP2a, a gene that encodes a homologue of the PBP2 (Fisher and Mobashery, 2016) or PBP2' which is resistant to drug action (Ferrer-González *et al*., 2017). This is based on the deep pocket location of the serine active site of the TP of PBP2a that is not accessible to β-lactams (Moon *et al*., 2018). Therefore, the enzyme can take over peptidoglycan (PG) biosynthesis in case the housekeeping PBP2 TP is inactivated. The PBP2 transglycosylase activity is necessary for peptidoglycan biosynthesis since the PBP2a moiety is non-functional (Pinho *et al*., 2001). Therefore, the biosynthesis of PG occurs as a combined effort of the two proteins when the TP of PBP2 is deactivated. Wolf *et al*., (2017) reported formation of a poorly cross-liked PG by growing MRSA strain in the presence of β-lactams. As a result, the formed PG exhibits more potent pro-inflammatory effects that could lead to more pathology during infection by MRSA strain under treatment by β-lactams (Wolf *et al*. 2017). In addition, the failed induction of the global accessory gene regulator (Agr) in some MRSA strains may be due to altered PG structure (Yang *et al*., 2019).

2.4.3 Expression of methicillin resistance

Penicillin-binding protein 2a (PBP2a) is mainly encoded by the *mec*A gene located within a distinct family but closely related to staphylococcal chromosome cassette (SCC) strands (Miragaia, 2018; Peacock and Paterson 2015; Saber *et al*. 2017). However, a distinct PBP2a named *Mec*C with *Mec*A residue identity of about 63% has been recently discovered. *Mec*C has been found in Europe predominantly in a single lineage (Paterson *et al*., 2014). Distinct MRSA strains have become endemic to specific geographical regions while some strains have spread

worldwide (Kyany'a *et al*. 2019; Liao *et al*. 2020). Indeed, the independent acquisition of SCC*mec* complex in the early 1960s by several *S. aureus* multidrug-resistant strains (resistant to tetracycline, penicillin, streptomycin and erythromycin) marked the most critical event *S. aureus* evolution (Crisostomo *et al*, 2001). This phenomenon led to *S. aureus* resistance to most commonly used antimicrobial drugs including members of the β-lactam family of antibiotics (Jevons, 1961). Currently, up to twelve known SCC*mec* types (I–XII) have been discovered. These SCC*mec* types have been classified according to the type of class of the *mec* complex and the cassette chromosome recombinase (ccr) complex. Types I, II and III constitute the large SCC*mec* elements primarily found in hospital environments (HA-MRSA) and harbour genes that confer resistance to several antibiotic classes (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC, 2009). The HA-MRSA strains are multidrug resistant, usually have large SCC *mec* elements and have abdicated virulence for increased levels of resistance to β-lactams (Chambers and Deleo 2009; Yang *et al*., 2019. These strains are frequently responsible for wound infections as well as systemic infections leading to bacteremia.

Moreover, types IV and V such as USA300 and USA400 are have smaller SCC*mec* elements and are mostly community associated MRSA (CA-MRSA), and rarely in some extensive HA-MRSA clones, for instance ST22-MRSA-IV, ST45-MRSA-IV and ST5-MRSA-VI. Community-associated MRSA are responsible for serious skin and soft tissue infections (SSSTI) in otherwise healthy individuals (DeLeo *et al*. 2010). These strains harbour small SCC*mec* cassette (Malachowa and DeLeo, 2010), are not multidrug resistant, and are capable of surviving on human skin (hence increased transmissibility and infectivity) and exhibit enhanced virulence

(DeLeo *et al*., 2010). The resultant SSSTIs often requires hospitalization. Low β-lactams resistance is typical of CA-MRSA (Yang *et al*. 2019). It also noteworthy that over the years, the distinction between the two epidemiological groups (HA-MRSA and CA-MRSA) has become blurred (Bal, *et al*., 2016).

All the SCC*mec* types contain *mec*A, except type XI, which harbors the homologue *mec*C that encodes the penicillin-binding protein 2a (PBP2a), a peptidoglycan transpeptidase (Hartman and Tomasz, 1984). PBP2a is exhibits extremely low affinity for most β-lactam drugs even in presence of adequate concentrations of β-lactam antibiotics that normally inhibit the function of the four usual *S. aureus* penicillin-binding proteins (PBP1, PBP2, PPB3 and PBP4).

A *mec*A variant, named *mec*C, has been identified in several *S. aureus* clones from human and animal isolates (Garcia-Alvarez *et al*., 2011). Penicillin-binding protein 2a name for *Mec*C was derived from the MRSA strain LGA251 from which it was first identified and encodes PBP2aLGA. *S. aureus* β-lactam resistance in strain LGA251 is comparable to the resistance mechanism in MRSA strains that carry *mec*A (Kim *et al*., 2012; Milheirico *et al*., 2017). However, in the LGA251 strain, methicillin resistance level varies according to *mec*C expression and on genes located in the genetic background of the strain. Furthermore, methicillin resistance mediated by *mec*B has been detected in *S. aureus* since 2018, although the actual mechanism responsible for resistance encoded by *mec*B remains unknown (Becker *et al*., 2018).

The *mec*A expression depends primarily on the gene regulators encoded by *mec*R1, *mec*R2 and *mec*I (Hiramatsu *et al*., 1992; Arede *et al*., 2012) as well as the *bla*Z, *bla*RI and *bla*I gene

regulators (Zhang *et al*., 2001). Furthermore, a high number of auxiliary or fem genes have been found to have significant influence on the phenotypic resistance (De Lencastre *et al*., 1999).

Previous studies indicate that the level of *mec*A transcription is associated to the level of methicillin resistance. To start with, the bacterial reaction to various stress conditions including heat shock, iron limitation, amino acid and fatty acids induced by the antibiotic mupirocin triggers has been found to increase PBP2a activity without affecting *mec*A transcription (Kim *et al*., 2013). Second, a study by Boyle-Vavra *et al*., (2006) reported that deactivation of a component of the regulatory system (vraS) involving VraS sensor protein and response regulator protein VraR (VraS–VraR) responsible for the regulation of the cell wall PG biosynthesis induced *mec*A transcription without increasing the level of PBP2a activity. Lastly, the chaperone foldase protein PrsA alters the levels of properly folded PBP2a in the membrane and, therefore, methicillin resistance without affecting *mec*A transcription (Jousselin *et al*., 2015). The crucial role of the stringent stress response in *mec*A expression has been demonstrated using different experimental approaches (De Lencastre *et al*., 1999; Kim *et al*., 2017).

In the prototype MRSA strains, expression of the *mec*A gene follows induction by exposure to the antibiotics. However, studies show that induction of mecA gene is not efficient following exposure to β-lactam drugs (Jensen and Lyon 2009). In many MRSA strains, the constitutive expression of *mec*A *have* been truncated by insertion sequences in *mec*IR genes. Furthermore, the *mec*A expression by β-lactamase can be also repressed by regulators BlaI and BlaR. Consequently, the expression nature of PBP2a can differ from strain to strain based on the

availability of Bla regulators and functional *Mec* The structure of peptidoglycan in MRSA could vary based exposure to antibiotic in strains where either one or both regulators are intact.

The expression of resistance by MRSA isolates to β-lactams is heterogeneous (Peacock and Paterson 2015). Therefore, in a culture derived from one colony most cells express low level of resistance while only a handful express high level resistance at a high level (Finan *et al*. 2002). Consequently, the transformation from mixed to uniform high-level resistance result from chromosomal mutations that increase directly or indirectly *mec*A gene transcription and increase the aggregate of PBP2a. *Mec*A gene introduction into a susceptible strain led to a mutation in the relA gene that triggered conversion from heterogeneous to homogeneous resistance to methicillin (Kim *et al*., 2013). Furthermore, the mutation of relA leads to nonfunctional RelA protein, induction of the stringent response and constitutive (p) ppGpp expression. Mutations in clinical isolates in several types of genes are linked to the emergence of homogeneous resistance, for instance relA, as well as rpoB that encodes the β-subunit of RNA polymerase (Dordel *et al*. 2014).

2.4.4 *Staphylococcus aureus* resistance against vancomycin

There are reports of enterococci capable in expressing resistance to high concentrations levels of vancomycin. The *van* genes are responsible for vancomycin resistance and encode enzymes that are inducible and take over the biosynthesis of PG precursors resulting in a lipid II molecule that has D-lactate replacing D-Ala5. The structure so formed has low affinity for vancomycin although it may be used as a transpeptidation substrate by PBP2 in the last stage of cell wall

biosynthesis. The responsible genes are found on mobile genetic elements (Courvalin 2006; Maya-Martinez *et al*., 2019).

Recently, there have been fears that MRSA would acquire vancomycin (Van) resistance genes horizontally from enterococci leading to severe SSSTIs and invasive infections becoming refractory to treatment by vancomycin. There have been reports of increasing vancomycin-resistant MRSA (Courvalin, 2006; Gardete and Tomasz 2014). Luckily, although vancomycin resistant MRSA has been on the increase, these strains have not been detected in the hospital environment (Courvalin, 2006; Gardete and Tomasz 2014). This is due to the fact that MRSA PBP2a cannot utilize lipid II with D-Ala-D-lactate after the introduction of the *van*A determinant into the homogeneous high-level methicillin resistant strain model, COL. In addition, the VRSA strains have very long lag phase before they can start growing in an inhibitory concentration levels of vancomycin in vitro. Lastly, the mobile genetic element harboring the van genes has been reported to be genetically unstable. Therefore, various factors have worked together to reduce the ability of VRSA to cause major infections resistant to treatment.

2.4.5 Vancomycin intermediate *Staphylococcus aureus* (VISA)

Vancomycin has been the cornerstone of therapy against MRSA strains. However, in the last decade, strains not susceptible to vancomycin have appeared either showing intermediate resistance (vancomycin intermediate *S. aureus* (VISA) or full resistance to vancomycin (vancomycin resistance *S. aureus* (VRSA). VISA was first reported in Japan in 1996 and was subsequently reported from other countries (Thitiananpakorn *et al*., 2020; Guo *et al*., 2020). Of note is that VISA or VRSA have emerged exclusively from MRSA, with a small number

showing heteroresistance (Morrisette *et al*., 2020; Lin *et al*., 2018). Furthermore, resistance does not appear to develop in a stepwise version, and VRSA does not develop from VISA, since VISA and VRSA show different mechanisms of resistance.

Vancomycin intermediate *Staphylococcus aureus* (VISA) have a minimum inhibitory concentration (MIC) of about 4–8 μg ml^{-1} in comparison to vancomycin-susceptible *S. aureus* (VSSA) that have an MIC of ≤2 μg ml−1 (Gardete and Tomasz 2014). In addition, Hetero-VISA (h-VISA), the intermediate in the development of VISA are variants with the majority of *S. aureus* in the population have an MIC of 2 μg ml^{-1} or less, hence defined as sensitive although they contain a subpopulation of cells with an of up to MIC of 4–8 μg ml^{-1}.

The mechanism the underlying insensitivity is an altered architecture and increase in thickness of the cell wall (Howden *et al*. 2010; Gardete and Tomasz 2014). The increased cell wall thickness increases the area of diffusion of the drug in order to reach lipid II, its lethal target found on the extra-cytoplasmic face of the membrane in division septum where active cell wall biosynthesis occurs. The reduced cross-linking in peptidoglycan provides an excess of D-Ala -D-Ala false targets which in turn impounds the drug, reducing diffusion to the membrane. In addition, abundant and bulky glycopeptide attached to the outer layers of the enlarged cell wall obstructs drug diffusion. Furthermore, in some VISA strains, a surge in the amount of D-Alanine substitution on teichoic acids may change the electric charge of the cell envelope leading to repulsion of the positively charged glycopeptides.

Vancomycin intermediate *Staphylococcus aureus* phenotype is mediated by various mutations in *S. aureus* genome. One such mutation is at *graR* gene encoding the response regulator of the

TCST GraSR as well as another in the RNA polymerase B subunit that globally functions by slowing growth rate and reducing autolysis. Although the typically phenotypic increase in thickness of the cell wall encountered in VISA was only demonstrated on exposure to vancomycin (Guo *et al*., 2020).

2.4.6 *Staphylococcus aureus* resistance to fluoroquinolones

Resistance to fluoroquinolones emerged soon after the introduction of ciprofloxacin, particularly among the HA-MRSA strains (Jones *et al*., 2004; Elnekave *et al*., 2019). Resistance is due to spontaneous mutations that alter amino acids in one or both enzymes that are necessary for DNA replication, DNA gyrase and topoisomerase IV. In *S. aureus*, resistance is conferred by point mutations primarily in subunit ParC (also known as Gr1A) or topoisomerase IVand secondarily in GyrB of DNA gyrase (Jones *et al*., 2004; Elnekave *et al*., 2019).

2.4.7 *Staphylococcus aureus* resistance to daptomycin

Various mechanisms have been suggested to explain the non-susceptibility to daptomycin (Mishra *et al*., 2012; Lasek-Nesselquist *et al*., 2019; Shariati *et al*., 2020). The first mechanism is related to the increase in the bacterial membrane positive surface charge, due to the increase of phospholipids in its outer layer. Secondly, the increased fatty acids composition in the alteration in the bacterial membrane alters the fluidity of the cell membrane. Another mechanism is related to the increased carotenoid pigment content. And the last possible mechanism of non-susceptibility is believed to be due to increased teicoic acid synthesis in the cell wall. Of note is that combinations of several of these factors are also possible.

The gene that encodes for lysyl-phosphatidyl glycerol synthetase enzyme is *mpr*F gene, is an enzyme involved in the phospholipid metabolism. This is a protein with two functional domains (Silverman *et al*., 2001; Shariati *et al*., 2020), which is responsible for the transfer of positively charged lysine molecules and adds them to phosphatidyl glycerol in the cell membrane (Bæk *et al*., 2015; Ernst and Peschel, 2011; Lasek-Nesselquist *et al*., 2019). Previous studies show that mutations in this gene can lead to an increased production of the lysyl-phosphatidyl glycerol. Consequently, the increased amount of this compound in the outer layer of the membrane leads to reduced susceptibility of *S. aureus* to daptomycin and cationic antimicrobial peptides (Friedman *et al*., 2006; Yang *et al*., 2010; Lasek-Nesselquist *et al*., 2019). Mutations in this region have been linked to daptomycin non-susceptibility both in clinical isolates and mutants obtained in vitro (Friedman *et al*., 2006). It is the only gene whose association to decreased daptomycin susceptibility has been demonstrated conclusively by gene deletion and complementation molecular studies.

2.4.8 *Staphylococcus aureus* resistance to Tetracyclines

Staphylococcus aureus resistance to tetracyclines is conferred by two mechanisms; active efflux of the drug encoded by plasmid-born genes, *tet*K and *tet*L and ribosome protection (the tetracycline target), encoded by the genes *tet*O and *tet*M (Ruhe *et al*., 2005; Martini *et al*., 2017; Hui-Ling *et al*., 2017).

2.4.9 *Staphylococcus aureus* resistance to aminoglycosides

Staphylococcus aureus resistance to aminoglycosides is due to horizontal transfer of mobile genetic elements responsible for expression of aminoglycoside modifying enzymes (Jensen and Lyon 2009; Khosravi *et al*., 2017; Seyedi-Marghaki *et al*., 2019). As a result, ribosome binding is abolished. Resistance to neomycin and gentamicin are conferred by a bifunctional acetyltransferase-phosphotransferase (*aacA-aphD*) which is encoded by Tn4001. In addition, neomycin resistance is conferred by a phosphotransferase (*aphA*) that is encoded by Tn5405) or an adenyltransferase (*aadD*) controlled by plasmid pUB110. Moreover, the plasmid is incorporated within the SCC*mec*II cassette which is found in some MRSA strains (Seyedi-Marghaki *et al*., 2019).

2.4.10 *Staphylococcus aureus* resistance to sulphonamides

Staphylococcus aureus resistance to sulfamethoxazole (SMX) emanates from amino acid substitutions in DHPS which is chromosomally encoded. DHPS presumably prevents the drugs from attaching to the enzyme. In a similar mechanism, the resistance to trimethoprim in clinical isolates is associated to either by amino acid substitutions in the chromosomally encoded DHFR or through horizontal acquisition of genes that encode DHFR enzymes which are resistant to inhibition and allow the blockade of chromosomal DHFR to be avoided (Griffith *et al*., 2018). In *S. aureus*, the change in trimethoprim resistant DHFR results from single amino acid substitution F98Y (Wróbel *et al*., 2020) in the DfrB resistance phenotype that is responsible for intermediate resistance. Three distinct horizontally obtained DHFRs that signal a high-level resistance have been elucidated, the first being DfrA represented by Tn4001 (Rouch *et al*. 1989) and DfrK

mainly found in livestock-associated staphylococci and lastly the DfrG determinant seldom found in human isolates (Kadlec and Schwarz 2009).

2.5 Antimicrobial susceptibility Testing (AST)

2.5.1 Agar Diffusion Method

The disk diffusion method was first discovered and standardized in 1956 by Bauer and Kirby's experiments, following optimization by changing physical conditions (Bauer *et al*., 1966). This method involves selection of the isolated bacterial colony, suspension into growth media, and standardization through a turbidity test. After standardization, the suspension is then cultured uniformly onto the solidified agar plate, and the paper impregnated with antibiotic is then carefully placed on the inoculated agar plate. Next, the disc impregnated with the antibiotic permitted time to diffuse through the solidified agar, resulting in formation of a zone of inhibition after an overnight incubation at 35-37 ^{0}C. Consequently, the diameter of the zone of inhibition formed around the antibiotic disc is measured; and the size of the zone of inhibition corresponds to the antibiotic concentration (Bauer *et al*., 1966; Chandrasekar *et al*., 2015).

Assessment and determination of the susceptibility of bacteria to antibiotics generally takes 16–24 h. This method has been adopted as the gold standard for confirmation of the susceptibility pattern of bacteria. In addition, this method is used routinely for testing of susceptibility in most clinical laboratories. Furthermore, the method is accepted widely because of its affordable cost as well as simple protocol that may be employed for testing of multiple targets at the same time (Chandrasekar *et al*., 2015). This method however, has a few important drawbacks that include lack of sufficient data for a significant number of bacterial strains (strains of Bacillus,

Corynebacterium and Pseudomonas), lack of automation for this method, with only semi-automation currently available (Sirscan), as well as poor performance when fastidious and slow-growing bacteria are analyzed (Hombach *et al*., 2018; Khan *et al*., 2019). Another weakness of the method is the impact many physiochemical factors like evaporation, pH, solubility, temperature and nutrient media have on its results. These limitations reduce its suitability when accurate diagnostics are desired (Balouiri *et al*., 2016).

2.5.2 Broth Dilution Method

Broth dilution method forms one of the earliest methods applied for antimicrobial susceptibility testing, starting from as early as the 1870s. This method allows the growth as well as the identification of bacterial species in a suspension (Schumacher *et al*., 2018). Early scientists such as Koch, Pasteur, Ehrlich, and Lister worked on the concept of macrodilution (Khan *et al*., 2019). The two types of dilution used commonly include microdilution and macrodilution, wherein agar and broth are the commonly used mediums. In broth dilution, consecutive two-fold serial dilutions (1, 2, 4, 8, and 12 µL) of antibiotics are prepared and dispensed into bacterial growth containing micro-centrifuge tubes, followed by making up the final volume through addition the medium and incubating overnight at 35-37 ^{0}C. Consequently, the growth examination is conducted in order to set the breakpoint through the culture medium turbidity (Jorgensen *et al*., 2005; Khan *et al*., 2019).

In agar dilution method, the bacterial cells are inoculated on the surface of agar medium containing antibiotics diluted into various concentrations. Rammelkamp and Maxon developed broth macro dilution, also known as the “tube dilution method”, regarded as the standardized dilution method applied for both minimum inhibitory concentration (MIC) and AST.

Furthermore, the breakpoints guidelines are recommended by the CLSI. The first recorded attempt at AST was made by Reymann and Schmith using agar medium as early as the 1940s (Khan *et al*., 2019). Microdilution results from miniaturization of the macrodilution method where AST is performed on 96-well microtiter plates that are disposable, with each well of the plates having a capacity of ~0.1 mL (Peng *et al*., 2017). Mechanized dispensers are used to dispense the antibiotics in order to avoid the handling error. Specialized optical instruments are used to assess the growth and MIC and this method is suitable for fastidious bacteria following standardization (Chandrasekar *et al*., 2015).

The requirement for large volumes of reagents is the main weakness of dilution methods. In addition, other potential limitations include: huge requirement for experimental space, it's tedious multiple dilution steps (macrodilution), possible risk of cross-contamination, inability of discriminating viable from nonviable bacteria long incubation time increases the possibility of false positive (Lallemand, *et al*., 2016), bacterial incompatibility for growth. Optimum testing parameters including media, pH, media, length of incubation and temperature constitute the additional challenges, and a control viability plate is mandatory in tests in order to achieve practical clinical application (Khan *et al*., 2019).

2.5.3 Epsilometer method (Etest)

Epsilometer testing (Etest) is another important method for detection of antibiotic resistance in bacteria that was developed by Bolmström and Eriksson in the late 1980s, (McLaughlin and Sue, 2018). In this method, pre-defined antibiotic concentrations are coated on Etest plastic strips. The strips have marked corresponding interpretive MIC ranges on the surface as well as on back.

