Staphylococcus aureus from Milk and Milk Products in Ethiopia: Prevalence, Enterotoxigenic Potential, Antibiotic Resistance and *spa* types

Staphylococcus aureus fra melk og melkeprodukter i Etiopia: Forekomst, enterotoksigenisk potensiale, antibiotikaresistens og *spa* typer.

Philosophiae Doctor (PhD) Thesis

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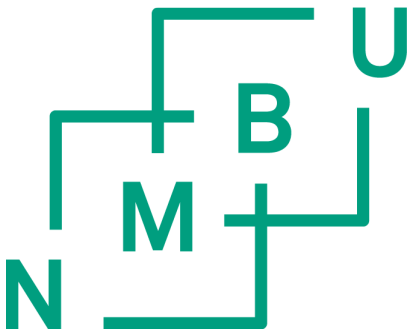

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Enquebaher Kassaye Tarekgne
Ås, Norway, April 2016

DEDICATION

To

My late Father

Memher Kassaye Tarekgne Zegta
(1920-2008 E.C.)

For his love, moral support and guidance to the right direction

ABBREVIATIONS

APC	Antigen presenting cells
BORSA	Borderline Oxacillin-Resistant
BPA	Baird-Parker Agar
CNS	Coagulase-negative staphylococcus
CPS	Coagulase positive staphylococcus
egc	enterotoxin gene cluster
IL	Interleukin
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
MDR	multidrug resistance
MHC	Major histocompatibility complex
PT	Pyrogenic toxin
qPCR	Quantitative real-time polymerase chain reaction
SE	Staphylococcal enterotoxin
SFP	Staphylococcal Food Poisoning
SaPI	*Staphylococcus aureus* pathogenicity Islands
SAgs	Super antigen
TBARD	Tigray Bureau of Agriculture and Rural Development
TSST-1	Toxic shock syndrome toxin
TCR	T-cell receptors
TNF	Tumor necrosis factors
TCRS	Two component regulatory system
16S rRNA	16S ribosomal ribonucleic acid

SUMMARY

Milk production in Ethiopia is increasing. However, due to unhygienic and traditional milk production and processing practices the issue of milk safety remains a challenge. If these safety issues are not properly addressed, the high nutritional composition and neutral pH of milk may convey many milk-borne pathogens, including *Staphylococcus aureus*, which imposes health risks to the consumer. *S. aureus* is an important food-borne pathogen globally as it can cause staphylococcal food poisoning (SFP) and also readily develops antibiotic resistance.

The objectives of this study were: i) to study the distribution of *S. aureus* and other *Staphylococcus* species in milk and milk products along the milk value chain of the studied area; ii) to assess the enterotoxigenic potential of the *S. aureus* isolates; iii) to evaluate the antimicrobial resistance profiles of the *S. aureus* isolates to 12 antibiotics commonly used in the studied area and verify the presence or absence of methicillin-resistant *S. aureus* (MRSA) iv) to assess the genetic relatedness of the enterotoxigenic and multidrug resistance (MDR) *S. aureus* strains using *spa* tying method; v) evaluate the performance of real time PCR (qPCR) targeting the *nuc* gene for quantification of *S. aureus* in bulk milk, in comparison with the plate count method.

Along the milk value chain, the prevalence of *S. aureus* ranged from 29.5% in traditional dairies to 48.2% in cafeterias and restaurants. The overall prevalence was 38.7% with mean count of 4.35 ± 0.97 Log CFU ml^{-1}. Odds ratio analysis indicated that samples from small-scale dairies (46.4%) were twice more likely to be contaminated by *S. aureus* than milk from the traditional dairies (29.5%) ($P < 0.05$, OR =2.07). Highest prevalence of *S. aureus* was registered in Shireendaselasse and the lowest in Maichew sampling areas ($p < 0.05$). Coagulase-negative staphylococci (CNS) were found in 51.6% of milk samples with mean count of 6.0 ± 1.21 Log CFU ml^{-1}. Ten species of CNS were identified and *S. epidermidis* was the most frequent.

Using a modified multiplex PCR method, 82 of 160 (51%) *S. aureus* isolates were found to harbor one or more enterotoxin genes Nine enterotoxin genes were identified; *sea* (n=12), *seb* (n=3), *sec* (n=3), *sed* (n=4), *seg* (n=49*), seh* (n=2), *sei* (n=40), *sej* (n=1), *tsst-1* (n=24), and the classical type of enterotoxin genes accounted for 27%. The most frequent gene association was observed between *sei* and *seg* and the *sea* and *seb* were frequently found associated with new types of enterotoxin genes. Eighteen enterotoxin genotypes were identified. The

enterotoxigenic *S. aureus* prevalence was higher ($P < 0.05$) in bulk milk than in the products and 32% of the samples contained > 5 Log CFU ml $^{-1}$ *S. aureus*.

To ascertain antibiotic susceptibility, the Kirby-Bauer disk diffusion method and PCR for the detection of either *mecA* or *mecC* genes were employed. From 160 *S. aureus* dairy isolates, 137 (86%) were resistant to one or more types of antibiotic of which 61 (45%) were multi drug-resistant (MDR) strains. The MDR strains showed 35-resistance patterns and 61% of isolates were resistant to 5 or more types of antibiotics. Resistance to penicillin G (69%) was most commonly encountered, followed by streptomycin (53%) and erythromycin (41%). Thirty-two (23%, 32/137) isolates were classified as borderline oxacillin-resistant *S. aureus* (BORSA) because they showed phenotypic oxacillin resistance without having either *mecA* or *mecC* genes and yet were susceptible to β-lactamase inhibitors.

spa typing of the enterotoxigenic and MDR *S. aureus* isolates from milk and milk products identified 22 *spa* types and 3 novel *spa* sequences, showing their wide genetic diversity. Greater *spa* type diversity was observed in bulk milk samples compared to milk product samples. No apparent correlation or pattern were observed between the *spa* types and the *se* genotypes or between the *spa* types and resistance pattern of the MDR strains. The *spa* types t314, t458 and t6218 were the most common and were widely distributed in three of eight localities of the studied area.

The performance of the qPCR assay in terms of amplification efficiency (91%) of the standard curve, repeatability (Standard Deviation (SD) = 0.12 - 0.3) and reproducibility (SD = 0.29 - 0.5) were within acceptable range. The primers could differentiate *S. aureus* from other *Staphylococcus* species. The detection limit was 18 copies of *nuc* gene/PCR. The qPCR assay (SCE ml $^{-1}$) showed higher cell count ($P< 0.05$) than the plate count (CFU ml $^{-1}$) and 29% samples that contained < 5 Log CFU ml $^{-1}$ *S. aureus* by the plating method were found to have > 5 Log SCE ml $^{-1}$ *S. aureus* using qPCR assay. The difference between the methods is likely to be due to detection of dead cells for the qPCR method and clustering of bacteria leading to that more bacteria form only one colony on the agar plates. Hence qPCR was not only faster, it also has benefits in cases when detection of dead and non-culturable *S. aureus* is desired.

This study showed the wide distribution of enterotoxigenic and MDR *S. aureus* isolates in milk and milk products. The isolates showed a diversified genetic background and may impose SFP risks as well as antibiotic resistance related problems for the dairy food consumer.

SAMMENDRAG

Melkeproduksjon øker i Etiopia. Imidlertid er mikrobiologisk trygghet av melk en utfordring på grunn av uhygienisk og tradisjonell melkeproduksjon og prosessering. Om disse sikkerhetsaspektene ikke blir tilstrekkelig adresserte, kan melkens høye næringsinnhold og nøytrale pH føre til at melk blir bærer av mange matbårne patogener, inkludert *Staphylococcus aureus.* Dette representerer en helserisiko for konsumenten. *S. aureus* er en viktig matbåren patogen fordi den kan forårsake stafylokokkintoksikasjon, og lett kan utvikle antibiotikaresistens.

Målene for dette studiet var: i) å studere distribusjon av *S. aureus* og andre *Staphylococcus* arter i melk og melkeprodukter langs verdikjeden i Tigray regionen; ii) å vurdere potensialet for enterotoksinproduksjon hos *S. aureus* isolatene; iii) å evaluere resistens hos *S. aureus* isolatene mot 12 ulike antibiotika som brukes ofte i Tigray regionen, samt verifisere tilstedeværelse av meticillin-resistente (MRSA); iv) å vurdere genetisk slektskap, ved hjelp av spa-typing, hos de enterotoksigene og antibiotikaresistente (MDR) isolatene; v) evaluere kvantifisering av *S. aureus* i melk ved hjelp av real time PCR (qPCR) basert på *nuc* genet, sammenlignet med platetelling.

utbredelse av *S. aureus* langs verdikjeden varierte fra 29.5% i melk hos tradisjonelle melkeprodusenter til 48.2% i melk og melkeprodukter ved kafeteriaer og restauranter. Utbredelsen målt for hele prøvemengden var 38.7%, med gjennomsnittstall 4.35 ± 0.97 Log CFU ml^{-1} av *S. aureus*. Odds ratio analyse viste at sannsynligheten for at prøver fra småskalameierier var kontaminert med *S. aureus* (46.4%) var det dobbelt av det fra tradisjonelle meierier (29.5%) ($P < 0.05$, OR =2.07). Høyest insidens av *S. aureus* ble funnet i Shireendaselasse og lavest i Maichew områdene ($p < 0.05$). Koagulase-negative stafylokokker (CNS) ble funnet i 51.6% av melkeprøvene med gjennomsnitt 6.0 ± 1.21 Log CFU ml^{-1}. Ti arter CNS ble identifisert og *S. epidermidis* var den mest vanlig.

Ved bruk av en modifisert multiplex PCR metode, ble det funnet en eller flere enterotoksingener i 82 av 160 (51%) *S. aureus* isolater. Ni enterotoksingener ble identifisert: *sea* (n=12), *seb* (n=3), *sec* (n=3), *sed* (n=4), *seg* (n=49*), seh* (n=2), *sei* (n=40), *sej* (n=1) og *tsst-1* (n=24). De klassiske enterotoksingenene utgjorde 27% av alle toksingenene. Den mest vanlige kombinasjonen ble observert mellom *sei* og *seg.* Genene *sea* og *seb* ble ofte assosiert med nye typer av enterotoksingener. Atten enterotoksin genotyper ble identifisert. Utbredelsen av enterotoksinogenisk *S. aureus* var høyere ($P <0.05$) i samlemelk på gården enn i meieriprodukter og 32% av prøvene inneholdt > 5 Log CFU ml^{-1} *S. aureus.*

Antibiotikaresistens ble studert ved bruk av Kirby-Bauer diskdiffusjon metoden, og PCR ble brukt for deteksjon av *mecA* og *mecC* gener. Fra 160 *S. aureus* isolater, var 137 (86%) resistente mot minst en type antibiotika og 61 (45%) av isolatene var resistente mot flere antibiotika (MDR). MDR isolatene viste 35 ulike resistensmønstrene og 61% av isolatene var resistente mot 5 eller flere antibiotika. Resistens mot penicillin G (69%) var det mest vanlige, etterfulgt av streptomycin (53%) og erythromycin (41%). Trettito (23%, 32/137) av isolatene ble klassifisert som heteroresistente *S. aureus* (BORSA) ettersom de viste fenotypisk oxacillin resistens uten å ha verken *mecA* eller *mecC* gener, men var følesomme til β-lactamase inhibitorer.

spa typing av enterotoksinogene og MDR isolater av *S. aureus* fra melk og melkeprodukter identifiserte 22 *spa* typer samt 3 nye *spa* sekvenser, dvs stor genetisk diversitet mellom isolatene. Større diversitet i *spa* type ble observert blandt melkeprøver enn i melkeproduktene. Ingen tydelig korrelasjon eller mønster kunne sees verken mellom *spa* typer og *se* genotyper eller mellom *spa* typer og resistensmønster for antibiotika. *Spa* typene t314, t458 og t6218 var de mest vanlige og var spredt bredt i 3 av de 8 studerte lokaliteter i Tigray.

Amplifiseringseffektiviteten av qPCR assayet var på 91 % i forhold til standardkurven, (Standard avvik (SD) = 0.12 - 0.3), og reproduserbarhet (SD = 0.29 - 0.5) var innenfor akseptable grenser. De valgte primerne differensierte mellom *S. aureus* og andre *Staphylococcus* arter. Deteksjonsgrensen var 18 kopier av *nuc* genet/PCR. qPCR (som SCE ml^{-1}) viste et høyere resultat ($P< 0.05$) enn platetelling (som CFU ml^{-1}) og 29% prøver som inneholdt < 5 Log CFU ml^{-1} *S. aureus* ved platetelling viste seg å ha > 5 Log SCE ml^{-1} *S. aureus* ved bruk av qPCR assayet. Årsaken til forskjellen var trolig deteksjon av døde celler i qPCR assayet og klumping av bakterier slik at en koloni på dyrkingsplater i en del tilfeller kom fra flere bakterier. qPCR metoden er derfor ikke bare en raskere analyse enn platedyrking, den har fordeler i de tilfellene man ønsker å detektere døde bakterier. Studiet viste bred forekomst av enterotoxigene- og MDR *S. aureus* isolater i melk og melkeprodukter. *S. aureus* isolatene viste svært ulik genetisk sammensetning og kan representere en risiko for matintoksikasjon for konsumenten i tillegg til problemer relatert til antibiotika resistens.

LIST OF PAPERS

PAPER I

Enquebaher Tarekgne, Siv Skeie, Knut Rudi, Taran Skjerdal and Judith A. Narvhus (2015). *Staphylococcus aureus* and other Staphylococcus species in milk and milk products from Tigray region, Norther Ethiopia. *Africa Journal of Food Science,* Vol. 9: 567-576.

PAPER II

Enquebaher K. Tarekgne, Taran Skjerdal, Siv Skeie, Knut Rudi, Davide Porcellato, Benjamin Félix and Judith A. Narvhus (2016). Enterotoxin gene profile and molecular characterization of *Staphylococcus aureus* isolates from bovine bulk milk and milk products of Tigray region, Northern Ethiopia. Accepted for publication in *Journal of Food Protection.*

PAPER III

Enquebaher K. Tarekgne, Taran Skjerdal, Siv Skeie, Knut Rudi, Davide Porcellato and Judith A. Narvhus (2016). Multidrug resistance including borderline-oxacillin resistance and *spa* typing of *Staphylococcus aureus* from milk and milk products of Tigray region, Northern Ethiopia. *Manuscript.*

PAPER IV

Enquebaher K. Tarekgne, Knut Rudi, Taran Skjerdal, Siv Skeie and Judith A. Narvhus (2016). Comparison of Real-time PCR Targeting *nuc* gene with Plate Count Method for Quantification of *Staphylococcus aureus* in bulk Milk. *Manuscript.*

1. INTRODUCTION

1. 1 Background and justification of the study

The milk production of Ethiopia in general and Tigray region in particular is increasing. The country showed 3 % increase in annual milk production in the past decade compared to 1.63-1.66 % of the previous two decades (Ahmed et al., 2004). Currently, the cow milk production of the country is planned to increase by 93% in the next five years (Shapiro et al., 2015). However, due to unhygienic and traditional milk production and processing practices the milk safety issue remains a challenge (Yilma et al., 2011). If milk safety and quality standards are not in place, the high nutritional composition and neutral pH of milk may convey many milk-borne pathogens and thereby constitute a public health risk to the consumers (Angulo et al., 2009). Currently, in Ethiopia, clinical and subclinical mastitis mainly caused by *S. aureus* is a major health problem (Abera et al., 2010; Lakew et al., 2009; Sori, 2011). In addition, milk-borne diseases such as brucellosis (Asmare et al., 2013; Berhe et al., 2007; Geresu et al., 2016; Jergefa et al., 2009), salmonellosis (Tadesse and Gebremedhin, 2015) and bovine tuberculosis (Ameni et al., 2007; Kelly et al., 2016; Shitaye et al., 2007; Wendmagegn et al., 2016) are widely reported from different dairy farms and livestock management systems in the different parts of the country. Moreover, a recent survey conducted in central Ethiopia reported that 31.8% of farmers consume raw milk (Makita et al., 2012) thus increasing the risk of milk-borne disease.

The World Health Organization (WHO) defined food-borne disease (FBD) as *"diseases or infections of toxic nature caused by, or thought to be caused by, the consumption of food or water"*. Accordingly, more than 250 FBD have been registered globally and in two thirds of foodborne outbreaks, bacteria are incriminated (Loir et al., 2003). Among these bacteria, *Staphylococcus aureus* (*S. aureus)* is the most common and is responsible for Staphylococcal Food Poisoning (SFP). SFP results from the ingestion of one or more preformed staphylococcal enterotoxins (SEs) in the staphylococcal-contaminated food. Ingestion of less than 1.0 µg enterotoxin causes SFP (Seo and Bohach, 2007). The disease is characterized by nausea, vomiting, acute prostration and abdominal cramps (Bennett and Hait, 2011). Milk and milk products are foodstuffs commonly associated with SFP (Cretenet et al., 2011).

SFP is among the most prevalent causes of gastroenteritis worldwide. In the United States, the 2006 annual report showed that *S. aureus* enterotoxication was ranked third among

bacterial food-borne outbreaks (CDC, 2009) while it was ranked as fourth in Europe (European Food Safety Authority, 2010). In China, a retrospective study (1994-2005) revealed that *S. aureus* was the second most common food-borne agent in homes (Wang et al., 2007). In developing countries, there is insufficient data on SFP. The poor disease reporting system and the lack of appropriate diagnostic facilities hinders the collection of sufficient data on the SFP situation. Although data on foodborne diseases in Africa is scanty, limited studies showed that *S. aureus* is one of the prevalent foodborne diseases in the region (De Waal and Robert, 2005). In Ethiopia, taking into consideration the poor hygiene during food production and preparation, the shortage of cooling facilities combined with the wide distribution of clinical and subclinical mastitis (Alemu et al., 2014; Duguma et al., 2014; Tolosa et al., 2015) a high incidence of SFP is likely.

Antimicrobial resistance is an ever-increasing threat to global public health. Nowadays, it is well documented that clinically important bacteria are not only characterized by a single drug resistance but also by multiple antibiotic resistance (Levy and Marshall, 2004). *S. aureus* is notorious for its ability to become resistant to antibiotics. The development of multidrug resistance in *S. aureus* is a global problem. *S. aureus* develops drug resistance more readily because of its ability to produce an exopolysaccharide capsule and the location in the microabscess limits access of the drug to the infecting cells (Jeljaszewicz et al., 2000). The emerging of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) and other antimicrobial resistant *S. aureus* strains in food of animals origin such as dairy products are of public health concern as these resistant strains could be transferred to humans and cause health problems (Verkade and Kluytmans, 2014). Hence, surveying and monitoring of the antimicrobial resistance of bacteria in food of animal origin is of paramount importance.

To protect consumers from microbial hazards it is of paramount importance to study and monitor the type, level and potential risk imposed by milk-borne pathogens such as *S. aureus* in the milk value chain. Such surveillance data may provide the basis for risk assessment studies with the ultimate goal of securing food safety through control and prevention of milk borne pathogens.

The purpose of this PhD research, which was conducted in the Tigray region in Northern Ethiopia, was to investigate the risks imposed to dairy food consumers by *S. aureus* of dairy isolates as a potential cause of SFP and source of antibiotic resistance. This research project has four sub-parts. Paper 1 describes the distribution of *S. aureus* and other *Staphylococcus* species in the milk value chain of the study area while paper 2 assessed the enterotoxin genes

profiles of *S. aureus* of the dairy isolates. Paper 3 studied the antibiotic resistance profile of the *S. aureus* isolates for 12 antibiotics, which are commonly used in the studied area, and verified the presence or absence of MRSA in the isolates. The genetic relatedness of the enterotoxigenic and multidrug resistance *S. aureus* was also assesseed by *spa* typing method. Finally, Paper 4 evaluated the performance of a SYBR Green I real time PCR assay for the quantification of *S. aureus* in raw cow milk and compared with the conventional plate count method.

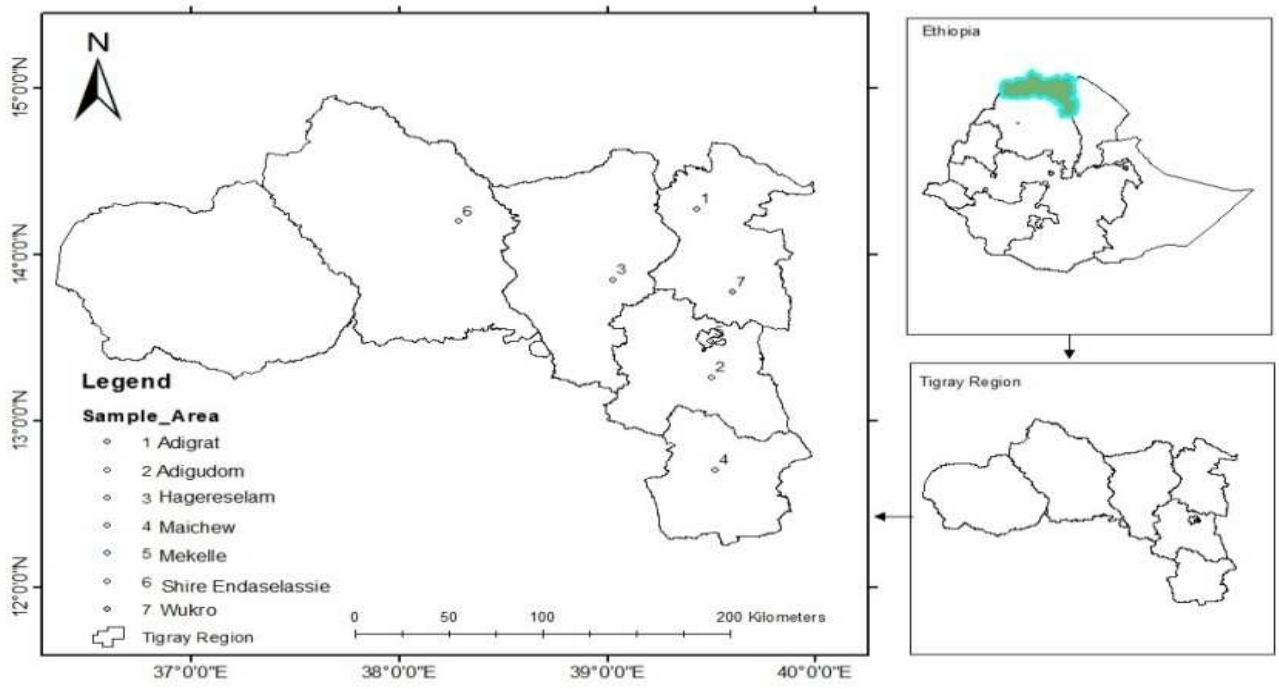
The first section of the thesis contains a short description of the project area, followed by literature reviews on biological characteristics of *S. aureus*, SFP, antibiotic resistance and the laboratory methods employed in the study. The main findings of the study are discussed in the consecutive section and finally the results of this study, paper 1- 4 are enclosed within.

1.2 Description of the studied area

1.2.1. The studied area

The study was conducted in the Tigray region of Northern Ethiopia. The region has a population of 4.3 million (CSA, 2013). According to the Tigray regional bureau of agriculture and rural development (TBARD), the livestock population of the region is estimated at cattle, 4 065,080, goats, 3,191,183, Equines, 585,999, camels, 35,946, poultry, 5,003,126 and bee colonies of 382 036 (TBARD, 2015).

For this study, seven towns/cities and their vicinities (Mekelle, Shireendasselase, Hagreselame, Adigudome, Wukro, Adigrate and Maichew) were purposely selected (non-probability sampling) in order to include the major towns/cities of the region with relatively large numbers of milk and milk product shop/ cooperatives, different geographical locations (south, north, west and east) and agro-climatic zones (highland, medium and lowland). The location of the region in Ethiopia, and the distribution of the sampling areas within the region with their metrological data are presented in Fig.1.



1. *em*=2509, mat=15.5, *2*. *em*=2107, *mat*=-na, 3. *em*=2663, *mat*=16.75, 4. *em*=2402, *mat*=17.11, 5. *em*=2221, *mat*= 18.32, 6. *em*=1732, *mat*= 21.7, 7. *em*=1783, *mat*=19.6 *em*=elevation (m), *mat* = mean annual temperature (^{0}C), na= not applicable

Fig. 1: Map of the project area: spatial distribution and metrological data of the sampling areas (source: Ethiopia metrological agency, 2013).

Samples were collected from the aforementioned seven sampling points following the milk value chain, starting from traditionally managed dairies at household level, small-scale dairy farms, milk collection centres/cooperatives, milk and milk product shops, market places, cafeteria and restaurants as well as from milk consumers at household level. Free informed consent was obtained from the milk and milk product owners, after explaining the research purpose, potential benefits, risks if any, and confidentiality of the research project. The value chain is described by Kaplinsky (2000) as "the full range of activities, which are required to bring a product or service from conception, through the intermediary phases of design, production, delivery to the final consumers, and final disposal after use".

1. 2. 2. Milk production system and milk processing practices

The milk production systems, generally in Ethiopia and particularly in Tigray region are classified into rural/traditional, peri-urban and urban production systems (Redda, 2001). The

traditional/rural dairy represents the milk production in the mixed and pastoral/agro-pastoral farming systems and accounts for 98 % of the total national production (Ketema, 2000). The majority of the milking cows here are the indigenous *zebu* breed. The peri-urban and urban production systems include small and large scale dairy farms with a business-oriented purpose and use exotic cross breed animals for milk production (Ahmed et al., 2004).

The country showed a 3 % increase in annual milk production in the past decade compared to 1.63 - 1.66 % of the previous two decades, and this trend is also predicted to continue as there is great livestock potential and a suitable climatic environment for dairying (Ahmed et al., 2004). Currently the government is planning to boost the annual cow milk production from the current 4,132 million litres of cows milk to 7,967 million litres in the next five years. This 93% increase in cows milk production will be achieved through upgrading the genetic potential of the lactating cow via artificial insemination, through improving the quality and quantity of feed as well as through improving the animal health service of the country (Shapiro et al., 2015). According to the TBARD, currently one small-scale dairy processing plant located at Mekelle is functional and another two at Mekeonne and Humera are under construction. They are expected to finalize soon (TBARD, 2015).

Like in all parts of Africa, traditional milk processing activities are common practice in Ethiopia. The *sour milk* or *Regoe* is prepared by natural spontaneous fermentation of bulk raw milk for 2-3 days at ambient temperature of 20 - 30 ^{0}C. After churning of the sour milk for around 2 hours using a traditional method, *butter* is separated (Fig 2).

Fig. 2. A typical traditional milk processing practice in the studied area

The remaining defatted sour milk is called *buttermilk or Awso*. It is a common practice to consume the buttermilk as it is, mixed with pepper and spices at household level. Cooking of the buttermilk (defatted sour milk) at 50-70 ^{0}C for few minutes separates the *Ethiopian cottage cheese* (*Ajiebo*) from the whey (*Maycheba*). Few farms owned by non-governmental organisations (NGOs) and the Mekelle milk processing plant, produce hard cheese. Besides, some is also brought from Addis Abeba, the capital city of the country. In most of the cases, the hard cheese is consumed at big Hotels and restaurants of the region.

Generally, the traditional milk production and processing activities has been shown to be unhygienic and consequently expose to microbial contamination (Yilma et al., 2007). Many milk-borne diseases that threaten the dairy food safety have been reported also in the region. Clinical and sub-clinical mastitis are common udder health problems in cows and small ruminates in the region (Gebrewahid et al., 2012; Yemane et al., 2015). Milk-borne diseases such as brucellosis and bovine tuberculosis were also reported at different prevalence in the region. Haileselassie et al. (2010) reported herd prevalence of bovine brucellosis at 63.6% from the western part of the region. Bovine tuberculosis was also documented at prevalence of 6.6 % at dairy cow level and 61. 3% in herd level in the region (Romha et al., 2013). All these conditions call for detail study on the milk- borne diseases in order to implement appropriate control measures.

2. LITERATURE REVIEW

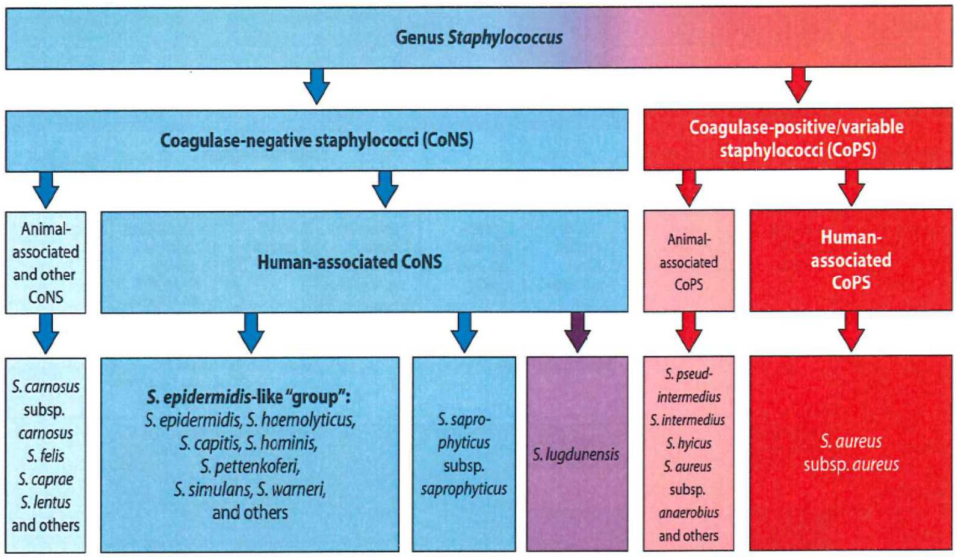
2. 1. Classification of *Staphylococcus* species

Staphylococci are small, spherical gram-positive bacteria having a diameter ranging from approximately 0.5 to 1.5 μm. They are catalase-positive (thus differentiated from enterococci and streptococci) having a DNA composition of 30 to 40 mol% guanine-plus-cytosine (G+C) content. They are non spore-forming, non motile and facultative anaerobic. Microscopically, cells are arranged in clusters resembling grapes. The *Bergeys Manual of Systematic Bacteriology* classified staphylococci in the family *Micrococcaceae*. This family includes the genera; *Micrococcus, Staphylococcus, Stomatococcus and Planococcus.* To date, the genus *Staphylococcus* is validly further subdivided into more than 47 species and 23 subspecies (Becker et al., 2014). They are present everywhere, in the air, dust, in surfaces, as well as in humans and animals. Due to human, animal and environmental contamination, many of them are present in food (Seo and Bohach, 2007).

Traditionally the *Staphylococcus* genus is classified into two broad categories as coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS) depending on the ability to produce the coagulase enzyme. Taking coagulase as major categorizing criterion, Becker et al. (2014) have classified the genus *Staphylococcus* into clinical and epidemiological important groups as shown in Fig 3. The CPS includes *S. aureus* and others staphylococci such as *S. intermedius* and *S. hyicus*. From SFP and other clinical perspectives, *S. aureus* is the most important species among the CPS. The majority of the CNS are found as food-associated saprophytes (Becker et al., 2014) and living in association with humans and animals forming a commensal relationship (Otto, 2010). Some CNS such as *S. xylosus* and *S. carnosus* are also used as starter cultures in meat and cheese preparation because of their positive impact on fermentation and good sensory flavour (Irlinger, 2008). On the other hand, CNS such as *S. epidermidis* and *S. haemolyticus* are reported to cause hospital-associated infection and post-operative wound complications in humans (Mazzariol et al., 2012).

A simplified system for bio-typing of the *S. aureus* strains depending on animal host origin was developed. By using the β haemolysin, staphylokinase, coagulation of bovine plasma and the crystal-violet tests, it was possible to biotype 604 of 809 *S. aureus* strains from humans, poultry, cattle, pigs, goats, rabbits and food into four eco variants typically associated with man, poultry, sheep and goat, cattle and the fifth non-specific biotypes (Devriese, 1984).

Fig 3. Classification of the genus *Staphylococcus* depending on presence or absence of coagulase



enzymes taking also into consideration the clinical and epidemiological importance of the genus Adapted from, Becker et al. (2014).

2.2. *Staphylococcus aureus* growth requirements

S. aureus grows at a wide temperature range between 6 - 48 ^{0}C with optimum of 37 ^{0}C. It tolerates a pH between 4 – 10 with optimum of 6 – 7; a salt concentration of 0 - 20 % with optimum of 0; and water activity (a_w) level range of 0.85 - 0.99 with optimum of 0.99 (Cretenet et al., 2011). However, the production of Staphylococcus Enterotoxin (SE) occurs at much narrower range than the growth of the bacteria (Tatini, 1973). Table 1 shows the physical requirement of *S. aureus* for growth and SE production.

Table 1. Physical requirements of *S. aureus* for growth and SE production

Factor	Optimal growth	Growth limit	Optimal SE production	SE production limit
Temperature	35-41 ^{O}C	6-48 ^{O}C	34-40 ^{O}C	10-45 ^{O}C
pH	6-7	4-10	7-8	5-9.6
Aw	0.99	0.85 $\underline{\geq}$ 0.99	0.99	0.86 $\underline{\geq}$ 0.99
NaCl	0%	0.20%	0%	0-10%
Redox potential (Eh)	> +200 mV	$\underline{\geq}$ +200 to >+200 mV	> +200 mV	$\underline{\geq}$ 100 to >+200 Mv
Atmosphere	Aerobic	Anaerobic-aerobic	Aerobic	Anaerobic-aerobic

Source; Anonymous et al. 2010 in Cretenet et al. (2011)

The ability of the *S. aureus* to grow at low water activity level (a_w) and in wide range of sodium chloride concentration allows the bacteria to survive in potentially dry and stressful environments such as the human nose, skin and on inanimate objects such as cloths and surfaces. This biological characteristic allows the bacteria to contaminate and grow in a wide range of foodstuffs including milk and dairy products (Kadariya et al., 2014; Loir et al., 2003; Meyrand et al., 1998). However, *S. aureus* is quite sensitive to microbial competition. This feature has been particularly well studied in fermented food products. Genigeorgis (1989) demonstrated that the higher the concentration of competing microorganisms in milk, the lower the rate of *S. aureus* growth and SE production. The negative growth effect of lactic acid starter bacteria on *S. aureus* is mainly due to lactic acid production, lower pH, hydrogen peroxide production, competition for nutrients and is sometimes due to the synthesis of antimicrobial substances, such as bacteriocins (Loir et al., 2003). *S. aureus* can also resist freezing temperature and can survive well in food stored below – 20 $^{\circ}$C, however, the viability is reduced at temperature of -10 $^{\circ}$C to 0 $^{\circ}$C. *S. aureus* has a relatively high heat resistance. The observed D-value (the value at which the initial concentration of bacteria cells would be reduced by 1 Log_{10} unit) was 4-6 -6.6 min. at 60 $^{\circ}$C when heated in broth. However, pasteurization temperature, 71.7 ºC 15 sec. readily kills *S. aureus* (Stewart, 2003).

2.3. *Staphylococcus aureus* cell components and virulence factors

2.3.1. *S. aureus* cell components

The genome, the cell wall and the cell capsule of *S. aureus* forms important cellular components of the pathogen.

A recent genome sequence study of *S. aureus* strain MI (HIP 5827) revealed that the chromosomal and plasmid genome size of *S. aureus* is 2,860,370 bp (G+C content 32.9%) and 55,980 bp (G+C content 29.%) respectively (Hishinuma et al., 2016). Another genome sequence study reported also that the genome of *S. aureus* was composed of a complex mixture of genes that mostly acquired through horizontal gene transfer mechanism. Moreover, the study identified three new classes of pathogenicity islands namely; the toxin-shock–syndrome toxin island family, the exotoxin islands and the enterotoxin islands. The exotoxin islands and the enterotoxin islands were found linked with other genes forming a cluster that encodes putative pathogenic factors (Kuroda et al., 2001).

About 50 % of the cell wall is composed of peptidoglycan layer by weight. Difference in the peptidoglycan structure of staphylococcal strains may contribute to variation in their capacity to disseminate intravascular coagulation and it is reported that the peptidoglycan layer has endotoxin-like activity (Kessler et al., 1991). Ribitol teichoic acid and lipoteichoic acid are important components of the cell wall that form the peptidoglycan layer (Fig 3). Penicillin-binding protein (PBP) structures are also located in the cytoplasmic membrane and are involved in the assembly of the cell wall (Lowy, 1998).

Capsular exopolysaccharides formed by some strains of *S. aureus* play important role in the pathogenesis and antibiotics resistance ability of the pathogen (Begun et al., 2007).

According to Lee (1996) 11 types of microcapsules could be produced by more than 90% of *Staphylococcus* species. Staphylococci that could synthesis types 5 and 8 are responsible for 75 % of human infections. Most methicillin resistant staphylococci (MRSA) have type 5 microcapsule.

2.3.2. *Staphylococcus aureus* virulence factors

S. aureus has more than 50 virulence factors, with a wide range of biological activities. They are responsible for a variety of toxin-mediated and suppurative diseases (Ferry et al., 2005). These virulence factors can be divided into two main categories as cell-surface- associated

(surface proteins) and secreted proteins (exotoxins) (Lowy, 1998). The different surface proteins and secreted proteins as well as their expression time during the growth phase of the bacteria are summarized Fig. 4.

Except for the toxin-mediated diseases such as toxic shock syndrome (TSS), the causation of staphylococcal infections is not due to a single virulence factor. The different virulence factors participate, in a stepwise manner, in the pathogenesis of the various diseases. In each step, one or more virulent factor may be involved (Ferry et al., 2005). First, a virulence determinant that aids the bacterium to adhere to the host surface or tissue, and virulence factors that avoid or invade the immune system of the host will be functional. For this purpose, the surface proteins such as protein A and collagen binding proteins will be expressed during the exponential growth phase. In the meantime, the second step, that involves the secretion of exotoxins or proteins that cause a harmful toxic effect to the host will follow (Costa et al., 2013).

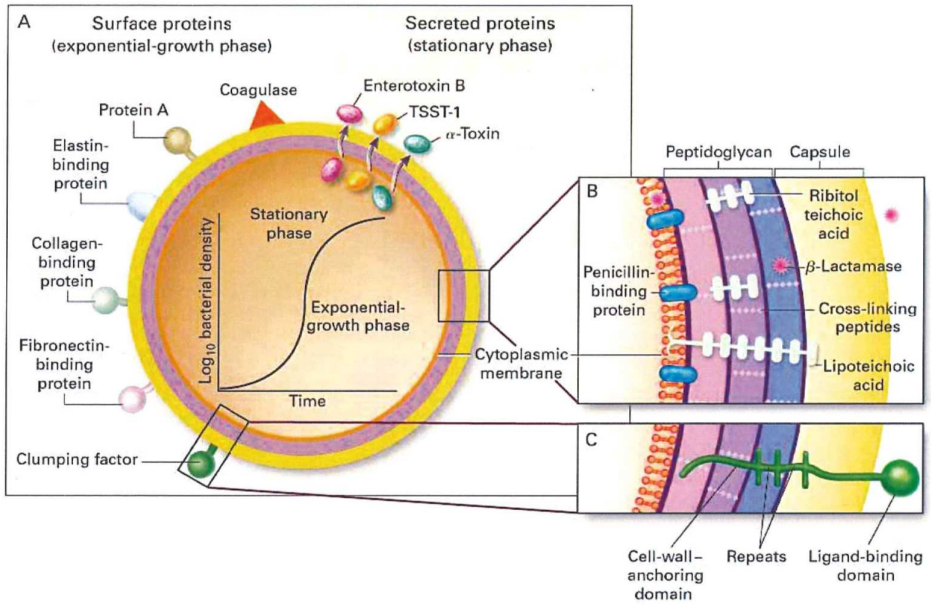


Fig 4. A. Types of surface and secreted proteins of *S. aureus* and the time of expression during the growth phase of the bacteria. B. The structures of the cell envelope that include the cytoplasmic membrane, the cell wall and the capsule. C. Typical structure of the surface proteins. They are made of repeats and firmly attached to the cell wall. Source: (Lowy, 1998).

The major virulence factors and their putative function as described by Costa et al. (2013) are the following. A. *Cell surface proteins*

Staphylococcal protein A: Is a 42 KD sized protein encoded by *spa* gene. The protein binds to IgG, thus interfering the phagocytosis activity of the host immune system.

Collagen-binding protein: Adherence to collagenous and cartilage parts of the host tissue.

Clumping factor proteins (CIfA and CIfB): Mediate clumping and adherence to fibrinogen in the presence of fibronectin. By doing so it brings about clumping of the blood plasma.

B. Secreted Factors/proteins

The secreted virulence factors include the superantigens, cytolytic toxins and various exotoxins.

Superantigens: Staphylococcal enterotoxins (SEA, SEB…etc) and the toxic shock syndrome toxin (TSST-1) are exotoxins secreted by some *S. aureus* strains that brings massive activation of T cells and antibody-presenting cells.

Cytolytic toxins: The *β*-hemolysin and *α*- hemolysins induce lysis of erythrocytes, monocytes and platelets whereas *panton-valentine leucocidin (PVL)* induces lysis of the leukocytes.

Various exoenzymes: *S. aureus* produces various enzymes such as *proteinase*, *lipases*, and *hyaluronidase*, that destroy the tissue and helps the bacteria to spread to the adjacent tissues. *β lactamase* is an enzyme that inactivates β-lactam group of antibiotics.

2.4. *Staphylococcus aureus* Enterotoxins

2.4.1. Nomenclature, structure and classification of enterotoxins

Staphylococcus enterotoxins (SEs) are exotoxins produced by *Staphylococcus aureus* during exponential growth or during the transition from exponential to stationary growth phase of the bacteria (Czop and Bergdoll, 1997; Lowy, 1998). Consumption of sufficient amount of one or more SEs causes Staphylococcal food poisoning (SFP) (Argudin et al., 2010). In addition to *S. aureus*, other *Staphylococcus* species, including CPS such as *S. intermedius* (Becker et al., 2001) and CNS such as *S. epidermis*, *S. cohnii*, *S. xylosus*, and *S. haemolyticus* can also produce the exotoxins (Bautista et al., 1988). However, nearly all cases of SFP are attributed to *S. aureus*-produced toxins (Seo and Bohach, 2007).

2.4.1.1. Nomenclature of SE

Although, all SEs have super-antigenic characteristics (Balaban and Rasooly, 2000), the naming of the SE depends on the their emetic activities (Lina et al., 2004). In order to be designated as a SE besides to super-antigen activity, the protein must produce emesis after oral administration to primates. Related toxins that either lack emetic properties or that have not been tested, should be designated “Staphylococcus enterotoxin-like” (SEI). For

nomenclature, dependent on the order of discovery, a sequential letter of the alphabet was assigned to each SE. However, for SEF, the nomenclature has been changed to TSST-1 (Hennekinne et al., 2011; Seo and Bohach, 2007).

To date, 22 SE and SEI are identified (Argudin et al., 2010; Hennekinne et al., 2011). The type, the general characteristic and mode of activities have been reviewed by Hennekinne et al. (2011) (Table 2).

Toxins which showed > 90% similar with the existing SE or SEIs are designated either by a number subtype or called variant. The SEA, SEB, SEC, SED and SEE are named as *classical* because they are mostly involved in SFP outbreaks and are classified in distinct serological types. The remaining are named as *new types* of SE and SEI (Argudin et al., 2010).

Table 2. Staphylococcal enterotoxins types to date, their molecular weight, genetic background and super antigenic and emetic characteristics. Source : Hennekinne et al. (2011)

Toxin type	General characteristics		Mode of activity		References
	Molecular weight (Da)	Genetic basis of SE	Super antigenic action [β]	Emetic Action [α]	
SEA	27 100	Prophage	+	+	Betley and Mekalanos (1985) Borst and Betley (1994)
SEB	28 336	Chromosome, plasmid, pathogenicity island	+	+	Jones and Khan (1986) Shafer and Iandolo (1978) Shalita *et al*. (1977) Altboum *et al.* (1985)
SEC_{123}	≈ 27 500	Plasmid,	+	+	Bohach and Schlievert (1987) Hovde et al. (1990) Altboum et al. (1985) Fitzgerald et al. (2001a)
SED	26 360	Plasmid	+	+	Chang et al. (1995) Bayles and Iandolo (1989)
SEE	26 425	Prophage	+	+	Couch et al. (1988)
SEG	27 043	enterotoxin gene cluster (*egc*), chromosome	+	+	Munson et al. (1998a) Jarraud et al. (2001)
SEH	25 210	Transposon	+	+	Su and Wong (1996) Ren et al. (1994)

					Noto and Archer (2006)
SEI	24 928	*Egc*, chromosome	+	(+)	Munson et al. (1998a) Jarraud et al. (2001)
SEIJ	28 565	Plasmids	+	nk	Zhang et al. (1998a)
SEK	25 539	Pathogenicity island	+	nk	Orwin et al. (2001)
SEIL	25 219	Pathogenicity island		-£	Orwin et al. (2003)
SEIM	24 842	*egc*, chromosome	+	nk	Jarraud et al. (2001)
SEIN	26 067	*egc*, chromosome	+	nk	Jarraud et al. (2001)
SEIO	26 777	*egc*, chromosome	+	nk	Jarraud et al. (2001)
SEIP	26 608	Prophage	+	nk†	Kuroda et al. (2001) Omoe et al. (2005)
SEIQ	25 076	Pathogenicity island	+	-	Jarraud et al. (2002) Diep et al. (2006)
SER	27 049	Plasmid	+	+	Omoe *et al*. (2003)
SES	26 217	Plasmid	+	+	Ono *et al.* (2008)
SET	22 614	Plasmid	+	+	Ono et al. (2008)
SEIU	27 192	*egc*, chromosome	+	nk	Letertre *et al*. (2003)
$SEIU_2$	26 672	*egc*, chromosome	+	nk	Thomas *et al.* (2006)
SEIV	24 997	*egc*, chromosome	+	nk	Thomas *et a*l. (2006)

[β]:+, Positive reaction
[α] +, positive reaction; (+) weak reaction; negative reaction; nk, not known
£; For SEIL, emetic activity was not demonstrated in *Mucaca nemestrina* monkey
† For SEIP, emetic activity was demonstrated in *Suncus murinus* but not in primate model.

2.4.1.2. Structure of SE

SEs and SEI are small globular or compact single chain proteins with molecular weight of 22,000-29,000 Da (Hennekinne et al., 2011; Seo and Bohach, 2007). Structural studies of SE and SEI revealed that all have similar three-dimensional topologies. The globular molecules have an overall ellipsoidal shape and are folded into two domains, the amino- terminal (Domain 1) and carboxy-terminal (Domain 2) which are connected by α-helixes (Fig 5). Domain 1, the N- terminal, contains residue near the N-terminal but not the N-terminal residues themselves. The folding conformation of this domain may have potential significance for the function of the toxin. The other prominent feature of domain 1 is that it

contains two cysteine (Cys 93 and Cys 110) residues, which are linked by disulfide bonds to form a cysteine loop. The cysteine loop is reported to be an important structure for conformation of the SE and is probably involved in the emetic activity (Loir et al., 2003). The domain 2 is the larger, carboxyl terminal and consists of four to five strand β-sheet that is packed against a highly conserved α-helix (Mitchell et al., 2000). The two domains form a shallow cavity that the T-cell receptor (TCR) binding site encompassed as well as the MHCII molecule binds to an adjacent site (Swaminathan et al., 1992). According to Deringer et al. (1997), the specificity TCR to each SE is determined by the amino acid residue located in the top of this shallow cavity. Structural and biochemical studies have indicated that some SEs needs zinc atom for proper functioning as well as for properly binding to MHCII (Seo and Bohach, 2007).

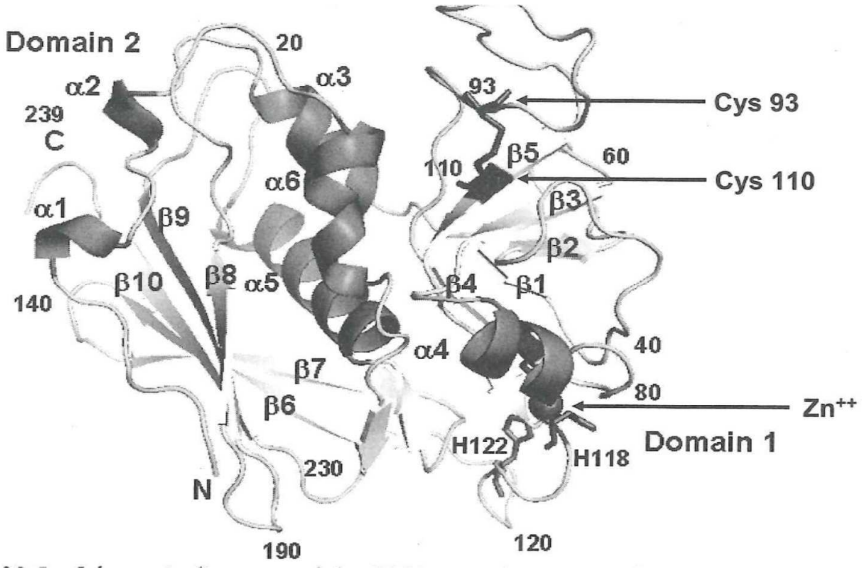


Fig 5. Crystal structure showing domain 1 and domain 2 of SEC. The cysteine loop formed by disulfide bond between Cys 93 and cys 110 is linked to the β5 stand. The zinc molecule is also attached to the SEC Structure. Source: Seo and Bohach (2007), pp 507.