For detection of antimicrobial resistance, a number of strips are incubated overnight after being dipped on a pre-inoculated agar plate. An elliptical zone of inhibition appear around the strips, which indicate the MIC at the point of intersection between the zone of inhibition and the edge of the strip (Khan *et al*., 2019). This method is accurate, reliable, and simple, hence convenient and appropriate hence has been approved the Food and Drug Administration (FDA)] (EFSA, 2019). This method is preferred over the disk diffusion as well as dilution method due to its convenience in MIC interpretations under various physical conditions in clinical laboratories for AST (Sartelli *et al*., 2016; Mercer *et al*., 2020).

Etest has been used to test many *S. aureus* strains as well as other clinical isolates and compared with other standardized methods, and the results reveal a good correlation within a range of 91%–99% (Riedel *et al*., 2014; Khan *et al*., 2019). Recently, MSSAMRSA isolates were examined in 2016 with an Etest method to establish the MIC of ceftaroline. The findings compared well with broth microdilution (BMD) with an outstanding agreement of over 95% (Skov *et al*., 2006; Cantón *et al*., 2019). One of the main advantages of the method lies in its sensitivity as well as the capability of detecting extended-spectrum beta-lactamase (ESBL), including in trace levels (Falagas and Karageorgopoulos, 2009). Additionally, studies show that accurate resistant *S. aureus* strains can easily be quantified easily in laboratories/hospitals since concentration gradient of antibiotics as marked on the Etest strip is stable.

The main limitations of the Etest are primarily related to its inconsistent and inaccurate behavior of the method for specific antibacterial agents, such as ciprofloxacin Penicillin, rifampicin and ofloxacin (Khan *et al*., 2019). Other demerits of the Etest method associated with routine analysis

are associated with expensive batch performance, pH-sensitive coated antibiotics, laboratory set up for proper plate inoculation, strip storage, and incubation (Balouiri *et al*., 2016).

2.5.4 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is a sensitive method used for identification of bacterial resistance that was initially introduced in 2000 (Singhal *et al*., 2015; Vrioni *et al*., 2018). This method is reliable due to its accuracy and sensitivity. Previous studies have shown its significance especially in discriminating MRSA, MSSA, as well as other bacterial strains whose resistance and susceptibility been evaluated through the spectral peak analysis (Singhal *et al*., 2015).

This method has capabilities to demonstrate minor expression differences in isogenic strains of *S. aureus* (Akindolire *et al*., 2015; Welker and Belkum, 2019). Matrix-Assisted Laser Desorption Ionization Biotyper, a newly identified antibiotic susceptibility test method provides a rapid assay (MBT-ASTRA) which is cost effective and more straightforward modulation of MALDI-TOF MS applied for both MIC and AST (Burckhardt and Zimmermann, 2017). The main limitation of MALDI-TOF MS is the expensive nature of the instrument as well as its maintenance.

2.5.5 Molecular Methods of antimicrobial susceptibility testing

Genotypic or molecular AST are efficient methods that are direct and do not involve the long incubation, tedious bacterial cultures and chances of contamination (Bard and Lee, 2018). Polymerase chain reaction (PCR), DNA chips, DNA microarray, and loop-mediated isothermal amplification (LAMP) constitute examples of genotypic techniques that are employed for the detection of antibiotic resistance (Khan *et al*., 2019). Mutational assessment of methicillin resistance in *Staphylococcus spp*., multi-drug (pyrazinamide, streptomycin, rifampin, fluoroquinilones and isoniazid) vancomycin resistance in *S. aureus* and multi-drug resistance in Mycobacterium spp. have been investigated through the various genomic techniques.

PCR is an example of a rapid and most efficient genotypic method for detection and quantification of bacterially transmissible genes. The study on application of PCR for diagnostic purposes was first reported by Saiki (Fluit *et al*., 2001). In general, PCR methodology includes denaturation cycles, annealing of the primers, as well as elongation of the primers catalysed by a thermostable DNA polymerase enzyme in a compatible buffer enriched with ions, nucleotides, and other compounds. In every cycle of amplification, the size of the target DNA molecule doubles. The resultant amplified target can then be confirmed for the presence of genetic determinants of resistance through electrophoresis, southern blotting, restriction fragment-length polymorphism, DNA fingerprinting, single-strand conformation polymorphism (SSCP), molecular beacons, and other DNA sequencing analysis methods (Fluit *et al*., 2001; Miller and Tang, 2009).

Generally, the genotypic methods can be attributed to the rapid, sensitive, specific and direct detection of genetic determinants of resistance. However, these techniques also suffer from

various drawbacks. To begin, these methods can only be used to detect potential/key resistance genes which are not mostly relevant as a result of coincidental mutations. Additionally, the antimicrobial agents need to be individually tested in a specific assay for detection. Next, patients with latent infections lack sensitivity, or when samples contain only a few organisms. Furthermore, not all the genetic mechanism for the resistance has been defined for all bacteria. Morever, false-positive results due to test sample contamination might be expected. Also, these methods require expensive machinery and reagents with specific maintenance conditions; finally, and most importantly, all the tools require prerequisite of skilled personnel (Khan *et al*., 2019; Wei *et al*., 2019).

CHAPTER 3: IDENTIFICATION OF *STAPHYLOCCOCUS AUREUS* FROM LIVESTOCK MILK FOR HUMAN CONSUMPTION IN ISIOLO AND MARSABIT

3.1 Introduction

Staphylococcus aureus is a significant public health pathogen responsible for toxin-mediated food poisoning, antibiotic resistance and invasiveness (Reddy *et al*., 2017; da Silva *et al*., 2020). The bacterium causes a wide range of infections, varying from superficial skin infections to severe and potentially fatal diseases (Sergelidis and Angelidis, 2017; Aires-de-Sousa, 2017; Wang *et al*., 2014). Furthermore, *S. aureus* is a common pathogen of ruminants such as cattle, goats, and sheep where it may lead to subclinical and clinical mastitis. *Staphylococcus aureus* can contaminate milk and other dairy products during farming and value addition process.

The main sources of contamination of raw milk by *S. aureus* are dairy animals with mastitis, colonization of the animal skin and mammary glands (Kalayu *et al*., 2020). One of the ways through which the bacteria spread into raw milk and other dairy products is through transfer from udder of the infected animals, consequently affecting the quantity and quality of the products. The pathogen can therefore become a serious economic burden for farmers and a significant problem along the dairy value chain (Ayele *et al*., 2017). Food handlers carrying *S. aureus* on their bodies may contaminate food (Ahmed, 2020) indicating that the presence of *S. aureus* in milk can be due to contaminations from skin of food handlers. Other causes of contamination of milk includes milking equipment, improper handling of animals and poor hygiene measures during value addition process of dairy products (Jorgensen *et al.*, 2005). Consequently, *S. aureus* has been isolated in raw milk, butter, cheese, clotted cream, and ice cream all over the world

(Jorgensen *et al*., 2005; Fagundes *et al.*, 2010; Jakobsen *et al.*, 2011; Gucukoglu, *et al.*, 2012; Rahimi and Alian, 2013; Kateete *et al.*, 2013; Asiimwe *et al*., 2017).

Raw milk contamination by *S. aureus* not only causes spoilage, but leads to staphylococcal food poisoning in humans if such products are consumed. Raw milk spoilage as a result of *S. aureus* contamination leads to losses in the dairy enterprises. Such food poisonings are as a result of consumption of preformed staphylococcal enterotoxins. Indeed, there has been an increase in hospitalizations and deaths as a result of food borne outbreaks that have been linked to *S. aureus* enterotoxins. For example, one of the largest staphylococcal food poisoning outbreaks involving 13,420 infected individuals was reported in Japan recently (Qian *et al*., 2019). Further, in Sicily, (Italy) cases of sporadic food poisoning showed that some pathogenic *S. aureus* strains were circulating in local farms with healthy animals free from any overt clinical signs (Vitale *et al*., 2018). On further analysis, up to 46% of the isolates carried a toxin gene, implying that food could be an important vector for the transmission of pathogenic *S. aureus* strains (Vitale *et al*., 2018). Similarly, food-borne infections caused by contaminated dairy foods by *S. aureus* were also frequently described in China (Rong *et al*, 2017). Currently, more than 20 Staphylococcal enterotoxins or enterotoxin-like proteins have been characterized (Fisher *et al*., 2018; Filipello *et al*., 2020).

In sub-Saharan Africa, more so in pastoralist communities that interact closely with livestock, *S. aureus* can contaminate milk and lead to serious illnesses in food and livestock production systems, general population as well as healthcare units (Njage *et al*., 2013: Gitau *et al*., 2014; Egyir *et al*., 2014b, 2014a; Akindolire *et al*., 2015; Maina *et al*., 2016). In addition, inadequate

investigation, under-reporting of the outbreaks and inadequate diagnostic facilities has led to unreliable reporting of staphylococcal food poisoning in these regions. Previous studies in Kenya indicate that *S. aureus* is responsible for up to 38% of reported foodborne disease outbreaks (Ombui *et al*., 2001). Moreover, a previous study by Ombui *et al*., (1992) had reported a prevalence of 74.2% for enterotoxins-producing *S. aureus* in raw milk sampled from Nairobi and its environs. Out of these isolates, three (4.17%) were reported to produce SEA on screening using latex agglutination test. Mathenge *et al*. (2015) also detected enterotoxigenic *S. aureus* strains in dairy and meat products in Nairobi County and its surroundings. However, in spite of these reports, the actual risk of staphylococcal food poisoning in the pastoral areas of northern Kenya generally remains unknown yet this information is crucial for mitigating foodborne illnesses.

Therefore, this study determined the prevalence of *S. aureus* in raw milk sampled from cows, goats, sheep, camels and pooled milk sample from the pastoralist households. In particular, this study determined the contribution of pooling of milk by pastoralists to the contamination of milk at household level. The information provided here could assist pastoralist in northern Kenya to mitigate against the contamination of milk by *S. aureus*.

3.2 Materials and methods

3.2.1 Study area and livestock population

This study was done in Isiolo and Marsabit counties in northern part of Kenya in June 2016 and February 2017 (Fig. 3.1). Both counties are part of the arid and semi-arid land (ASAL) areas of the country and are inhabited by pastoralists whose livelihoods are mainly dependent on livestock.

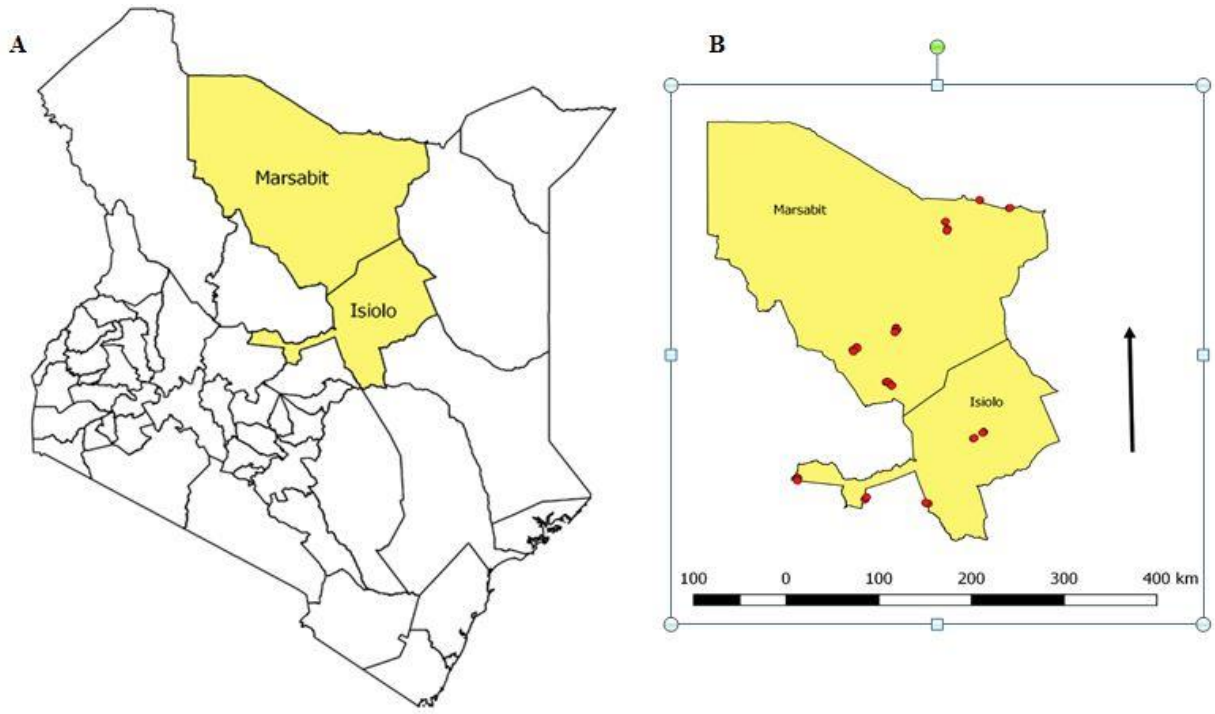

Figure 3.1. A map of Kenya showing the counties where the samples were collected and questions administered; B) shows specific maps of Isiolo and Marsabit counties

3.2.2 Study design

A cross-sectional study design with a household being a unit of analysis was used. Four wards (Burat, Kinna, Merti and Oldonyiro) in Isiolo county and six wards in Marsabit (Karare, Korr, Laisamis, Moyale, Sololo and Turbi) county were conveniently selected as the study sites whereas households within these areas were randomly identified using systematic sampling method along transects defined by feeder roads. In this process, every firth household that kept animals (cattle, sheep, goats and camels) of interest in this study were recruited obtaining a total of 188 households.

3.2.3 Sample collection

From each household one pooled milk sample consisting of milk from multiple lactating animals and randomly selected individual lactating animals were collected from animals kept in those households. A total of 603 milk samples from 57 zebu cattle, 346 Galla goats, 8 red Maasai and dorper sheep, 4 one-humped camels (*Camelus dromedaries*) and 188 pooled were collected from Isiolo and Marsabit counties. After selected households consented to participate in the study, a total of 603 milk samples were collected of which 305 were from Isiolo and 299 from Marsabit. About 10 ml of milk was aseptically collected into a sterile 15 ml falcon tube after disinfection of the udder using cotton swabs moistened with 70% ethyl alcohol. Individual milk samples were collected mid-stream from all the teats of the lactating cattle, sheep, goats and camels. Additionally, a pooled milk sample from each household was collected into a 50 ml sterile falcon tube identified using unique barcode identifiers. Aliquotes of milk samples were placed into cryovials and stored at -20^0C pending laboratory analysis. A questionnaire was administered to capture animal and households

data. Information on milk processing before consumption was collected and households' geo-referenced using Garmin ETrex hand held GPS units.

3.2.4 Isolation and identification of *Staphylococcus* Species

Ten microliters of milk samples from each collection site was inoculated to selective medium mannitol salt agar (MSA) and incubated at 37^{o}C for 24- 48 h. Growth of yellow colonies on this medium surrounded by yellow zones was considered a presumptive positive for *S. aureus* (Kateete *et al.* 2010)

The presumptive *S. aureus* colonies were sub cultured onto 5 % sheep blood agar and incubated at 37^{o}C for 24 h to get a pure culture. Identification of *S. aureus* and other staphylococci was performed using the following tests: Morphology following Gram staining, catalase activity, mannitol fermentation, coagulase activity and *β*-hemolysis. The isolates were confirmed by amplification of *S. aureus* specific staphylococcal terminase gene (satm).

3.4.5 Extraction of *Staphylococcus aureus* DNA

Genomic DNA extraction was performed using Invitrogen DNeasy DNA extraction protocol for bacterial cultures. Bacterial DNA was extracted according to the protocol provided by the manufacturer (Invitrogen DNeasy ®). Colonies were harvested and suspended in 180 µL lysozyme digestion buffer and incubated at 37^{0}C for 30 minutes. Twenty microlitres of Proteinase K was added followed by 200 µL of PureLink™ genomic lysis/binding buffer. After incubation at 55^{0}C for 30 minutes and addition 200 µl of 96-100% ethanol, DNA was bound to silica-gel-membrane in a brief centrifugation step. The inhibitors of PCR such as the proteins and divalent cations were completely removed in two washing steps, leaving pure nucleic acid

behind, which was eluted in the elution buffer. The DNA was stored at 4°C in refregirator pending subsequent analysis. The DNA quantity and purity was assessed spectrophotometrically at 260-280 nm, with NanoDrop ND-1000 full spectrum UV-Vis spectrophotometer**.**

3.2.6 Detection of *Staphylococcus aureus* by PCR

Primers that target the staphylococcal terminase gene (MH678720) were designed using the Primer Blast tool (www.ncbi.nlm. nih.gov/tools/primer-blast/) for detection of *S. aureus*. Oligonucleotide forward primer was 5'-TAACCCCTCATCACCTCCGT-3'and the reverse primer was 5'-ACTGCAAAGCAAGCACGTTT-3'. The annealing temperature was determined using a gradient PCR on the Veriti 96-well thermal cycler (Applied Biosystems, Foster city, CA, USA). A 25µl reaction volume contained 12.5 µl of 1X dreamTaq mastermix (Fermentas, Thermo Scientific, USA) 10000nM of each forward and reverse primers, 1 µl of DNA template and 9.5 µl nuclease free water. Optimized PCR conditions were: 95°C for 3 min; 35 cycles of 95°C for 30 sec; 57°C for 60 sec; and 72°C for 1 min with a final extension at 72°C for 10 min. Amplified products were detected using gel electrophoresis. DNA extracted from *S. aureus* subsp. *aureus* Rosenbach (ATCC® 25923^{TM}) was used as the positive control and nuclease-free water as the negative control in all the analyses done.

Some PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH Hilden, Germany) and taken for sequencing at Macrogen Inc (Macrogen Europe Meibergdreef, Amsterdam, The Netherlands). Sequences obtained were edited and analysed using the BLASTn tool to confirm identity of the isolates.

3.2.7 Data management and analysis

The milk samples and their corresponding *S. aureus* isolates were labelled and barcoded. Thereafter, data derived from the bacterial isolates were entered in Microsoft access. Data from the bacterial isolates and milk samples from individual animals and households were merged, and then imported to the R software. Descriptive statistics of the variables were determined by calculating the proportions of milk samples contaminated with *S. aureus* using the R console. Pearson chi-square test was used to determine any statistically significant differences in the proportion of *S. aureus* isolated from pooled milk samples and those from individual lactating animals at 95% confidencce interval.

3.3 Results

3.3.1 *Staphylococcus aureus* phenotypes detected raw milk

Two hundred and fifty presumptive *S. aureus* were isolated from 603 milk samples by culture on Mannitol salt agar (MSA). On biochemical tests, 223 (89.2%, 95% CI: 84.74–92.47%) of the isolates were catalase positive, 151 (60.4%, 95% CI: 54.22–66.26%) isolates were β- hemolytic while 129 (51.6%, 95% CI: 45.43–57.72%) isolates were coagulase positive (Table 3.1) (Fig. 3.1). Out of these, 85 (14.09%, 95% CI: 11.55–17.1%) isolates were confirmed to be *S. aureus* using amplification of satm gene. Among the 85 isolates, 43 isolates (14.38%, 95% CI: 10.86%–18.81%) were from Marsabit and 42 isolates (13.82%, 95% CI: 10.39%–18.15%) were from Isiolo counties (Table 3.1).

With respect to species distribution, 17 (40%) pooled milk samples and 6 (14%) individual milk samples from cattle in marsabit County were contaminated with *S. aureus*. In Isolo county, 5 (12%) pooled and 10 (24%) individual milk samples from cattle were positive for *S. aureus* isolates. Nine pooled milk samples and 9 (21%) individual milk samples from goats in Marsabit county were contaminated with *S. aureus* as compared to 15 (36%) individual and 10 (24%) pooled milk samples in Isiolo counies. Among the sheep, 2 (5%) pooled milk samples were contaminated in Marsabit and from Isiolo County. None of the four milk samples from camels was contaminated with *S. aureus*.

Pearson chi-square test revealed a significantly (p=0.0001) higher proportion of *S. aureus* isolated from pooled milk samples (23.94%, 95% CI: 18.40–30.52%) than those from individual lactating animals (9.64%, 95% CI: 7.12–12.86%).

In Marsabit county, there was a significantly higher (p=0.0001) proportion of isolates from pooled milk (25.44%, 95% CI: 18.34–34.14%) as compared to individual lactating animals (7.37%, 95% CI: 4.44–11.99%). The trend was similar in Isiolo County where proportion of isolates from pooled milk samples (21.62%, 95% CI; 13.77–32.27%) was higher as compared to milk from individual lactating animal (11.56%, 95% CI: 8.01–16.39%) (p=0.03).