TSST-1: It is a single polypeptide protein having a molecular weight of 22,000 Da and has a isoelectric point (pI) of 7.2 Although TSST-1 has a high percentage of hydrophobic amino acids, it is highly soluble in water. Generally, the toxin is resistant to heat and proteolytic enzymes such as trypsin. There are no cysteine residues in the structure of TSST-1. Antigenically, TSST-1 is distinct from other pyrogenic toxin super antigens (PTSAgs) and

does not have significant primary sequence homology to other known proteins, including SE and SEI (Fig 5). The mode of attachment of the TSST-1 with the TCR has some differences, in comparison with the mode of attachment of the SEB and SEC. The former is attached on the top back of the TCR where as the SEs attached to the top front of the TCR. Besides, the TSST-1 appears to insert much more into the peptide groove of the major histocompatibility complex (MHC) molecule, occluding many of the contact sites between the MHC class II and the TCR. This may exert some differences on the super-antigen characteristics of the TSST-1 and the SE (Dinges et al., 2000).

2.4.1.3. Classification of SE

Many studies have shown that there is primary amino acid sequence homology among SEs and SEIs. The homology of the amino acid sequence among SEs could reach 33- 42 % (Loir et al., 2003) or/and up to 22- 33 % (Balaban and Rasooly, 2000). Another study reported also that 15% of the SE residues are conserved (Dinges et al., 2000). Depending on the primary amino acid sequence the SE, SEI and TSST-1 are divided into four groups as shown in Fig 6.

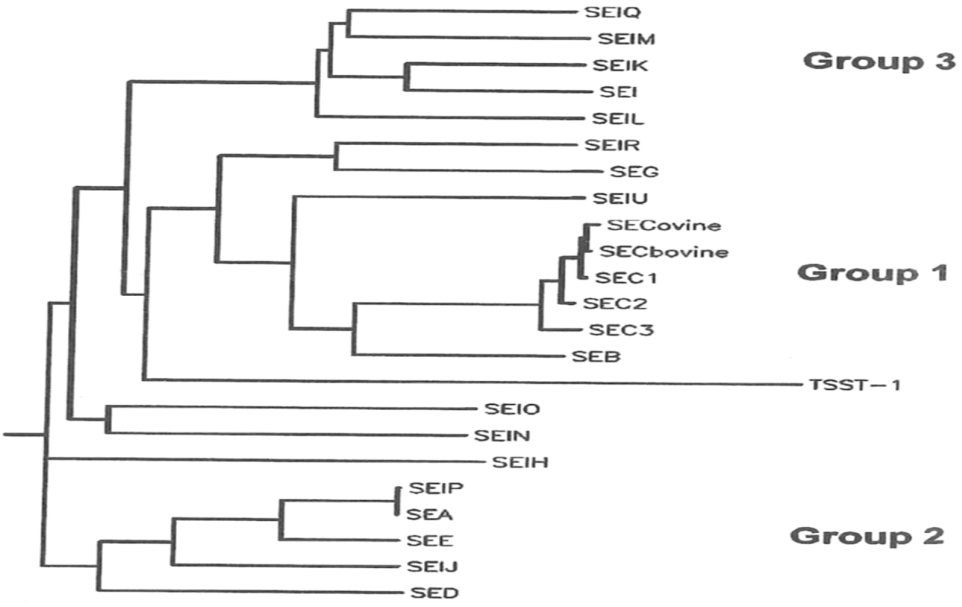


Fig. 6. Molecular relatedness of the currently identified SE, SEI and TSST-1 as expressed by cluster analysis of the amino acid sequences. Adapted from Seo and Bohach (2007).

Not only have the primary amino acid sequences of SE similarity to each other, but show remarkable relatedness with pyrogenic toxin (PT) families. For example, Balaban and Rasooly (2000) reported that the primary amino acid sequences of SEC and SEB showed around 51- 81 % relatedness with streptococcal toxin, which is in the PT family.

2.4.2. Physiochemical property of SE

SE are hygroscopic peptides that are easily soluble in water and salt solutions. They have an isoelectric point (pI) of 7.0-8.6 (Loir et al., 2003). The SEs are more stable in many respects than most proteins. They are highly heat resistant, and this heat resistance appears to be greater in food than in laboratory culture media (Bergdoll and Wong, 2006). This characteristic is most important in terms of food safety in that it imposes a significant challenge for the food industry (Balaban and Rasooly, 2000). Anderson et al. (1996) reported that the biological activity of SEA was retained after the toxin was exposed to 121 ^{0}C for 28 min. in mushrooms. According to (Balaban and Rasooly, 2000), the heat stability of SE seems to depend on the media in which the toxin is present, the pH, salt concentration and other environmental factors. They are also highly resistant to freezing and drying. They are also resistant to low pH and proteolytic enzymes such as pepsin and trypsin and this allows the SE to be functional in the gastrointestinal tract (Hennekinne et al., 2011; Loir et al., 2003). Although pepsin can digest the enterotoxins at pH value of 2.0 and below (Bergdoll, 1970) this acidic level does not exist in the stomach under normal condition, particularly in the presence of food.

The mechanism by which the SE causes emesis is not clearly elucidated but a simian model study showed that SE affects the intestinal epithelium to result in the stimulation of the local neural receptor of vagus nerves and sympathetic nerves that transfer impulses to medullar emetic center to result in emesis (Sugiyama and Hayama, 1965).

As described in the previous section, one characteristic of the toxins is the presence of two cysteine residues near the center of the molecule that are joined by a disulfide bond, forming what is referred to as the *cystine loop* (Merlin, and Amy, 2006; Seo and Bohach, 2007). According to Loir, Baron and Gautier (2003) these cystine loops are required for proper conformation of the SE and are probably involved in the emetic activity of the SE. However, others commented this hypothesis as some SE such as SEI, do not have the loop but nevertheless exhibit weak emetic activities (Hennekinne et al., 2011). They suggested that the cysteine loop may not be directly involved in the emesis but may stabilize the critical conformation of the SE which is important for this action (Hovde et al., 1994).

The SE belongs to a family of the so-called *pyrogenic toxins* (PTs) originating from *Staphylococcus* and *Streptococcus* species. Pyrogenic toxins include SEs, Toxic Shock Syndrome Toxin (TSST), exfoliatin A and B and *Streptococcus* pyrogenic toxins. These toxins share some structural, functional and sequence similarities (Balaban and Rasooly, 2000; Dinges et al., 2000; Seo and Bohach, 2007). The one feature in common to all PTs, including SEs, is their unique ability to act as super antigens (SAgs).

To exert their biological action, the super-antigens must interact with epithelial cells of the intestine through transcytosis to pass the epithelial cell barrier. Then the induction of the inflammatory state will continue after activation of the cell (Hennekinne et al., 2011).

SAgs are molecules that have the ability to stimulate an exceptionally high percentage of T cells. The mechanism by which this occurs distinguishes them from mitogens and conventional antigens (Ags). SAgs are bifunctional molecules that interact with major histocompatibility complex class II (MHCII) molecules on Ag-presenting cells (APCs) (Fig 7). Unlike interactions involving the conventional Ags, this interaction does not require processing and occurs outside of the MHCII peptide-binding groove. The MHCII molecules–SAg complex interacts with the T-cell receptor (TCR). The interaction in the TCR is also nonconventional and relatively nonspecific; for most SAgs, binding occurs at a variable (V) location on the TCR β-chain (the Vβ region). Since SAgs bind outside the area on the TCR used for Ag recognition, they activate a much higher percentage of T-cells, which is above the order of antigen specific activation. The high proliferation of T-cells also stimulates the production of high cytokines such as interleukin (IL)-1, IL-2, tumor necrosis factor (TNF) α, β and interferon (IFN)-γ., which are responsible for toxic shock syndrome clinical signs such as fever, hypotension and organ failure (McCormick et al., 2001). SEB
is reported as a potent super antigen that is considered as potential microbiological weapon of warfare and terrorism (Greenfield et al., 2002).

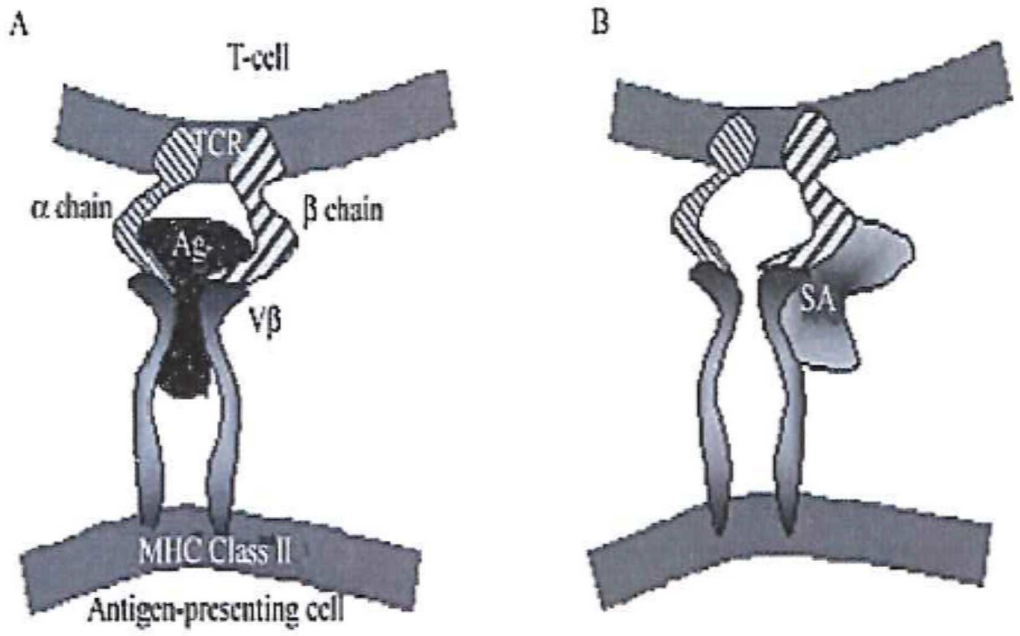


Fig 7. Activation of T-cells by conventional antigen (A) and super antigen (B) . There is a difference in the attachment of the two types of antigen to the MHC Class II and T- cell receptors (TCR). This leads to difference in the T- cell production. Adapted from : Balaban and Rasooly (2000) in (Loir et al., 2003).

Hoffman (1990) described that the enterotoxins have been labelled super antigens because they can activate as many as 10% of the mouses T-cell repertoire, whereas conventional antigens stimulate less than 1% of all T cells.

A high correlation exists between the super antigen and emetic characteristics of SE since, in most cases, genetic mutations resulting in a loss of super antigen activity results in loss of emetic activity (Harris et al., 1993).

2.4.3. Location of genes encoding SE

All *se* and *sei* genes are carried by accessary genetic elements. Most of them are mobile genetic elements, which can spread among strains of *S. aureus* through horizontal gene

transfer. This phenomena enables the bacteria to modify their ability to cause diseases and thereby contributes to the evolution of the bacteria (Argudin et al., 2010). The mobile genetic elements where the *se* and *sel* genes located includes plasmids, prophages, *Staphylococcus aureus* pathogenicity Islands, and vSa genomic islands. The genetic location of each *se* and *sel* genes in the chromosome and in mobile genetic elements is presented in Table 2.

Argudin et al. (2010) reviewed the genetic location of enterotoxin genes in different mobile genetic elements of *S. aureus* in a relatively detailed manner. The main points of the review are summarised as follows:

Plasmids: The two most common plasmids in *S. aureus* which are known to carry *se* and *sel* genes are *pIB485* and *pF5*. They were identified from *S. aureus* strain KSI1410 and Fukuoka. It is reported that they are well-characterized plasmids.

Prophages: The temperate tailed *S. aureus* phages belong to the *Siphoviridae* family and carry *se* and *sel* genes. It is also documented that apart from *se* and *sel* genes carriage, the bacteriophages also carry other virulent factors involved in evasion of the innate immunity.

Staphylococcus aureus Pathogenicity Islands (SaPI): They are common and widely distributed mobile genetic elements in *S. aureus*. They range in size from 15-17 Kb. They carry and transfer *se* and *sel* genes including the genes encoding *tsst-1*.

vSa Genomic Islands: These genetic elements are exclusively present in *S. aureus* isolates. They have a locus that is inserted to the bacterial chromosome. These genetic elements are acquired by horizontal gene transfer although there is no evidence that they move. There are two major types of vSa genomic islands, the vSaβ and vSaα having a size ranging from 20-30 kb. These two structures have a Saul type I restriction-modification system. The system will digest DNA transferred from different lineage while transferred DNA of the same lineage will allow and protected from restriction. Due to these characteristics the Saul type I system is considered as key player in monitoring of lineage evolution.

Both vSaβ and vSaα structures of the genomic island contain many gene clusters inside. The vSaβ carries a gene cluster that harbors many *se* and *sel* genes and is called enterotoxin gene cluster (*egc*). This cluster carries a variable number of *se* and *sei* genes linked together forming an operon (Jarraud et al., 2001). The *egc* is suggested to have been created from ancestral *se* gene through random duplication and gene recombination that could be able to produce various toxins having different biological activities (Thomas et al., 2006). The

dynamic evaluation of this gene cluster is considered as a nursery of *se* and *sei* genes that is observed by the presence of many variants of this cluster (Fitzgerald et al., 2001b; Jarraud et al., 2001). The different *egc* variants, the types of *se* and *seI* genes carried by this structure and the strain of *S. aureus* that acquires this cluster is presented in Fig 8.

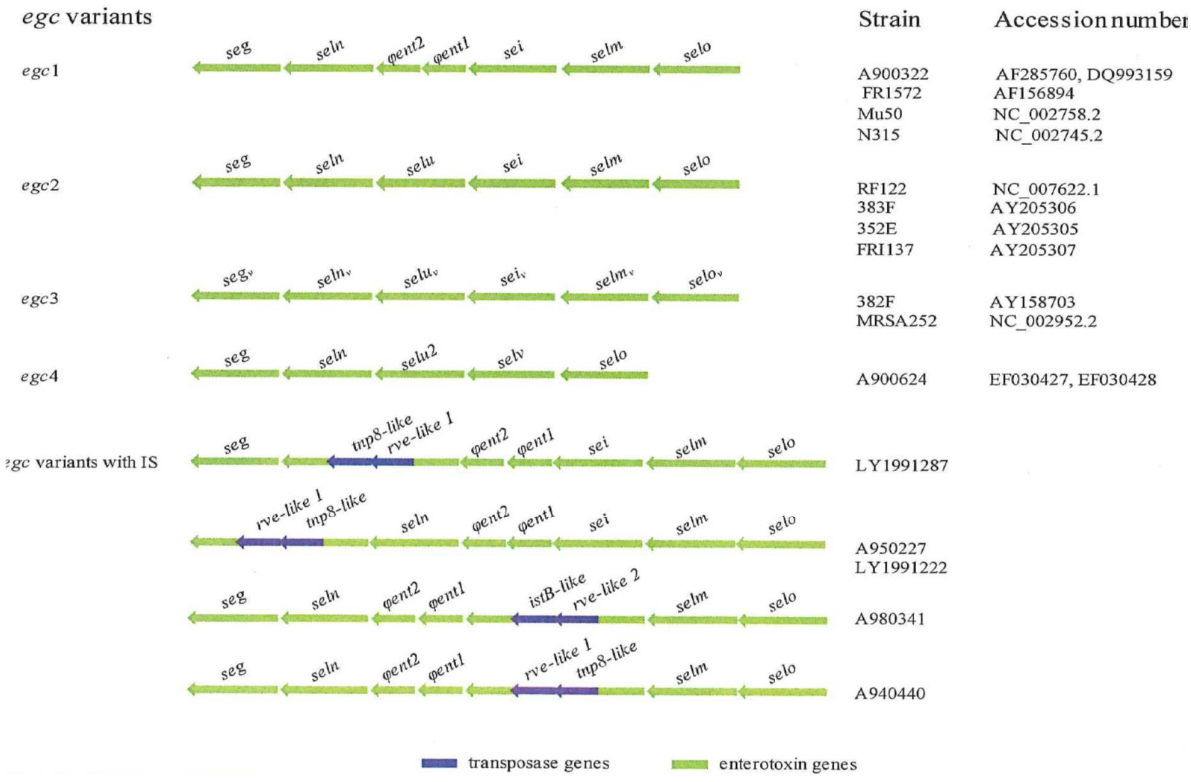


Fig 8: The different structures of the *egc* clusters variants. Modified from Thomas et al. (2006) and Collery et al. (2009) and based on the accession numbers indicated to the right of the Figure. Adopted from Argudin et al. (2010)

2.4.4 Regulation of SE Production

Depend on its phase of growth, and in response to the changing environment, *S. aureus* is able to switch on and off different sets of genes. In recent years, a great deal has been discovered about the regulatory system that control these responses, but much still remains to be understood. In general terms, proteins facilitating adhesion and invasion of *S. aureus* are manufactured during the exponential phase of bacterial growth, while enzymes and exotoxins, including the SEs, are synthesized in the post-exponential phase of growth (Lowy, 1998).

These processes are controlled by complex networks of regulatory genes. These complex regulatory genes can be categorized in two major broad groups*: the two-component regulatory systems (TCRS)* and the *global regulatory system* (Cheung et al., 2004). The TCRS include also the accessory gene regulator (*agr*) and the staphylococcal accessory elements (*sae*). The *agr* is the main gene regulatory system in the expression of virulence factors in *S. aureus*. The *agr* locus regulates more than 70 genes of which 23 are related to virulence factors. However, not all SEs expression are regulated by *agr*. For example SED and SEJ, which have more similarity in primary amino acid sequence, and yet the expression of SED is regulated by *agr* whereas the expression of SEJ is not (Zhang et al., 1998b). The expression of SEA is not regulated by *agr* (Tremaine et al., 1993). On the other hand, Costa et al. (2013) noted that all the aforementioned regulatory networks exert their influence in interactive manner, not singly, to ensure that specific virulence genes are expressed when required.

According to Seo and Bohach (2007) cell density, availability of nutrients and environmental signals temporarily control the expression of virulence factors. The density-sensing *agr* system of *S. aureus* could enable the bacteria to respond to environmental changes with typical bacterial two components sense and response characteristics (Novick, 2003). Environmental signals, such as high salt concentration, pH, and sub-inhibitory concentration of antibiotics, affect the production of extracellular proteins (Herbert et al., 2001). For example the production of SEB and SEC has been inhibited in the presence of glucose as a result of inhibitory effect of *agr* expression due to low pH resulted from glucose metabolism (Regassa et al., 1992). It is well-documented that neutral pH is the optimum condition for production of SE (Table 1).

2.4.5. Frequency of enterotoxigenic strains from *S. aureus* isolates

Many studies have investigated the occurrence of *se* genes in *S. aureus* from various sources in different geographical locations. Overall, *se* genes are prevalent in most *S. aureus* populations, but certain differences in prevalence and distribution of *se*-gene profiles may exist depending on the source of isolates and geographical regions (Larsen et al., 2002). Among *Staphylococcus aureus* strains isolated from food samples, the percentage of enterotoxigenic strains is estimated to be around 25% (Loir, Baron and Gautier, 2003). However, estimations vary considerably from one food to another and from one area to another area. A prevalence of 52.2% enterotoxigenic *S. aureus* were reported from bovine milk in Norway (Jørgensen et al., 2005b) while in Korea 50% of the *S. aureus* isolates from

ready-to –eat- food were enterotoxigenic (Kim et al., 2011). In USA 95 % of the *S. aureus* isolates from a bakery involved in SFP were enterotoxigenic (Hait et al., 2014).

2.5 Staphylococcal food poisoning and other diseases

2.5.1 Staphylococcal food poisoning

2.5.1.1 Epidemiology

SFP is one of the most common food-borne diseases worldwide. It is the most reported food-borne disease in the United States (Kadariya et al., 2014) and is the leading cause of food-borne outbreaks in the European Union (EFSA, 2013). The true incidence of SFP is underestimated for many reasons among which misdiagnosis, unreported minor outbreaks, improper sample collection and laboratory examination have been noted (Argudin et al., 2010). In developing countries, there is not sufficient data on SFP due to the poor disease reporting system. However, considering the poor hygienic conditions during food production and processing, coupled with absence /shortage of cooling facilities, a high prevalence is likely.

The ability of *S. aureus* to grow in wide range of growth environments, including in a potentially dry and stressful environment, favours growth in many foodstuffs (Loir et al., 2003) including in salted food products (Qi and Miller, 2000; Scott, 1953). Meat and meat products, poultry and egg products, salads, bakery products, especially cream-filled pastries, cakes and sandwich filing have been frequently implicated in SFP (Argudin et al., 2010; Hennekinne et al., 2011).

Milk and milk products are also foodstuffs commonly associated with SFP (Cretenet et al., 2011; Seo and Bohach, 2007). Retrospective analysis of SFP outbreaks indicated that milk and milk products are usually involved in SFP. An extensive SFP outbreak that affected 13,420 people in Japan (Asao et al., 2003), the largest SFP registered in USA (Evenson et al., 1988a) the SFP outbreak in a christening party in Germany (Fetsch et al., 2014) and the recent outbreak of SFP in Swiss boarding school (Johler et al., 2015) are a few of many outbreaks that were caused by the consumption of *S. aureus*-contaminated milk and milk products.

Humans and animals are reservoirs of *S. aureus* on their skin and mucous membranes (Hennekinne et al., 2011). According to Kluytmans and Wertheim (2005), around 10-35 % and 20-75% of humans are persistent and intermittent carriers of *S. aureus*, respectively. Although *S. aureus*-colonized persons do not show the clinical signs of the diseases, they are important sources of contamination to other persons and also to food. The transfer of *S. aureus*

from the carrier to another person or food occurs through direct contact, indirectly through skin fragments or through respiratory droplets. It is also reported that most SFP outbreaks occur due to human contamination (Seo and Bohach, 2007; Wertheim et al., 2005).

Cows with mastitis are also a common source of *S. aureus* in raw milk (Kadariya et al., 2014). A cow with clinical or subclinical staphylococcal mastitis can excrete up to 8 Log CFU ml $^{-1}$ of *S. aureus* (Peles et al., 2007). Many SFP outbreaks have occurred where the sole source of contamination of the bulk milk was from a mastitic cow. In Brazil 328 individuals were affected with SFP where the source of *S. aureus* was mastitic cows (do Carmo et al., 2002).

Improper handling of food pre- and post- cooking is incriminated as important risk factor for SFP outbreaks. Post-cooking environmental surfaces that came in contact with food and even gloves worn by food employees, if not changed frequently, could serve as source of *S. aureus* contamination (Lues and Van Tonder, 2007; Syne et al., 2013). Retrospective analysis of SFP outbreaks and other studies indicate also that errors during food processing and preparation may lead to SFP outbreaks. These errors includes i) insufficient time and temperature during initial cooking ii) prolonged exposure of foods at room temperature iii) slow cooling of prepared food iv) inadequate refrigeration, v) preparing food for extended period of time prior to serving vi) Insufficient cleaning of food processing equipment and storage of food in a contaminated environment (Bennett et al., 2013). In addition, prolonged use of warming plates when serving food may favour the multiplication of *S. aureus* and hence production of SE (Bryan, 1976).

2.5.1.2. Clinical signs

SFP is caused by consumption of food that contains one or more types of SEs produced by enterotoxigenic strains of *S. aureus.* The disease is characterized by a short incubation period (an average of 4.4 h), nausea, violent vomiting, abdominal cramps, headache and diarrhoea. The amount of toxin ingested and the susceptibility of the person influences the severity of the clinical signs (Hennekinne et al., 2011). SFP is usually a self-limiting illness but death occasionally occurs with case fatality rate ranging from 0.03% for general public to 4.4% for more susceptible populations such as children and the elderly. Death results from severe dehydration and electrolyte imbalance (Holmberg and Blake, 1984b).

Depending on individual sensitivity and body weight, the amount of toxins required to produce SFP in a person can vary. However, the scientific community agree that the dose of 0.1-1 µg/kg body weight will cause SFP illness in humans. The basal level 1 nanogram of SE

per gram of contaminated food can cause the SFP symptoms and 1-5 µg of ingested toxin has been associated with many outbreaks (Seo and Bohach, 2007). Outbreaks have also been observed for lower concentrations; 0,5 ng/ml in milk (Evenson et al., 1988a). Although the mortality from SFP is low (Burgey et al., 2016), the actual impact of the intoxication is large due to loss of working days and productivity, hospital expenses and economic loss for restaurants and food industries (Kadariya et al., 2014; Murray, 2005).

Alone or in combination with other SE or SEI, SEA is the most common enterotoxin involved in SFP worldwide, followed by SED, SEB and SEC (Argudin et al., 2010; Johler et al., 2013; Johler et al., 2015; Kérouanton et al., 2007; Li et al., 2015). The extraordinary high resistance of the SEA to proteolytic enzymes (Bhatia and Zahoor, 2007; Holmberg and Blake, 1984a) may contribute to the high prevalence of SEA in most SFP outbreaks (Argudin et al., 2010). Although SEA is commonly found in SFP outbreaks, SEB produce more severe clinical symptoms of SFP than SEA. This may be related with the higher amount of toxin produced by SEB than SEA. Although the production of SE depends on the strain of the *S. aureus,* SEB and SEC are generally produced in highest quantities; up to 350 µg /ml while SEA, SED and SEE produce as little as 100 *n*g/ml (Seo and Bohach, 2007).

2.5.1.3. Diagnosis of SFP

The development of typical SFP clinical signs such as projectile vomiting, diarrhoea, etc starting within 30 min. after consumption of food may suggest *Staphylococcus* intoxication. If the clinical signs are also manifested in two or more people who consume the food, then it should be tentatively diagnosis as SFP (Bergdoll and Wong, 2006). However for confirmation, microbiological, immunological and molecular examinations of food or vomit, alone or in combination, should be carried out. The purpose of this is either for isolation and enumeration of *S. aureus* or for detection of SE in food remnants. An overview of the current assays for characterization of SFP outbreaks has been reviewed by Hennekinne et al. (2011) (Fig 9)

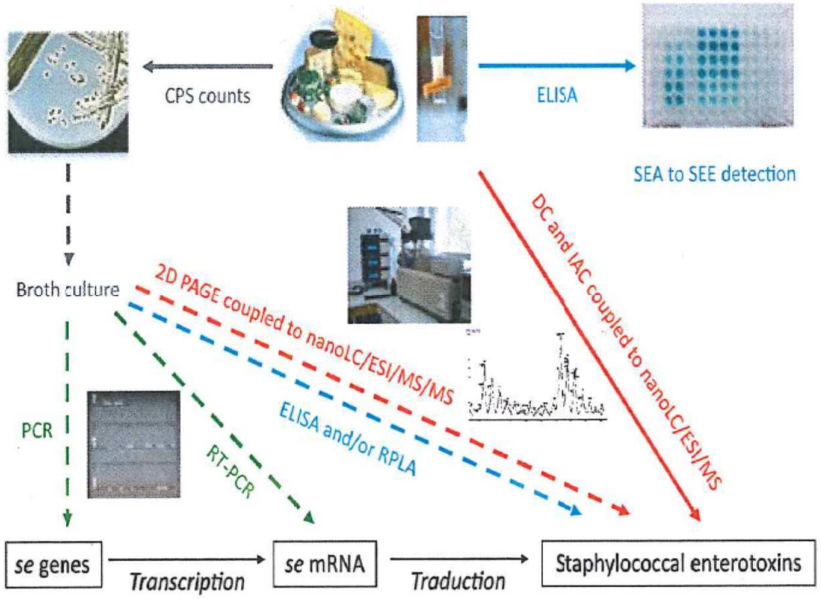


Fig 9. The general microbiological, immunological, molecular and physiochemical analytical methods employed for diagnosis of SFP. Source: (Hennekinne et al., 2011)

2.5.1.3.1. Isolation and enumeration of S. aureus

Isolation, identification and enumeration of *S. aureus* from the remnants food is an important step in the diagnosis of SFP. For isolation and enumeration of CPS including *S. aureus* from food remnants, Baird–Parker agar differential and selective media (EN/ISO 6888-1) or Rabbit plasma fibrinogen agar (EN/ISO 6888-2) are employed. According to European microbiological criteria, raw milk cheeses should be analysed according to EN/ISO 6888-2, while other milk and milk products can be analysed using either 6888-1 or 6888-2 (EU regulation 2073/2005). The Rabbit plasma fibrinogen agar is however challenging to prepare and apply in field studies, and Baird-Parker medium is therefore often used in this kind of research studies. Numbers of at least Log 5 CFU gram $^{-1}$ of *S. aureus* from the food is generally confirmatory of SFP (Hennekinne et al., 2011).

Outbreaks of SFP could also occurred from consumption of cooked or processed food products, in these cases *S. aureus* may die during the process. However, the thermostable SEs (Balaban and Rasooly, 2000; Hennekinne et al., 2011) which are responsible for the disease, may be investigated by one of the following methods.

2. 5.1.3.2. Biological method

The rhesus or cynomologus young monkey has been successfully used for detection of SE. Oral administration of SE causes emesis which is thus a biological detection of the toxin. However, due to the cost of the monkeys, expenses of keeping them, ethical aspects and the development of resistance of the monkey to SE were reported as drawback of the method (Bergdoll and Wong, 2006).

Even though this method is useful in research studies of toxicity of e.g new toxins, this method is not applicable for analysis of toxins in food on routine basis, for analysis of many samples or in outbreak investigations.

2. 5.1.3.3. Immunological methods

A commonly used immunological method for detection of SEs from isolates of Staphylococcus is the micro-slide gel double diffusion, reversed passive latex agglutination (RPLA). The sensitivity of this technique is approximately 0.01- 0.02 µg/ml and it is possible to detect low enterotoxin-producing *S. aureus* strains. As 0.1- 0.2 µg of enterotoxin A, the most common SE identified in SFP, is necessary to produce illness (Evenson et al., 1988b), and hence the application of this sensitive diagnostic technique for detection of SE during outbreak investigation is possible (Bergdoll and Wong, 2006).

For food samples, enzyme-linked immunosorbent assays (ELISA) are most used. The official methods in Europe are based on the Ridascreen kit and Vidas methodology. It is possible to detect SE below the level of 0.001 µg/g of contaminated food (Freed et al., 1982).

There are some limitations and drawbacks of the immunological method for detection of SE in food. It is very difficult and expensive to prepare a highly-purified antibody against the SEs, which are required for the immunological test (Hennekinne et al., 2011). The specificity (false positive) of the immunological assays may be decreased if the food contains peroxidase, Protein A or other endogenous substances. Only enterotoxins SEA-SEE can be detected by immunological methods as antibodies for the other SE or SEI are not yet commercially available (Bennett and Hait, 2011).

2. 5.1.3.4. Molecular method

The molecular assay includes the extraction of *S. aureus* DNA either directly from food or from a cultured broth and then testing for the presence of enterotoxin genes. It is possible to examine the presence of many enterotoxins genes simultaneously with one PCR run using multiplex PCR. However, presence of the genes does not automatically imply that toxins are produced in the food, and this is the main drawback of the method. On the other hand, the

assay reduces time and also gives valuable information about the enterotoxigenic potential of the *S. aureus* (Hait et al., 2014; Kim et al., 2011; Løvseth et al., 2004; Morandi et al., 2007). Besides, for the detection of enterotoxin genes, it is also possible to quantify *S. aureus* in the dairy food and other foodstuff by real time PCR assay (Alarco'n et al., 2006; Hein et al., 2005). This may be an important step in diagnosis and risk-assessment studies of SFP. It is also reported that with using the reverse-transcription- real time PCR (RT-qPCR) assay, it is possible to determine the SE gene expression level of *S. aureus* isolates in different food types by quantifying the mRNA (Duquenne et al., 2010; Lee et al., 2007).

2. 5.1.3.5.Mass spectrometry method

As alternative approach, to overcome some technical limitations observed in the immunological method, mass spectrometry technology has been employed for detection and quantification of SEs in different food matrixes. By combining immune-capture and protein standard absolute quantification strategy, mass spectrometry assay has been able to detect and quantify SEA in food matrixes to a level of 1.47 ± 0.05 ng/g of food (Dupuis et al., 2008). For the first time, absolute quantitative mass spectrometry has been also applied successfully for characterization of SE in coconut pearls SFP outbreak (Hennekinne et al., 2009). In apple juice, taken as model food matrix, Callahan et al. (2006) demonstrated the detection, and quantification of SEB using the mass spectrometry assay. On the other hand, the cost incurred per analysis by mass spectrophotometry as compare to immunological assay was reported as expensive. However, due to low method development cost and some technical advantages (such as applies for detection of all SEs and SEI and high sensitivity) the mass spectrometry is considered as good alternative (Hennekinne et al., 2011).

2.5.1.4. Control of SFP

S. aureus is ubiquitous organism and 20- 75% of humans are carriers of the organism (Kluytmans and Wertheim, 2005). Therefore, food which has not been refrigerated and has been handled by humans, has a high probability of contamination with *Staphylococcus* species. Cooking of food after handling by humans is an important step in the control of SFP. However the food should not be kept unrefrigerated for long period of time as *S. aureus* is able to multiply at room temperature and SE may be produced, but not degraded at a later time by cooking temperatures (Bergdoll and Wong, 2006). The ideal refrigeration and cooking temperature of food should be below 5 ^{O}C and greater than 60 ^{O}C, respectively, as *S. aureus* can multiply and produce SE at temperatures in the range of 4 ^{O}C – 46 ^{O}C.

As the mastitic udder has been reported as important source of *S. aureus* contamination in dairy products (Kadariya et al., 2014; Loir et al., 2003; Seo and Bohach, 2007) controlling mastitis at the dairy farm level is a very important step in the control of SFP.

Public awareness of proper food handling, processing and preparation, and disinfection of equipment used in food processing and preparation are important measures to control SFP (Hennekinne et al., 2011; Weese et al., 2010). Analysis of epidemiological data from Europe, Northern America, Australia, and New Zealand showed that a substantial cause of food borne disease was due to improper food preparation practices in the consumer's home (Redmond and Griffith, 2003). Adherence to Hazard Analysis and Critical Control Point (HACCP), Good Manufacturing Practice (GMP) and Good Hygienic Practice (GHP) developed by World Health Organization (WHO) is reported to be helpful to control *S. aureus* food intoxications (Syne et al., 2013).

2.5.2. Other S. aureus infections in humans and animal

S. aureus is major human pathogen responsible for a variety of toxin-mediated and suppurative diseases. In the last 20 years the number of community-acquired and hospital–acquired staphylococcal infections has increased and the treatment scheme has become very difficult due to the emergence of multidrug resistant strains (Lowy, 1998).

2.5.2.1. Staphylococcal toxic shock syndrome (TSS)

TSS is caused by diffusion of TSST-1 toxin into the blood stream. Multiplication of *S. aureus* in a localized infected area of the body produces TSST-1 which enters the vascular system and exerts these generalized symptoms (Murray, 2005). TSS is an acute onset, multisystem illness characterized by five clinical symptoms namely, fever (> 38.9 ^{0}C), hypotension (systolic pressure < 90 mm Hg), a diffuse erythematous rash, variable multi-organ involvement (vomiting, or diarrhoea at onset, mucous hyperaemia, acute renal failure, etc) and cutaneous desquamation (occurs up to 2 weeks after onset). The mortality rate is about 5 % (Ferry et al., 2005).

2.5.2.2. Staphylococcal scalded skin syndrome (SSSS)

It is a superficial blistering skin disorder caused mainly by toxin exfoliating B toxin. SSSS typically affects neonates and infants but can also occur in predisposed adults. In localized form – bullous impetigo (mainly caused by the toxin ETA), the skin damage is restricted to

the site of infection. In generalized SSSS, which affects the entire body surface, exfoliative toxin spreads through the bloodstream from the focal site of infection (Ferry et al., 2005)

Besides skin diseases, *S. aureus* is responsible for suppurative soft tissue, respiratory, bone, joint and endocardial disorders (Libman and Arbeit, 1984). Suppurative collection at these sites serves as potential foci for recurrent infections (Musher et al., 1994). *S. aureus* endocarditis is characterized by rapid onset, high fever, and frequent involvement of normal cardiac valves (CHAMBERS et al., 1983).

2.5.2.3. Staphylococcus septic shock

One of the most frequent causes of septic shock is *S. aureus*. It is a disorder due to uncontrolled inflammatory response to the staphylococcal toxins. Superantigen toxins trigger T- proliferation, which may in turn lead to massive Th 1 cytokine production such as TNF-α, IL-1β, IL-6 and IFN (Ferry et al., 2005). The overall mortality rate of *S. aureus* septic shock is 40-60% (Astiz and Rackow, 1998).

2.5.2.4 Intra-mammary infection of lactating cows

S. aureus is well known for being a causative agent of sub-clinical mastitis in cows and other lactating animals such as buffalo (Kant et al., 2015; Prabhu et al., 2015). Sub-clinical mastitis, besides causing a significant reduction in milk production, has also public health importance as *S. aureus* can transmit to humans via the milk value chain (Halasa et al., 2007; Hameed et al., 2006).

2. 6 Antibiotic resistance of *S. aureus*

2.6.1. Antibiotic resistance problem and causes

Antimicrobial resistance is an ever-increasing global public health threat. Nowadays, it is well documented that clinically important bacteria are not only characterized by a single drug resistance but also by multiple antibiotic resistance (Levy and Marshall, 2004). The WHO 2014 annual report indicated that resistance to common antibiotics has reached alarming levels in many parts of the world indicating that many of the available treatment options for common infections in some settings are becoming ineffective (WHO, 2014).

The ever-increasing antimicrobial resistance problem is not only associated with potential treatment failure but also brings a fundamental alteration in the microbial ecosystems of humans, animals and the environment. In addition, it also increases the infection rates and facilitates the emergence of more virulent bacterial pathogens that could lead to more severe infections (Barza et al., 2002).

Globally, the widely accepted cause of antibiotic resistance is the over use and misuse of antibiotics. In developing countries the situation is escalating in that besides the increasing use of antibiotics and their ready availability without prescription, poor sanitation condition around premises aid the spread of resistant strains (Levy and Marshall, 2004).

One of the biggest issues in antimicrobial resistance is the use of antibiotics in animals. There is a continuous debate on the association between antimicrobial use in the production of food animals and the emergence of resistant organisms in humans. In veterinary medicine, antibiotics are given for treatment of contagious and infectious animal diseases including clinical and subclinical mastitis at dairy farm level. However, besides human and veterinary use, antibiotics are widely used in animal husbandry and other agricultural practices and this has significantly exaggerated the antibiotic resistance problem globally (Aarestrup, 2005). Of these practices, the most serious is the continuous administration of sub-therapeutic doses of antibiotics as growth promoters for food animals. This practice favours emergence and propagation of a large number of resistance genes (Marshall and Levy, 2011). Commensal bacteria could constitute a risk by being a reservoir of resistance genes. Resistant commensal bacteria of food animals may pass on their resistance to zoonotic bacteria and reach the intestine tract of humans (van den Bogaard and Stobberingh, 2000). Presumably the *mec* region of the *mecA* gene of *S. aureus* was originally transferred from CNS (Brakstad and Mæland, 1997).

2.6.2. Biological causes of antibiotic resistance

When antibiotics come into contact with microorganisms, they may inhibit or kill susceptible microorganisms while microorganisms with a resistance genetic determinant will be selected by the antibiotics and survive (Levy, 1994, 2002). This selective pressure of antibiotics enables that microorganism to survive as a resistant strain. Not only will the resistant microorganisms multiply and give rise to resistant offspring but also they can transfer the resistance gene horizontally to other microorganisms in different hosts and geographical locations (Levy and Marshall, 2004). These conditions propagate and amplify antibiotic resistance problems elsewhere. This selective pressure of antibiotics is a centre of the resistance problem, but the spread of the resistance genes through horizontal gene transfer also plays a significant role.

The misuse and extended use of antibiotics in humans, veterinary medicine, agriculture and in stock farming as growth promoters are some selective pressures that encourage the bacteria to develop antimicrobial drug resistance at an increasing rate (Andremont, 2015).

Prolonged use of antimicrobial agents (for more than 10 days) not only lead to development of resistance to a single antibiotics but also to many types of antibiotics, the so-called multidrug resistance (MDR) (Levy et al., 1976). According to Levy and Marshall (2004) this indicates that the different resistant genes are carried by the same genetic material such as plasmids or transposons. However, they pointed out that it is not clear why prolonged use of single antibiotics brings about multiple resistance characteristics.

Bacteria including *S. aureus* acquire resistance genes in two main ways.

1) The genes responsible for antibiotic resistance are carried by mobile genetic elements (MGE) such as bacteriophages, plasmids, naked DNA or transposons and transfer of this genetic material occurs through horizontal gene transfer. In the same lineage the transfer of MGE occurs at high frequency and the major transfer system is reported to be generalized transduction, although conjugation can occur, followed by transformation at much lower frequency (Lindsay, 2013). Some transposons /naked DNA contains integrons, more complex genetic material that contain a site for integrating different antibiotic resistance genes and gene cassettes in tandem for expression from a single prompter (Bennett, 1999; Hall et al., 1999).
2) Resistance could also occur through mutation of the chromosome. This type of antibiotic resistance is characterized by step-wise progression from low level to high level (Schneiders et al., 2003; Wang et al., 2001).

There is also another type of resistance called intrinsic type of resistance. This type of resistance is an innate ability of a bacteria species to resist activity of a particular antimicrobial agent through inherited structural or functional characteristics. For example, Gram negative bacteria are resistant to vancomycin (CDC, 2016).

2.6.3. Mechanism of antibiotic resistance

Microorganisms develop antimicrobial resistance characteristics through different mechanisms (Fig 10). Some produce an enzyme that destroys the antibiotics. Enzymes such as β-lactamase disintegrate penicillin and cephalosporin. Other enzymes also modify the structure of the antibiotics so that it will no longer be effective. Such resistance mechanisms are observed against chloramphenicol and aminoglycoside antibiotics. Some resistance mechanisms also interfere with the transportation of the antibiotics inside the cell by pumping out the antibiotics from the cell to the outside. Such mechanisms of resistance has emerged for tetracycline, chloramphenicol and fluoroquinolones (Coyle, 2005; Levy and Marshall, 2004). One type of resistance mechanism can be expressed by many types of

genes and also for one type of antibiotic more than one type of resistance mechanisms can be expressed (Levy et al., 1999; Martínez-Martínez et al., 1998).

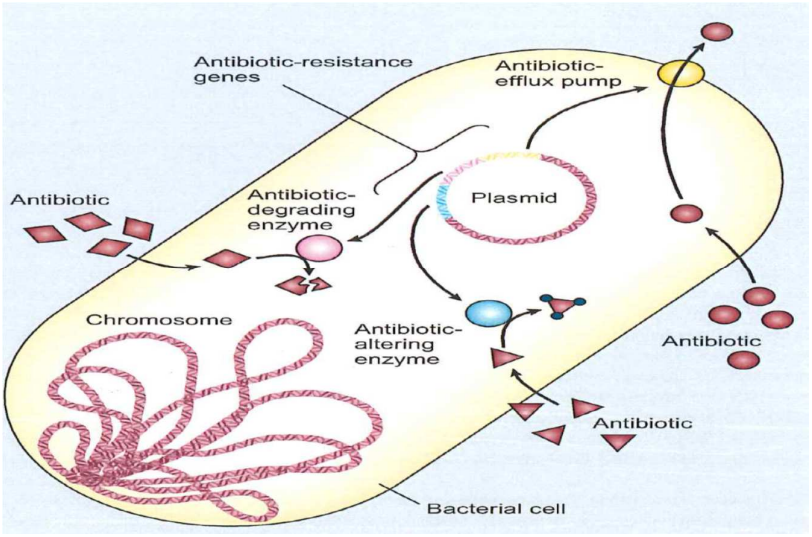


Fig 10. Antimicrobial resistance mechanism of microorganisms. Source: Levy and Marshall (2004)

2.6.4. Antibiotic resistance of *S. aureus*

S. aureus is notorious for its ability to become resistant to antibiotics. The development of multidrug resistance of *S. aureus* is a global problem. *S. aureus* develops drug resistance more readily because of its ability to produce an exopolysaccharide barrier and their location in the micro abscess that limit access of the antimicrobial (Jeljaszewicz et al., 2000).

Of all the resistance traits *S. aureus* has acquired since the introduction of antimicrobial chemotherapy in 1930s , methicillin resistance is clinically the most important, since a single genetic element confers resistance to the most commonly prescribed class of antimicrobials - the beta-lactam antibiotics, which include penicillin, cephalosporin and carbapenems (Grundmann et al., 2006).

Hospital- associated MRSA is currently a major health problem among hospitals patients and health workers. It is an endemic problem globally except in Scandinavian countries and in The Netherlands, where they are controlled by a “search and destroy” strategy (Kluytmans, 2010). In addition to this, a community-associated MRSA is currently emerging which is epidemiologically linked to food animals. The first livestock associated MRSA was isolated from the milk of a mastitic cow (Devriese et al., 1997) Since then, the prevalence of livestock-associated MRSA in food animals is increasing. Verkade and Kluytmans (2014) reported that MRSA type CC398 is spread extensively in food animals and in people in close contact

with these animals. They described direct contact with animals, environmental contamination, as well as eating or handling contaminated food as the main routes where the livestock associated MRSA can transfer to humans. Surveillance studies indicate also that in the last 15 years the epidemiology of MRSA has changed. It has become an emerging organism in livestock settings around the world including Europe, Asia and Northern America (Smith and Pearson, 2011).

Globally, there are many reports that indicate the presence of MRSA in milk and milk products. A 4% herd prevalence from Minnesota dairy, USA (Haran et al., 2012) and 1.3% of milk and dairy products from Italy (Carfora et al., 2015) were reported as contaminated with MRSA. Furthermore, 16.6% of Turkish cheese (Can and Celik, 2012), 32.1% of the *S. aureus* collected from milk of Brazilian dairy farms (Oliveira et al., 2015), and 5.3% of dairy *S. aureus* isolates in Egypt (Kamal et al., 2013), are but a few examples in which dairy food and isolates were found to be contaminated with MRSA. The emergence of livestock-associated MRSA and other multi-drug resistant *S. aureus* strains in food of animal origin such as dairy products is of public health concern as these resistant strains could be transferred from food to humans. The transfer could be effected by one of the following ways: i) by means of antibiotic residues in food ii) through the transfer of resistant food-borne pathogens iii) through the ingestion of resistant parts of the original food microflora and resistance transfer to pathogenic microorganisms (Verkade and Kluytmans, 2014). Hence, surveying and monitoring of antimicrobial resistance in food of animal origin is of paramount importance (Dunne and Belkum, 2014).

The methicillin resistance trait of *S. aureus* is attributed to the penicillin-binding proteins (PBP2a), which is encoded by the chromosomal *mecA* gene. This protein has a low affinity to the lactam antibiotics such as penicillin, oxacillin and methicillin.

The mechanism of action of β-lactam antibiotics is that they bind to penicillin-binding proteins (PBPs) of the bacteria which are important for synthesis of the cell wall of the bacteria by acting as peptidoglycan transpeptidase. When the β-lactam antibiotics inactivate PBPs, the synthesis of the cell wall of the bacteria will be inhibited and that leads to the disintegration of the bacterial cell wall (susceptible to lactam antibiotics). MSSA have PBP1, PBP2, PBP3 and PBP4 which could be inactivated by β-lactam antibiotics. MRSA are able to produce a protein called PBP2a, which is encoded by a *mec*A gene that has low affinity to β-lactam antibiotics. To inactivate the PBP2a, a 2-10 times higher penicillin concentration than PBP2 is required or 20 times higher penicillin concentration than needed for inactivation of PBP1 is required. The staphylococcal cassette chromosome (SCC*mec*) is a mobile genetic

element that carries the central determinants for broad-spectrum β-lactam resistance encoded by the *mecA* gene. The SCC*mec* contains a *mec* complex that includes the *mecA* gene and one or two regulatory genes and a cassette chromosome recombinase (*ccr*) (Deurenberg and Stobberingh, 2008a). The SCC*mec* are classified into 7 types by hierarchical system as SCC*mec* I, SCC*mec* II, SCC*mec* III, SCC*mec* IV, SCC*mec* V, SCC*mec* VI, SCC*mec* VII, SCC*mec* VIII. Types are defined by a combination of the type of *ccr* gene allotype and the class of the *mec* gene complex (International Working Group, 2009).