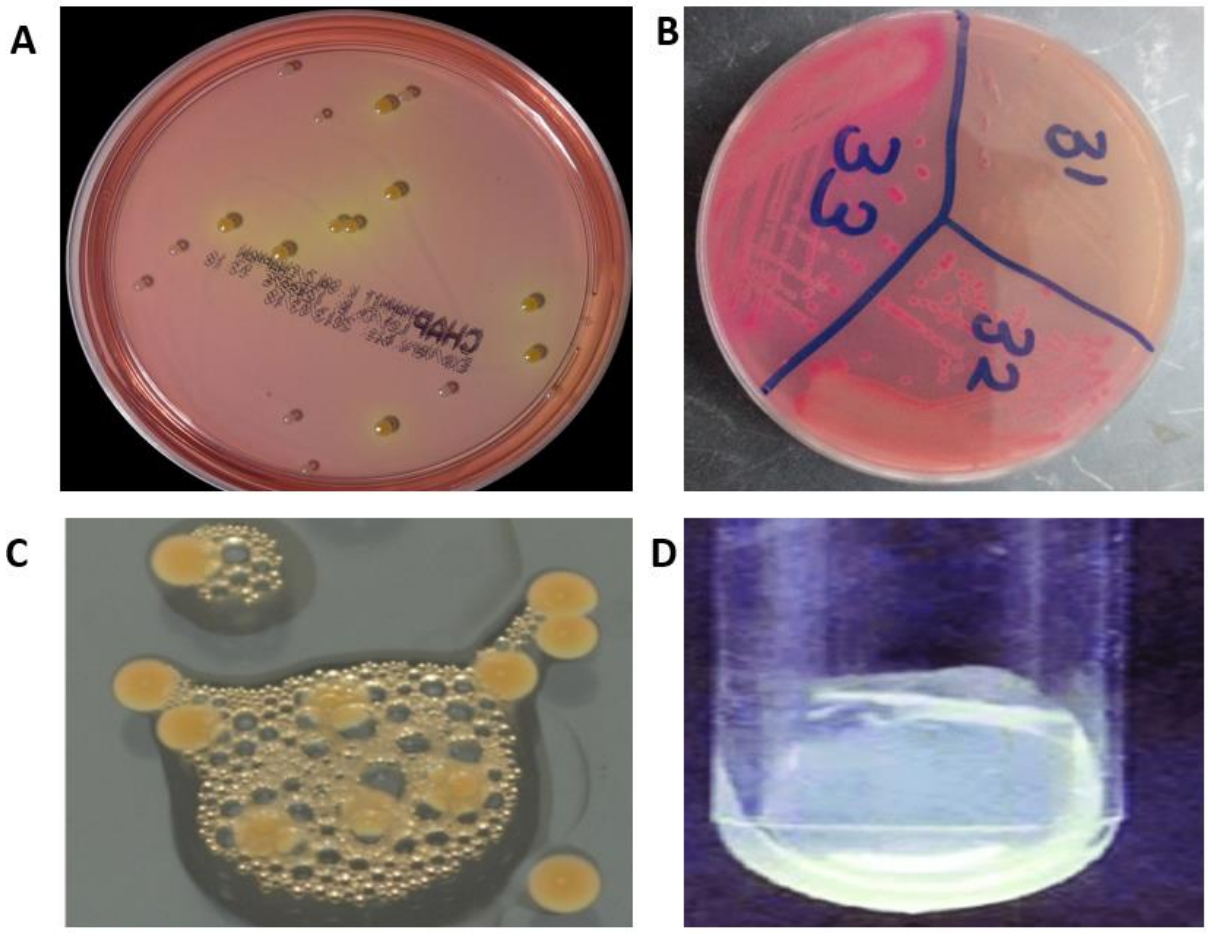

Figure 3.1. Various biochemical tests used for the identification of *S. aureus*. A-growth on mannitol salt agar (MSA), B-beta-hemolysis test using sheep blood agar, C- positive catalase test and D –positive tube coagulase test.

3.3.2 *Staphylococcus aureus* confirmed by sequencing

Staphylococcus aureus analyzed by culture and biochemical tests were confirmed by PCR and sequencing. The primer targeting the terminase gene amplified the fragment yielding a specific band corresponding to approximately 517bp (Fig. 3.2). The sequenced PCR products were confirmed to be *S. aureus* gene by Blastn analysis using Genbank of the NCBI database. The blastn results

revealed that the genes were homologous to *S. aureus* DNA Pathogenicity Island, one of the genetic determinants responsible for pathogenicity of the bacterium. The nucleotide identities of the sequenced isolates as compared with the homologues above were between 95% and 98%. Subsequently, this result confirmed that the 85 (14%) isolates obtained from the milk samples were actually potentially pathogenic *S. aureus*. The accession numbers of the sequenced terminase gene of *S. aureus* are available in the Genbank under the accession numbers MH678717-MH678720. Blastx analysis revealed that the translated amino acid sequences were homologous to the *S. aureus* terminase small sub unit protein revealing sequence identity of 100 %. (Fig. 3.3).

Table 3.1 Prevalence of *S. aureus* in raw milk in Isiolo and Marsabit counties.

County	**MSA**	***β*-hemolysis**	**Catalase-test**	**Coagulase-test**	**PCR**
Overall	250 (41%)	151(60%)	223 (89%)	129 (52%)	**85 (66%)**
Isiolo	150 (49%)	104 (69%)	136 (91%)	78 (52%)	42 (54%)
Marsabit	100 (33%)	47(47%)	87(87%)	51(51%)	43 (84%)

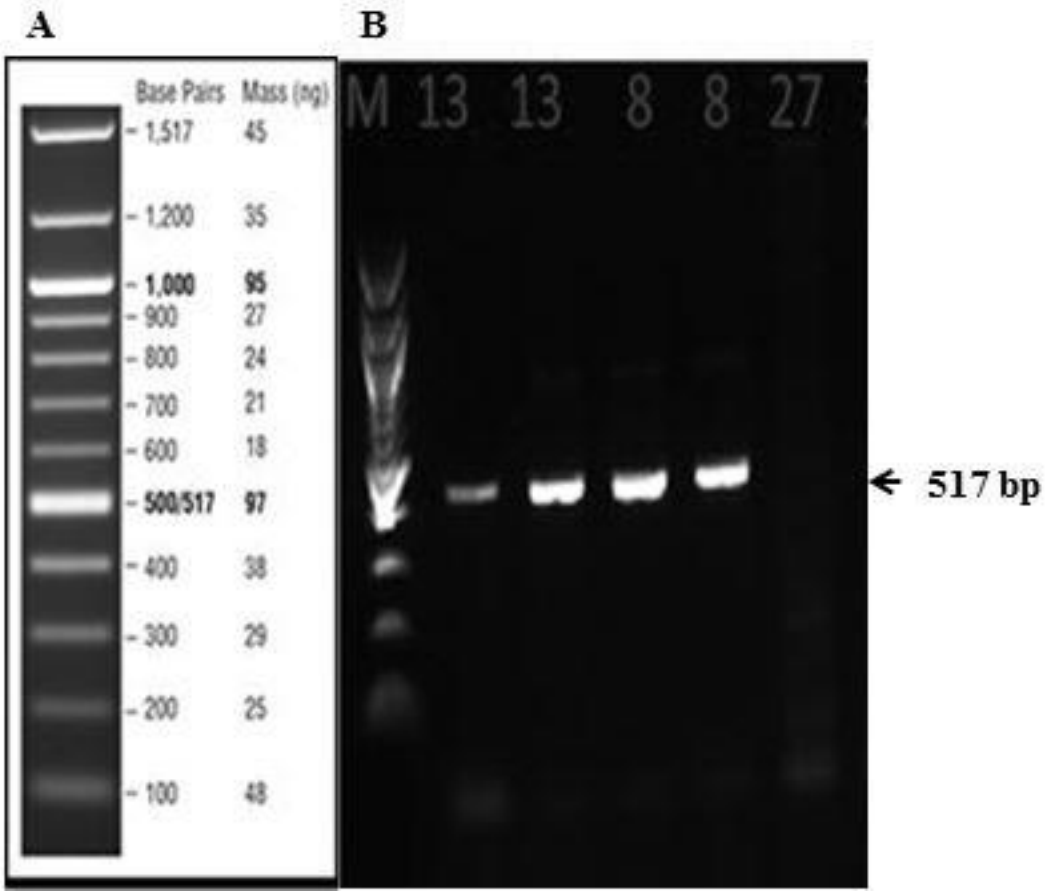

Figure 3.2. PCR amplification of *S. aureus*-terminase gene from representative isolates obtained from milk samples. The amplification of the gene is seen by presence of a specific band corresponding to approximately 517bp.

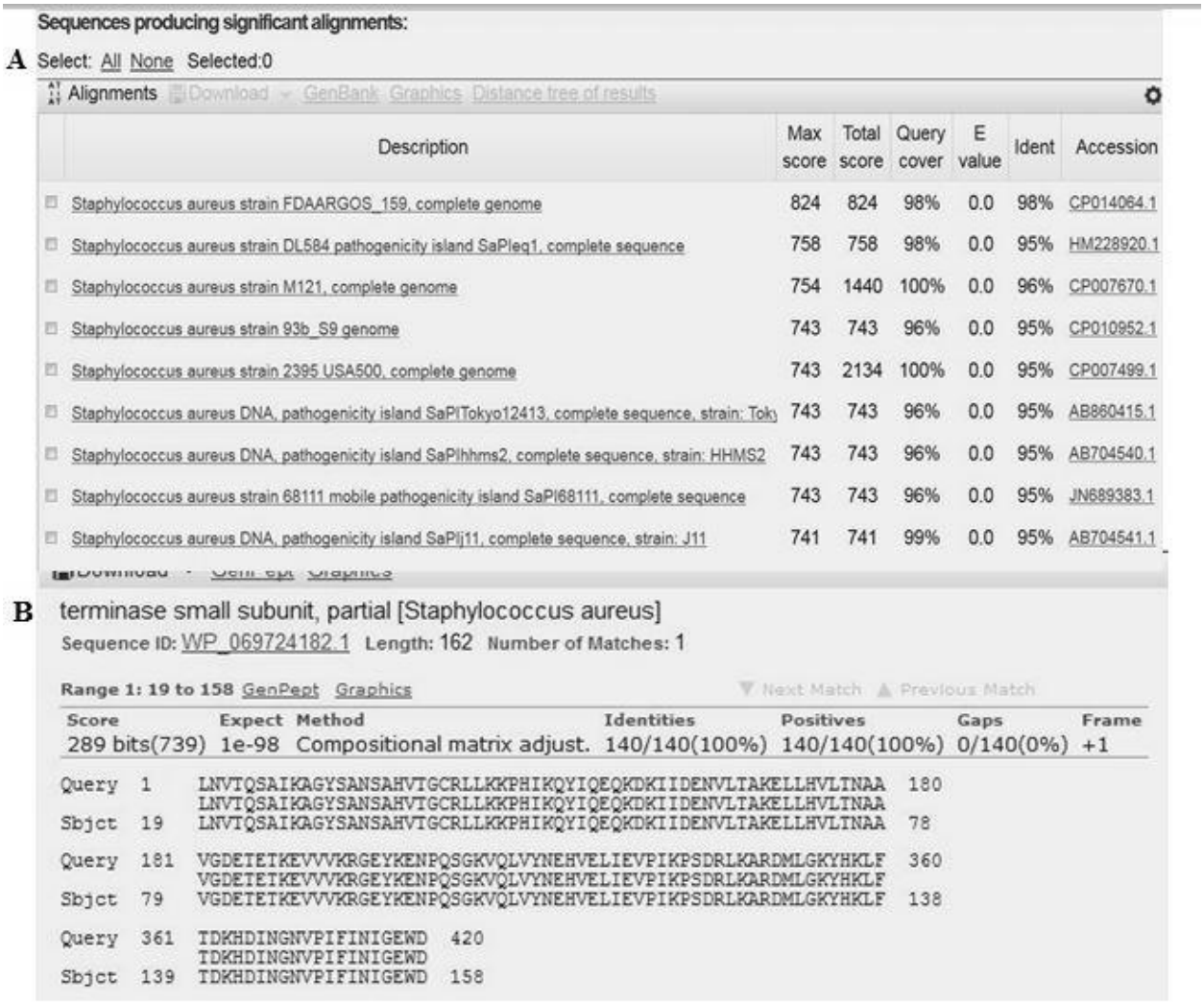

Figure 3.3 Confirmation of *S. aureus* by Blastn analysis. (A) - *S. aureus* confirmed with nucleotide identities of 95% to 98% to our sequenced amplicons, (B) -translated amino acid sequences with 100% identities to those present in GenBank

3.4. Discussion

This study determined the presence of potentially pathogenic *S. aureus* in raw milk intended for human consumption in northern Kenya. *Staphylococcus aureus* β-hemolysin is one of the cytotoxic molecules responsible for its pathogenicity (Dinges *et al*., 2000). These exotoxins together with coagulase are known to be some of the virulence factors responsible for its ability to cause infections (Hennekinne *et al*., 2012). In this study, a number of *S. aureus* isolated from milk were β-hemolytic and coagulase positive indicating that these bacterial isolates could be potentially pathogenic.

Terminase gene was used in this study to identify potentially pathogenic *S. aureus* from the milk samples because it forms one of the core genes in the staphylococcal pathogenicity islands (SaPIs), which is a mobile genetic element responsible for the bacterial virulence (Malachowa and DeLeo, 2010). This study found that the *S. aureus* sequences were homologous to *S. aureus* DNA Pathogenicity Island with the corresponding translated amino acids sequences being homologous to the *S. aureus* terminase small sub unit protein suggesting that these isolates were pathogenic.

In this study, the overall occurrence of *S. aureus* in the analyzed samples was 14% for milk samples in Marsabit and Isiolo Counties. This prevalence was lower than that of Asiimwe *et al*. (2017) who reported a prevalence of 20.3% for *S. aureus* detected in bulk can-milk and 12.1% in sour milk consumed in pastoral areas of Uganda. The prevalence was also lower than that reported by Mathenge *et al*. (2015) in which an overall prevalence of 36% in meat and milk products was found in Nairobi county and its surroundings.

Higher prevalence rates in milk were also reported in various studies done in other countries including Turkey (Kiymet *et al*., 2010), USA (Lubna *et al*., 2015), Zimbabwe (Gran *et al*., 2003) and in Malaysia (Chye *et al*., 2004). Other studies have reported *S. aureus* prevalence ranging from 51% to 91% in bulk milk samples (Jørgensen *et al*., 2005; Katholm *et al*., 2012; Walcher *et al*., 2014).

Another study in Greece reported *S. aureus* prevalence of 17.6% in raw goat milk, prevalence higher than our finding. Similarly, a study in china reported a contamination rate of *S. aureus* in pork industry of 26% (130/501) (Zhang *et al*, 2018). In Italy, a *S. aureus* prevalence of 53.5% (153/286) was detected in of the bulk tank milk (Giacinti *et al*, 2017), as well as 46% (47/104) of the bulk tank milk samples in the United States (Merz *et al*. 2016). In addition, a higher contamination rate of *S. aureus* was 76.9% (60/78) in bulk tan milk samples in Italy (Spanu et al, 2013) was demonstrated in another study. However, Xing *et al*. (2016) found a prevalence of 1.5% (1/67) of *S. aureus* in raw goat milk of healthy goats in Shaanxi province as well as a prevalence of 7.5% (60/781) in a final product of goat milk powder in the processing plant environments in 2012–2013. Thus, measures to control *S. aureus* contamination in raw milk including goat milk should be adopted. Indeed, in this study, most the raw milk samples studied were collected from goats. The pastoral community in northern Kenya relies on goat milk during drought since small ruminants (Goat and sheep) are more resilient and hence can withstand the harsh arid and semi-arid conditions in addition to their reduced feed requirement.

Data from small ruminants' farms in this study show higher *S. aureus* and MRSA prevalence estimates compared to those previously reported in ovine and caprine bulk tank milk in Greece

(Pexara et al., 2016). The reported isolation frequencies of *S. aureus* and MRSA in small ruminants' milk in other countries are quite variable, ranging from 43.1% to 76.9% and from 0% to 2%, respectively (Cortimiglia *et al*., 2015; Giacinti *et al*., 2017; Spanu e*t al*., 2013). The aforementioned differences in the reported *S. aureus* and MRSA prevalence estimates among different studies may be attributed to the sensitivity of the detection methods used i.e., selective isolation of MRSA vs. targeting *S. aureus* and testing of isolated S. aureus for MRSA phenotypic traits (Lakhundi *et al*., 2018)

Overall, the proportion of *S. aureus* isolated from pooled milk samples was significantly ($p=0.0001$) higher than those from individual lactating animals. During pooling of milk, there is risk of contamination from human skin carrying *S. aureus* thereby resulting in higher contamination level as seen above. Alternatively, pooling of milk under poor hygienic conditions involving use of contaminated utensils could have been responsible for the relatively high contamination. For individual milk samples, contamination by *S. aureus* isolates may have been due to clinical and subclinical mastitis resulting from intramammary infections caused by the bacterium. Usually the bacterium is known to enter the udder through the teat canal from the surrounding environment (Smith *et al*., 2005) and this can act as a source of infection and milk contamination. Nevertheless, other modes of contamination cannot be ruled out and further studies are needed to establish the other possible sources of contamination of milk with *S. aureus*.

Increased awareness of pastoralists is necessary in order to minimize contamination of milk through improved hygiene practices as well as through diagnosis and treatment of infected animals. Pastoralists in northern Kenya live in geographically different ecosystem and practice

livestock production system different from other systems in other regions of the country. It is possible that this difference in geographical ecosystem and production systems could be responsible for the disparities in the contamination levels seen with the other previous studies.

Nevertheless, this claim needs to be confirmed by performing further studies because other factors could have been responsible for the high level of Staphylococcal-contamination. The high level of *S. aureus* detected in raw milk may also be as a result of contamination by milk-handlers potentially harboring the bacterium. Indeed, it has been reported that up to 40% of the human population are colonized by *S. aureus* in their nares and skin. (Van Belkum *et al*., 2009; Crago *et al*., 2012).

The detection of *S. aureus* from raw milk was higher in goat milk than from other species of livestock in this study. This is important since information on risks associated with consumption of goat milk is scarce in this region and the country at large. This could be ably due to improper hygiene and poor management practices among the pastoralists. This comes at the wake of increased demand for goat milk mainly due to its differentiated nutritional properties when compared with cow milk (Miller and Lu, 2019; Sepe and Argüello, 2019). In addition, the potential of goat milk to develop distinctive products, especially cheeses has been widely explored for in recent times in other countries as well (Miller and Lu, 2019; Sepe and Argüello, 2019). Goat dairy products have high added value due to their unique taste and perception as a healthy food among consumers (Medina *et al*., 2011; Jaafar *et al*., 2018; Gallier *et al*., 2020). For example, various types of cheeses have been prepared from raw goat milk; hence contamination of milk with *S aureus* raises significant concern to public health. Indeed, previous studies have

shown goat milk and goat milk products to be potential source of food borne pathogens, such as enterotoxigenic *S. aureus* (Silanikove *et al*., 2010; Rola *et al*., 2014). Therefore, guaranteeing adequate hygienic standards of the goat herd, the environment, as well as efficient control during the dairy value chain is required to ensure the quality and safety of these products.

CHAPTER 4: CHARACTERIZATION OF THE VIRULENCE FACTORS ASSOCIATED WITH PATHOGENICITY OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM NORTHERN PASTORAL REGION OF KENYA

4.1 Introduction

Staphylococcus aureus (S. aureus) is known to widely cause foodborne illnesses in humans with most of the outbreaks resulting from consumption of many food products including milk obtained from different parts of the world (Aragon-Alegro *et al*, 2007; Chiang *et al*, 2008; Zouharova and Rysanek, 2008; Chaalal *et al*., 2018). *Staphylococcus aureus* can contaminate several food products including pork, beef, mutton, poultry, eggs and milk during farming, and value addition process. The bacterium is normally found in the skin and nasal cavities of humans and animals. About 30 % of humans are thought to be intermittent carriers while the other 20% are known to be permanent carriers (Van Belkum, *et al*, 2009). Food handlers carrying *S. aureus* on their bodies may contaminate food (Crago *et al.*, 2012) indicating that the presence of *S. aureus* in milk can be due to contaminations from skin of food handlers. The contamination of milk with the bacterium can also be from milk of animals with intra-mammary infections (Kümmel *et al.*, 2016), and from the environment as a result of poor hygienic conditions during milking (Dittmann *el al*., 2017).

Staphylococcal food intoxication is dependent on a single type of virulence factor that is responsible for the production of heat stable staphylococcal enterotoxins (SEs) by certain strains of *S. aureus* (Fisher *et al*., 2018). There is a strong association between the ability of *S. aureus* strains to produce one or more of the SEs and the occurrence of staphylococcal food poisoning.

Currently, about 23 SEs have been identified and out of these, five enterotoxins namely *SEA, SEB, SEC, SED,* and *SEE* are considered to be the classical enterotoxins (da Silva *et al*., 2020). The five *SEs* are encoded by specific enterotoxin genes such as *sea, seb, sec, sed*, and *see* respectively. Some of these enterotoxins are produced by *S. aureus* when the bacterium grows in unpasteurized raw milk. Pasteurizing raw milk, which involves heat-treatment, normally eliminates *S. aureus* from raw milk. However, once the *S. aureus*-enterotoxins have been produced, they can withstand high temperatures of pasteurization (Asao *et al.*, 2003). For example, SEs such as *SEA* is known to be highly resistant to heat treatment and retain their biological activity after exposure to a high temperature of 121°C for 28 minutes (Seyoum *et al.*, 2016). Therefore, if raw and unpasteurized milk contaminated with the SEs is consumed they can cause food poisoning sometimes resulting in deaths.

Of late, there has been increasing incidences of staphylococcal food poisoning worldwide resulting in serious food safety concerns (Pu *et al.,* 2011). For example, in 2012 enterotoxins-producing *S. aureus* was reported in European Union where it caused 346 foodborne disease outbreaks (FBOs) representing 6.4 % of all the outbreaks documented (Macori *et al.*, 2016). In the United States, staphylococcal food poisoning has been reported to account for approximately 241,000 illnesses annually. Some of these cases required hospitalization and were part of the 56,000 foodborne illnesses in the United States (Scallan *et al*., 2011; Byrd-Bredbenner *et al.*, 2013). In China, approximately 20–25% of reported bacterial foodborne illnesses have been reported to be caused by *S. aureus* isolated from retail food outlets in Shaanxi (Wang *et al*., 2014).