The presence of *mecA* has been the "golden standard" for detection of methicillin resistant *S. aureus*. However recently a novel *mecC* (mecA $_{LGA251}$) gene, which has 70 % nucleotide homology with the conventional *mecA* has been investigated from MRSA of bovine and human isolates which have a typically livestock lineages; CC130, CC705 and ST 425 (García-Álvarez et al., 2011). They were reported from western Switzerland (Basset et al., 2013) Austria (Kerschner et al., 2015) UK (García-Álvarez et al., 2011) and Denmark and their epidemiology is associated with rural areas and livestock settings (Petersen et al., 2013).

Some *S. aureus* strains have neither *mecA* nor *mecC* gene but showed partial or full resistance to methicillin and oxacillin. McDougal and Thornsberry (1986) have reported the presence of such *S. aureus* strains and they named them as borderline oxacillin-resistant *Staphylococcus aureus* (BORSA). There are many reports that indicate the existence of BORSA from food as well as from clinical isolates. Wang et al. (2015) reported the emergence of MRSA without *mecA* gene in Chinese dairy farms and reported them as the predominate strain. In Portugal, out of 148 *S. aureus* food isolates including milk and dairy products, 38% (n=56) were found to be oxacillin resistant but without *mecA* gene except one (Pereira et al., 2009). According to Brakstad and Mæland (1997) three mechanism by which these strains develop resistance to methicillin/oxacillin have been explained:

1. Hyper-production of β-lactamase enzyme. This is reported as a major reason for BORSA
2. Modified penicillin-binding protein 1, 2, and 4 to reduce the binding capacities with the β-lactam antibiotics than the same proteins in susceptible strains
3. Production of methicillinase enzymes, mostly observed in clinical isolates

Unlike MRSA, when oxacillin is administered in combination with β-lactamase inhibitors the BORSA become fully susceptible to oxacillin in agar diffusion testing. This shows that lactamase inhibitors are helpful to differentiate BORSA from MRSA (Chang et al., 1995; Liu and Lewis, 1992; McDougal and Thornsberry, 1986; Montanari et al., 1990; Sierra-Mader et al., 1988)

Leahy et al. (2011) hypothesized that BORSA have developed as a result of antibiotic pressure. As MRSA developed the *mecA* gene to resist the antibiotic pressure, BORSA also developed the above-mentioned mechanism to challenge antibiotic pressure.

Two disease outbreaks caused by BORAS in a dermatological unit of human medicine have been reported in Denmark. Molecular typing of the BORSA by pulsed field gel electrophoresis (PFGE) and *spa* typing showed no genetic relatedness among the lineage (Balslev et al., 2005; Thomsen et al., 2006). It has been reported also that detection of BORSA influences the choice of antibiotics treatment and correct identification in clinical cases is important (Leahy et al., 2011; Maalej et al., 2012).

Sometimes, it is possible to encounter MRSA which has the *mecA* gene but is phenotypically oxacillin susceptible as the gene is not expressed. Such strains are termed as heterogeneous MRSA. On the other hand, homogenous MRSA strains have the *mecA* gene fully expressed and showed complete resistance to oxacillin (Coyle, 2005).

2.6.5. Classes of antibiotics and mode of action

The action of antibiotics can be classified into two - as bacteriostatic agents and as bactericidal agents. Bacteriostatic types of antimicrobial agents such as tetracycline inhibit the multiplication and growth of the microorganisms. When the antimicrobial agent is removed, the microorganisms recommence multiplication. Bactericidal agents such as fluoroquonoline disintegrate and kill the microorganisms and the action is irreversible (Coyle, 2005). There are at least six mechanisms of action by which antimicrobials exert their action to the target microorganisms. These include inhibition of cell wall synthesis, inhibition of protein synthesis either the 30S ribosomal subunit or the 50S ribosomal subunit proteins, inhibition of DNA synthesis, competitive inhibition of folic acid synthesis, inhibition of RNA synthesis and the use of other DNA inhibitors. The major classes of antimicrobial agents with their mode of action are presented in Table 3 (Annexed). It is adapted and modified from Moore (2015) and Levy and Marshall (2004).

2.7. Laboratory methods used in this study

2.7.1. Conventional plate count method

Baird-Parker agar (BPA) is a medium that was developed by Baird-Parker (1962) and is used as a selective and differential medium for isolation of CPS from food. It is also recommended by the International Organization for Standardization (ISO) for isolation and enumeration of CPS from food (ISO, 2015). The selectivity of BPA medium to *Staphylococcus* species is due

to the presence of lithium chloride and potassium tellurite in the agar base. After incubation at 35 ^{O}C for 24-48 hours, all *Staphylococcus* species grow as black colonies in PBA medium and in addition, *S. aureus* shows a halo clear zone around the colony. The reduction of potassium tellurite to tellurium gives the colony a black colour with all *Staphylococcus* species while the clear halo zone around *S. aureus* colonies is due to lecithinase activity of *S. aureus* which breaks down the added egg yolk thus causing a clear zone. A clear halo zone around the black colony is a differential characteristic feature for *S. aureus* in the medium. The PBA supplement with egg-yolk tellurite emulsion has opalescent and canary yellow colour. In the present study, the BPA medium supplemented with egg-yolk tellurite emulsion was used for isolation and identification of *Staphylococcus* species including *S. aureus* from milk and milk products.

2.7.2. 16S rRNA gene sequencing

The identification of bacteria to species level by 16S rRNA gene sequencing is in line with the principle that phylogenetic relationship of all living things could be determined by comparing the stable part of the genetic code such as 23S rRNA or 16S rRNA (Woese et al., 1985; Woese, 1987). The use of 16S rRNA gene for identification of bacteria in clinical microbiology has been recommended by Clarridge (2004) for the following reasons: i) The 16S rRNA gene is a critical component of the cell function. It is needed for synthesis of enzymes that are vital for life. Hence it is a highly-conserved gene which tolerates mutation more frequently and therefore, could act as molecular chronometer that can show the long-term variation among bacterial species. It can mark evolutionary distances and relatedness of the organisms ii) It is a long gene (around 1550 bp) with variable and conserved regions (Fig 11) having enough interspecific polymorphisms that can provided distinguishing and statistically valid measurements. iii) In GenBank, the largest nucleotide sequence data base, the 16S rRNA gene sequence has been determined for a very large number of strains iv) The 16S rRNA is universal gene present in all bacterial species. Therefore, using this gene, it is possible to determine the genetic relationship among the bacteria.

The work flow for identification of a bacteria to species level by sequencing the 16S rRNA includes extraction of DNA, PCR amplification of the 16S rRNA gene using a universal primer, verification of the amplicon with gel electrophoresis, purification of the PCR product, sequencing of the 16S rRNA, bioediting the sequence result and blast the sequence in the

global database such as GenBank, EMBL, DDJB, ribosomal database and EzTAXON data base.

In the present study all *Staphylococcus* species identified were confirmed to species level by sequencing the 16S rRNA gene.

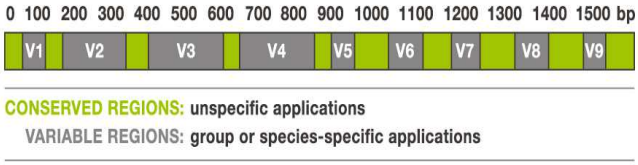


Fig. 11. 16S rRNA gene illustrating the conserved (Green) and variable (Gray) region. Source: (Alimetrics, 2012).

Although sequence trumps phenotype (Clarridge, 2004), the method may have limitations due to the present of multiple copies of 16S rRNA that may evoke problems during the identification.

2.7.3. Multiplex PCR

Multiplex PCR is a modification of PCR, which enables simultaneous amplification of many target or loci of interest in one reaction by using more than one pair of primers. This assay has been applied for many purposes including pathogen identification, DNA testing for deletion, mutation, and polymorphisms, quantitative assay and reverse transcription PCR, genotyping applications where simultaneous analysis of multiple markers is required. For a successful multiplex PCR assay, optimization of many reaction parameters is necessary. Henegariu et al. (1997) have developed a step-by-step protocol for successful multiplex PCR assay that focuses on optimization of the relative concentrations of the primers at various loci, the concentration of the PCR buffer, the cycling temperature and the balance between the magnesium chloride and deoxynucleotide.

Multiplex PCR has many advantages over conventional PCR. It save time and money as it gives many results in a single PCR reaction. Besides, it is also possible to include internal controls in order to detect the presence of PCR inhibitors in the reaction or each amplicon provides an internal control for the other amplified fragment.

However, multiplex PCR is not without limitations. Formation of primer-dimers as result of complex primer-primer reactions and some time expenditure is necessary for optimization of the multiplex PCR reaction.

2.7.4. Real time PCR (qPCR)

Real-time quantitative PCR (qPCR) is a culture independent molecular method that can detect and quantify pathogens from different foodstuffs. Compared to the culture-based conventional methods, qPCR is reported to be fast, sensitive and more specific (Postollec et al., 2011). The qPCR is a recent modification of the conventional PCR. It enables quantification of specific nucleic acids in a complex mixture even if the initial amount is very low. The quantification of the DNA is performed by monitoring the amplification of the target sequence in the *real time*, using a fluorescent technology. The qPCR can quantify amplicon production at the exponential phase of the PCR reaction in contrast to measuring the amount of product at the end-point of the reaction as is the case in conventional PCR (Fraga et al., 2014). The exponential increase of the amplicons (amplification products) can be monitored at every cycle (in real time) using a fluorescence reporter. The increase in fluorescence is plotted against the cycle number to generate the amplification curve, from which a quantification cycle (Cq) value can be determined. The Cq corresponds to the number of cycles for which the amount of fluorescence (hence of template) is significantly higher than the background fluorescence.

Several detection chemistries are now available however in food microbiology, the two more popular are the non specific DNA binding dye (eg SYBR Green I) and the strand-specific probes 5‘ nuclease assay (eg Taqman or Molecular Beacons). The principle behind the SYBR Green family of dyes is that they undergo 20-100 fold increase in their fluorescence upon binding with dsDNA that is detected by qPCR machines detector. Thus, as the amount of dsDNA increases in the reaction mix, there will be a corresponding increase in the fluorescence signal. One limitation of the dye is that it does not distinguish dsDNA of the target amplicons from any other dsDNA in the reaction. However, this problem can be resolved by gel electrophoresis and melting point analysis of the PCR products.

TaqMan probes are sequence- specific oligonucleotides with two fluorophores labelled at either end. One fluorophore is termed the quencher (3’ end, MGB or TAMRA) and the other end is the reporter (5’ end, FAM, JOE, NED.etc). When the PCR amplification takes place, the probe is displaced and cleaved. Hereby the fluorochrome is released from the quencher

and fluorescence is emitted. The emitted fluorescence is measured in each PCR cycle. Thus, there is a corresponding increase in fluorescence that is correlated with the specific amplification of the target sequence. Besides its specificity, the other advantages of the strand-specific probe system is that multiple probes can be combined or multiplexed, and thus information about several target sequences can be obtained from one reaction. However, it is more expensive than SYBR Green I dye.

The application of qPCR for detection and enumeration of *S. aureus* and other pathogens in milk and milk products has been reported. The qPCR assay has been used for detection and enumeration of *S. aureus* from dairy products (Pilla et al., 2013; Studer et al., 2008) as well as for detection and enumeration of other pathogens such as *Listeria* (Vanegas et al., 2009), *E. coli* (Singh et al., 2009), *Bacillus cereus* (Wehrle et al., 2010) from dairy products and other foodstuffs.

There are two types of real time quantification methods. Absolute quantification and relative quantification.

Absolute quantification: allows the quantification of a single target sequence and expresses the final result as an absolute value (viral load in copies/ml). This method depends on comparison of the Cq value of the samples with standard curve generated from amplification of a known/reference *S. aureus* gene.

Relative quantification: compares the levels of two different target sequences in a single sample and expresses the final result as a ratio of these targets.

2.7.5. *spa* typing

Molecular typing of pathogens such as *S. aureus* is important for two main reasons. The first is to know the genetic micro-variation at strain and lineage level during outbreak investigation, which is useful to trace the source and understand the transmission. The second is to apply the genetic macro-variation for phylogenetic and population based studies (Narukawa et al., 2009; O`Hara et al., 2016).

There are two main molecular typing methods: “band- based” and “sequence –based” The sequence-based molecular typing has advantages over the band-based method because the data is exchangeable (Deurenberg and Stobberingh, 2008b). *spa* typing is one of the sequence-based molecular typing methods developed by Frénay et al. (1996) for molecular typing of methicillin-resistant staphylococcus (MRSA). The *spa* typing method depends on sequence variation of the polymorphic X –region of the protein A gene (*spa*). This region is

composed of mainly 24 bp length repeats, which shows polymorphism due to point mutation, deletions and duplications (Kahl et al., 2005; Shopsin et al., 1999).

There are many molecular methods for typing pathogens. The choice of method depends on the purpose for which the typing will be used (Goering et al., 2013; O`Hara et al., 2016). Pulsed-field gel electrophoresis (PFGE) is the golden standard for *S. aureus* typing. However, some studies reported that the method faced technical problems for long term macro-epidemiological studies of MRSA (Blanc et al., 2002), in addition that the method is laborious and has technical difficulties that require inter-laboratory standardization (Cookson et al., 1996). On the other hand, the sequence-based *spa* typing shows sensitivity comparable to PFGE and yet it is rapid and easy to handle with a lower cost (Narukawa et al., 2009). The *spa* typing is also reported to show a highly comparable result with multi-locus sequence typing (MLST) in macro-epidemiology and evolutionary studies of *S. aureus* when combined with Based Upon Repeat Pattern (BURP) clustering algorithm (O`Hara et al., 2016). Generally, the sequence-based *spa* typing is becoming a preferred and promising method for macro-epidemiology and evolutionary investigations and is even considered as a front line tool in epidemiological typing (Koreen et al., 2004; Strommenger et al., 2008).

Due to easy performance, interpretation and exchange of results as well as due to its low cost the *spa* typing method is widely in use and obtains increased popularity. However, some studies suggested that, as *spa* typing is a single-locus-based method, it should preferably used in combination with additional markers such as staphylococcus cassette chromosome *mec* typing or resistance or virulence gene detection (Hallin et al., 2007). Other reported limitations of *spa* typing is that 1-2 % of strains may be designated as “non-typeable” by this method if there is a rearrangement in the IgG region of the gene where the forward *spa* primer is located (Votintseva et al., 2014). Moreover, sometimes the *sp*a typing/BURP method lacks discriminatory power. This may happen due to the presence of the same or related *spa* loci or *spa* repeat successions in different clonal lineages, which may be caused by gene recombination event (Robinson and Enright, 2004; Strommenger et al., 2008).

3. OBJECTIVES

3.1. General objective

To evaluate the potential food safety risks imposed by *S. aureus* isolates from milk and milk products through characterization of enterotoxigenic potential, antimicrobial resistance and genetic relatedness.

3.2. Specific objectives

- To study the occurrence and distribution of *S. aureus* and other *Staphylococcus* species in bulk milk and milk products along the milk value chain of the study area (Paper 1)

- To assess the enterotoxigenic potential of *S. aureus* from milk and milk products, and to characterize the genetic relatedness of the enterotoxigenic isolates using *spa* typing (Paper 2).

- To assess the antimicrobial resistance profiles of *S. aureus* from milk and milk products to 12 antibiotics commonly used in the study area, to verify the presence or absence of MRSA in the isolates and then to evaluate the genetic relatedness of the resistant isolates using *spa* typing (Paper 3).

- To evaluate the performance of SYBR Green 1 based real-time PCR (qPCR), targeting the *nuc* gene, for quantification of *S. aureus* in bulk milk, and to compare with conventional culture dependent method (Paper 4).

4. MAIN RESULTS AND GENERAL DISCUSSION

S. aureus is one of the most important food-borne pathogens due to its toxin-mediated virulence factors and propensity to develop antibiotic resistance. Milk and milk products are commonly contaminated by this pathogen. Therefore, studying and understanding the pathogen in these foods is of public health concern.

4.1. The distribution of *S. aureus* and other *Staphylococcus* species

The main purpose of Paper 1 was to assess the distribution and occurrence of *S. aureus* and other *Staphylococcus* species in bulk raw milk and milk products along the milk value chain. All isolates in the study were identified to species level by sequencing the 16S rRNA gene.

The overall point prevalence of *S. aureus* in milk and milk product samples was 38.7%, with mean count of 4.35 ± 0.97 Log CFU ml^{-1}. Along the milk value chain, the distribution of *S. aureus* ranged from from 29.5% in milk from a traditional dairy to 48.2% in milk and milk products in cafeterias and restaurants. The prevalence was significantly higher in raw milk than in milk products. Furthermore, odds ratio analysis of the results indicated that samples from small-scale dairies (46.4%) were twice as likely to be contaminated by *S. aureus* compared to the traditional dairy samples (29.5%) ($P < 0.05$, OR =2.07). Significantly higher *S. aureus* contamination rate was registered in the Shireendaselasse sampling area and the lowest was in Maichew area. Of all the *S. aureus* positive milk samples, 34% were found to contain > 5 Log CFU ml^{-1} of *S. aureus*. Coagulase-negative staphylococci (CNS) were identified in 51.6% of samples with mean count of 6.0 ± 1.21 Log CFU ml^{-1}. Ten species of CNS (*S. epidermidis*, *S. warneri*, *S. cohnii*, *S. hemolyticus*, *S. carnosus*, *S. sciuri*, *S. hominis*, *S. devriesei*, *S. succinus* and *S. caprae*) were identified and *S. epidermidis* was the most frequent.

The overall result of Paper 1 showed that *S. aureus* is widely distributed in milk and milk products in the studied area. From a public health point of view this finding is significant, as the isolates could harbor enterotoxigenic genes which are responsible for staphylococcal food poisoning (SFP) (Argudin et al., 2010; Bergdoll and Wong, 2006; Hennekinne et al., 2011) and carry antibiotic resistance genes that could be transferred to humans through the food chain (Verkade and Kluytmans, 2014; Verraes et al., 2013). The enterotoxigenic potential and antibiotic resistance profiles of the isolates were investigated in Papers 2 and 3, respectively.

Clinical and subclinical mastitis are major problems in milk production systems in Ethiopia, as indeed they are in all parts of the world where milk production occurs (Duguma et al., 2014; Tolosa et al., 2015; Yemane et al., 2015). It is therefore likely that one source of contamination of *S. aureus* in milk is mastitis. It is well documented that the mastitic cow and humans are two major sources of *S. aureus* in milk and milk products (Kadariya et al., 2014; Loir et al., 2003; Seo and Bohach, 2007). Peles et al. (2007) have reported that cows with clinical and subclinical staphylococcal mastitis can excrete up to 8 Log CFU ml^{-1} of S. aureus to the milk and the occurrence of SFP where the sole source of *S. aureus* is mastitic cow has been documented (do Carmo et al., 2002). However, the susceptibility of the lactating cow to mastitis may vary, among other factors, due to the breed of cow (Govignon-Gion et al., 2015; Iraguha et al., 2015). Accordingly, in the present study, a higher *S. aureus* contamination rate (46.4%) was observed in milk from the small-scale dairy farms where the exotic lactating cows are the dominant breed compared to prevalence in milk from the traditional dairies (29.5%) where the dominant milking cows are the endemic *zebu* breed. When introducing exotic animals in order to increase milk production, the susceptibility of the animal to diseases should be considered.

Another possible source of *S. aureus* contamination in milk and dairy products can be humans. Around 10- 35% and 20-75% of humans are persistent or intermittent carriers of *S. aureus*, respectively (Kluytmans and Wertheim, 2005). As milk production and processing activities in the studied area are unhygienic (Yilma et al., 2007), humans can be considered as a possible source for contamination of milk and milk products. Another possible indicator for involvement of humans in contamination of dairy food could be the more frequent contamination observed in the milk value chain from traditional dairy (29.5%) to the cafeteria and restaurants (48.2%). As 98% of the milk supply is from traditional dairies (Ketema, 2000), the observed increase in contamination along the milk value chain could be due to a human source. Through skin fragments and respiratory droplets, *S. aureus* can transfer from humans to food (Wertheim et al., 2005). Of course, another possible reason for this increase in contamination could be the multiplication of the pathogens during milk storage and transportation as there are no cold chain facilities in these settings. A molecular epidemiological study could verify the actual source by comparing the genetic relatedness of the *S. aureus* in the milk and milk products with the *S. aureus* of strains of human and bovine origin (Deurenberg and Stobberingh, 2008b; Stefani and Agodi, 2000).

Comparison of the presence of *S. aureus* in milk in the different sampling localities showed greatest prevalence of *S. aureus* contamination in Shireendaselasse localities and least in

Maichew localities. This difference may be associated with poor hygiene during production and processing of the milk and milk products. Furthermore, differences in climate (annual mean temperature of Shire is 21.6 °C and Maichew 17.1 °C) and community-associated factors such as demography and public sanitation may greatly influence *S. aureus* contamination (Kim et al., 2011).

In the present study, the lower *S. aureus* contamination rate observed in milk products compared to raw bulk milk may be related to heat treatment (50-100 °C) and the natural fermentation process undertaken during the traditional milking processes. These two factors may kill or significantly reduce the numbers of *S. aureus* in the milk products. *S. aureus* is a poor competitor when mixed with other microbes such as lactic acid bacteria (Genigeorgis, 1989). When *S. aureus* grows together with lactic acid bacteria, its growth and SE production will be reduced due to competition for nutrients, as well as influenced by developing low pH, the presence of H_2O_2, and possibly bacteriocins (Loir et al., 2003).

The high prevalence and numbers of CNS in the milk and milk products may also indicate poor hygiene and sanitation conditions during milk production and processing and this needs future attention for improvement. Furthermore, although all the species of CNS identified are regarded as part of the normal flora of the cow and the environment, the presence of *S. epidermidis* at high frequency and numbers compared to other CNS may suggest that there may also be considerable human contamination in the milk value chain as *S. epidermidis* is part of the normal flora of human skin (Ahlstrand et al., 2014; Becker et al., 2014; Otto, 2009; Villari et al., 2000).

The finding that 34% of the milk samples contained > 5 Log CFU ml $^{-1}$ of *S. aureus* is important with relation to SFP, as this level is considered critical for production of SE (Food and Drug Adminstration, 2012). However, the true risk of SFP could be assessed only after evaluating the enterotoxigenic potential of the *S. aureus* as described in Paper 2. According to the result of paper 2, among the *S. aureus* isolates collected during this study 51.5% were found to carry one or more enterotoxin genes.

4.2. Enterotoxigenic potential of the *S. aureus* isolates

Paper 2 describes the enterotoxigenic potential of the *S. aureus* isolates from milk and milk products of the studied area. The objectives of the study were to investigate the enterotoxigenic potential of *S. aureus* isolates from milk and milk products and to evaluate the genetic relatedness of the enterotoxigenic strains using *spa* typing. A modified multiplex PCR assay (Løvseth et al., 2004) was employed for detection of the enterotoxigenic genes (*se)*.

To increase the number of *S. aureus* dairy isolates for the enterotoxigenic genes study, in addition to the 120 isolates obtained from the prevalence study (Paper 1), an additional 40 *S. aureus* strains were isolated from milk and milk products, during the second field trip to the study area. Hence in total, 160 *S. aureus* isolates from milk and milk product were subjected for enterotoxin gene determination using modified multiplex PCR.

From these 160 *S. aureus* isolates from milk and milk products, 82 (51%) were found to harbor one or more enterotoxin genes. Thirty-two percent of these isolates were identified in samples having >5 Log CFU ml^{-1} *S. aureus* count. The enterotoxigenic *S. aureus* prevalence was higher in bulk milk (70%) than the products (30%). Odds ratio (OR=1.85, 95% CI 0.9706-3.548) analysis showed that raw milk is 1.8 times more likely to contain enterotoxigenic *S. aureus* than milk products. Nine types of enterotoxin genes were identified; *sea* (n=12), *seb* (n=3), *sec* (n=3), *sed* (n=4), *seg* (n=49*)*, *seh* (n=2), *sei* (n=40), *sej* (n=1), *tsst-1* (n=24), and the classical type enterotoxin genes accounted for 27%. Out of the total enterotoxigenic *S. aureus* isolates, 53.9% were found to harbor two or more types of *se* genes (the remaining *se*-positive isolates had only one *se*) and overall 18 enterotoxin genotypes were documented. The most frequent gene association was shown between *sei* and *seg* and *sea* and *seb* were also frequently found associated with new types of enterotoxin genes.

The finding that 51% of *S. aureus* dairy isolates were enterotoxigenic, and 32% of these were present in samples with > 5 Log CFU ml^{-1} *S. aureus* count indicate a potential risk of SFP to the consumers in the study area. If the quantification had been carried out using qPCR assay, as described in Paper 4, the estimated numbers would have been higher than this. According to the qPCR study (Paper 4) 29% of samples which had been reported as having less than 5 Log CFU ml^{-1} *S. aureus* with the plate count method were found to have > 5 Log SCE ml^{-1} when analysed using qPCR.

The remaining 68% enterotoxigenic *S. aureus* with < 5 Log CFU ml^{-1} in milk and milk products could also grow to the critical level due to shortage or absence of cold chain facilities

in the study area. The growth of enterotoxigenic *S. aureus* above > 5 Log CFU ml^{-1} is critical as it is around this level that SE may be produced.

The general susceptibility of the individuals to the toxin and type of the SE produced could influence the severity of SFP symptoms (Seo and Bohach, 2007). But lower concentrations, as low as 144 ± 50 ng, were found to be responsible for a SFP outbreak in a dairy product in USA (Evenson et al., 1988b).

Seventy-three percent of the identified enterotoxin genes, including the *tsst-1*, were shown to be the new types of *se* genes. Although the involvement of the newly-identified SE in the SFP is not widely described like the classical types of SE, there are many reports that show the emetic and super-antigenic characteristics of these toxins. The *seg* and *sei*, which were the frequently identified *se* in the present study, are well characterized and reported to produce emesis at 80 µg and 150 µg kg^{-1} dose in a monkey model (Munson et al., 1998b). Omoe et al. (2002) demonstrated that *S. aureus* with *seg* gene produced SEH capable of causing food poisoning and other staphylococcal super antigen-related diseases. Furthermore, SEH has been implicated in SFP outbreaks in Norway, following consumption of mashed potato to which raw milk had been added (Jørgensen et al., 2005a) and in Japan after consumption of reconstituted milk (Ikeda et al., 2005).

The association between *sei* and *seg* is interesting and may be related to their actual location in the genetic material of *S. aureus.* Both are located in an accessary genetic element called *vSa* genomic island (Argudin et al., 2010). In this genetic element, they form a chromosomal operon in a tandem orientation known as an enterotoxin gene cluster (*egc*). In addition to these, the three genes, *selo, selm, seln* and two pseudogenes, the *ψ enta 1and ψ enta 2,* are found in this cluster (Jarraud et al., 2001). This cluster is consider as a nursery of *se* and *sei* genes due to its dynamic evolutionary nature which is observed by the presence of different *egc* variants (*egc 1, egc 2, egc 3* variant,etc) (Collery et al., 2009; Fitzgerald et al., 2001b; Jarraud et al., 2001).

Our study showed that 27% of the isolates harboured the classical type of enterotoxin genes alone (18%) or in association with the new type of enterotoxins (82%). The majority of SFP outbreaks are caused by the classical type of enterotoxins alone or together with a new type of SE/SEI (Argudin et al., 2010; Hennekinne et al., 2011; Loir et al., 2003; Tang et al., 2011). Global retrospective analysis of the SFP outbreaks indicate that SEA is the most common toxin involved in the SFP outbreaks, followed by SED and SEB (Argudin et al., 2010; Johler et al., 2015; Kérouanton et al., 2007; Li et al., 2015). In the present study, among the total identified classical *se* genes, 55% were *sea* thus suggesting a likely high risk of SFP if control

measures are not in place. It has been suggested that the extraordinary high resistance of SEA to proteolytic enzymes in the gut may help this toxin to frequently be involved in SFP outbreaks (Balaban and Rasooly, 2000; Bergdoll, 1988).

The high prevalence of enterotoxigenic *S. aureus* in raw milk samples compared to the milk product samples may require attention particularly in areas where consumption of raw milk is common. A study report indicated that 31.8% of farmers in Ethiopia consume raw milk (Makita et al., 2012).

Many researchers have reported the presence of enterotoxigenic CNS in milk (Oliveira et al., 2011) and in milk products such as cheese (Rall et al., 2010). SFP outbreaks cause by *S. epidermidis* have been also reported (Breckinridge and Bergdoll, 1971). Postollec et al. (2011) pointed out that there is localized part in the genome of CNS that is involved in coding and expression of SEs. Therefore, the finding of a high incidence of CNS in milk and milk products in the study area (Paper 1) merits further investigation on the enterotoxigenic potential of CNS. Among the 10 species of CNS identified, *S. epidermidis*, *S. cohnii*, and *S. hemolyticus* have been reported to produce SE (Bautista et al., 1988).

4.3. Antibiotic resistance profiles of the *S. aureus* isolates

The antibiotic resistance profiles of the 160 *S. aureus* dairy isolates, and their genetic relatedness using *spa* typing were assessed in Paper 3. Verification work for presence or absence of MRSA was also conducted. The Kirby-Bauer disk diffusion method was used for testing phenotypic antimicrobial susceptibility to 12 types of antibiotics that are commonly in use in the studied area. In addition, PCR was used for the detection of either *mecA* or *mecC* which are considered as verification for the presence of MRSA.

Of the tested 160 *S. aureus* dairy isolates, 86% were resistant to one or more types of antibiotics and of these 45 % (61/137) were multi-drug resistant (MDR) strains. In these MDR strains, 35 resistance patterns were observed and 61% were resistant to 5 or more types of antibiotics. A higher frequency of resistance was observed for penicillin G (69%), streptomycin (53%) and erythromycin (41%). Thirty-two isolates were classified as borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) because they showed oxacillin resistance phenotypically without having neither *mecA* nor *MecC* gene and yet were susceptible to amoxicillin/clavulanic acid, which is a β-lactamase inhibitor. Of the total BORSA strains, 91% were multi-drug resistant.

Many studies have reported that antibiotic resistance can be transferred to humans via the food chain. (Mayrhofer et al., 2004; Verraes et al., 2013). The intestinal tract of humans can be colonized by *S. aureus* (Ray et al., 2003; Vollaard and Clasener, 1994) and the number of *S. aureus* can be particularly high in infants (Lindberg et al., 2000) and immunocompromised people (Ray et al., 2003). Consumption of milk and milk products contaminated with MDR *S. aureus* could enable the establishment of strains bearing many resistance genes in the intestinal tract. Further, through horizontal gene transfer, the resistance genes could be transferred to the normal gut microflora and to other pathogenic bacteria, if present (Lindsay, 2013). The development of MDR commensal gut microflora may result in a significant health problem to the individual as the members of the gut microflora could in turn transfer the multidrug resistance genes to pathogenic bacteria that may infect the individual. Milk-borne pathogenic zoonotic diseases such as brucellosis, salmonellosis, and tuberculosis are common in Ethiopia, including in the study area (Asmare et al., 2013; Geresu et al., 2016; Haileselassie et al., 2010; Kelly et al., 2016; Romha et al., 2013; Tadesse and Gebremedhin, 2015). Therefore the presence of MDR *S. aureus* in dairy food may increase the risk of the transfer of resistance genes to these pathogens.

The MDR *S. aureus* strains that may colonise the intestinal tract of the dairy food consumer could also be involved in invasive staphylococcal infections (ISI) that affect other organs (lung, heart, bone, etc.) and thereby lead to high mortality and morbidity (Lowy, 1998; Song et al., 2013). Food-originated ISI, caused by MRSA, has been documented in the Netherlands with the result of high morbidity and five fatalities (Kluytmans et al., 1995).

Although the conventional *mecA* or *mecC* gene-mediated MRSA was not identified in the dairy isolates, oxacillin-resistant strains, with different mechanisms of action, were noted. High β-lactamase production that can hydrolyze the lactam group (McDougal and Thornsberry, 1986), plasmid-mediated methicillinase production (Montanari et al., 1996) and modification of penicillin-binding proteins that do not bind oxacillin effectively (Nadarajah et al., 2006; Tomasz et al., 1989) are the mechanisms by which BORSA may show phenotypic oxacillin resistance. The selective pressure from antibiotics is suggested as the main reason for development of such strains (Leahy et al., 2011). Two skin disease outbreaks caused by BORSA were reported in Denmark (Balslev et al., 2005; Thomsen et al., 2006).

The MDR *S. aureus* strains in the milk and milk products may have originated from the cow or from the humans around. As it has been mentioned above, mastitis causes by *S. aureus* is major health problem in the majority of the dairy farms in the studied area. Prolonged and inappropriate use of antibiotics for treatment of the disease may lead to development of MDR

strains. In addition, human MDR *S. aureus* carriers could contaminate the dairy food along the milk value chain (Paper 1). Studies conducted in Central and Southwest Ethiopia showed that nasal carriage of *S. aureus* in farm workers was 33% (Mekuria et al., 2013) and 23% (Kejela and Bacha, 2013) in school children and other community members were found to harbour MDR *S. aureus* strains.

Generally it has been elucidated that the major cause of antibiotic resistance at cell level is the selection pressure of the antibiotics on the bacteria, and furthermore, the horizontal gene transfer plays an important role in the spread of the resistance genes (Levy and Marshall, 2004). There is much misuse and prolonged use of antibiotics both in human and veterinary medicine in the study area. According to authorized personnel in the study area, the use of antibiotics without prescription, and administration of the wrong dose for the wrong number of days by unauthorized personnel is common.

4.4 Correlation between enterotoxigenic and MDR *S. aureus*

Out of the 82 enterotoxigenic isolates of *S. aureus*, 53 (65%) were found to be MDR strains. It is possible to get such results as the genes that encode the SE and antibiotic resistance are carried by mobile genetic elements that can move from one bacteria to other via horizontal gene transfer. However, the two virulence determinants are not located on the same mobile genetic element. According to Ortega et al. (2010), two well-defined classes of mobile elements carry the two types of virulence genes. The staphylococcal pathogenicity island (SaPI) carries only enterotoxin genes whereas staphylococcal chromosome cassette methicillin-resistance islands (SCC*mecs*) carry genes for resistance determinants.

In line with our finding, other studies also reported the presence of antibiotic resistance in enterotoxigenic *S. aureus* isolates from dairy and other foodstuffs (Normanno et al., 2007; Pereira et al., 2009). As SFP is not treated by antibiotics, antibiotic resistance in enterotoxigenic strains may not be a significant problem. However some studies reported that enterotoxin-producing strains of MRSA may cause nosocomial antibiotic-associated diarrhoea (Boyce and Havill, 2005).

Twenty-eight percent (15/53) of the MDR *S. aureus* strains harbored the *tsst-1* gene (Papers 2 and 3). This gene is responsible for non-menstrual related toxic shock syndrome (NMTSS). An infection caused by enterotoxigenic *S. aureus* bearing the *tsst- 1* gene could cause NMTSS (Murray, 2005) The disease is characterized by multi organ failure, hypotension and other shock symptoms and yet, according to Stevens et al. (2006) NMTSS is treated with high

doses of antibiotics. In light of these facts, the presence of MDR *S. aureus* with *tsst-1* gene constitute a possible risk to milk and milk product consumers in the study area.

4.5 Molecular characterization of isolates using *spa* typing

In the present study, the sequence-based, *spa* typing method was utilized to assess the genetic realatedness of the enterotoxigenic (paper 2) and multidrug resistant (paper 3) *S. aureus* isolates from milk and milk products. The assay is easy to perform, has a low cost and shows comparable performance with multilocus sequence typing (MLST). The method has therefore been suggested for use in macro-epidemiological and evolutionary studies of *S. aureus* (O`Hara et al., 2016). Strommenger et al. (2008) also considered the method as a front line tool in epidemiological typing of *S. aureus*.

4.5.1 *spa* typing of the enterotoxigenic *S. aureus*

Spa typing of the enterotoxigenic *S. aureus* identified 22 *spa* types and three novel *spa* sequences, however, no apparent relationship or pattern was observed between *spa* type and *se* genotypes. The genetic relatedness among the *spa* types, the number of isolates and types of *se* in each *spa* types is explained by a minimum spanning tree (MST) in Paper 2 (Annexed). Of the total 25 *spa* types, 13 were identified from bulk milk, 3 from milk products and the remaining 9 from both sample types. The t314 (n=17, 20.7%), t458 (n= 15, 18.3%) and t6218 (n=8, 9.8%) were the most common *spa* types and were widely distributed in three of the seven localities of the studies area.

4.5.2. *spa* typing of the MDR *S. aureus*

Out of the 20 *spa* types and 3 novel *spa* sequences identified from the MDR strains, t314, t458, and t6218 were also the most frequent and were mainly found in Shireendaselasse, Mekelle and Wukro localities, respectively. No specific association or relationship was apparent between *spa* types and the 35 resistance patterns. Genetic diversity among *S. aureus* isolates in the milk and milk product was observed.

The distribution of the 22 *spa* types and 3 novel *spa* sequences showed that there was genetic diversity among the enterotoxigenic and MDR *S. aureus* strains. This genetic diversity was also reflected in the two types of the dairy samples. Intra bulk milk *spa* types variation was significant (13 types in bulk milk samples). All these observations may suggested that there is a wide range of source of *S. aureus* contamination in milk and milk products across the study area. On the other hand, the absence of any specific pattern of correlation between *spa* types and *se* genotypes and resistance pattern of the antibiotics may indicate that this is no

specific risk of SFP and development of antibiotics resistance by a specific *spa* type in the studied area.

Molecular characterization of enterotoxigenic and MDR *S. aureus* strains with *spa* typing is a public health issue because it is important during an outbreak investigation to identify the source, and to understand the transmission, and pattern of infections. If there is a SFP outbreak in an area, comparison of the *spa* types with the suspected sources may help to track the source of infection and to understand the mode of transmission. Moreover, molecular characterization is of importance for epidemiological surveillance of enterotoxin and antibiotic resistance genes in order to understand the phylogenetic evolution of the *S. aureus* lineages across countries and frontiers (Koreen et al., 2004; Stefani and Agodi, 2000; Strommenger et al., 2008).

From a macro-epidemiological surveillance perspective, the t314, t458 and t6218 are the most common *spa* types reported in present study. Likewise, similar *spa* types from enterotoxigenic *S. aureus* have been reported from different parts of the world. The t314, t127 and t325 *spa* types were reported from five African cities, Cameroon, Madagascar, Morocco, Niger and Senegal and t314 was the most common type (Breurec et al., 2011). Moreover, the *spa* type t127 harboring *seh* was reported from Korea, (Hwang et al., 2010) and Switzerland (Hummerjohann et al., 2014). The *spa* type, t127, which is the 5th most common *spa* type in this study, was registered as one of the top ten registered clones in the global frequency of *S. aureus* on the Radom *spa* database server (Ridom, 2004). In Ghana, the *spa* types t314, t311 and t355 were also identified from clinical MDR *S. aureus* strains.

Although *spa* typing has many advantages, the method is not without limitation. Besides, to the least discriminatory power (Robinson and Enright, 2004) and some strains may be designated as “non-typeable” by this method (Votintseva et al., 2014). Others have commented also that, as *spa* typing depends on a single locus, it is therefore preferable to use the method in combination with other virulent gene markers such as SCC*mec* or *se* genes (Hallin et al., 2007).

4.6 Comparison of qPCR and Plate count method

Enterotoxigenic *S. aureus* produce SEs when the population exceeds > 5 Log CFU ml $^{-1}$ in the contaminated food. Hence from food safety perspective, it important to monitor the level of *S. aureus* in the food with a reliable, sensitive and fast assay. The purpose of paper 4 was

to evaluate the performance of the culture independent SYBR Green I based qPCR assay that targets the *nuc* gene for quantification *S. aureus* in bulk milk and compare the outcome with plate count method (Paper1).

In this study, milk artificially contaminated with the reference *S. aureus* strain and 72 naturally-contaminated milk samples selected from study 1 (Paper 1) were analysed.

Performance indicators of the qPCR assay: The amplification efficiency and the coefficient of determination (R^2) of the standard curve were 91% and 0.98, respectively. The detection limit of the assay was 18 copies of *nuc* gene/PCR. The repeatability and reproducibility of the assay, as expressed by standard deviation, were in the range of 0.12-0.30 and 0.29-0.5, respectively. With wide quantification cycle (Cq) difference, the primers could differentiate *S. aureus* (Cq = 13.83 ± 0.93) from other *Staphylococcus* species (Cq = 30.34 ± 2.65).

Quantification result: In artificially contaminated milk samples, the coefficient of correlation between CFU ml $^{-1}$ count and *S. aureus* cell equivalent (SCE) ml $^{-1}$ count was 0.95. The performance of the qPCR assay as evaluated by the PCR efficiency, detection limit and precision were within acceptable limits. The primers could differentiate *S. aureus* from other *Staphylococcus* species by a wide difference in Cq value. However, in samples when the level of the *S. aureus* was low, the Cq value increased and could potentially overlap the Cq value of other *Staphylococcus* species. To overcome this problem the amplification cycles were reduced from 40 to 35. This reduction in cycle numbers has shown some effect on the detection limit of the assay.

In naturally contaminated milk samples, qPCR gave in general a higher result than the plate count method and this increased, by 29%, the proportion of samples with an amount *of S. aureus* over 5 Log cells ml^{-1}. This may be due to several reasons. The presence of intact DNA from dead cells as well as the presence of viable but not cultivable (VNC) cells, would only be detected by qPCR assay (Chen et al., 1997; Mäntynen et al., 1997; Montville and Matthews, 2007). Moreover, one colony on a plate may originate from more than one cell and, in addition, some primers involved in molecular assay may target multiply copy genes (16s rRNA) that could increase the apparent count (Hein et al., 2001). However, the detection and quantification of dead cells, like in the present qPCR assay, is important because it may provide a retrospective analysis of the food for *S. aureus* exposure (Hein et al., 2005; Postollec et al., 2011). The identification of 29% more samples containing *S. aureus* with > 5 Log SCE ml $^{-1}$ count by qPCR assay could indicate an increased probability of SEs production (Food and Drug Adminstration, 2012) in the milk samples assuming that 51 % of the *S. aureus* are enterotoxigenic (Paper 2). Once the SEs are produced they will persist in the food as they are

thermostable and resistant to freezing and low pH (Hennekinne et al., 2011; Loir et al., 2003). The heat stability of SEs imposes a challenge for the food industry as the bacterial cells may die but the toxin remains in the food (Balaban and Rasooly, 2000).

Generally, not only does qPCR give quantified data regarding the total viable and non-viable *S. aureus* in milk, which is important for microbial risk assessment study, but also generates the data within a short period of time. This speed is a great advantage during diagnosis of food poisonings outbreaks.

In addition, it has been demonstrated that the qPCR assay is an efficient alternative to the plate count method in monitoring intermammary infection (Graber et al., 2007; Studer et al., 2008), which is relevant to the study area where clinical and subclinical mastitis is a major problem. Furthermore, it has been reported that the SYBR Green based qPCR is a rapid and reliable method for detection and quantification of *S. aureus* harbouring the enterotoxin gene cluster (*egc*) in raw milk. It has been suggested as a promising diagnostic tool in the control of SFP in milk and milk products (Fusco et al., 2011).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

S. aureus and other *Staphylococcus* species were found widely distributed in milk and milk products along the milk value chain of the studied area. The prevalence of *S. aureu*s ranged from 29.5% in traditional dairies to 48.2% in cafeterias and restaurants. The overall prevalence was 38.7% with a mean count of 4.35 ± 0.97 Log CFU ml^{-1}. The *S. aureus* prevalence was significantly higher ($P < 0.05$) in bulk milk (47%) than in milk products (28.8%). CNS were found in 51.6% of samples with a mean count of 6.0 ± 1.21 Log CFU ml^{-1}. Ten species of CNS were identified and *S. epidermidis* was the most frequent.

Of the total *S. aureus* isolates from milk and milk products 51 % were found harbouring one or more *se* genes forming 18 enterotoxin genotypes. Nine enterotoxin genes, *sea, seb, sec, sed, seg, seh, sei, sej* and *tsst-1* were identified of which 27% were the classical types. Among the total enterotoxigenic *S. aureus* isolates, 32% were present above the critical 5 Log CFU ml^{-1} count, which indicates a potentially high risk of SFP.

Antibiotic resistance was common among *S. aureus* isolates from milk and milk products, thus raising a public health concern as antibiotics resistance could transfer to humans trough food chain. Of the total isolates, 86% (137/160) showed resistance to one and more antibiotics and, of these, 45% (61/137) were MDR showing 35 types of resistance profiles. Of the total MDR strains, 61% showed resistance to five or more classes of antibiotics. MRSA were not detected but BORSA, strains which are phenotypically oxacillin resistant having different mechanism of resistance, were identified. The most frequently found resistance was for penicillin G (69.4%), streptomycin (53%) and erythromycin (42%).

Among the total 61 MDR *S. aureus* strains, 65% (53/61) were found to carry one or more enterotoxin genes. This may increase the risk of SFP and antibiotic resistance-related complications in a single *S. aureus* isolates.

spa typing of the enterotoxigenic and MDR *S. aureus* isolates from milk and milk products revealed 22 *spa* types and 3 novel *spa* sequences thus showing the diverse genetic background of the isolates. Greater *spa* type diversity was documented in milk than the milk products, which may be associated with the diversified source of contamination. No apparent correlation or patterns were observed neither between the *spa* types and the *se* genotypes nor between the *spa* type and antibiotic-resistance patterns of the MDR strains. The t314, t458 and t6218 were the most common spa types and were found in three of seven localities of the study area.

Generally, this study showed the wide distribution of enterotoxigenic and MDR *S. aureus* isolates from milk and milk products and the strains had a diversified genetic background. This may impose a risk to dairy food consumers due to the likelihood of food intoxication and antibiotic resistance problems. Control interventions to curb the current situation and further research to fill gaps identified for further understanding the situation are important.

5. 2 Future perspectives

Research gaps identified during this work will be the focus of future research:

1. Molecular epidemiological studies on enterotoxigenic, MDR, and BORSA *S. aureus* isolates in order to understand their source and transmission to the milk and milk products. Besides, further molecular characterize of the isolates in order to understand the epidemiological and phylogenetic relatedness with clinical MRSA isolates around is important.

2. Study the factors and determinants that influence the production of SE by enterotoxigenic strains of *S. aureus* in milk and milk products. The study will also investigate the toxin-producing potential of the new type of *se* genes.

3. Study on enterotoxigenic potential of CNS as well as the role of CNS as a reservoir of antibiotic resistance genes, particularly in relation to dairy setting are important.

6. APPENDICES

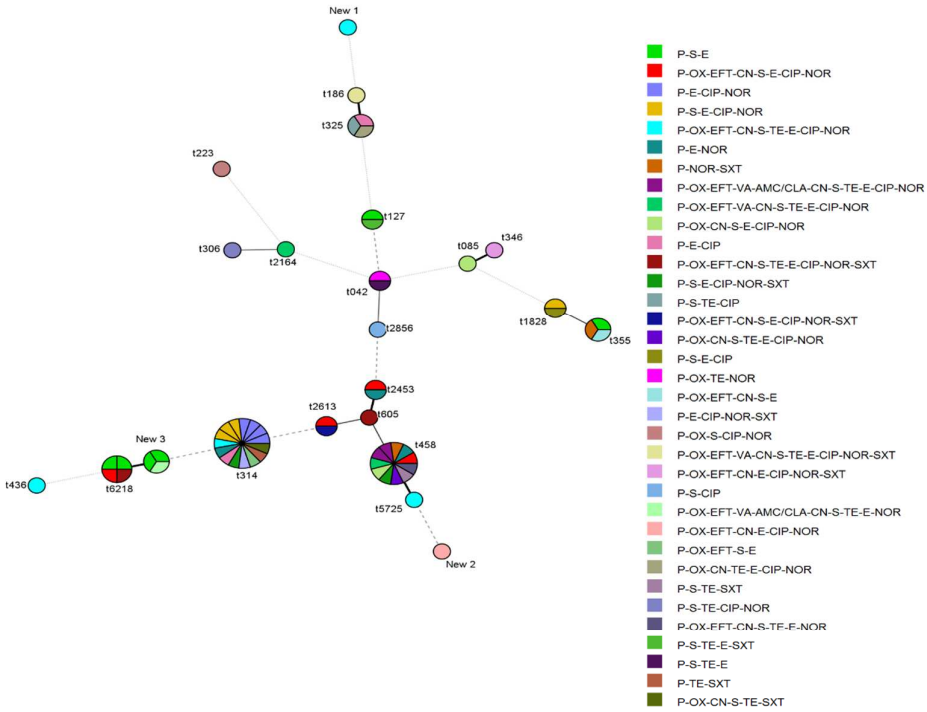


Figure 2. Minimum spanning tree (MST) of 23 *spa* types according to antibiotics resistance pattern of the 61 MDR *S. aureus* from milk and milk products. In 61 MDR *S. aureus* 35 resistance patterns were documented. The 35 different resistance pattern are represented by the different colors on the right side of the Figure.. The size of the circles represent number of MDR isolates within the *spa* type and the distance between the circles represent the genetic relatedness. The more they are nearer to each other, the more they have genetic relatedness. The colors inside the circles represent the resistance pattern exhibited by the *spa* type.