In sub-Saharan Africa, especially in communities that interact closely with livestock like pastoralist communities, *S. aureus* can cause serious illnesses in general population, healthcare units, as well as in food and livestock production systems (Njage *et al.*, 2013: Gitau *et al.,* 2014; Egyir *et al*., 2014a, 2014b; Akindolire *et al*., 2015; Maina *et al*., 2016). Furthermore, under-reporting, inadequate investigation of the outbreaks and inadequate diagnostic facilities has led to unreliable data on staphylococcal food poisoning in these regions. In Kenya, *S. aureus* is responsible for up to 38 % of reported foodborne disease outbreaks (Ombui *et al* 2001). Moreover, another study by Ombui *et al*. (1992) also reported a prevalence of 74.2 % for enterotoxins-producing *S. aureus* in raw milk sampled from Nairobi and its environs. Out of these isolates, three (4.17%) were found to produce *SEA* on screening using latex agglutination test. Mathenge *et al* (2015) also identified enterotoxigenic *S. aureus* strains in meat and dairy products in Nairobi County and its surroundings. However, in spite of these reports, the actual risk of staphylococcal food poisoning in the pastoral areas of northern Kenya generally remains unknown yet this information is crucial for the management of foodborne illnesses. The unpasteurized raw milk regularly consumed by the pastoral communities in northern Kenya could contain potentially pathogenic *S. aureus,* which harbour enterotoxin genes responsible for the production of heat stable SEs.

Therefore, this study determined the risk of contamination of raw milk of cows, goats, sheep and camels with enterotoxigenic *S. aureus.* The study also established whether the potentially pathogenic isolates harbour enterotoxin genes, which encode the heat-stable SEs responsible for the foodborne illness. The information provided here could assist pastoralist in northern Kenya to mitigate outbreaks associated with fatal Staphylococcal food poisonings.

4.2. Materials and methods

4.2.1 Study area and livestock population

This study was carried out in Isiolo and Marsabit counties in northern Kenya. The study design is as earlier described earlier in chapter 3.

4.2.2 Study design

A cross-sectional study design was used to determine bacteriological analysis of *S. aureus* in milk with households as unit of analysis. Within each household, one pooled milk sample was collected as well as up to three samples from randomly selected lactating animals (Chapter 3).

4.2.3 Sample collection

Sampling was done as previously reported (Chapter 3). Briefly, 10ml of raw milk was drawn aseptically from the udder into a sterile 15ml falcon tube midstream following disinfection by cotton wool moistened by 70% ethyl alcohol. Further, about 50 ml of pooled household milkfrom the recruited households were collected into a sterile falcon tube uniquely identified by barcodes. Consequently, aliquots of milk samples were placed into cryovials and transported on ice to the Department of Public Health, Pharmacology and Toxicology, University of Nairobi for analysis and storage. A questionnaire was administered to capture animal and households data

4.2.4 Extraction of *Staphylococcus aureus* DNA

Genomic DNA extraction was performed using Invitrogen DNeasy DNA extraction protocol for bacterial cultures as reported in chapter 3 (section 3.4.5). Briefly, bacterial DNA was extracted according to the protocol provided by the manufacturer. Colonies were harvested and suspended

in 180 µL lysozyme digestion buffer, incubated at 37^0C for 30 minutes followed by the addition of Proteinase K and then ethanol. DNA was bound to silica-gel-membrane in a brief centrifugation step followed by two washing steps, leaving pure nucleic acid behind, which was eluted in the elution buffer.

4.2.5 Molecular detection of staphylococcal enterotoxin genes by PCR

The isolates confirmed to be potentially pathogenic *S. aureus* using PCR were further evaluated to determine whether they harbor enterotoxin genes responsible for milk-borne food poisoning. Oligonucleotides primers used in a previous study (Table 4.1) were used for the amplification of the enterotoxin encoding genes (Mehrotra, *et al.,* 2000). Synthesis of oligonucleotides was done by Macrogen Inc (Macrogen Europe Meibergdreef, Amsterdam, The Netherlands). The primers were used for the amplification of *sea, seb, sec, sed* and *see* genes. Some amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH Hilden, Germany) and taken for sequencing at Macrogen Inc (Macrogen Europe Meibergdreef, Amsterdam, The Netherlands). Sequences obtained were edited and analysed using the BLASTn tool to confirm identity of the isolates. The sequenced genes were then subjected to Blastx analysis to confirm whether the amplified genes encoded enterotoxin proteins.

Table 4.1. Primers used for the PCR amplification of *S. aureus* enterotoxin genes

Gene	Primer name	Oligonucleotide sequence (5'- 3')	Amplicon size (bp)
sea	GSEAR1[1]	5'- GGTTATCAATGTGCGGGTGG-3'	102
	GSEAR2[1]	5'- CGGCACTTTTTTCTCTTCGG – 3'	
seb	GSEBR1[1]	5'- GTATGGTGGTGTAACTGAGC – 3'	164
	GSEBR2[1]	5'- CCAAATAGTGACGAGTTAGG- 3'	
sec	GSECR1[1]	5'- AGATGAAGTAGTTGATGTGTATGG-3'	451
	GSECR2[1]	5'- CACACTTTTAGAATCAACCG-3'	
sed	GSEDR1[1]	5'- CCAATAATAGGAGAAAATAAAAG-3'	278
	GSEDR2[1]	5'- ATTGGTATTTTTTTTCGTTC-3'	
see	GSEER1[1]	5'- AGGTTTTTTCACAGGTCATCC-3'	209
	GSEER2[1]	5'- CTTTTTTTTCTTCGGTCAATC-3'	

Primers adopted from Mehrotra, et al., (2000)

4.2.6 Data management and analysis

Data on *S. aureus* enterotoxin gene carriage were entered in Microsoft access and merged with individual animal milk and pooled milk data from households and then imported to the R software. Descriptive statistics of the variables were determined by calculating the proportions of *S. aureus* isolates habouring various enterotoxins using the R console. Pearson chi-square test was used to determine any statistically significant differences in the proportion of *S. aureus* habouring enterotoxin genes from pooled milk samples and those from individual lactating animals at 95% confidencce interval.

4.3. RESULTS

4.3.1 Isolation and confirmation of *S. aureus*

The total number of samples collected, isolated and confirmed is as described in chapter 3 (results section). Briefly, a total of 85 isolates were confirmed to be *S. aureus* by PCR. Among the 85 isolates, 43 isolates (14.38%, 95% CI: 10.86% - 18.81%) were from Marsabit and 42 isolates (13.82 %, 95%CI: 10.39% - 18.15%) were from Isiolo counties. Overall, pooled milk samples were significantly (p= 0.0001) more contaminated by *S. aureus* (23.94 %, 95% CI: 18.40-30.52%) when compared to milk from individual lactating animals (9.64%, 95% CI: 7.12-12.86 %).

4.3.2 Enterotoxin genes detected by genetic analysis

Overall, 63 (74.11%, 95% CI: 63.91-82.24 %) of the 85 *S. aureus* were found to harbor at least one gene of the enterotoxin genes. Among the genes that code classic enterotoxins, *see* gene was the most frequent, carried by 51 (60%) isolates, followed by *sea* 22 (25%). The s*eb* gene was detected in 9 (10.6%) isolates only while *sed* gene was not detected in all the *S. aureus* evaluated (Table 4.2). Of the *S. aureus* isolates (85), 21 (25%) strains harbored more than one enterotoxin gene and more than half of the isolates harbored at least one of the enterotoxin coding genes. There was no significant difference (p= 0.47) in the proportion of enterotoxins detected in *S. aureus* from individual milk samples (17.8%, 95% CI: 14.45- 21.8%) as compared with *S. aureus* pooled milk samples and (15.43%, 95% CI: 10.96-21.28%)

Sequencing and blastx analysis revealed that these enterotoxin genes were homologues to the enterotoxin sequences of *S. aureus* revealing high amino acid identities of 76 % and 91 % for enterotoxin A and E respectively. The presence of enterotoxins C and B was also confirmed.

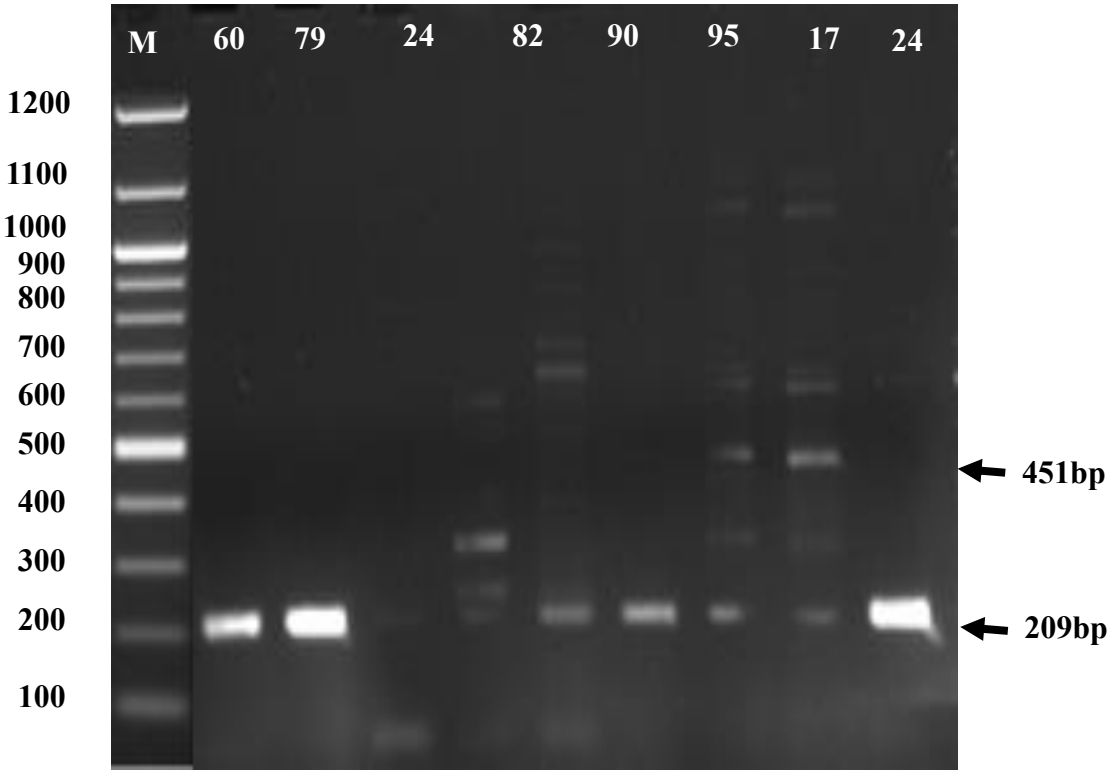

Figure 4.1 Conventional PCR amplification of *S. aureus* enterotoxigenic genes. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane M is DNA marker. Lanes 95 and 27 show bands corresponding to enterotoxigenic genes with 451 bp (*sec*). Lanes 60, 79, 82, 90, 95 and 24 shows bands corresponding to 209bp (*see*) as shown above.

Table 4.2. Enterotoxin genes of *S. aureus* isolates detected by PCR and sequencing

Enterotoxin gene	Overall (%, n=85)	Marsabit (%, n=43)	Isiolo (%, n=42)
1. *see*	51(60)	25(58.1)	26(61.9)
2. *sea*	22(25.9)	13(30.2)	9(21.4)
3. *sec*	18(21.2)	11(25.6)	7(16.7)
4. *seb*	9 (10.6)	9(20.9)	0(0)
5. *sec/see*	11(12.9)	7(16.3)	4(9.5)
6. *seb/see*	4(4.7)	4(9.3)	0(0)
7. *sea/sec*	8(9.4)	6(14)	2(4.8)
8. *sea/sec/see*	5(5.9)	3 (7)	2 (4.8)
9. *sea/seb/sec*	1(1.2)	1(2.3)	0(0)

4.3.4. *Staphylococcus aureus* enterotoxin genes detected from raw milk of various species

With respect to milk from various livestock species, high levels of enterotoxin genes were detected in *S. aureus* isolates from goat milk (44 %) followed by cattle (25 %) and sheep (2 %) in that order (Table 4.3). In Marsabit county, enterotoxin genes sec and seb in raw milk of cattle. *S. aureus* isolates from raw milk samples from cattle in Marsabit haboured enterotoxin genes of 56%, 31%, and 13% of *see*, *sec* and *seb* respectively. Isolates from goats milk haboured the following enterotoxin genes: *see (52%) sec (32%)* and *seb (16%).*

The prevalence of *S. aureus* enterotoxins in isolates from cattle milk were 55%, 27%, and 18% for *see, sea* and *sec* respectively. On the other hand, *S. aureus* isolates from milk samples from goats haboured enterotoxin genes at a frequency of 50%, 36% and 21% of *see, sea* and *sec* respectively (Table 4.3).

Table 4.3. The proportion of *S. aureus* isolates harbouring enterotoxin genes in milk from various livestock species of in Isiolo and Marsabit counties

	Overall	**Marsabit**				**Isiolo**				
	Number (%)	*Sea*	*seb*	*sec*	*sed*	*see*	*sea*	*seb*	*sec*	*sed*	*see*
Cattle	27(27)	0	5(19)	2(7)	0(0)	9(33)	3(11)	0(0)	2(7)	0(0)	6(22)
Goats	45(45)	10(22)	0(0)	5(11)	0(0)	16(36)	4(9)	0(0)	3(7)	0(0)	7(16)
Sheep	2(2)	0	0	0(0)	0(0)	0(0)	1(50)	0(0)	0(0)	0(0)	1(50)
Camels	0	0	0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Pooled milk	26(26)	3(12)	6(23)	4(15)	0(0)	0(0)	1(4)	0(0)	2(8)	0(0)	12(46)

4.4 Discussion

In this study, the genetic determinants that are responsible for the production of enterotoxins from the *S. aureus* isolates were detected. At least one type of *S. aureus* enterotoxin gene *(SE)* was detected in 74.11 % of the isolates, similar to a previous study done in Kenya by Mathenge *et al* (2015). Furthermore, other similar studies have detected high levels of *S. aureus* harboring enterotoxin genes in milk and dairy products, one in Italy (Morandi *et al.* 2007), and two in Japan (Omoe *et al*.,2002; Katsuda *et al.,* 2005) thereby corroborating with the findings of this study. A previous study done in Switzerland reported a high prevalence (65.2 %) of *S. aureus* isolates carrying one or more enterotoxin gene (Scherrer *et al*., 2003); a finding closer to that observed in the current study. Therefore, from these reports, it appears that enterotoxin- producing *S. aureus* is gaining global significance and may not just be a problem of pastoral communities alone. Creation of awareness on good hygienic practices during milk handling is therefore recommended. Pasteurization of raw milk is also encouraged in the region to avoid the risk.

The *see* gene exhibited the highest prevalence (60 %) in this study. It is carried by a prophage (Cao *et al*., 2012) and can be easily disseminated among *Staphylococcus* Spp. strains. In another study, Normanno *et al,* (2005) suggested that the classical *sea* is the most frequently observed enterotoxin gene in enterotoxigenic strains of *S. aureus*; however in this study it was the second highest (22 %) of the strains. This is probably due to the fact that different strains from different foods carry different enterotoxins, as observed elsewhere (Asiimwe *et al.,* 2017). A previous study in Nairobi, Kenya revealed that 4.17 % of *S. aureus* isolated from raw milk produced SEA on latex agglutination test (Ombui *et al*., 1992). Staphylococcal enterotoxin A is frequently associated with food poisoning since it is toxic at low concentrations (Morandi *et al*., 2007).

Enterotoxin A is produced at the beginning of the exponential phase and its expression is not regulated by the accessory gene regulator (*agr*), different from enterotoxins B, C, and D, which depend on the *agr* system for maximum expression (Balaban and Rasooly, 2000: Hennekinne *et al.,* 2011).

The *sec* gene is located on pathogenicity islands and can be divided into three subtypes (sec1, sec2, and sec3) based on antigenic differences and on the animal host associated with it. Some studies suggest that the heterogeneity of enterotoxin C is related to selection for modified *sec* sequences that facilitate the survival of *S. aureus* in their respective hosts (Smyth *et al*, 2005). In the present study, *sec* was the third most common classical enterotoxin after *see* and *sec.*

The *SED* gene was not detected in any of the strains studied. The *SED* gene is located on plasmid pIB485 (Hennekinne *et al.,* 2011) and enterotoxin D is the second most common toxin associated with food poisoning (Balaban and Rasooly, 2000). A small amount of this enterotoxin is able to cause illness, mainly in children and the elderly (Aydin *et al.,* 2011). Nonetheless, the absence of *sed* in the strains studied here suggests that it is scarcely related with *Staphylococcus* Spp. isolates from raw milk in Northern Kenya and consequently low risk of causing food poisoning. In this study, 21 isolates of the 85 (25 %) had more than one enterotoxin gene. Based on various studies, *se* genes can be located on plasmids (*sed* and *sej*, phages (*sea* and *see*), pathogenicity islands (*seb* and *sec* and chromosomes (*seg*, *sew,* and *sej*); therefore, several *se* genes can be harbored by enterotoxigenic *S. aureus* strains (Asiimwe *et al.*, 2017). This study reports the first case of staphylococcal enterotoxin genes in milk from the pastoral region of northern Kenya.

The detection of *S. aureus* enterotoxin genes isolated from milk of goats is important since information from this region of the country is scarce. Enterotoxigenic *S. aureus* was high in milk samples from goats (45 %) followed by Cattle (27 %) and sheep (2 %) (Table 4.3). The presence of the SEs was probably due to improper hygiene and poor management practices among the pastoralists. High prevalence of *sea* and *see* genes in goat milk in this study may be difficult to understand, however, improving the hygienic conditions of the milking environment and/or utensils may reduce the prevalence of *S. aureus* in milk and prevent its transmission to humans. (Abo- Shama, 2014)

Raw milk is one of the leading foods contaminated with *S. aureus* (Aydin *et al*., 2011). The risk of infections and staphylococcal food poisoning increases when food contaminated with *S. aureus* is not cooked properly or when the bacterium contaminate some ready-to-eat food by cross contamination. It is reported that about 95% of staphylococcal food poisoning (SFP) are associated with the classical SEs namely *SEA, SEB, SEC, SED*, and *SEE* (Aydin *et al.*, 2011). Indeed, some of the genes encoding the classical SEs were detected in many *S. aureus* isolates identified in this study indicates that these SEs may be produced by the bacterium thereby accumulating in raw milk and cause food poisoning especially if such a product is stored at inappropriate temperature.

CHAPTER 5: DETECTION OF ANTIMICROBIAL RESISTANT *STAPHYLOCOCCUS AUREUS* PHENOTYPES IN RAW MILK FROM THE PASTORAL NORTHERN KENYA

5.1 Introduction

Staphylococcus aureus is a common resident in the skin and mucous membranes of humans and animals such as primates and livestock. The bacterium can cause a variety of infections ranging from simple superficial skin abscesses to more severe, invasive and potentially fatal diseases (Sergelidis and Angelidis, 2017; Aires-de-Sousa, 2017). In ruminants, *S. aureus* is a leading cause of clinical and subclinical mastitis as well as endometriosis. In some cases, *S. aureus* may spread from the udder of infected animals into raw milk and other dairy products thereby affecting the quality of the products. This, coupled with the cost incurred in prevention of infections has made the pathogen one of the most significant economic burdens to dairy farmers and public health (Seegers *et al*., 2003).

Control of *S. aureus* infections in animals and humans is mainly through antimicrobial therapy. Due to extensive use of antibiotics in dairy farms, new resistant strains have appeared in addition to increased presence of drug residues in milk. Antimicrobial resistance (AMR) is indeed one of the most serious public health threats of this century (Kirchhhelle *et al*., 2020; Prestinaci *et al*., 2015; O'Neill, 2016; Wernli *et al*., 2017). Antimicrobial resistance has been attributed to antimicrobial use, overuse and misuse in animal and human medicine leading to increased selective pressure for resistant pathogens. Expanded intensive livestock production has led to increased disease burdens hence increased global antimicrobial use in human and food animals. (Van Boeckel *et al*., 2017).

Most of the antimicrobial agents used for the treatment of human infections are also used in veterinary medicine (Toutain, *et al*., 2016). For example, tetracyclines, beta-lactams, streptogramins, linconsamides, fluoroquinolones, sulfonamides, rifamycins, macrolides, and aminoglycosides have been used to manage *S. aureus* infections in human and animals (Vestergaard, *et al*., 2019). This has led to increasing cases of antimicrobial resistant- *S. aureus* resulting from either overuse and/or misuse of the drugs during livestock production (Kateete *et al.*, 2013). The extensive use of antimicrobial agents has resulted in selective pressure that is responsible for the emergence and spread of antimicrobial-resistant- *S. aureus* including the multidrug resistant isolates (Gomes and Henriques, 2016).

Penicillin is the drug of choice for treatment of *S. aureus* infections in animal and humans (Siriken *et al.,* 2016: Wang *et al.,* 2018), however, resistance to penicillin has developed over the years due to production of beta lactamase enzymes. In order to counteract this mechanism, methicillin was developed and unfortunately, methicillin-resistant *S. aureus* (MRSA) strains have appeared (Siriken *et al.,* 2016). Furthermore *S. aureus* resistance to methicillin is mediated by overproduction of the penicillin-binding protein (PBP) 2a, an altered PBP with extremely low affinities for beta-lactam antibiotics. PBP2a is encoded by *mec*A, a gene that is not present in susceptible isolates (Siriken *et al.*, 2016). Originally, MRSA was restricted to hospitals (Hospital associated MRSA, HA-MRSA). However, the epidemiology of MRSA has changed fundamentally in recent years with emergence of the pathogen in the community (community-acquired MRSA, or CA-MRSA), considerably increasing the importance of this pathogen (Guimarães *et al*., 2017). Currently MRSA is considered a major cause of hospital-acquired and community-acquired infections that are difficult to treat (Gopal and Divya, 2017). They also

exhibit multi-drug resistance to antibiotics often used in the treatment of mastitis and other staphylococcal infections (Wang *et al.,* 2018: Loncaric *et al*., 2019).