Table 3 Major classes of antibiotics and their mechanism of action

Antibiotics mechanism of action	Class	Class/mechanisms	Drugs
Inhibition of cell wall synthesis	Penicillin (Bactericidal: Block cross linking via competitive inhibition of the transpeptidase enzyme)	Penicillin	Penicillin G, Benzathine penicillin G
		Aminopenicillins	Ampicillin, Amoxicillin
		Penicillinase-resistant penicillins	Methicillin, Nafcillin, Oxacillin, Cloxacillin, Dicloxacillin
		Antipseudomonal penicillins	Carbenicillin, Ticarcillin, Piperacillin
	Cephalosporins Bactericidal: Inhibits bacterial cell wall synthesis via competitive inhibition of the transpeptidase enzyme)	1st generation	Cefazolin, Cephalexin
		2nd generation	Cefoxitin, Cefaclor, Cefuroxime
		3rd generation	Ceftriaxone, Cefotaxime, Ceftazidime, Cefepime
	Other cell wall inhibitors	Vancomycin (Bactericidal: disrupts peptidoglycan cross-linkage)	Vancomycin
		Beta-lactamase inhibitors (Bactericidal: blocking cross-linking)	Clavulanic Acid, Sulbactam, Tazobactam
		Carbapenems	Imipenem, Meropenem, Doripenem, Ertapenem
		Aztreonam	Aztreonam
		Polymyxins	Polymyxin B, Polymyxin E
		Bacitracin	Bacitracin
	Anti-30S ribosomal subunit	Aminoglycosides (Bactericidal: Irreversible binding to 30S)	Gentamicin, Neomycin, Amikacin, Tobramycin, Streptomycin

Inhibition of Protein Synthesis		Tetracycline (Bacteriostatic: blocks tRNA)	Tetracycline, Doxycycline, Minocycline Demeclocycline
	Anti-50S ribosomal subunit	Macrolides**,** (Bacteriostatic: reversibly binds 50S)	Erythromycin, Azithromycin Clarithromycin
		Chloramphenicol, (bacteriostatic)	Chloramphenicol
		Lincosamide**,** (Bacteriostatic: inhibits peptidyl transferase by interfering with amino acyl-tRNA complex)	Clindamycin
		Linezolid**,** (variable)	Linezolid
		Streptogramins	Quinupristin, Dalfopristin
Inhibition of DNA syntheses	Fluoroquinolones (Bacteriocidal: Inhibits DNA gyrase enzyme, inhibiting DNA synthesis)	1st generation	Nalidixic acid
		2nd generation	Ciprofloxacin, Norfloxacin, Enoxacin Ofloxacin, Levofloxacin
		3rd generation	Gatifloxacin
		4th generation	Moxifloxacin, Gemifloxacin
Competitive inhibition of folic acid synthesis	Folic acid synthesis inhibitors	Trimethoprim/Sulfonamides (Bacteriostatic: inhibition with PABA)	Trimethoprim/Sulfamethoxazole (SMX) Sulfisoxazole, Sulfadiazine
		Pyrimethamine	Pyrimethamine
Inhibition of RNA synthesis	RNA synthesis Inhibitors	Rifampin (bactericidal: inhibits RNA transcription by inhibiting RNA polymerase)	Rifampin
Other DNA Inhibitors	Other DNA Inhibitors	Metronidazole, (Bacteridical: metabolic biproducts disrupt DNA)	Metronidazole (Flagyl)

Adapted and modified: from Moore (2015) and Levy and Marshall (2004)

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8. ENCLOSED PAPER 1-IV

PAPER 1

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Full Length Research Paper

Staphylococcus aureus and other *Staphylococcus* species in milk and milk products from Tigray region, Northern Ethiopia

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***Staphylococcus aureus* is an important pathogen that can cause Staphylococcal Food Poisoning (SFP). Milk and dairy products are frequently contaminated by this bacterium. In this study, 310 samples (168 bovine raw milk and 142 dairy products) were collected in the Tigray region of Northern Ethiopia, with the objective of detection and enumeration of *S. aureus* and other *Staphylococcus* species. Baird-Parker agar for isolation and 16S rRNA gene sequencing for species identification were employed. *S. aureus* was found in 38.7% of samples with mean count of 4.35 +/- 0.97 log $_{10}$ CFU ml $^{-1}$. The prevalence of *S. aureus* was significantly greater in raw milk samples (47%) than in dairy products (28.8%). Of all *S. aureus* positive samples, 34.2% contained $\geq$5 log$_{10}$ CFU ml^{-1}. Samples from cafeterias and restaurants showed the greatest prevalence of *S. aureus* (P<0.05) compared to other sampling points. Samples from small-scale dairies were twice more likely to be contaminated by *S. aureus* than traditionally managed dairies (P<0.05, OR=2.0). Coagulase-negative staphylococci (CNS) were found in 51.6% of the samples, with mean count of 6.0±1.21 log$_{10}$ CFU ml^{-1}. Ten species of CNS were identified and *S. epidermidis* (36.13%) was the most frequent. The frequency of isolation found in this study indicates that *S. aureus* and other *Staphylococcus* spp. may impose public health hazard in dairy products. Therefore, further studies on the enterotoxigenic potential of the isolates, and molecular epidemiology to trace the sources of the contamination are recommended.**

Key words: Coagulase negative staphylococcus (CNS), dairy products, Ethiopia, *Staphylococcus aureus*, raw milk.

INTRODUCTION

Staphylococcal Food Poisoning (SFP) is among the most prevalent causes of gastroenteritis worldwide. In the

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United States, the 2006 Centre for Disease Control (CDC) annual report showed that *Staphylococcus aureus* enterotoxication was ranked third among bacterial food-borne outbreaks (CDC, 2009), while it was ranked as fourth in Europe (European Food Safety Authority, 2010). In China, a retrospective study (1994 to 2005) revealed that *S. aureus* was the second most common food-borne agent in homes (Wang et al., 2007). In developing counties like Ethiopia, there is little available data on the incidence of SFP. However, considering the poor hygiene conditions during production & processing of foods, as well as the shortage of cooling facilities, a high occurrence of SFP is likely. *S. aureus* grows at a wide temperature range between 6 to 48°C with optimum of 35 to 41°C. It tolerates a pH between 4 to 10 with optimum of 6 to 7, a salt concentration of 0 to 20%, and a water activity (aw) level of 0.83 to 0.99 with optimum at 0.99 (Cretenet et al., 2011).

These growth characteristics enable the bacterium to grow in a wide range of foodstuffs including milk and dairy products (Loir et al., 2003; Meyrand et al., 1998). Although, *S. aureus* is ubiquitous in nature, humans and animals are the primary reservoirs. Around 50% of healthy individuals harbour the bacteria in their nasal passage, throat and skin (Bergdoll and Wong, 2006; Hennekinne et al., 2011) whereas the mastitic cow is a common source of *S. aureus* in raw milk (Kadariya et al., 2014; Loir et al., 2003). *S. aureus* has many potential virulence factors and staphylococcus enterotoxin (SE) is one of among several responsible for food poisoning. Ingestion of less than 1.0 µg enterotoxin causes SFP (Seo and Bohach, 2007). To date, 21 SE and SEI (Staphylococcus Enterotoxin Like: SE that lack emetic properties) have been identified and designated as SEA-SEE (classical), SEG-SEI (new), SEIJ-SEIV (new) (Argudin et al., 2010; Bennett and Hait, 2011; Hennekinne et al., 2011; Ortega et al., 2010). Recent research reported that among *S. aureus* food isolates, 57% up to 72.8% harbour the classical and/or new SE genes (Akineden et al., 2001; Rosec and Gigaud, 2002).

The milk production systems in Ethiopia are classified into rural/traditional, peri-urban and urban production systems (Redda, 2001). The traditional/rural dairy represents the milk production in the mixed and pastoral/agro-pastoral farming systems and accounts for 97% of the total national production. The majority of the milking cows here are the indigenous *zebu* breed. The urban and peri-urban production systems include small and large scale dairy farms with a commercially oriented purpose and use exotic cross breed animals (Ahmed et al., 2003). Like all parts of Africa, traditional milk processing is a common practice in Ethiopia. The naturally sour/fermented milk (Rego) and buttermilk (Auso) are made from raw bulked milk. Butter and Ethiopian cottage cheese (Ajibo) are made by churning of soured milk and heating of sour buttermilk, respectively. The traditional milk preparation activities have been shown to be unhygienic and consequently expose to microbial contamination (Yilma et al., 2007).

Due to favourable agricultural policy and gradual improvement in living standards of the population, there is an on-going expansion of small-scale dairy farmers around the major cities of Ethiopia. The country showed 3 % increase in annual milk production in the past decade compared to 1.63-1.66 % of the previous two decades. This trend is also predicted to continue as there is great livestock potential and a suitable climatic environment for dairying (Ahmed et al., 2004).

However, if milk safety and quality standards are not in place, the high nutritional composition and neutral pH of milk may convey many food-borne pathogens and thereby constitute a public health challenge to consumers (Angulo et al., 2009). In the country clinical and subclinical mastitis mainly caused by *S. aureus* are a major challenge in the majority of Ethiopian dairy farms (Abera et al., 2010; 2009; Sori, 2011), moreover, a recent survey conducted in central Ethiopia also reported that 31.8% of farmers consume raw milk (Makita et al., 2012) that increased the risk of milk borne contaminations.

Therefore, in order to protect consumers from microbial hazards it is of paramount importance to study and monitor the type and level of pathogenic microbes such as *S. aureus* in the milk value chain. Such surveillance data may provide a basis for risk assessment study as well as give a foundation for the establishment of national milk quality standards that currently do not exist in Ethiopia. In light of this, there is a knowledge gap concerning the occurrence and distribution of *S. aureus* and other *Staphylococcus* species in the milk value chain in the Northern part of Ethiopia. The present study has therefore been designed to fill this gap.

MATERIALS AND METHODS

Project area and origin of samples

The study was conducted in Tigray region, Northern Ethiopia. Seven sampling areas (Mekelle, Shireendasselase, Hagreselame, Adigudome, Wukro, Adigrate and Maichew) were selected purposely in order to cover the major cities (with relatively large number of milk and milk product shop or cooperatives), geographical location (south, north, west and east) and agro climatic zones (highland medium and lowland). The spatial distribution of the sampling areas in the region and their metrological data are presented in Figure 1 and Table 1, respectively.

Study design and sampling points

A cross sectional study was conducted from August, 2012 to January, 2013. Samples were collected randomly from small-scale dairy farms, traditionally managed dairies at household level, milk collection centres/cooperatives, milk and milk product shops, market places and cafeteria and restaurants. A free informed consent was obtained from the milk and milk product owners, after explaining the research purpose, potential benefits, risks if any, and

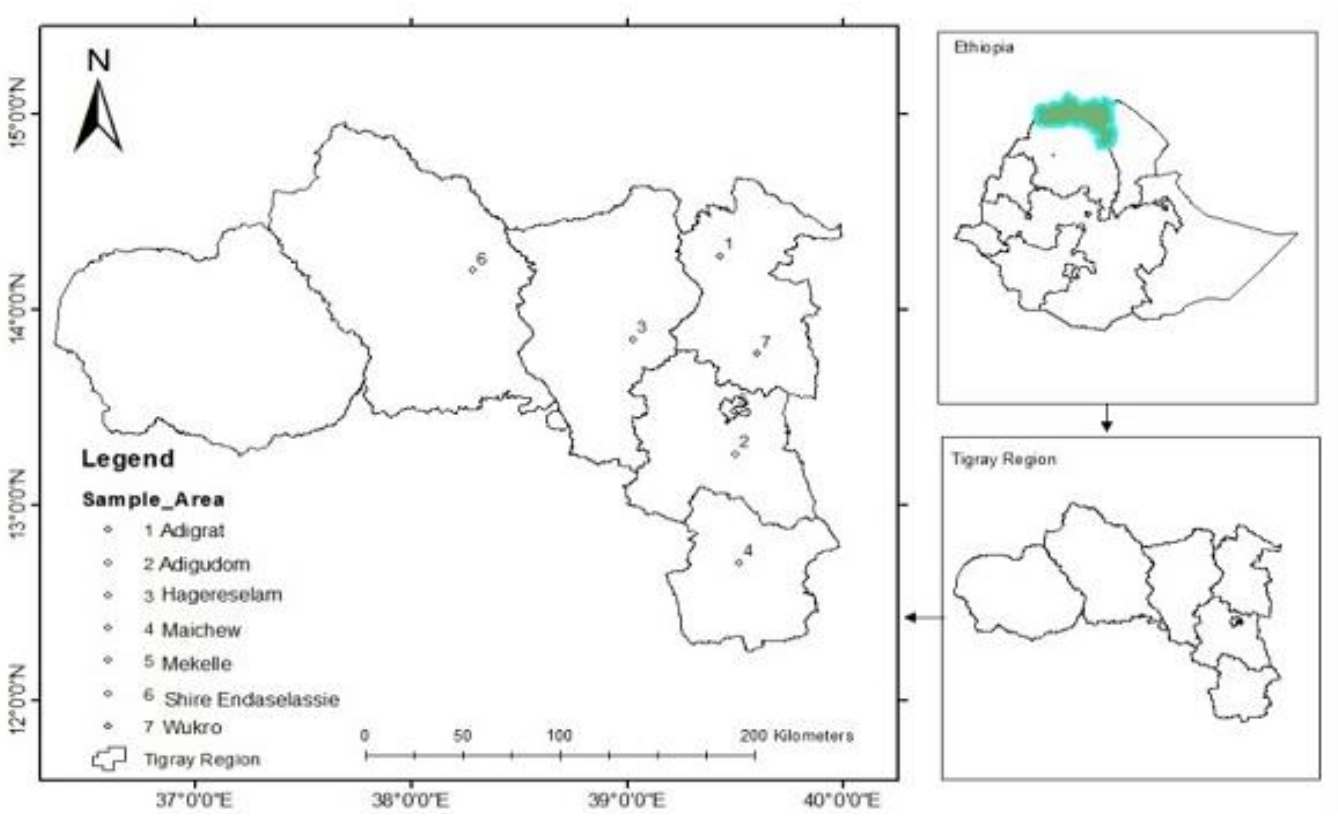

Figure 1. Map of the project area: Spatial distribution of the sampling sites.

Table 1. Metrological data of the sampling areas.

Code	Name	Elevation (m)	Mean annual temperature (°C)
1	Adigrat	2509	15.51
2	Adigudom	2107	-
3	Hagereselam	2663	16.75
4	ichew	2402	17.11
5	Mekelle	2221	18.32
6	Shire Endaselassie	1732	21.57
7	Wukro	1783	19.6

Source: Ethiopian meteorology agency, 2013.

confidentiality of the research project.

Sampled milk and products

A total of 310 samples (168 bovine raw bulk milk and 142 milk product) were collected. These were: raw bovine bulk milk (n= 168), naturally soured/fermented raw milk (n=51), butter milk (n=44), butter (n= 32), Ethiopian cottage cheese (n=7), Cheese (n= 4) and Cake made from milk (n=4).

Sampling methodology

From each sampling unit, 100 ml of mixed raw bulk milk or 100 g of milk product was collected in a sterile container and maintained at 4°C.

Microbial analysis

Milk and milk products

From the raw milk samples, tenfold serial dilutions were made. While for the milk products, 11 g of the product was mixed with 99 ml peptone water (Sigma-Aldrich, Switzerland) and blended for 2 min in a stomacher (Stomacher® 400, UK) before further dilutions. The isolation and enumeration of *S. aureus* were performed as per Ahmed and Carolyn (2003) with some modifications. Briefly, 100 µl of each tenfold dilution of the samples was spread with a sterile

bent glass rod into duplicate Baird-Parker agar (Oxoid, England) supplemented with egg yolk tellurite (Merck, Germany). Typical and non-typical colonies were differentially counted after incubation of the plates at 37°C for 48 h. Circular colonies of 2 to 3 mm diameter, jet-black to gray-black in colour and surrounded by an opaque halo and clear zone were considered as a typical *S. aureus* colonies while gray colonies without halos or clear zones were considered as non-typical.

Both typical and non typical colonies were gram-stained and tested for catalase activity (3% H_2O_2, VWR/International). Moreover, to ensure the purity of the colony before further biochemical tests, 3 to 4 colonies from each type of colony were sub-cultured into Brain Heart Infusion (BHI) broth (Oxoid, England) and incubated overnight at 37°C. From the overnight culture, the following tests were performed as per Benntt and Lancette (2001) recommendations: tube coagulase test on rabbit plasma with EDTA (Remel, Lenexa, KS, USA); DNase test on agar with toluidine blue (Sigma-Aldrich, USA), and anaerobic fermentation of 1% mannitol (Sigma-Aldrich, USA).

Pure cultures were preserved with 85% glycerol in cryovials (Sarstedt, Nümbrecht, Germany) and stored at – 20°C until they were transported to Norwegian University of Life Sciences (NMBU), Norway for further molecular work. At NMBU, they were stored at -80 until analysis.

Genetic identification

The isolates of *Staphylococcus* species were cultured on Baird Parker agar, supplemented with egg yolk tellurite, followed by sub-culturing of a single colony in BHI to obtain a pure culture.

DNA extraction

From the over-night BHI culture, DNA was extracted by GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as per the manufacturer's instructions with some minor modifications. In summary, 1 ml of the broth was centrifuged at 16 363 x g for 2 min. The pellet was washed twice with 0.9% aqueous NaCl solution, homogenized with 200 µl of lysosome solution and incubated at 37°C for 90 min. The suspension was treated with 20 µl each of RNase and proteinase K, followed by 200 µl of lysis solution C, and incubated at 55°C for 60 min. The lysate was treated with 200 µl of ethanol and transferred into the new binding column and centrifuged at 3968 x g for 1 min. The content was washed twice with washing solution 1 and concentrated wash solution and the eluate was discarded. The column was put into a new 2 ml tube and the DNA was collected with elution buffer solution. The concentration (ng/ µl) and quality (260/280 and 260/230 ratio) of the collected DNA was determined by NanoDrop® - 2000 spectrophotometer (Thermo Fischer Scientific Inc. Waltham MA, USA). The genomic DNA was stored at -20°C until use.

16S rRNA gene sequencing

According to the recommendation by ClarridgeIII (2004), the final identification of the isolates to species level was performed by sequencing the 16S RNA gene. The oligonucleotide sequences: Forward:5'TCCTACGGGAGGCAGCAG3'Reverse: 5' CGGTTACCTTGTTACGACTT 3' primers described by Vebø et al. (2011) were used to amplify the 16S rRNA gene with expected 1200 bp product. The amplification was conducted with a final PCR reaction volume of 50 µl. It contained, 5 µl of 10x Thermopol™ reaction buffer (BioLabs, New England, USA), 0.25 µl Taq Polymerase of 5,000 µ/ml (BioLabs), 1 µl of each primer of 10 pMol con, 1 µl of 10 Mm deoxynucletide triphosphate mixture (Sigma-Aldrich, USA) 2 µl of genomic DNA and finally adjusted to final volume of 50 µl with milliQwater. The amplification was carried out in C1000 ™Thermal cycler (BIO RAD laboratories) programmed to initial denaturation of 95°C for 1 min, 30 cycles of 95°C for 30 sec., 55°C for 30 sec. 68°C for 80 s and a final extension period at 68°C for 5 min.

After amplification, the expected PCR product (1200 bp) was verified by gel-electrophoresis (Figure 2) and purified by QIAquick ® PCR purification kit (QIAGEN®, Oslo, Norway) as per the company's procedures. The purified PCR products were mixed with the same primer and sent to GATC Biotech AG (European Genome and Diagnostic Centre, Konstanz, Germany) for sequencing as per the company´s instructions. The sequence results were bioedited (BioEdit version 7.0.0) and compared in GenBank using the nucleotide BLAST algorithm (http://www.ncbi.nlm.gov/blast). Maximum identification at species level was considered with result scores of 99 to 100% that appear in the first row.

Statistical analysis

Descriptive statistics (mean, max., min. SD, median) and log-transformed counts was calculated using Microsoft Excel (Windows version 8.1 version). Epi info™ version 7.1.3.10 (CDC, Atlanta, USA) was utilized to analyse rates, confidence intervals (CI) and significant associations between explanatory variables (type of samples, different localities, sampling points) and the contaminations. Difference with P-values <0.05 was considered as statistically significant.

RESULTS

S. aureus in milk and milk products

The overall point prevalence of *S. aureus* in both raw milk and milk products was 38.7% (95% CI 33.4 to 44.2%) with a mean count of 4.35 ± 0.97 log $_{10}$ CFU ml $^{-1}$. A significant difference (P< 0.05) in the occurrence of *S. aureus* was observed between raw bulk milk, 47.0%, (95% CI 39.63 - 54.5%) and milk products, 28.9%, (95% CI 22 – 36.8%) (Table 2).

S. aureus in dairy products in different localities

The prevalence of *S. aureus* in products in the different geographical localities of the project area is shown in Figure 3. The highest prevalence (56.38%, 95% CI 46.3 to 65.9%) was observed in Shire area and the lowest (8.69 and 95% CI 2.41 - 26.7%) in Maichew area (P<0.05).

S. aureus at different sampling points in the milk chain

Analysis of the prevalence of *S. aureus* in relation to sampling points in the milk supply chain showed that the prevalence was significantly (P<0.05) higher in samples from cafeterias and restaurants than in samples from other sampling points (Table 3). Odds ratio analysis of two dairy production systems indicated that milk samples

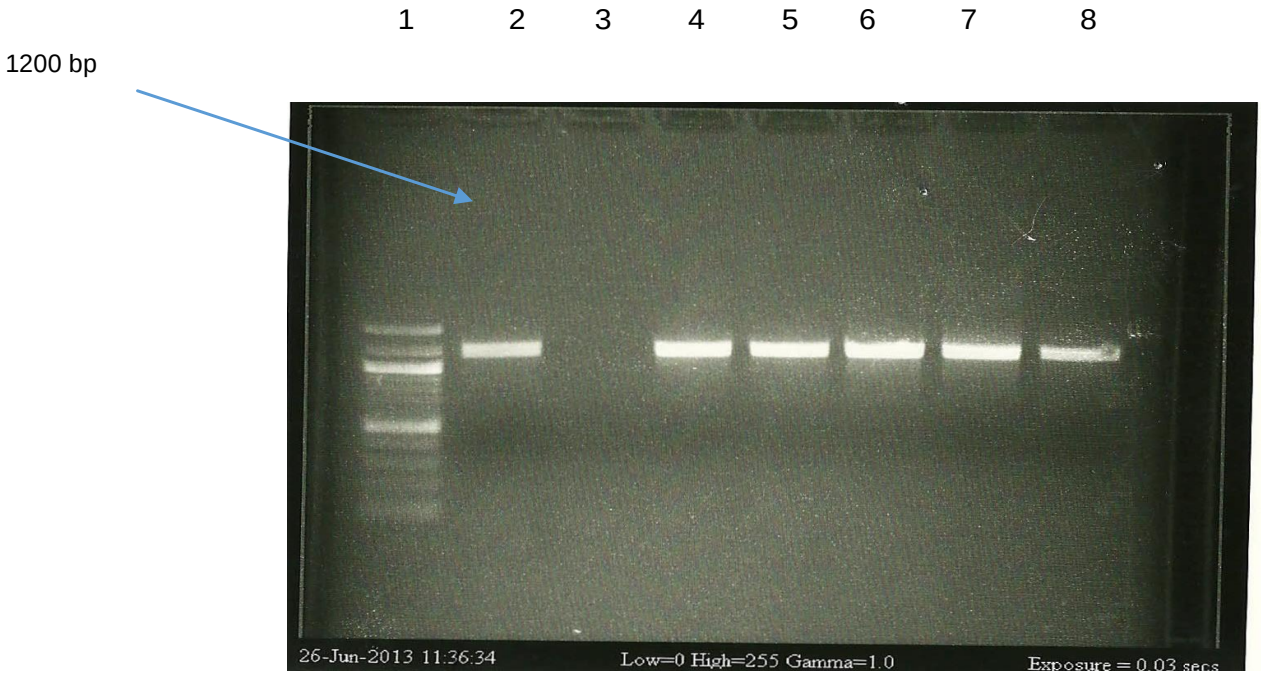

Figure 2. Gel electrophoresis of PCR product of 16 S RNA gene amplified with universal primer (lane 1= 100 bp DNA ladder, lane 2, 4, 5, 6, 7, 8= Amplified PCR product (1200 bp) positive samples, lane 3=negative control.