Multiple antibiotic resistant *S. aureus* strains have been isolated from milk obtained from cattle, beef and human samples in Kenya (Shitandi and Sternesjö 2004; Gitau *et al* 2014: Mathenge *et al*., 2015) and other parts of the world (Wang *et al* 2018: Igbinosa *et al* 2016: Kamau *et al*, 2013; Asiimwe *et al* 2017; Pesavento *et al*., 2007: Gitau *et al* 2014: Garvey *et al.,* 2019). Thus, food-related bacteria have the potential to serve as a reservoir of antimicrobial resistance genes with the potential of transferring these determinants to other commensal or pathogenic bacterial species in the human gut. Various studies have focused on the spread of resistant *S. aureus* in clinical environments (Bradley *et al*, 2017: Garvey *et al,* 2018: Garvey *et al*., 2019: Funaki *et al*., 2019), whereas few studies have investigated resistant strains of *S. aureus* in food.

In pastoral areas of East Africa such as northern Kenya, people rely on animals and their products for subsistence and income. This promotes close contact between livestock and humans with a high chance of transmission of multi-drug resistance (MDR) microorganisms of public health concern such as MRSA (Kateete *et al.*, 2013; Kamau *et al.*, 2013; Kasozi *et al.*, 2014; Asiimwe *et al.,* 2017). Due to scarcity of human and animal health care services in these pastoral areas, infections are poorly detected, and hence inappropriate treatments often provided. A large proportion of cases are treated using over-the-counter medications often prescribed by unqualified professionals (Lamuka *et al.*, 2017). This overuse and misuse of the antibiotics has resulted into a surge in multidrug resistance microorganisms, now a growing problem in farms, healthcare settings and at the community level (Kateete *et al.*, 2011; Kateete *et al*., 2013; Seni *et*

al., 2013). Reports of spillovers of AMR bacteria to wildlife from domestic sources have also been reported in other studies (Porrero *et al*., 2013).

In Kenya, some of the major antibiotics used in both human and livestock treatment include penicillins, erythromycins, oxytetracyclines, streptomycins and gentamycin among others (Mitema *et al*., 2001: Mitema and Kikuvi, 2004: Lamuka *et al.*, 2017). Despite the use of antibiotics, limited investigations have been conducted on the prevalence and spread of antimicrobial resistance *S. aureus* phenotypes including MRSA in raw milk obtained from counties such as Marsabit and Isiolo where majority of the population are pastoralists.

This study investigated the phenotypic antimicrobial susceptibility patterns of *S. aureus* strains isolated from raw milk from various lactating livestock species in Isiolo and Marsabit counties in Northern Kenya. The study also correlated antimicrobial usage with emergence of MDR *S. aureus* and the genes responsible.

5.2. Materials and methods

5.2.1 Study area

This study was carried out in Isiolo and Marsabit counties in northern Kenya as described earlier in chapter 3.

5.2.2 Study design

A cross-sectional study design was used to determine bacteriological analysis of *S. aureus* in milk with households as unit of analysis. Within each household, one pooled milk sample was collected as well as up to three samples from randomly selected lactating animals. The study design is as described earlier (Chapter 3).

5.2.3 Sample collection

Sampling was done as previously reported (Chapter 3). Briefly, 10ml of raw milk was drawn aseptically from the udder into a sterile 15ml falcon tube midstream following disinfection by cotton wool moistened by 70% ethyl alcohol. Further, about 50 ml of pooled household milk from the recruited households were collected into a sterile falcon tube uniquely identified by barcodes. Consequently, aliquots of milk samples were placed into cryovials and transported on ice to the Department of Public Health, Pharmacology and Toxicology, University of Nairobi for analysis and storage. A questionnaire was administered to capture animal and household's data

5.2.4 Isolation and identification of *S. aureus*

Staphylococcus aureus isolates were cultured from the samples using Mannitol Salt Agar (MSA). Biochemical methods and polymerase chain reaction (targeting staphylococcal terminase gene) were used to verify species and pathogenicity of the isolates as described in chapter 3.

5.2.5 Antimicrobial use

Data on the commonly used and/or sold antimicrobials in the study area was collected from wholesalers and veterinary pharmacies. Consolidated sales records were obtained from 10 veterinary pharmacies in Marsabit and 13 in Isiolo counties during the year 2017. Quantities of antimicrobial vials and packs, concentrations of antimicrobial preparations, antimicrobial class, whether single molecule, or combined were recorded. Furthermore, the quantities in grams or kilograms of active antimicrobial ingredients in drug preparations were calculated and entered a MS excel spread sheet. Antimicrobial agents were then classified according to the pharmacological class, for instance as oxytetracyclines, beta lactams, aminoglycosides, macrolides or potentiated sulphonamides.

5.2.6 Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method (Hudzicki, 2019) according to Clinical and Laboratory Standards Institute guidelines using Mueller-Hinton Agar (Oxoid, Hampshire, England) (CLSI, 2014). An inoculum of *S. aureus* equivalent to 0.5 McFarland standards was streaked on the Agar plate. The *S. aureus* subspecies *aureus* Rosenbach (ATCC® 25923™) was used as the reference organism for quality control assurance (CLSI, 2014). The following antibiotic disks (Oxoid, Hampshire, England) were

dispensed on the Agar plate; oxacillin (30µg), ampicillin (10µg), erythromycin (15µg), kanamycin (30µg), tetracycline (30µg), clindamycin (2µg), ciprofloxacin (5µg), and cephalexin (30µg). Oxacillin was used as a surrogate for methicillin resistant *S. aureus* (MRSA) phenotypes . The antibiotic disks dispensed on the inoculated agar plates were incubated at 37ºC for 16-18 hours. The diameter zones of inhibition were measured using a Vernier caliper. The readings were recorded as either susceptible, intermediate, or resistant based on the interpretative breakpoints by the Clinical Laboratory Standards Institute guidelines (CLSI, 2014). Test results were accepted only when the zone of inhibition for the control strain fell within the acceptable ranges. Oxacillin was used as surrogate for methicillin in detection of MRSA strains. Multidrug resistant *S. aureus* isolates were determined when the isolates were resistant to at least three of the antibiotic classes.

5.2.7 Data management and analysis

Antimicrobial use and the phenotypic antimicrobial resistance data were merged with individual animal milk and pooled milk data from households and then imported to the R software. Descriptive statistics was determined by calculating the proportions of resistance phenotypes and various antimicrobial classes used in Marsabit and Isiolo counties. Pearson's correlation coefficient was determined to measure the strength of the relationship between AMU and the prevalence of *S. aureus* resistance phenotypes using R version 3.5.1 software. Tetracyclines penicillins, aminoglycosides and macrolides were included in this analysis.

5.3 Results

5.3.1 Isolation and confirmation of *S. aureus*

The total number of samples collected, isolated and confirmed is as described in chapter 3 (results section). Briefly, a total of 85 isolates were confirmed to be *S. aureus* by PCR.

5.3.2 Antimicrobials use

Overall, tetracycline was the most commonly used antibiotics at 95 % (4,168 kg) in both Isiolo and Marsabit. Other commonly used antimicrobials were sulfonamides 1.6 % (70 kg), aminoglycosides 1.1% (49.7 kg), beta-lactams 1.1% (46.4 kg) and macrolides 0.9 % (39.4 kg). Trimethoprim was the least used 0.01 % (0.52 kg), probably due to the fact that it is used as a synergist with sulfonamides (Table 2). In Isiolo County, tetracycline (3,491.1) was the most commonly used antibiotic followed by sulfonamides (65.6 kg), aminoglycosides (42.7 kg), penicillin (33.8 kg), macrolides (tylosin) (33.2 kg) and trimethoprim (0.4 kg). Similarly, tetracycline was the most commonly used in Marsabit (677 kg). However, as opposed to Isiolo county, penicillins (12.6 kg) was the second most used antibiotic. The other antibiotics used in Marsabit in order of preference included aminoglycosides (7 kg), macrolides (6.2 kg) sulfonamides (4.4 kg) and trimethoprim (0.12 kg) (Table 2). Antimicrobial use was generally higher in Isiolo than in Marsabit for all the drugs with exception of trimethoprim.

Table 5.1. Quantities (kg) of antimicrobial agents used in Isiolo and Marsabit counties during the year 2017

	Antibiotic usage kg (%)		
Antimicrobial agent	**Isiolo**	**Marsabit**	**Combined**
Oxytetracyclines	3491.1 (95.2)	677 (95.7)	4168.1 (95.2)
Sulfonamides	65.6 (1.8)	4.4 (0.6)	70 (1.6)
Streptomycin	42.7 (1.2)	7 (1)	49.7 (1.1)
Penicillin	33.8 (0.9)	12.6 (1.8)	46.4 (1.1)
Tylosin	33.2 (0.9)	6.2 (0.9)	39.4 (0.9)
Trimethoprim	0.4 (0.01)	0.12 (0.02)	0.52 (0.01)

Sulfonamides: sulfadiazine and sulfadimidine.

5.3.3 *Staphylococcus aureus* resistance phenotypes

Overall, 88 % of the *S. aureus* isolates were resistant to at least one of the antibiotics including oxacillin, ampicillin, erythromycin, kanamycin, tetracycline, clindamycin, ciprofloxacin, and cephalexin. Of these resistant isolates, 51 % were isolated from raw milk of livestock in Isiolo while 49 % were from raw milk of livestock in Marsabit. Seventy four percent of the isolates from Marsabit were resistant to tetracycline, 51 % to ampicillin, 37 % to oxacillin, 28 % to clindamycin, 25 % to cephalexin, 16 % to erythromycin and kanamycin and 9 % to ciprofloxacin (Table 5.2). Furthermore, some 30 % of the isolates were MDR phenotypes. In Isiolo County, 83 % of the isolates were resistant to tetracycline. This was followed by ampicillin at 64 % with rest of the isolates resistant to oxacillin (29 %), clindamycin (7 %), cephalexin (7 %), erythromycin (5 %) and kanamycin (5%). None of the isolates were resistant to ciprofloxacin. The profiles of the resistant phenotypes observed in Isiolo were similar to that of Marsabit with 7 % of the isolates being classified as MDR *S. aureus*.

The resistance phenotypes from the individual and pooled milk samples are shown in Table 5.4. *S. aureus* isolates from individual milk samples from cattle in Marsabit showed resistance of 100% and 83% to tetracycline and ampicillin while isolates from pooled cattle milk showed resistance of 41%, 35% and 29% to oxacillin, tetracycline and ampicillin respectively (Table 5.2). However, *S. aureus* isolates from individual milk samples from goats in Marsabit showed resistance of 56%, 100%, and 44% to oxacillin, tetracycline and ampicillin respectively. On the other hand, *S. aureus* isolates from pooled milk samples from goats showed resistance of 33%, 100% and 67% to oxacillin, tetracycline and ampicillin respectively (Table 5.4).

In Isiolo County, *S. aureus* isolates from individual cattle milk samples showed resistance of 90% and 50% to tetracyclines and ampicillin, while isolates from pooled cattle milk samples showed resistance of 40%, 100% and 60% to oxacillin, tetracyclines and ampicillin respectively (Table 5.2). However, *S. aureus* isolates from individual milk samples from goats showed resistance of 27%, 73%, and 87% to oxacillin, tetracycline and ampicillin respectively. On the other hand, *S. aureus* isolates from pooled milk samples from goats showed resistance of 30%, 90% and 50% to oxacillin, tetracyclines and ampicillin respectively (Table 5.2).

In overall, *S. aureus* isolates from the two counties showed high resistance to tetracycline and ampicillin.

Table 5.2. Prevalence of *Staphylococcus aureus* resistant phenotypes from Marsabit and Isiolo counties

Antimicrobial Agent	**Antimicrobial Resistance**		
	Marsabit n (%)	**Isiolo n (%)**	**Overall Resistance**
Tetracycline	32 (74.4)	35 (83.3)	67 (79)
Ampicillin	22 (51.2)	27 (64.3)	49 (57)
Oxacillin	16 (37.2)	12 (28.6)	28 (33)
Clindamycin	12 (28)	3 (7.1)	15 (18)
Cephalexin	11 (25.6)	3 (7.1)	14 (16)
Kanamycin	7 (16.3)	2 (4.8)	9 (11)
Erythromycin	7 (16.2)	2 (4.8)	9 (11)
Ciprofloxacin	4 (9.3)	-	4 (5)
Multi-drug resistance	13 (30)	3(7)	16 (19)

5.3.4 Multidrug resistant *Staphylococcus aureus*

A total of 16 (30%) *S. aureus* isolates from Marsabit and 12 (7%) from Isiolo were resistant to more than three classes of antibiotics tested indicating that these isolates were MDR. Out of these, 15 (94%) were MRSA. Table 5.3 shows the resistance phenotypes of the multi-drug resistance isolates detected in this study. As shown in Table 5.3, up to 15 (94%) of the multi-drug resistance isolates were MRSA. Only one methicillin susceptible *S. aureus* (MSSA) isolate was multi-drug resistant. The other MDR-SA isolates were also resistant to tetracyclines (88 %), clindamycin (81 %), Kanamycin (44 %), ciprofloxacin (19 %) and erythromycin (19 %). As shown in Table 5.3, all the MDR-SA harbored *tet*K while the other isolates contained *bla*Z (81 %), and *aph* (75 %) genes. The other isolates contained *msr*A (38%), *tet*M (31 %), *aac-aph* (31 %) and *mec*A (25 %). Therefore, *tet*K gene was predominant genetic determinant for the tetracycline resistance phenotypes in most *S. aureus* isolates indicating that it could be the main gene responsible for tetracycline resistance. Thirty one percent of the multidrug resistant *S. aureus* phenotypes were resistant to oxacillin, tetracycline and clindamycin followed by 19% that were resistant to oxacillin, tetracycline, clindamycin and kanamycin (Table 5.3).

Table 5.3. Multidrug resistant *Staphylococcus aureus* including MRSA from Marsabit and Isiolo Counties

Antimicrobial agents	**Number of Isolates**
Oxa, Tet, Clind, Cip, Kan	1
Oxa, Tet, Clind, Kan	3
Oxa, Tet, Clind, Cip	1
Oxa, Tet, Kan, Cip	1
Oxa, Tet, Ery	2
Oxa, clind, Kan	1
Oxa, Tet, Clind	5
Oxa, Clind, Ery	1
Tet, Clind, Kan	1

*****MRSA, Oxa (Oxacillin), Tet (Tetracycline), Clind (Clindamycin), Cip (Ciprofloxacin), Kan (Kanamycin), Ery (Erythromycin)

Table 5.4. Prevalence of *Staphylococcus aureus* resistant phenotypes from various livestock species from Marsabit and Isiolo counties.

County	Species	Sample	*S. aureus*	Oxa	Tet	Ery	Clind	Cip	Amp	Ceph	Kan
Marsabit (n=43)	Cattle	Individual	6	0	6(100)	0(0)	0(0)	0(0)	5(83)	1(16)	0(0)
		Pooled	17	7(41)	6(35)	3(17)	4(24)	1(6)	5(29)	4(24)	4(24)
	Goats	Individual	9	5(56)	9(100)	2(22)	4(44)	2(22)	4(44)	3(33)	2(22)
		Pooled	9	3(33)	9(100)	2(22)	3(33)	1(11)	6(67)	2(22)	1(11)
	Sheep	Individual	0	0	2(67)	0	0	0	0	0	0
		Pooled	2	1(50)	0	0	1(50)	0	2(100)	1(50)	0
	Camels	Individual	0	0	0	0	0	0	0	0	0
		Pooled	0	0	0	0	0	0	0	0	0
Isiolo (n=42)	Cattle	Individual	10	2(20)	9(90)	0	1(10)	0	5(50)	0	0
		Pooled	5	2(40)	5(100)	0	0	0	3(60)	0	0
	Goats	Individual	15	4(27)	11(73)	2(13)	2(13)	0	13(87)	3(20)	2(13)
		Pooled	10	3(30)	9(90)	0	0	0	5(50)	0	0
	Sheep	Individual	2	1(50)	1(50)	0	0	0	1(50)	0	0
		Pooled	0	0	0	0	0	0	0	0	0
	Camels	Individual	0	0	0	0	0	0	0	0	0
		Pooled	0	0	0	0	0	0	0	0	0

5.3.5 Correlation of AMU to *S. aureus* resistance phenotypes

The correlations between AMU and phenotypic antimicrobial resistance for *S. aureus* resistance to penicillins (oxacillin, ampicillin), tetracyclines (oxytetracycline), aminoglycosides (Kanamycin) and macrolides (erythromycin) were determined. There were positive correlations between oxytetracycline usage to oxytetracycline resistance (r=0.71), penicillin to oxacillin resistance(r=0.61), penicillin to ampicillin resistance (r=0.92) and aminoglycoside to kanamycin resistance (r = 0.63) even though there were no significant differences. However, there was a negative correlation between macrolide usage and detection of erythromycin resistance (r = -0.06, p = 0.9).

Table 5.5. Pearson's correlation coefficient measuring the correlation between antimicrobial use and *S. aureus* phenotypic antimicrobial resistance

Antimicrobial group	Resistant gene	Number (n)	Pearson's correlation (r)	*P* value
Tetracyclines	Oxytetracycline	82	0.71	0.5
Penicillins	Oxacillin	67	0.61	0.58
	Ampicillin	45	0.92	0.25
Aminoglycosides	Kanamycin	35	0.63	0.59
Macrolides	Erythromycin	20	-0.06	0.96

Pearson's (r) value above + 0.5 indicates a large association between AMU and resistance genes

[2] There was no association between tylosin use and erythromycin resistance

5.4. Discussion

This study established the phenotypic antimicrobial resistance profiles of *S. aureus* from individual animal and pooled raw milk samples from northern Kenya. The contamination of raw milk along the dairy value chain by antimicrobial resistant *S. aureus* is of global public health importance. This is because of the challenges posed by difficulty in the treatment of drug resistant milk-borne *S. aureus* infections and foodborne illnesses (Hennekinne *et al*., 2012; Sergelidis *et al*., 2017). Moreover, the occurrence of genetic determinants responsible for the emergence of AMR *S. aureus* including the MRSA in milk is a possible means of spread of antimicrobial resistance especially when the contaminated milk is consumed by humans (Igbinosa *et al*., 2016). The emergence of the drug resistant *S. aureus* isolates including the MDR-MRSA has been linked to increased usage of antimicrobials in human and veterinary medicine.

Extensive use of antimicrobial drugs was observed in this study, a practice correlated with the high level of resistance by the milk-borne *S. aureus*. Apart from oxytetracycline usage, sulfonamides, beta-lactams, aminoglycosides and macrolides usage was common among the pastoral communities, a trend consistent with previous findings of in the study sites nearly two decades ago (Mitema *et al*., 2001). Furthermore, a recent study reported the extensive use of tetracycline and to some extent penicillins as well as aminoglycosides in Isiolo (Lamuka *et al*., 2017). Similar antimicrobial usage patterns have also been reported in the USA and China (Chu *et al*., 2013). Previous studies have shown that the extensive usage of antimicrobials including oxytetracycline and penicillins may have contributed to the high level of resistance especially to tetracycline and penicillins by the *S. aureus* isolates (Lamuka *et al*., 2014). Indeed, some studies have linked emergence of antimicrobial resistant *S. aureus*, including the MDR-MRSA to

extensive usage of antimicrobial drugs in human and veterinary medicine (Valsangiacomo *et al*., 200; Grema *et al*., 2015). Furthermore, tetracyclines and penicillins have been excessively used for the prevention and treatment of mastitis and other livestock infections in Kenya as well as other countries such as Uganda, Egypt, Algeria, Tunisia and Nigeria (Igbinosa *et al*., 2016; Al-Ashmawy *et al*., 2016; Lamuka *et al*., 2017; Asiimwe *et al*., 2017; Klibi *et al*., 2018; Titouche *et al*., 2019; Ahmed *et al*., 2020).

The emergence of MDR strains of *S. aureus*, including MRSA, that cause infections in animals and humans has become a growing public health concern (Li *et al*., 2015). In this study, 88 % and 90% isolates from Marsabit and Isiolo counties respectively were resistant to one or more antimicrobial agents used in this study. Our findings agree with a recent study by Dai *et al*., (2019) in china that found varying degree of *S. aureus* resistance to 10 antimicrobials in 97.06% of *S. aureus* isolates from raw goat milk samples. In the same study, 52.94% of the isolates were multi-drug resistant to three or more antimicrobial agents as compared to 33% in this study (Dai *et al*., 2019). In Nigeria, Igbinosa *et al*., (2016) reported *S. aureus* resistance of 100% to 7/11 of antibiotics used in that study, including methicillin, penicillin, amoxicillin, cloxacillin, clindamycin, chloramphenicol and trimethoprim-sulfamethaxazole. In addition, these findings mirrors well with a report on antimicrobial resistance of *S. aureus* isolates from dairy foods by Wang *et al*., (2017) in China and other similar studies in other countries that found high proportion of resistant *S. aureus* in animal source foods (Wang *et al*., 2014: Lv *et al*., 2014; Kraushaar *et al*., 2017: Ge *et al*., 2017). The high level of antibiotic resistance observed in this study could be a response to the selective pressure of antimicrobials, a phenomenon that has been reported earlier (Gomes and Henriques, 2016). Furthermore, rational management and appropriate usage of antimicrobial compounds in food-producing livestock has been reported to

be important in the control and prevention of drug resistant isolates (Jessen *et al*., 2017: Loncaric *et al*, 2019). The fast-rising emergence of *S. aureus* antimicrobial resistance may threaten the currently available antibiotics and complicate treatment outcomes for serious bacterial infections in human and veterinary medicine.

Thirty-three percent of the *S. aureus* isolates were phenotypically resistant to oxacillin indicating the presence of milk-borne MRSA. In this regard, it appears that milk-borne MRSA was more prevalent in pastoral communities in Kenya as compared to other regions such as Algeria (17.7%), Tunisia (6.6%), Korea (2.8 %), Switzerland (1.4 %), Finland, (1.5 %), Japan (1.5 %), in Southwest Germany (5.1–16.7 %), Belgium (7.4 %,), in Mexico (18.1 %) and Brazil (22 %) (Klibi *et al*., 2018; Titouche *et al*., 2019; Moon *et al*., 2007: Huber *et al*., 2010; Gindonis *et al*., 2013; Hata *et al*., 2010; Spohr *et al*., 2011; Vanderhaeghen *et al.*,2010; Adame-G´omez *et al*., 2018; Funaki *et al*., 2019). The prevalence is however lower than that reported in raw milk in Uganda (56.1%) (Asiimwe *et al*., 2017), Nigeria (100%) (Igbinosa *et al*., 2016) and Egypt (53 and 87.5%) (Al-Ashmawy *et al*., 2016; Ahmed *et al*., 2020). Ninety-four percent of the MDR isolates were MRSA. Previous studies show that MRSA are multi-dug resistant, with resistance to the commonly used antibiotics such as penicillins, aminoglycosides, macrolides, sulfonamides and tetracyclines, most of which are commonly used in the treatment of mastitis (Lamuka et al., 2017; Zuhair, 2017; Asiimwe et al., 2017; Ahmed et al., 2020).

Further, the MRSA isolates were resistant to more than one class of antibiotics and formed 94% of MDR *S. aureus.* Wang *et al*., (2008) and Loncaric *et al* (2019) observed that MRSA are multi-drug resistant to various antibiotics such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, among others which are often used in the treatment of mastitis

since the usage of antibiotics correlates with the emergence and maintenance of antibiotic resistant traits within pathogenic strains (Pesavento *et al.*, 2007:).

In the current study, *S. aureus* isolates from individual milk samples from goats in Marsabit showed resistance of 56%, 100%, and 44% to oxacillin, tetracycline and ampicillin respectively. On the other hand, *S. aureus* isolates from pooled milk samples from goats showed resistance of 33%, 100% and 67% to oxacillin, tetracycline and ampicillin respectively. This is the first study to report presence of antimicrobial resistance *S. aureus* in milk in Kenya. The proportion penicillin resistant *S. aureus* isolates reported in this study are lower than the 79.41% *S. aureus* resistant to penicillin G in a study involving raw goat milk by Qian *et al*., (2019) in China.

CHAPTER 6: ANALYSIS OF THE GENETIC BASIS OF ANTIMICROBIAL RESISTANCE PHENOTYPES OF *STAPHYLOCOCCUS AUREUS* IN NORTHERN PASTORAL AREAS OF KENYA

6.1. Introduction

Staphylococcus aureus (*S. aureus*) is a ubiquitous gram-positive bacterium found in the environment including air, water and the skin surfaces including mucus membranes of animals and humans (Tong *et al*., 2015; Bradley *et al*., 2017). Although some strains are non-pathogenic, others can cause life threatening infections in human and animals (Tong *et al*., 2015). The bacterium is specifically important in exemplifying the adaptive evolution of bacteria in the antibiotic era (Pantosti *et al*., 2007). For instance, *S. aureus* developed resistance to penicillin within the first decade of introduction of penicillin, with 28% of isolates showing resistance at the Boston city Hospital (Maranan *et al*., 1997). Currently, a vast majority of hospital acquired and community acquired *S. aureus* are resistant to penicillins (Chen *et al*., 2017). Since the introduction of penicillins, several new antibiotics have been developed, but *S. aureus* has exhibited a unique ability to quickly respond to each new antibiotic by development of new resistance mechanism (Kaur *et al*., 2015; Guo *et al*., 2020).

Staphylococcus aureus resistance to various early antibiotic classes is mediated by genetic determinants acquired through horizontal DNA transfer. This process of resistance acquisition is beneficial to microorganisms, with a possibility of acquiring a pre-assembled all-inclusive arsenal encoding resistance against various antimicrobials (Pantosti *et al*., 2007). For instance, horizontal gene transfer has been incriminated for *S. aureus* resistance against methicillin and vancomycin (Pantosti *et al*., 2007; Brody *et al*, 2008). Furthermore, endogenous resistance obtained through the process of random mutation and selection under pressure provide a major

route for resistance development against fluoroquinolones, vancomycin, daptomycin, linezolid and many earlier antibiotics. Through these mechanisms, *S. aureus* have become resistant to a wide range of antimicrobials thereby presenting major public health challenges to humans (Kateete *et al*, 2013; Vestergaard, *et al*., 2019). In particular, there are documented reports of *S. aureus* manifesting resistance phenotypes to beta-lactams such as penicillins and cephalosporins, as well as tetracyclines, fluoroquinolones, macrolides, and aminoglycosides (Faires *et al*., 2010). Consequently, cases of prolonged hospital admissions, reduced therapeutic efficacy of the antimicrobial agents, increased virulence, high cost of treatment and even mortalities have been reported (Li and Webster, 2018).

Staphylococcus aureus can develop resistance to beta-lactams by degrading the antibiotics through the production of β-lactamases. The β-lactamases that hydrolyze penicillins and cephalosporins are known to be encoded by *Bla*Z, genes. Alternatively, some strains of *S. aureus* may acquire an altered penicillin binding protein 2a' (PBP 2a') encoded by *mecA* gene (Si˙ri˙ken *et al*., 2016). The *S. aureus mec*A genes encode resistance phenotypes to methicillin yielding the methicillin resistant *S. aureus* (MRSA). MRSA are usually known to be resistant to a wide range of antibiotics commonly used for the treatment of livestock and humans thereby complicating treatment outcomes (Lobanovska and Pilla 2017). For example, MRSA had been reported to cause about 72,444 invasive infections in the United States in 2014 with most of the infections classified as healthcare-associated (HA) while the rest were community-associated (CA-) MRSA (CDC, 2014).

The multidrug-resistant phenotype is a characteristic of the MRSA strains (Egyir *et al*., 2014a; Herrmann *et al*., 2013). The *mec*A gene encoding the MRSA phenotypes is usually present in the staphylococcal cassette chromosome *mec* (SCC*mec*) (Si˙ri˙ken *et al*., 2016). The SCC*mec*

subtypes I, II, III, IV and V are known to harbour other antimicrobial resistance genetic determinants such as *tet*K, *tet*M, *bla*Z, *aac (6')/aph (2'')*, *aph (3')-IIIa*, *msr*A, and *erm*A (Katayama *et al*., 2000). These genetic determinants are believed to be responsible for the emergence of multidrug resistant MRSA. The *tetK*, *tetM gene* are known to confer resistance phenotypes to tetracyclines whereas *aac (6')/aph (2'') aph (3')-IIIa* genes confer resistance to the aminoglycosides. The presence of AMR genes in mobile genetic elements such as SCC*mec* is a precursor to horizontal gene transfer of the resistance gene amongst *S. aureus* isolates in clinical set-up and along the milk food chain (Igbinosa *et al*., 2016).

Recently, there has been an increase in reported cases of MRSA contamination in foods of animal origin detected at international airports in the Europe Union (EU). For example, a study by Rodríguez-Lázaro et al (Rodríguez-Lázaro *et al*., 2017) reported MRSA contamination of foods of animal origin confiscated from passengers on flights from 45 non-European Union (EU) countries at various international airports in EU. The contamination was also reported in foods from open markets close to EU land borders. Furthermore, increase in MRSA contamination in milk has been reported in many countries including China, Italy and Uganda (Pesavento *et al*., 2007; Kasozi *et al*., 2014; Liu *et al*., 2017; Wang *et al*., 2018). What is not clear is whether the MRSA isolates harbor resistant genetic determinates of resistance to various antibiotics. It is generally believed that the high level of antimicrobial phenotypic resistance is mediated by genetic determinates of resistance obtained through endogenous resistance development as a result of antimicrobial overuse or horizontal gene transfer. Therefore, this study investigated the genetic basis of phenotypic resistance patterns observed in the two counties and related the AMU to emergence of *S. aureus* resistance genes in *S. aureus* contaminating raw milk of sheep, cattle, goats and camels in households from Isiolo and Marsabit in northern Kenya.

6.2. Materials and methods

6.2.1 Study area

This study was carried out in Isiolo and Marsabit counties in northern Kenya. Both counties are part of the arid and semi-arid lands (ASAL) areas of Kenya and are populated by pastoralists whose livelihoods are mainly dependent on consumption and trade of cattle sheep, goats and camel and their products. Isiolo county lies between latitudes 2'N and 0 50'S and longitudes 39 50'E and 36 50'W while Marsabit county lies between latitudes 02°45’ N and 04°27’ N and longitudes 37°57’ E and 39°21’ E.(KNBS, 2015a; GoK, 2017).

6.2.2 Study design and sampling

A cross-sectional study design was used to determine bacteriological analysis of *S. aureus* in milk with households as unit of analysis. Within each household, one pooled milk sample was collected as well as up to three samples from randomly selected lactating animals.

6.2.3 Sample collection

Sample collection was conducted as reported earlier in chapter 3 (sample collection section).

6.2.4 Isolation and identification of *S. aureus*

Staphylococcus aureus isolates were cultured from the samples using Mannitol Salt Agar (MSA). Biochemical methods and confirmation by polymerase chain reaction (targeting staphylococcal terminase gene) were used to verify the species and pathogenicity of the isolates as described in chapter 3.

6.2.5 Extraction of Genomic DNA

Genomic DNA extraction was performed using PureLink™ DNA extraction Kit (Invitrogen CA, USA) according to the manufacturer's instructions for extraction of DNA from Gram-positive bacteria (see chapter 3).

6.2.6 PCR detection of *S. aureus* resistance genes

Staphylococcus aureus isolates found to be highly resistant were tested for the presence of genetic determinants responsible for antibiotic resistance. Genes encoding for resistance genes *mecA, tetK, tetM, blaZ, aac (6')/aph (2'')*, *aph (3')-IIIa*, *msrA* and *ermA* were designed using the Primer Blast tool (www.ncbi.nlm. nih.gov/tools/primer-blast/) and optimized and used in the study (Table 6.1). All PCRs were performed on Veriti thermocycler (Applied Biosystems, USA), and each run included a negative control and an appropriate positive control isolated in this study. The reactions were run in duplicate. A 25µl PCR mixture containing 1µl of DNA template, 1X Dream Taq master mix (Fermentas, Thermo Scientific, USA), 10 pmol of each reverse and forward primers and 9.5µl of nuclease free water was prepared. PCR was performed at 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, determined annealing temperatures for each primer set for 60 sec, and 72°C for 1 min with a final extension at 72°C for 10 min. The PCR products were analyzed by gel electrophoresis (Figure 3). The amplified bands of interest were excised, purified and sequenced (Macrogen Co., Korea. Sequences obtained were edited and analyzed by BLASTn tool (www.ncbi.nlm.nih.gov/BLAST) to confirm identity of the isolates. The sequences obtained were also submitted to GenBank and given the accession numbers MH763655 (*aph*) and MH636811 (*mecA*).

Table 6.1 Nucleotide sequences and amplicon sizes for the *S. aureus* gene-specific oligonucleotide primers used in PCR in this study

Gene	Primer	Oligonucleotide sequence (5'-3')	Annealing temperature	Size of amplified product (bp)
mecA	mecA-F	TGGCCAATACAGGAACAGCA	54	844
	mecA-R	ACGTTGTAACCACCCCAAGA		
tetK	tetK-F	TCTGCTGCATTCCCTTCACT	58	451
	tetK-R	GCCCACCAGAAAACAAACCA		
TetM	tetM-F	CCGTCACGCTGTTGTTAGGA	54	370
	tetM-R	TTCATCGCCACGTTATCGCT		
blaZ	blaZ-F	ACACCTGCTGCTTTCGCTAA	54	314
	blaZ-R	ACACTCTTGGCGGTTTCACT		
aph (3')-IIIa	aph (3')-F	ATCGAGCTGTATGCGGAGTG	56	328
	aph (3')-R	TGTCATACCACTTGTCCGCC		
msrA	msrA F	AACAGTTGAAACGGTTGGCG	56	819
	msrA R	TTTGCACCTACGAGCGCTAT		
ermA	ermA-F	TCTGCAACGAGCTTTGGGTT	57	243
	ermA-R	TGCTTCAAAGCCTGTCGGAA		
aac (6')/aph (2'')	aac/aph-F	CAGAGCCTTGGGAAGATGAAGT	62	294
	aac/aph-R	GGTATGCCCTTATTGCTCTTGGA		

6.2.8 Data management and analysis

Antimicrobial use and *S. aureus* genotypic antimicrobial resistance data were merged with individual animal milk and pooled milk data from households and then imported to the R software. Descriptive statistics was determined by calculating the proportions of *S. aureus* resistance genes and various antimicrobial classes used in Marsabit and Isiolo counties. Pearson's correlation coefficient was determined to measure the strength of the relationship between AMU and the prevalence of *S. aureus* resistance genotypes using R version 3.5.1 software. The analysis included *mec*A/*blaZ* (for penicillins), *tet*K, *tet*M, (for tetracyclines), *aac* (*6''*)/*aph* (*2''*), *aph* (*3'*)-*IIIa* (for aminoglysosides), and macrolides *msr*A/*erm*A (for macrolides).

6.3 Results

6.3.1 *Staphylococcus aureus* phenotypes detected in raw livestock milk

A total of 85 isolates were subsequently confirmed to be *S. aureus* by PCR as reported in detail in chapter 3.

6.3.2 Antimicrobial resistance determinants haboured by *S. aureus*

Overall, eight genes including *mec*A, *aac (6')/aph (2'')*, *tet*K, *tet*M, *aph (3')-IIIa*, *blaZ*, *msrA*, and *erm*A were analysed by PCR. The PCR analysis identified genetic bands corresponding to 844 bp for *mec*A, 451 bp for *tet*K, 370 bp for *tet*M, 314 bp for *blaZ,* 328 bp for *aph (3')-IIIa,* 819 bp for *msrA,* 243 bp for *ermA* and 294 for *aac (6')/aph (2'')*(Fig 6.1 and 6.2)). Most phenotypes that were resistant to tetracyclines haboured tetK gene (95% CI: 92.59-100.41%) as opposed to *tet*M (19%). Further, most isolates resistant to beta lactams haboured more *blaZ* (78.8%) gene as opposed to *Mec*A (38.8%). On the other hand, most isolates resistant to aminoglycosides haboured *aac (6')/aph (2'')* (52.9%) as opposed to *aph (3')-IIIa* (41.2%). Most phenotypes resistant to macrolides haboured *msrA* (23.5%) as opposed to *ermA* (7%) (Table 6.2).

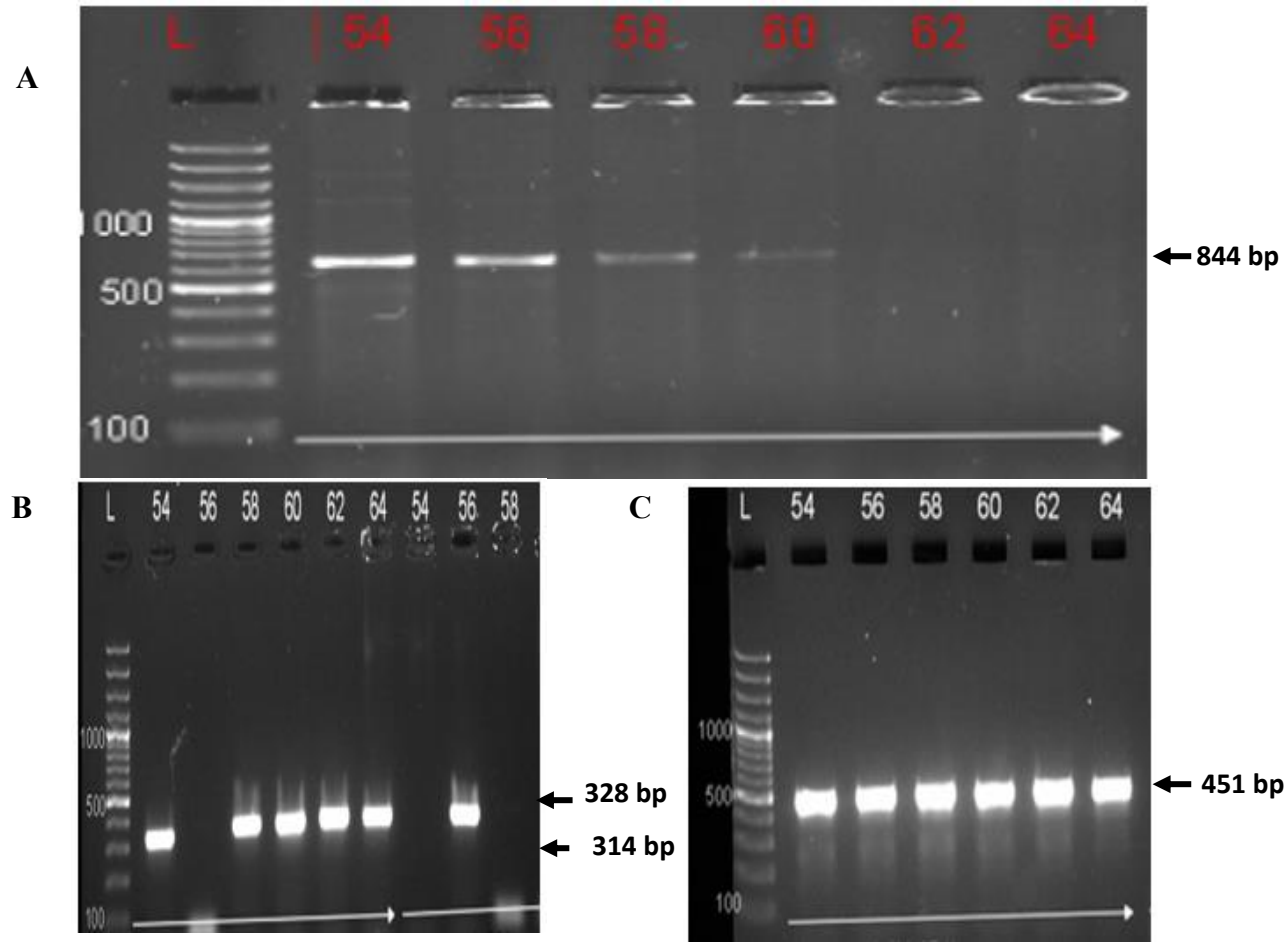

Figure 6.1 Conventional PCR amplification of *S. aureus* antibiotic resistance genes. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane L is DNA marker. In panel (A) - lane 54, 56, 58 and 60 are positive samples for *mecA*. Panel B lane 54, 56, 58, 60, 63, 64 are positive for *blaZ*, Lane 56 is positive for *aph (3')-IIIa*. Panel (C) lane 54, 56, 58, 60, 63 and 64 is positive for *tetK*. The lines indicate the identity and position of the amplicons.

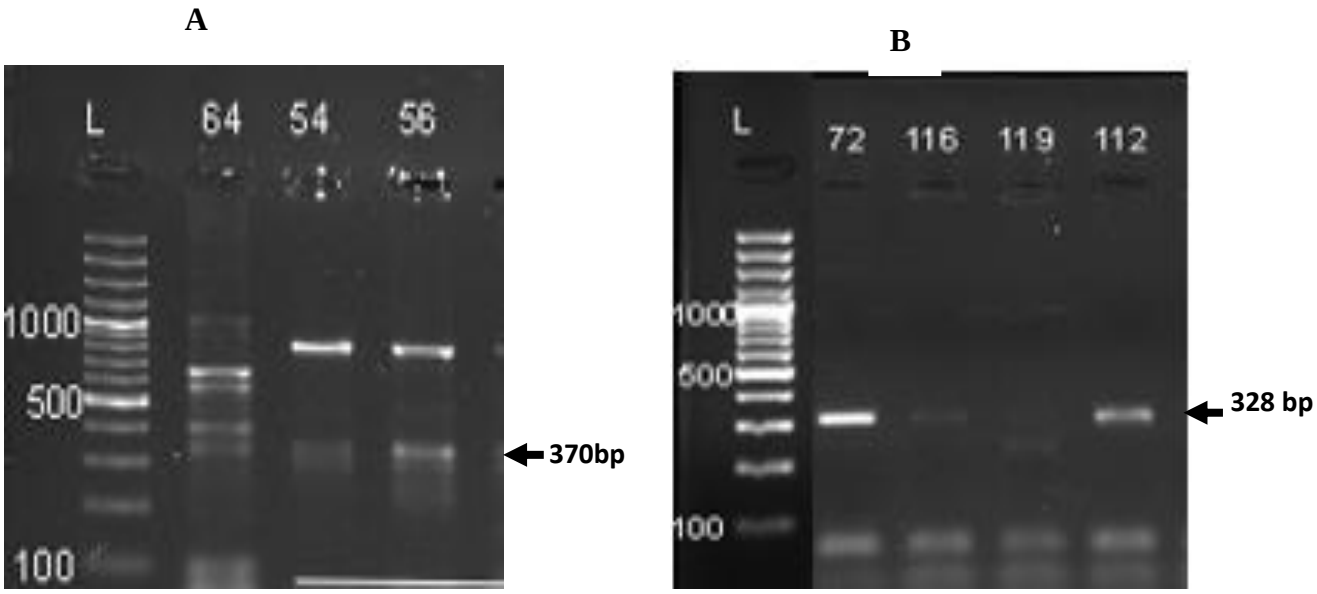

Figure 6.2 Conventional PCR amplification of *S. aureus* antibiotic resistance genes. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane L is DNA marker. In panel (A)- lane 64,54 and 56 are positive samples for tetM. Panel (B) lane 72, 116 and 112 are positive for *aac (6')/aph (2'')*. The arrow indicates the position of the amplicons.

Table 6.2. The various *S. aureus* resistance genes detected against antimicrobial agents used in Marsabit and Isiolo counties

Antibiotic	**Genotype profile**	**Marsabit**	**Isiolo**	**Overall**
		Number (%)	Number (%)	Number (%)
Tetracyclines	*tet*K	42 (97.7)	40 (95.2)	82 (96.5)
	*tet*M	5 (11.6)	11(26.2)	16 (18.8)
Beta lactams	*blaZ*	31 (72)	36 (85.7)	67 (78.8)
Oxacillin	*Mec*A	11 (25.6)	22 (52.4)	33 (38.8)
Aminoglycosides	*aac (6')/aph (2'')*	20 (46.5)	25 (59.5)	45 (52.9)
	aph (3')-IIIa	28 (65.1)	7 (16.7)	35 (41.2)
Erythromycin	*msrA*	9 (20.9)	11 (26.2)	20 (23.5)
	ermA	2 (4.7)	4 (9.5)	6 (7)

6.3.3 Multidrug resistant *Staphylococcus aureus*

Sixteen isolates were resistant to more than three classes of antimicrobial agents used. They were therefore classified as multidrug resistant isolates. Most MDR (94%) isolates were also MRSA. (Table 6.3). Molecular determinants of resistance were analysed among the MDR isolates to understand the genes responsible for the MDR phenotypes. As shown in Table 6.3, 94%, 81%, 75% and 25% of the MDR isolates were found to harbour *tetK*, *blaZ*, *aph*, *tetM* and *msrA* resistance genes respectively (Table 6.3).

Table 6.3. Multidrug resistant *Staphylococcus aureus* isolated in Marsabit and Isiolo Counties

Isolate ID	**Antimicrobial agents**	**Resistant genes**
M90*	Oxa, Tet, Clind	*tetK, blaZ, aph, msrA, aac-aph*
I 119*	Oxa, Tet, Clind	*tetK, blaZ, mecA, tetM, msrA,*
M54*	Oxa, Tet, Clind, Cip	*tetK, blaZ, aph, mecA, msrA*
M52*	Oxa, Tet, Clind, Kan	*tetK, blaZ, aph, tetM, msrA,*
I 43*	Oxa, Tet, Clind	*tetK, blaZ, mecA, aac-aph*
M85*	Oxa, Tet, Kan, Cip	*tetK, msrA, aph, aac-aph*
M74*	Oxa, Tet, Clind, Kan	*tetK, ermA, aph, aac-aph*
M79*	Oxa, Tet, Clind	*tetK, blaZ, aph, aac-aph*
M57*	Oxa, Tet, Clind, Cip, Kan	*tetK, blaZ, aph, tetM*
I 122	Tet, Clind, Kan	*tetK, blaZ, aph, tetM*
M71*	Oxa, Tet, Clind, Kan	*tetK, blaZ, aph, tetM*
M42*	Oxa, Tet, Clind	*tetK, blaZ, mecA*
M18*	Oxa, Tet, Ery	*tetK, aph, msrA*
M60*	Oxa, clind, Kan	*tetK, blaZ, aph*
M66*	Oxa, Tet, Ery	*tetK, blaZ, aph*
M95*	Oxa, Clind, Ery	*tetK, blaZ,*

*****MRSA, Oxa (Oxacillin), Tet (Tetracycline), Clind (Clindamycin), Cip (Ciprofloxacin), Kan (Kanamycin), Ery (Erythromycin)

6.3.4 Correlation of AMU to *S. aureus* resistance genotypes

There was a positive correlation (r > 0.6) between AMU and presence of the various *S. aureus* resistance genes (Table 6.4). These correlations were not statistically significant, except for *tet*M detection and oxytetracycline usage. There was a positive correlation (r = 0.98) between penicillin usage and presence of *mec*A and blaZ (r = 0.86) genes.

Oxytetracycline usage was also correlated with the presence of *tet*K/tetM genes (r = 0.62/1) and aminoglycoside usage with detection of *aac (6')/ aph (2'')* genes (r = 0.76). Macrolide usage was also positively correlated with *S. aureus* isolates with *ermA*/*msrA* genes (r = 0.94/0.77) responsible for the resistance. However, there was a negative correlation for *aph (3') - IIIa* gene (r=-0.13) (Table 6.4).

Table 6.4 Pearson's correlation coefficient measuring the correlation between antimicrobial use and *S. aureus* antimicrobial resistance gene prevalence in the study sites

Antimicrobial group	Resistant gene	Number (n)	Pearson's correlation (r)	*P* value
Tetracyclines	*tet*K	82	0.62	0.58
	*tet*M	16	1	<0.001[1]
Penicillins	*bla*Z	67	0.86	0.34
	*mec*A	45	0.99	0.09
Aminoglycosides	*aac (6')/ aph (2'')*	35	0.76	0.44
	aph (3') - IIIa	33	-0.13	0.92[2]
Macrolides	*msr*A	20	0.77	0.44
	*erm*A	6	0.94	0.44

Pearson's (r) value above + 0.5 indicates a large association between AMU and resistance genes

[1] large positive correlation with significant difference observed for tetracycline use and *tet*M

[2] There was no association between aminoglycoside use and *aph (3') – IIIa*

6.4 Discussion

This study established the phenotypic and genotypic antimicrobial resistance profiles of *S. aureus* from individual animal and pooled raw milk samples from northern Kenya.

Screening of the isolates for genotypic determinants of resistance revealed that tetracycline resistance gene *tet*K had the highest frequency of detection at 96.5% followed by *bla*Z (78.8%), *aac (6')/aph (2'')* (52.9%), *aph (3')-IIIa (*42.7%), *MecA (*38.8%*), msrA (*23.5%*), tet*M *(*18.8%*), and erm*A *(*7%). This trend is similar to that observed in phenotypic test suggesting expression of resistance genes in most isolates. The presence of resistance genes is important since horizontal transfer of resistance in bacteria has been reported to occur between different animal species, within humans, from animals to humans, and from humans to animals (Igbinosa *et al*., 2016).

As a result of evolution, *S. aureus* has acquired tetracycline resistant genes (*tet*) such as *tet*M, *tetL, tet*O and *tetK* (Ullah *et al*., 2012). The acquisition of plasmid-located *tet*L and *tet*K genes aids in the active efflux of tetracycline out of the bacterial cells (Ray *et al*., 2017). As at 2006, the number of MRSA isolates harbouring *tet*K gene were reported to be 74.0 % while tetM were only 13%. In this study, *tet*K was detected in 84% while *tet*M was found in 16% of all the isolates that haboured *tet* gene (Lim *et al*., 2012). Most of the tetracycline-resistant *S. aureus* harbours both *tet*M and *tet*K genes (Lim *et al.,* 2012), though in this study most resistant isolates haboured *tetK* gene.

BlaZ (serine β-lactamase) inhibits the functions of β-lactam antibiotics such as penicillin by formation of acyl-enzymeintermediate and was the second highest resistance gene reported in this study. Furthermore, the enzyme is a lipoprotein that aids in protecting PBP2 (penicillin-binding protein 2) released from the bacterial cell to the surrounding medium (Lowy, 2003).

Genes responsible for inactivation of aminoglycosides *aac (6')/aph (2'')* and *aph (3')-IIIa* were also detected at 52.9% and 42.7% respectively. The inactivation process could be through release of enzymes such as acetyltransferases (AAC) and aminoglycoside phosphotransferase (APH) (Ramirez and Tolmasky, 2010). In addition, the *aac(6')-Ie-aph (2'')* gene is the commonest aminoglycosides resistant gene in MRSA and may be absent or present in combination with *tet* genes (Fatholahzadeh *et al.,* 2009).Therefore, the gene plays a protective role and facilitates the spread of MRSA among the dairy products. In this study the two genes were present in combination.

Some isolates were found to harbour *ermA* and *msrA* genes that confer resistance to macrolides such as erythromycin. The *erm* genes mediate the modification of 23S rRNA by N^6-demethylation of an adenine residue (Fines and Leclercq, 2000). In a separate study, Mwova (2016) detected *erm* genes in enterococci isolated from caged baboons in Kenya. Most importantly, the *erm* gene may be transferred horizontally since it associates with mobile genetic elements. The *msr* genes encode for macrolide efflux pump which is ATP-dependent and mediate resistance through the exclusion of the antibiotic from MRSA cells.

Methicillin resistance gene (*mec*A) was detected in 38.8 % of the isolates. *Mec*A gene is located on the staphylococcal cassette chromosome *mec* (SCC *mec*) and encodes for a 78-kDa penicillin binding protein (PBP2a) which causes decreased affinity to methicillin and all beta-lactam antibiotics (Siriken *et al.,* 2016). In this study, there were more isolates that were positive for *mecA gene* than were phenotypically resistant ones. A similar observation where *S. aureus* isolates have been found to be positive for *mec*A and PBP2a but phenotypically susceptible to oxacillin have been reported in other studies (Pu *et al*., 2014: Guimarães *et al*., 2017) and these isolates are referred to as oxacillin-susceptible *mecA*-positive *S. aureus* (OS-MRSA) (Guimarães

et al., 2017). This could be due to failure of expression of some resistance genes. Another postulated alternative mechanism for resistance could be mutations of the endogenous penicillin-binding proteins (PBP) in *mecA* and *mecC* negative strains (Ba *et al.,* 2014).

MRSA has not been detected in raw milk before in the Kenya and this is significant due to their zoonotic potential. The detection of MDR strains resistant to multiple antimicrobials administered to both animals and humans in the study area was also significant, which is largely pastoralist characterized by close interaction between pastoralists and their livestock. The risk of antimicrobial resistant *S. aureus* disease outbreaks is therefore high, with narrow treatment options for clinicians and veterinarians. Identification of all potential sources and reservoirs of MRSA in the dairy value chain in the study site is advised since this is essential for its eradication (Spohr *et al*., 2011).

CHAPTER 7: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

Milk is a highly nutritious food which provides excellent nutritional requirement for humans of all ages (Górska-Warsewicz *et al*., 2019). However, raw milk is extremely susceptible to spoilage by microbes if produced under unhygienic conditions (Soomro *et al*., 2002; Machado *et al*., 2017). Therefore, this study determined the presence of enterotoxigenic and antimicrobial resistant *S. aureus* in raw milk intended for human consumption in northern Kenya. The contamination of raw milk along the dairy value chain by enterotoxigenic *S. aureus* that are resistant to commonly used antimicrobials is of great importance to the global public health. This is because of the challenges posed by difficulty in the treatment of drug resistant milk-borne *S. aureus* infections and foodborne illnesses (Hennekinne *et al*., 2012; Sergelidis *et al*., 2017). Moreover, the occurrence of genetic determinants responsible for the emergence of AMR *S. aureus*, including the MRSA in milk is a possible means of spread of AMR especially when the contaminated milk is consumed by humans (Igbinosa *et al*., 2016).

The overall occurrence of *S. aureus* in the analyzed samples was 14% for milk samples in Marsabit and Isiolo Counties. Although this prevalence was lower than that of Asiimwe *et al*., (2017) (20.3%) for *S. aureus* detected in bulk can-milk consumed in pastoral areas of Uganda and Mathenge *et al*. (2015) (36%) in meat and milk products in Nairobi county, it is significant considering that the milk is consumed raw. Previous studies reported higher prevalence rates in milk in other countries including Turkey (Kiymet *et al*., 2010), USA (Lubna *et al*., 2015), Zimbabwe (Gran *et al*., 2003) and in Malaysia (Chye *et al*., 2004).

The proportion of *S. aureus* isolated from pooled milk samples in this study was significantly higher than those from individual lactating animals. Other than poor hygiene during the milking

process, it is hypothesized that during pooling of milk, there is an increased risk of contamination from human skin carrying *S. aureus* thereby resulting in higher contamination level as seen in this study. Furthermore, pooling of milk under poor hygienic conditions involving use of contaminated utensils could have resulted in the relatively high contamination. For individual milk samples, contamination by *S. aureus* isolates may have resulted from clinical and subclinical mastitis resulting from intramammary infections caused by the bacterium. *Staphylococcus aureus* is usually known to enter the udder through the teat canal from the surrounding environment (Smith *et al*., 2005) and this can act as a source of infection and milk contamination. Consequently, increased awareness of pastoralists is necessary in order to minimize contamination of milk through improved hygiene practices as well as through diagnosis and treatment of infected animals.

In this study, a number of *S. aureus* isolated from milk were β-hemolytic and coagulase positive indicating that these bacterial isolates could be potentially pathogenic. In addition, the terminase gene used to identify *S. aureus* from milk samples forms one of the core genes in the staphylococcal pathogenicity islands (SaPIs), which is a mobile genetic element responsible for the bacterial virulence (Malachowa and DeLeo, 2010). Furthermore, genetic determinants that are responsible for the production of enterotoxins from the *S. aureus* isolates were detected in 74.11% of the isolates. Although there were no reported cases of staphylococcal food poisoning during the study period, the *S. aureus* detected in this study could cause serious infections under suitable conditions of growth and toxin production. Therefore, contamination of milk in the study site poses serious public health risk to the pastoralist communities that may consume raw milk.

Overall, 88% of the *S. aureus* isolates were resistant to at least one of the antibiotics, including oxacillin, ampicillin, erythromycin, kanamycin, tetracycline, clindamycin, ciprofloxacin, and cephalexin. This finding is significant considering that *S. aureus* infections and staphylococcal food borne outbreaks are controlled using antibiotics. Indeed, *S. aureus* is well known for its ability to rapidly develop resistance to commonly used antibiotics thereby presenting major public health challenges to humans (Kateete *et al*., 2013; Vestergaard *et al*., 2019). Consequently, cases of prolonged hospital admissions reduced therapeutic efficacy of the antimicrobial agents, increased virulence, high cost of treatment and even mortalities are likely to be reported from staphylococcal infections as earlier reported (Li *et al*., 2018).

Although *S. aureus* resistance is acquired through chromosomal mutation and horizontal transfer of genes from outside sources, antibiotic selection plays an important role (Asiimwe *et al*., 2017; Gomes and Henriques, 2016). In this study, high levels of tetracycline resistance were observed, similar to previous studies (Kateete *et al*., 2013). The emergence of the drug-resistant *S. aureus* isolates, including the MDR-MRSA has been linked to increased usage of antimicrobials in human and veterinary medicine (Valsangiacomo *et al*., 2000; Grema *et al*., 2015). Indeed, extensive use of antimicrobial drugs was observed in this study, a practice which correlated with the high level of resistance to tetracyclines and penicillins by the milk-borne *S. aureus*. In addition to oxytetracycline usage, sulfonamide, beta lactams, aminoglycoside, and macrolide usage was common among the pastoral communities, a trend consistent with previous findings of the study sites nearly two decades ago (Mitema *et al*., 2001). Furthermore, a recent study reported the extensive use of tetracycline and to some extent penicillins as well as aminoglycosides in Isiolo (Lamuka *et al*., 2017).

In addition to genetic mutations and antibiotic selection pressure, the spread of AMR-*S. aureus*

can be aided by various mobile genetic elements (Igbinosa *et al*., 2016; Vestergaard *et al.,* 2019). Therefore, identification of genetic determinants associated with resistance phenotypes is essential for understanding the molecular mechanisms responsible for the milk-borne *S. aureus* phenotypic resistance. The Majority of *S. aureus* isolates harbored a high proportion of tetK gene (96.5%) followed by blaZ (78.8%). Other genetic determinants detected were *aac (6')/aph (2")* (52.9%), *aph (3')-IIIa* (42.7%), *Mec*A (38.8%), *msr*A (23.5%), *tet*M (18.8%), and *erm*A (7%).

The genetic profiles of the resistant isolates mirrored the profiles of the resistance phenotypes indicating that the genes harbored by the milk borne *S. aureus* could have been responsible for the resistance phenotypes observed. The detection of these resistance genes in the *S. aureus* isolates is significant since these genetic elements can be transferred horizontally between bacteria that infect different animal species and humans (Igbinosa *et al*., 2016).

Up to 33% of the *S. aureus* isolates were phenotypically resistant to oxacillin indicating the presence of milk-borne MRSA. In this regard, it appears that milk-borne MRSA was more prevalent in pastoral communities in Kenya as compared with other regions such as Algeria (17.7%), Tunisia (6.6%), Korea (2.8%), Switzerland (1.4%), Finland, (1.5%), Japan (1.5%), Southwest Germany (5.1– 16.7%), Belgium (7.4%,), Mexico (18.1%), and Brazil (22%).5–9,25,49–56 The prevalence is however lower than that reported in raw milk in Uganda (56.1%),5 Nigeria (100%), 25 and Egypt (53 and 87.5%) (Al-Ashmawy *et al*., 2015; Ahmed *et al*., 2020). Ninety-four percent of the MDR isolates were MRSA. Previous studies show that MRSA are MDR, with resistance to the commonly used antibiotics, such as penicillins, aminoglycosides, macrolides, sulfonamides, and tetracyclines, most of which are commonly used in the treatment of mastitis (Asiimwe *et al*., 2017; Lamuka *et al*., 2017; Funaki *et al*., 2019; Ahmed *et al*., 2020;).

Methicillin resistance gene (*mec*A) was detected in 38.8% of the isolates. Furthermore, *mec*A gene is located on the SCC*mec* and encodes for a 78-kDa PBP (PBP2a), which causes decreased affinity to methicillin and most of beta-lactam antibiotics (Wanjohi *et al*., 2013). The SCC*mec* can also transport determinants of resistance for other antimicrobials, virulence determinants, and other genes necessary for the survival of *S. aureus* in stressful conditions (Katayama *et al*., 2000). Therefore, in a single event of genetic acquisition, SCC*mec* can turn susceptible staphylococci into virulent MDR pathogens that are well adapted to thrive in an infection situation, particularly in the hospital environment.

In the current study, there were more isolates that were positive for *mec*A gene than were phenotypically resistant, an observation similar to another study, which documented presence of oxacillin-susceptible *mec*A-positive *S. aureus* (Asiimwe *et al*., 2017). This could be due to failure of expression of some resistance genes. Another possible alternative mechanism for resistance could be mutations of the endogenous PBPs in *mec*A- and *mec*C negative strains (Ba *et al*., 2014).

It should be noted that MRSA has never been isolated from raw milk of livestock in Kenya before. Therefore, the detection of the milk-borne MRSA is significant because, for a long time, it has been believed that MRSA are acquired only at community level or during hospital admission. Indeed, this finding provides further proof of the changed epidemiology of MRSA.

The only other possible documented source of infection of humans is during interaction with dogs since MRSA has been isolated from dog wounds in Kenya (Njoroge *et al*., 2018). The detection of MDR strains resistant to multiple antimicrobials administered to both animals and humans in the pastoral communities was also significant because of close interaction between pastoralists and their livestock. The risk of AMR *S. aureus* infections, including the MDR-

MRSA is therefore high. Subsequently, identification of all potential sources and reservoirs for MDR-MRSA transmission, including the environment for the dairy value chain in pastoral communities, is recommended since this is essential for its eradication (Spohr *et al*., 2011).

This study revealed that AMU was generally positively correlated with the phenotypic resistance and the detection of *mec*A, *blaZ*, *tet*K, *tet*M, *aac (6')/aph (2")*, and *erm*A/ *msr*A genes responsible for the resistance to a range of antibiotics, indicating an apparent relationship with the high prevalence of isolates with resistance phenotypes. For all the antimicrobial classes, Pearson's correlation coefficient was above 0.6 (although there was no significant difference, except for tetM, $p = 0.05$) suggesting that AMU was apparently linked to the AMR pattern observed in this study. A previous study also found a link of AMU to the emergence of AMR profiles (Chantziaras *et al*., 2014). Judicious use of antimicrobials is therefore recommended among the pastoralist communities in Kenya.

Although the current study achieved its objectives, possible limitations of the approach should be considered. For example, only four camel milk samples were collected from the study areas because pastoral communites had moved their livestock to other inaccesible areas due to the prevailing drought experienced during the sampling period. Another limitation of the current study is the ommission of AMU data from informal supply chain since only data from formal drug supplies was considered. It should however be noted that informal antimicrobial sources could be in existance in the study areas owing to the porous borders with neighboring countries. Finally, the antimicrobial data obtained in this study indicated that potentiated sulphonamides were among the most commonly used antimicrobials. However potentiated sulphonamides (trimethoprim-sulfamethoxazole (TMP-SMZ)) are not commonly used for control of staphylococcal infections because *S. aureus* thermonuclease releases thymidine from host DNA

in the damaged host tissue rendering the TMP-SMZ inactive (Proctor, 2008). Therefore no phenotypic or genotypic antimicrobial resistance tests were included for TMP-SMZ in this study.

7.2 CONCLUSIONS

Based on the findings of this study, it is concluded that:

1. Milk consumed by pastoralists in Marsabit and Isiolo Counties is contaminated with *S. aureus.*
2. The *S. aureus* isolates harbour enterotoxigenic genes hence potentially pathogenic
3. Raw milk samples from Marsabit and isiolo counties are contaminated with multidrug resistant MRSA
4. The *S. aureus* resistant phenotypes isolated in milk harbour genetic resistance determinants responsible for antimicrobial resistance phenotypes.
5. There was a correlation between *tet*M detection and *S. aureus* antimicrobial resistance pattern.

7.3 RECOMMENDATIONS

- Hygienic handling and pasteurization of milk is recommended before consumption to reduce the risk of exposure to enterotoxigenic *S. aureus*
- Regular surveillance on contamination of milk by enterotoxigenic *S. aureus* is recommended in order to monitor the risk of food poisoning
- There is need for continuous surveillance and monitoring of MDR *S. aureus* including MRSA in the pastoral areas of Northern Kenya

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APPENDICES

APPENDIX A: A QUESTIONNAIRE ON COMMON ANIMAL DISEASES ENCOUNTRED, TREATMENT AND ANTIBIOTICS USE IN DISEASE CONTROL AT MARSABIT AND ISIOLO COUNTIES, KENYA

Dear participant,

Am **Isaac Omwenga**, a PhD student at the department of Public Health, Pharmacology and Toxicology, University of Nairobi carrying out a study on "**Pathotyping and antimicrobial resistance-characterization of *Staphylococcus aureus* in milk for human consumption in Marsabit and Isiolo Counties, Kenya".**

As one of the stake holder in milk production system, you have been selected to participate to this exercise voluntarily. The information you will generate is purely for academic purposes and will be treated with utmost confidentiality and will not be used whatsoever to incriminate your business enterprise. This study will help to improve the marketability of your dairy product (milk) and avoid public health risk associated with consumption of milk contaminated by multi-drug resistant enterotoxin producing *Staphylococcus aureus*.

Consent Form

I certify that I am the owner or authorized agent (manager) of a herd of dairy cattle maintained at the following address:

I certify that I have read this statement and I voluntarily agree to participate in this study. I have received a copy of this consent form.

---------- ----------

Signature of herd owner/ Print Name

Manager

Date---------- Phone----------

---------- ----------

Signature of person Obtaining Consent Print Name

Date ---------- Phone----------

1. What are the common livestock diseases encountered in your farm……
   ……
   ……
   ……...?

2. What are the common drugs used to treat animals in your farm (including antibiotics)

   ……

   ……

   ……?

3. Do you consult any animal health practitioner when your animals are sick? YES/NO

4. What dosage do you use in various species and ages of animals?

   Cattle……

   Calves……

   Goats……

   Kids……

   Sheep……

   Lambs……

   Camels……

   Camel calf……

5. What is the average length of treatment……...?

6. How many animals are milking?

   Cattle……

   Goats……

   Sheep……

   Camels……

7. Have you had any cases of mastitis…….?

8. How do you manage mastitis

   ……..?

9. Have the milking animals been treated in the last three months? YES/NO If yes, what medicines were they given.....?
10. Do you observe any withdrawal period after administering drugs to the milking animals? YES/NO. If yes, how long is the withdrawal period…….
11. Do you boil your milk before consumption? YES? NO If yes, how do you do it……

    ……

    ……

    ……...
12. Have you ever had a health problem related to milk consumption? YES/NO If yes, what where the symptoms/clinical signs?

    .....

    .....

    .....
13. Did you seek medical attention……?

APPENDIX B: Sequence blasting results (*mec*A) (Accession No: MH636811)

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Staphylococcus sciuri subsp. sciuri strain TXG-24 mecASs complex region	1393	1393	98%	0.0	98.97%	KX774480.1
Staphylococcus sciuri SCBM1 mecA1 gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA1, complete CDS	1393	1393	98%	0.0	98.97%	NG_047950.1
Staphylococcus aureus strain 28C penicillin-binding protein (mecA) gene, partial cds	1393	1393	98%	0.0	98.97%	KF058902.1
Staphylococcus aureus strain 8A penicillin-binding protein (mecA) gene, partial cds	1393	1393	98%	0.0	98.97%	KF058901.1
Staphylococcus aureus strain 272B penicillin-binding protein (mecA) gene, partial cds	1393	1393	98%	0.0	98.97%	KF058900.1
Staphylococcus sciuri strain SCBM1 PBP2a-like protein (mecA) gene, complete cds	1393	1393	98%	0.0	98.97%	AY820253.1
Staphylococcus sciuri strain 09-LEM-1/3 penicillin binding protein 2a (mecA) gene, complete cds	1201	1201	98%	0.0	94.49%	JX094435.1
Staphylococcus sciuri subsp. rodentium DNA, methicillin-resistance gene region, strain: ATCC 700061	1162	1162	98%	0.0	93.60%	AB547235.1
S.sciuri mecA1 gene, strain K3(MM2)	1151	1151	98%	0.0	93.34%	Y13052.1
Staphylococcus sciuri strain FDAARGOS_285 chromosome, complete genome	1118	1118	98%	0.0	92.57%	CP022046.2
Staphylococcus sciuri strain Slemani/Kurdistan/2017 PBP2a family beta-lactam-resistant peptidoglycan (mecA1) gene, partial cds	1112	1112	98%	0.0	92.45%	MG706986.1
Staphylococcus sciuri subsp. sciuri strain NCTC12103 genome assembly, chromosome: 1	1112	1112	98%	0.0	92.45%	LS483305.1
Staphylococcus sciuri ATCC 29062 mecA1 gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA1, complete CDS	1112	1112	98%	0.0	92.45%	NG_047949.1
Staphylococcus sciuri subsp. sciuri DNA, methicillin-resistance gene region, strain: ATCC 29062	1112	1112	98%	0.0	92.45%	AB547234.1
S.sciuri mecA gene & ORF's 450, 145 & 179	1112	1112	98%	0.0	92.45%	Y09223.1

APPENDIX C: *S. aureus mec*A translated amino acid sequences Accession number: MH636811

PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA1 [Staphylococcus aureus]
Sequence ID: WP_037589174.1 Length: 666 Number of Matches: 1
▸ See 1 more title(s)

Range 1: 24 to 638 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
1159 bits(2997)	0.0	Compositional matrix adjust.	597/615(97%)	610/615(99%)	1/615(0%)	+1

```
Query  1    SKNSQINDTLDAIEDKNVKQVFKDSTYQSKNDNGEVEMTDRPIKIYDSLGVKAINIKDRD  180
            SK+ +IN+T+DAIEDKNVKQVFK+STYQSKNDNGEVEMTDRPIKIYDSLGVK INIKDRD
Sbjct  24   SKDKKINETIDAIEDKNVKQVFKNSTYQSKNDNGEVEMTDRPIKIYDSLGVKDINIKDRD  83

Query  181  IKKVSKNKKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGMQKNQSINI  360
            IKKVSK+KKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGM+KNQSINI
Sbjct  84   IKKVSKDKKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGMKKNQSINI  143

Query  361  EPLKSERGKILDRNNVELATTGTAHEVGIVPNNVSTSDYKAIAEKLDLSESYIKQQAEQD  540
            EPLKSERGKILDRNNVELATTGTAHEVGIVPNNVS++DYKAIAEKLDLSESYIKQQ EQD
Sbjct  144  EPLKSERGKILDRNNVELATTGTAHEVGIVPNNVSSNDYKAIAEKLDLSESYIKQQTEQD  203

Query  541  WVKDDTFVPLKTVQNMNQDTKRFVEKYHLTTQETESRQYPLEEATTHLLGYVGPINSEEL  720
            WVKDDTFVPLKTVQNMNQDTK FVEKYHLTTQETESRQYPLEEATTHLLGYVGPINSEEL
Sbjct  204  WVKDDTFVPLKTVQNMNQDTKNFVEKYHLTTQETESRQYPLEEATTHLLGYVGPINSEEL  263
```

penicillin-binding protein, partial [Staphylococcus aureus]
Sequence ID: AGU99975.1 Length: 610 Number of Matches: 1

Range 1: 1 to 610 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
1187 bits(3070)	0.0	Compositional matrix adjust.	610/610(100%)	610/610(100%)	0/610(0%)	+1

```
Query  10   SQINDTLDAIEDKNVKQVFKDSTYQSKNDNGEVEMTDRPIKIYDSLGVKAINIKDRDIKK  189
            SQINDTLDAIEDKNVKQVFKDSTYQSKNDNGEVEMTDRPIKIYDSLGVKAINIKDRDIKK
Sbjct  1    SQINDTLDAIEDKNVKQVFKDSTYQSKNDNGEVEMTDRPIKIYDSLGVKAINIKDRDIKK  60

Query  190  VSKNKKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGMQKNQSINIEPL  369
            VSKNKKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGMQKNQSINIEPL
Sbjct  61   VSKNKKQVTAKYELQTNYGKINRDVKLNFIKEDKDWKLDWNQSVIIPGMQKNQSINIEPL  120

Query  370  KSERGKILDRNNVELATTGTAHEVGIVPNNVSTSDYKAIAEKLDLSESYIKQQAEQDWVK  549
            KSERGKILDRNNVELATTGTAHEVGIVPNNVSTSDYKAIAEKLDLSESYIKQQAEQDWVK
Sbjct  121  KSERGKILDRNNVELATTGTAHEVGIVPNNVSTSDYKAIAEKLDLSESYIKQQAEQDWVK  180
```

APPENDIX D: *S. aureus mec*A blast results and amino translated amino acid sequences

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
penicillin binding protein PBP2A [Staphylococcus aureus]	1278	1278	96%	0.0	100.00%	AYA60753.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]	1278	1278	96%	0.0	100.00%	WP_078066067.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]	1277	1277	96%	0.0	99.84%	WP_111122758.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]	1276	1276	96%	0.0	99.84%	WP_086031948.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus hominis]	1276	1276	96%	0.0	99.84%	WP_103268303.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]	1276	1276	96%	0.0	99.84%	WP_000721310.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]	1276	1276	96%	0.0	99.84%	WP_048518303.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus hominis]	1276	1276	96%	0.0	99.69%	WP_081261151.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus epidermidis]	1276	1276	96%	0.0	99.84%	WP_134288489.1
Penicillin-binding protein MecA [Staphylococcus petrasii subsp. jettensis]	1276	1276	96%	0.0	99.84%	SUM42526.1
penicillin binding protein 2 prime, methicillin resistance determinant MecA [Staphylococcus aureus M0515]	1276	1276	96%	0.0	99.84%	EVA14240.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus pseudintermedius]	1276	1276	96%	0.0	99.84%	WP_110158838.1
PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus petrasii]	1276	1276	96%	0.0	99.84%	WP_103298748.1

PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA [Staphylococcus aureus]

Sequence ID: WP_078066067.1 Length: 669 Number of Matches: 1

▸ See 24 more title(s)

Range 1: 25 to 669 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
1278 bits(3306)	0.0	Compositional matrix adjust.	645/645(100%)	645/645(100%)	0/645(0%)	+1

```
Query  70    ASKDKEINNTIDAIEDKNFKQVYKDSSYISKSDNGEVEMTERPIKIYNSLGVKDINIQDR  249
            ASKDKEINNTIDAIEDKNFKQVYKDSSYISKSDNGEVEMTERPIKIYNSLGVKDINIQDR
Sbjct  25    ASKDKEINNTIDAIEDKNFKQVYKDSSYISKSDNGEVEMTERPIKIYNSLGVKDINIQDR  84

Query  250   kikkvsknkkRVDAQYKIKTNYGNIDRNVQFNFVKEDGMWKLDWDHSVIIPGMQKDQSIH  429
            KIKKVSKNKKRVDAQYKIKTNYGNIDRNVQFNFVKEDGMWKLDWDHSVIIPGMQKDQSIH
Sbjct  85    KIKKVSKNKKRVDAQYKIKTNYGNIDRNVQFNFVKEDGMWKLDWDHSVIIPGMQKDQSIH  144

Query  430   IEKLKSERGKILDRNNVELANTGTAYEIGIVPKNVSKKDYKAIAKELSISEDYIKQQMDQ  609
            IEKLKSERGKILDRNNVELANTGTAYEIGIVPKNVSKKDYKAIAKELSISEDYIKQQMDQ
Sbjct  145   IEKLKSERGKILDRNNVELANTGTAYEIGIVPKNVSKKDYKAIAKELSISEDYIKQQMDQ  204

Query  610   NWVQDDTFVPLKTVKKMDEYLSDFAKKFHLTTNETESRNYPLGKATSHLLGYVGPINSEE  789
            NWVQDDTFVPLKTVKKMDEYLSDFAKKFHLTTNETESRNYPLGKATSHLLGYVGPINSEE
Sbjct  205   NWVQDDTFVPLKTVKKMDEYLSDFAKKFHLTTNETESRNYPLGKATSHLLGYVGPINSEE  264

Query  790   LKQKEYKGYKDDAVIGKKGLEKLYDKKLQHEDGYRVTIVDDNSNTIAHTLIEKKKKDGKD  969
            LKQKEYKGYKDDAVIGKKGLEKLYDKKLQHEDGYRVTIVDDNSNTIAHTLIEKKKKDGKD
Sbjct  265   LKQKEYKGYKDDAVIGKKGLEKLYDKKLQHEDGYRVTIVDDNSNTIAHTLIEKKKKDGKD  324

Query  970   IQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYDVYPFMYGMSNEEYNKL  1149
            IQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYDVYPFMYGMSNEEYNKL
Sbjct  325   IQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYDVYPFMYGMSNEEYNKL  384
```

APPENDIX E: *S. aureus tet*K blast results and amino translated amino acid sequences

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Staphylococcus aureus strain NRS384-rpoB-H481N-SCV plasmid pNRS384_1, complete sequence	699	699	87%	0.0	100.00%	CP043390.1
Staphylococcus aureus strain NRS384-rpoB-H481N-NCV plasmid pNRS384_1, complete sequence	699	699	87%	0.0	100.00%	CP043393.1
Staphylococcus aureus strain NRS384 plasmid pNRS384_1, complete sequence	699	699	87%	0.0	100.00%	CP043387.1
Staphylococcus aureus subsp. aureus plasmid p6530, complete sequence	699	699	87%	0.0	100.00%	MK933276.1
Staphylococcus aureus subsp. aureus plasmid p6306, complete sequence	699	699	87%	0.0	100.00%	MK933274.1
Staphylococcus aureus pT181 tet(K) gene for tetracycline efflux MFS transporter Tet(K), complete CDS	699	699	87%	0.0	100.00%	NG_048200.1

Staphylococcus aureus subsp. aureus USA300_FPR3757 pUSA02 tet(K) gene for tetracycline efflux MFS transporter Tet(K), complete CDS

Sequence ID: NG_055987.1 Length: 1515 Number of Matches: 1

Range 1: 384 to 761 GenBank Graphics — Next Match — Previous Match

Score	Expect	Identities	Gaps	Strand
699 bits(378)	0.0	378/378(100%)	0/378(0%)	Plus/Minus

```
Query  2    TCTTGAAATATGTTTAATAAAAATCACAAAAAAGATTGTGAAGAGTATTAAAAAAGTCCA  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  761  TCTTGAAATATGTTTAATAAAAATCACAAAAAAGATTGTGAAGAGTATTAAAAAAGTCCA  702

Query  62   ATTATAATTTGTCGTAAATAACATAAAACATATAATACTTATAGACATTAAAACAATACC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  701  ATTATAATTTGTCGTAAATAACATAAAACATATAATACTTATAGACATTAAAACAATACC  642
```

APPENDIX F: *S. aureus ermA* blast results and amino translated amino acid sequences

Sequences producing significant alignments Download Manage Columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Staphylococcus aureus subsp. aureus strain Gv51, complete genome	171	338	31%	3e-38	93.16%	CP012015.1
Staphylococcus aureus strain AR_0467 chromosome, complete genome	169	336	30%	1e-37	93.86%	CP029658.1
Staphylococcus aureus strain Cx6 erythromycin resistance protein subunit A (ermA) gene, complete cds	167	167	31%	4e-37	92.44%	KX638955.1
Staphylococcus aureus strain Cx4 erythromycin resistance protein subunit A (ermA) gene, complete cds	167	167	31%	4e-37	92.44%	KX638954.1
Staphylococcus aureus strain Cx5 erythromycin resistance protein subunit A (ermA) gene, complete cds	167	167	31%	4e-37	92.44%	KX638951.1
Staphylococcus aureus strain DL4 erythromycin resistance protein subunit A (ermA) gene, complete cds	167	167	31%	4e-37	92.44%	KX638948.1
Staphylococcus aureus strain Cx3 erythromycin resistance protein subunit A (ermA) gene, complete cds	167	167	30%	4e-37	93.10%	KX638947.1

Staphylococcus aureus strain Cx5 erythromycin resistance protein subunit A (ermA) gene, complete cds

Sequence ID: KX638951.1 **Length:** 732 **Number of Matches:** 1

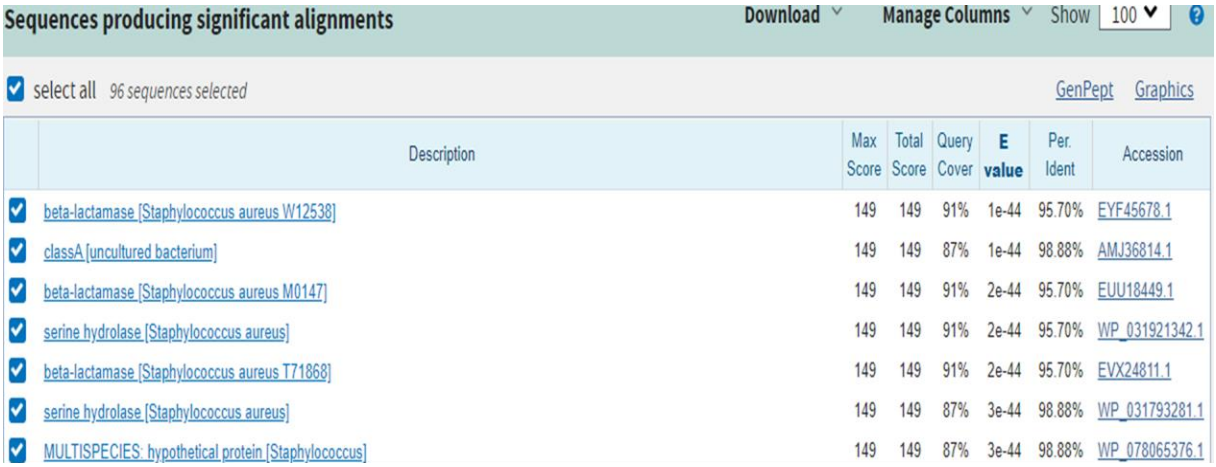
Range 1: 445 to 563 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
167 bits(90)	4e-37	110/119(92%)	4/119(3%)	Plus/Plus

```
Query  10   GACATGGATAT--ATATGCTC--AAAAGTACCACCACTATATTTTCATCCTAAGCCAAGT  65
            || ||||||||  | |||||||  ||||||||||||||||||||||||||||||||||||
Sbjct  445  GAGATGGATATAAAAATGCTCAAAAAAGTACCACCACTATATTTTCATCCTAAGCCAAGT  504

Query  66   GTATACTCTGTATTGATTGTTCATGAACGACATCAACCATTGATTTCAAAGAAAGACTA  124
            ||| |||||||||||||||||| ||||||||||||||||||||||||||| |||||
Sbjct  505  GTAGACTCTGTATTGATTGTTCTTGAACGACATCAACCATTGATTTCAAAGAAGGACTA  563
```

APPENDIX G: *S. aureus blaZ* blast results and amino translated amino acid sequences



Sequences producing significant alignments Download Manage Columns Show 100

select all *96 sequences selected* GenPept Graphics

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
beta-lactamase [Staphylococcus aureus W12538]	149	149	91%	1e-44	95.70%	EYF45678.1
classA [uncultured bacterium]	149	149	87%	1e-44	98.88%	AMJ36814.1
beta-lactamase [Staphylococcus aureus M0147]	149	149	91%	2e-44	95.70%	EUU18449.1
serine hydrolase [Staphylococcus aureus]	149	149	91%	2e-44	95.70%	WP_031921342.1
beta-lactamase [Staphylococcus aureus T71868]	149	149	91%	2e-44	95.70%	EVX24811.1
serine hydrolase [Staphylococcus aureus]	149	149	87%	3e-44	98.88%	WP_031793281.1
MULTISPECIES: hypothetical protein [Staphylococcus]	149	149	87%	3e-44	98.88%	WP_078065376.1

beta-lactamase [Staphylococcus aureus W12538]

Sequence ID: EYF45678.1 Length: 97 Number of Matches: 1

Range 1: 5 to 97 GenPept Graphics Next Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
149 bits(376)	1e-44	Compositional matrix adjust.	89/93(96%)	90/93(96%)	0/93(0%)	+3

```
Query  18   ELSKKNKNFLLDLMFNNKNGDTLIKDGVPKDYKVADKSGQAITYASRNDVAFVYPKGQSE  197
            +LSKKNKNFLLDLMFNNKNGDTLIKDGVPKDYKVADKSGQAITYASRNDVAFVYPKGQSE
Sbjct  5    KLSKKNKNFLLDLMFNNKNGDTLIKDGVPKDYKVADKSGQAITYASRNDVAFVYPKGQSE  64

Query  198  PIVLVIFTnkdnksdkpndkLISETAKSVYFFF  296
            PIVLVIFTNKDNKSDKPNDKLISETAKSV   F
Sbjct  65   PIVLVIFTNKDNKSDKPNDKLISETAKSVMKEF  97
```