Table 2. Prevalence and mean count of *S. aureus* in raw bulk milk and milk products.

Type of sample	n	Number of positives	% prevalence (95% CI)	Mean count ± SD ($\log_{10}$ CFU ml^{-1})
Milk				
Raw bulk milk	168	79	47 (39.6-54.6)	4.34±0. 98
Milk products				
Naturally soured/fermented raw milk	51	13	25.4 (15.6-38.9)	4.02±1.06
Butter milk	44	14	31.8 (20-46.6)	4.47±0.94
Butter	32	8	25 (13.3-42.1)	4.89±0.87
Ethiopian cottage cheese	7	2	28.6 (8.22-64.1))	3.31±0.43
Cheese	4	2	50 (15-85)	4.46±0.91
Cakes made from milk	4	2	50 (15-85)	4.32±1.58
Sub total	142	41	28.9 (22-36.8)	4.34±0.99
Total	310	120	38.7 (33-44.2)	4.35±0.97

from small- scale dairies (46.42%) were twice more likely to be contaminated by *S. aureus* than samples from traditional dairies (29.49%) ($P< 0.05$, OR = 2.07).

Classification of *S. aureus* counts

Out of the total 120 *S. aureus* positive samples 41 (34.2 %) contained ≥ 5 $\log_{10}$ CFU ml^{-1} *S. aureus*. Classification of samples based on their *S. aureus* count is shown Table 4.

Identification of coagulase-negative staphylococcus (CNS) and other microorganisms

Based on the microbiological and 16 S r RNA gene sequence results, out of the total 310 milk and milk product samples, 276 (89%) were found contaminated

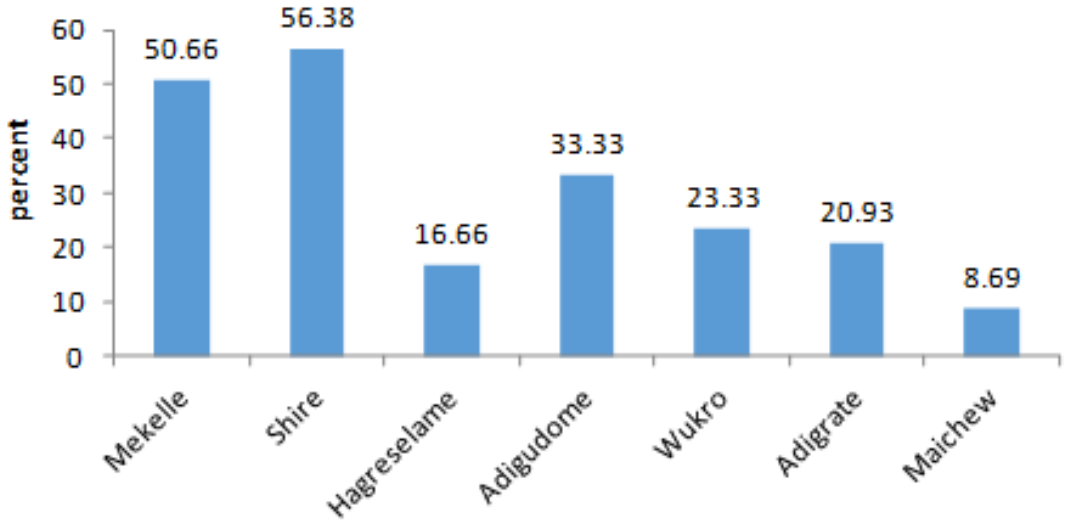

Figure 3. Point prevalence (%) of *S. aureus* in different localities of the project area.

Table 3. Distribution of *S. aureus* at different sampling points in the milk supply chain.

Sampling site	Total sampled	Positive for *S. aureus*	% (95 % CI)
Small scale dairy farms	56	26	46.4 (34-59.3)
Traditional dairies	139	41	29.5 (22.5-37.6)
Milk and milk product shops, milk collection center/Cooperatives	32	13	40.6 (25.5-57.7)
Cafeterias and Restaurants	83	40	48.2 (37.8 -58.8)
Total	310	120	-

Table 4. Classification of *S. aureus* positive samples based on *S. aureus* count ($\log_{10}$ CFU ml^{-1}).

Sample type	≤ 3 $\log_{10}$ CFU ml^{-1}	> 3 - <5 $\log_{10}$ CFU ml^{-1}	5 $\log_{10}$ CFU ml^{-1}
Raw milk	-	50	29
Naturally fermented milk	1	9	3
Buttermilk	-	11	3
Butter	-	4	4
Cottage cheese	1	1	-
Cheese	-	1	1
Cakes made from milk	-	1	1
Total	2 (1.67 %)	77 (64.2%)	41 (34.2%)

with one or two types of either *Staphylococcus* species or other microorganisms (*Enterococcus* and *Macrococcus* species). From the 276 positive samples, 318 isolates were identified. Coagulase-negative staphylococci (CNS) were found in 51.61% of the samples with a mean count of 6.07±1.21 $\log_{10}$ CFU ml^{-1}. Ten different species of CNS were identified and *S. epidermidis* was the dominant species (36.13%). The type of identified isolates and their respective microbial load is summarized in Table 5.

DISCUSSION

S. aureus is one of the most important food-borne microorganisms, responsible for SFP. Consumption of 100 *ng* of *Staphylococcus* enterotoxin (SE) produced by

Table 5. Prevalence and microbial count of *Staphylococcus* species and others microorganisms from milk and milk products.

Identified species	Isolates (n)	%	Microbial count Log $_{10}$ CFU ml $^{-1}$			
			Mean	Max	Min	SD
Coagulase positive staphylococcus (CPS) Species						
Staphylococcus aureus	120	38.7	4.35	5.95	3	0.97
Coagulase negative staphylococcus (CNS) Species						
Staphylococcus epidermidis	112	36.13	6	8.81	3	1.26
Staphylococcus cohnii	16	5.16	5.6	7.8	4.07	1.01
Staphylococcus haemoltyicus	10	3.22	6.78	8.61	5.72	0.85
Staphylococcus sciuri	6	1.94	6.99	7.81	6.07	0.82
Staphylococcus warneri	5	1.61	6.28	7.81	5.07	0.94
Staphylococcus hominis	5	1.61	6.27	7.81	5	1.1
Staphylococcus succinus	2	0.65	5.48	5.65	5.32	0.17
Staphylococcus carnosus	2	0.32	6.17	6.81	5.54	0.63
Staphylococcus caprae	1	0.32	7.81	-	-	-
Staphylococcus devriesei	1	0.32	6.81	-	-	-
Sub-total	**160**	**51.61**	**6.07**	**8.81**	**3**	**1.21**
Other microorganisms						
Enterococcus faecalis and other *Enterococcus* species	36	11.61	6.0	7.81	4.5	0.99
Macrococcus caseolticus	2	0.65	6.49	6.81	6.17	0.32
Sub-total	**38**	**12.26**	**6.04**	**7.81**	**4.57**	**0.98**
Total isolates	**318**	-	**5.41**	**8.81**	**3**	**1.38**

enterotoxigenic strains causes SFP (Hennekinne et al., 2011; Seo and Bohach, 2007). This amount of toxin is produced when the enterotoxigenic *S. aureus* population is greater than 5 $\log_{10}$ CFU ml $^{-1}$ (Food and Drug Adminstration, 2012; IESR, 2001). In the present study, 34.4% of the positive samples contained >5 log $_{10}$ CFU ml $^{-1}$ of *S. aureus* which implies that there was high probability for production of SE. Once SE is produced, it will remain structurally stable and biologically active as it is thermo-stable, and also resistant to low pH, freezing, and to the action of different enzymes. Normal pasteurization does not denature the toxin (Bergdoll and Wong, 2006; Hennekinne et al., 2011; Loir et al., 2003).

S. aureus could multiply in wide range of growth limiting determinants such as temperature, aw, NaCI and gaseous atmosphere (Cretenet et al., 2011; Kadariya et al., 2014) and hence samples that contained < 5 log $_{10}$ CFU ml $^{-1}$ of *S. aureus* could also impose a public health hazard. An increase in time or temperature before consumption could lead to further proliferation of the pathogen and the production of toxins by enterotoxigenic strains if any.

The point prevalence of *S. aureus* in the Shire area is statistically higher (P< 0.5) than the Maichew area. This difference may be attributed to differences in the milk hygiene situation during production, transportation, milk processing or storage. In addition to this situation, the relatively higher annual mean temperature of Shire (21.6°C) may encourage greater growth of the pathogens than Maichew, where the temperature is cooler (17.3°C). In agreement with this suggestion, Luca et al. (1997) reported that *S. aureus* is more prevalent during the warmest months of the year suggesting the impact of temperature on the prevalence of the pathogen.

This study shows that *S. aureus* was statistically more prevalent (P<0.05) in raw bulk milk than in the milk products. This may be related to the fact that during the traditional milk product preparation such as natural sour milk, butter milk and Ethiopian cottage cheese, the milk usually undergoes a natural spontaneous fermentation and/or is exposed to heat treatment, usually in the range of 50°C to 100°C for 50 to 70 min (Yilma et al., 2007), which may kill the *S. aureus*. Fermentation process may also reduce the growth of *S. aureus* as it competes poorly with other microbes like lactic acid bacteria. Whenever there are competing microorganisms in the milk, the growth, and SE production of *S. aureus* will be decreased as a result of low pH and presence of H_2O_2 and possibly

other antimicrobial substances (Hennekinne et al., 2011; Loir et al., 2003).

The prevalence of *S. aureus* was significantly higher in cafeteria and restaurant samples than from other sampling points. This situation may occur because the initial *S. aureus* load from the farm may multiply during transportation, as cold chain facilities are not available in all sampling points, or more *S. aureus*, from human sources, may contaminate the milk because of poor personal and/or equipment hygiene during the value chain. Although a molecular epidemiological study is required to verify the source, the obtained results indicates that there is need to improving food handlers and equipment hygiene as well as application of cold chain facilities in the milk value chain in order to protect the consumer from milk-borne hazards.

The higher prevalence of *S. aureus* in small-scale dairy farms compared to the traditionally managed dairy ($P<0.05$) may be associated with the type of milking cows available in the respective farms. In the traditional dairy, the local indigenous *zebu* breed is usual, whereas in small-scale dairy farms, cross-bred animals are the dominant milking cows. *S. aureus* is the major causative agent of sub-clinical mastitis in dairy cows (Akineden et al., 2011; Petersson-Wolfe et al., 2010), and consequently a major source of raw milk contamination (Kadariya et al., 2014). However, the susceptibility of the dairy cows to mastitis varies between breeds. Pure dairy cows are 6.3 times and crossbred cows are 3.1 times more likely to be exposed to mastitis than the local *zebu* breed (Katsande et al., 2013). Therefore, this genetic difference may be a further reason for the difference in the occurrence of *S. aureus* in the two dairying systems.

S. aureus is one of the most important food-borne pathogens in Africa as well as in other parts of world. In Zimbabwe, a prevalence of 75.8% with mean count of 5.4 $\log_{10}$ CFU ml^{-1} in raw milk and 40% with mean count of 4.7 $\log_{10}$ CFU ml^{-1} in processed milk was reported (Mhone et al., 2011), which is higher than this study. Poor hygienic practices during production and /or processing may give rise to this situation. In Nigeria, *S. aureus* was reported in the range of 25 to 45% in locally produced fermented milk, *Nono* (Nnadi, 2006) while in Pretoria, South Africa, 40% of the milk samples collected from milk shops were found to be contaminated with *S. aureus* (O`Ferrall-Berndt, 2003). Milk collected from large and small-scale dairy farms in Kenya for a multidrug resistance study reported a 30.6% prevalence of *S. aureus* (Shitandi and Sternesj, 2004). The aforementioned similar prevalence of *S. aureus* in milk and milk products indicates that *S. aureus* is one the most important public health concerns and warrants further attention in the improvement of food safety in Africa.

In Europe, Asia and USA, *S. aureus* has become one of the most important milk-borne pathogens. *S. aureus* was reported at a prevalence of 62% at dairy farms in Minnesota, USA, (Haran et al., 2011) and at prevalence of 26% from milk-producing herds in Ireland (Murphy et al., 2010). In Italy, 43% of raw milk intended for Caprino cheese making (Foschino et al., 2002) and 68% of raw milk from the Reconcavo area, Brazil (Oliverira et al., 2011) was positive for *S. aureus*. In India, a prevalence ranging from 61.7 to 65.6% was reported in raw cow's milk (Lingathurai and Vellathurai, 2010; Singh et al., 2010). Similar to this study, all the above surveys showed the importance of *S. aureus* in the milk supply chain of the respective areas.

In this study, the presence of 51.61% CNS with a mean count of 6.07±1.21 $\log_{10}$ CFU ml^{-1} in the samples indicated that the hygienic condition of the milk and milk products, during production, transportation, processing and storage was poor and needs attention for improvements. In a similar research conducted in Sudan by Suliman and Mohamed (2010), out of 644 raw milk samples, 44.7% were positive for CNS, which is lower than that of this study, however, similar to this study finding, the dominant species was *S. epidermidis*. In addition to the above hygiene-related implications, the finding of high percentage of CNS in the milk and milk product may have some risk associated with Staphylococcal Food Poisoning. Recent research outputs reported the enterotoxinogenic potential of CNS (Guimarães et al., 2013; Loir et al., 2003; Madhusoodanan et al., 2011; Park et al., 2011; Vera et al., 2010) and the involvement of CNS in SFP. Podkowik et al. (2013), confirmed the presence of localized elements in the genome of some CNS which are involved in the coding and production of SE. A food-borne disease outbreak caused by *S. epidermidis* was also reported in USA (Breckinridge and Bergdoll, 1971). These facts necessitate further screening of the CNS for enterotoxinogenic potential.

Conclusions

The study showed that *Staphylococcus* spp. in general, and *S. aureus* in particular, are common in milk and milk products in the study area and may impose a public health hazard. From a food safety point of view, food handlers at different points in the milk value chain should be educated on how to reduce contamination of milk and milk products from staphylococci species and other pathogens through personal and equipment hygiene as well as through provision of cold-chain equipment in the milk supply chain. On the other hand, farmers should also be educated on methods of reducing raw milk contamination from the environment and from the cow (mastitis) itself. Governmental regulatory bodies should also propose a standard for microbial limits for milk and milk products that reach the consumer and the milk processing plants. Further study on the enterotoxigenic potential of *S. aureus* and the other coagulase-negative

staphylococcus (CNS) to assess the risk of SFP should be undertaken. Molecular epidemiological studies aimed to trace the source of *S. aureus* in milk and milk products will be of paramount importance in the control strategy.

Conflict of Interests

The authors have not declared any conflict of interests.

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PAPER II

ENTEROTOXIN GENES OF *STAPHYLOCOCCUS AUREUS* FROM DAIRY ISOLATES

Enterotoxin gene profile and molecular characterization of *Staphylococcus aureus* isolates from bovine bulk milk and milk products of Tigray region, Northern Ethiopia

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ABSTRAC

Staphylococcal food poisoning (SFP) is important foodborne disease worldwide and milk and milk products are commonly associated with SFP outbreaks. The objectives of this study were to investigate the distribution of enterotoxin genes in *Staphylococcus aureus (S. aureus)* from raw cow milk and milk products and to assess their genetic background with *spa* typing method. Of the 549 samples (297 bulk milk and 162 products) collected from Tigray region, Northern Ethiopia, 160 (29.1%) were positive for *S. aureus* out of which 82 (51%) were found to harbor enterotoxin genes by a modified multiplex PCR. Nine enterotoxin genes (*se*) were identified; *sea* (n=12), *seb* (n=3), *sec* (n=3), *sed* (n=4), *seg* (n=49*), seh* (n=2), *sei* (n=40), *sej* (n=1), *tsst-1* (n=24), and the classical type of genes accounted for 27%. Of the 82 enterotoxigenic isolates, 41.5% and 12.4% harbored two and more *se* genes, respectively. The highest gene association was observed between *sei* and *seg* while *sea* and *seb* were always found together with the new types of *se* genes. Altogether, 18 genotypes of toxin genes were identified and 32% of the samples contained > 5 log CFU ml $^{-1}$ *S. aureus* count. *spa* typing identified 22 *spa* types and three novel *spa* sequences which showed high genetic diversity of the isolates. No apparent relationship was observed between *spa* type and *se* genes. Out of the 25 *spa* types, 13 (52%) were from raw milk, 3 (12%) from products and 9 (36%) from both samples. The t314 (20.7%, n=17), t458 (18.3%, n=15) and t6218 (9.8%, n=8) were the most common *spa* types identified and were widely distributed in three out of the eight localities of the studied area. This is the first study from Tigray region that reported the high distribution of enterotoxigenic *S. aureus* from dairy food having diversified genetic background. The study may provide valuable data for microbial food safety risk assessment, molecular epidemiology and phylogenetic studies of *S. aureus* in Ethiopia.

Staphylococcal food poisoning (SFP) is one of the most important foodborne diseases worldwide *(17, 47)*. It is caused by consumption of food that contains one or more types of staphylococcal enterotoxins (*SEs*) produced by enterotoxigenic strains of *Staphylococcus aureus (S. aureus).* One nano gram of staphylococcal enterotoxin (SE) per gram of contaminated food can cause SFP symptoms *(47)*, however outbreaks have been observed for lower concentrations, 0,5 ng/ml in milk *(14)*. The disease is characterized by a short incubation period (an average of 4.4 h), nausea, violent vomiting, abdominal cramps, headache and diarrhea. SFP is usually a self-limiting illness but death occasionally occurs with case fatality rates ranging from 0.03% for general public to 4.4% for more susceptible populations such as children and the elderly. Death results from severe dehydration and electrolyte imbalance *(18)*. Although the mortality from SFP is low *(9)*, the actual impact of the intoxication is large due to loss of working days and productivity, hospital expenses and economic loss for restaurants and food industries *(24, 37)*.

Milk and milk products are commonly associated with SFP *(11)*. An extensive SFP outbreak that affected more than 13000 persons in Japan *(5)*, and the recent outbreak of SFP at a Swiss boarding school that affected 14 school students *(21)* are examples of outbreaks that were caused by consumption of contaminated milk and milk products.

Humans and animals are reservoirs of *S. aureus* on their skin and mucosal membranes *(17)*. It is reported that, 10-35% and 20-75% of humans are persistent and intermittent carriers of *S. aureus*, respectively *(28)*. Cows with mastitis are also a common source of *S. aureus* in raw milk *(24)*. *S. aureus* can also be introduced to food by contaminated equipment used in food processing. Furthermore, inadequate refrigeration, preparation of food in advance, prolonged use of warming plates when serving foods and poor personal hygiene are favorable conditions that promote staphylococci growth and the production of SEs in food *(47)*. The main rule is that enterotoxigenic *S. aureus* must grow to a population greater than 5 log per g of contaminated food before sufficient SEs are produced to result the food intoxication *(15)*.

SEs are small proteins that range in size from 22 to 28 kDa. They are highly stable and resistant to many gastric proteolytic enzymes such as pepsin or trypsin. They are soluble in water and saline solution. They

are also highly resistant to heat, such as 127 ℃ for 15 min., freezing and drying *(17, 31)*. The heat stability of SE imposes a challenge in processed food as the bacteria may have been killed but the toxin remains *(6)*. To date, 22 SEs and SE*I*s (SE that lack emetic activity or have not been tested) excluding molecular variants, have been identified *(4)*. The *classical* SEs include SEA, SEB, SEC (SEC1, SEC2, SEC3, SEC ovine and SEC bovine variants), SED and SEE. The involvement of the toxins in SFP has been clearly elucidated and have distinct serological types. The *new* types of SE and SE*I*I includes SEG, SEH, SEI, SER, SES, SET and SE*I*J, SE*I*K, SE*I*L SE*I*M, SE*I*N, SE*I*O, SE*I*P, SE*I*Q, SE*I*U SE*I*U2 and SE*I*V. The toxin formerly designated as SEF was renamed as toxic shock syndrome toxin (TSST) because it lacks emetic activity *(7)*. Studies have shown that 57-72 % of *S. aureus* food isolates, harboured the classical and/or new SE genes *(1, 44)*.

Molecular typing of pathogens such as *S. aureus* is important for two main reasons. The first is to know the genetic micro variation at strain and lineage level as this is useful during outbreak investigation to trace the source and understand the transmission. The second is to apply the genetic macro variation for phylogenic and population based studies *(38, 39)*. There are many molecular methods for typing pathogens. The choice of method depends on the purpose for which the typing will be used *(16, 39)*. Although pulsed-field gel electrophoresis (PFGE) is the golden standard for *S. aureus* typing, the sequence-based *spa* typing shows comparable sensitivity to PFGE and yet it is rapid and easy to handle with less cost *(38)*. The *spa* typing method depends on sequencing of the polymorphic 24 bp variable-number tandem (VNTR) within the X region of the *S aureus* specific Staphylococcus protein A. *spa* typing has been considered as a frontline tool in epidemiological typing of *S. aureus (51)*.

Due to the present Ethiopian agricultural policy and the gradual increase in living standards there is an expansion of small-scale dairy farms in and around the major cities of Ethiopia. The country has shown 3% increase in annual milk production in the past decade compared to 1.6% in the previous two decades. The production of cows milk in Ethiopia may increase by 93% in the next five years *(48)*. However, cows on the majority of dairy farms commonly suffer from clinical and sub-clinical mastitis *(3, 13, 54)*. Milk

processing activity of the country in general and the Tigray region in particular is traditional and unhygienic *(57)* and 31.8% farmers were reported as consumers of raw milk *(33)*. A recent study conducted in the project area, reported point prevalence of *S. aureus* at 38.7% from milk and milk products *(53)*. However, the study did not describe the enterotoxigenic potential of the isolates. All these conditions call for further study on milk hygiene in order to protect consumers from milk borne pathogens such as *S. aureus*.

The objectives of this study were to investigate the distribution of enterotoxin genes in *S. aureus* isolates from milk and milk products in the Tigray region, Northern Ethiopia by multiplex PCR and to characterize the genetic background of the enterotoxigenic *S. aureus* using *spa* typing. This hazard identification and molecular typing work may serve as an input in the microbial food safety risk assessment, epidemiological investigations and phylogenetic studies of *S. aureus* in Ethiopia.

MATERIALS AND METHODS

Collected sample types and size. A total of 549 milk and milk products were collected from Tigray region, Northern Ethiopia, from August 2012 to May 2014. They were collected from eight cities/towns and their vicinities, of the region, namely Mekelle (n=147), Shireendaselase (n=139), Wurko (n=60), Adigudome (n=52), Hagreselame (n=21), Maichew (n=53), Adigrate (n=43) and Abi-adi (n=34). The cities/towns were selected purposely in order to cover: i) major cities/towns of the region with relatively larger number of dairy farms, milk and milk product shops and dairy cooperatives, ii) geographical locations (south, north, east and west) and agroclimatic zones (highland, medium and lowlands). The sampling points included dairy farms, milk collection centers, milk and milk product shops, restaurants and cafeterias. Samples were collected by the previously reported procedure *(53)*. The samples comprised raw, bulk milk (n=297), buttermilk (n=64), butter (n=58), sour milk (spontaneously fermented milk) (n=97), Ethiopian cottage cheese (n=14), cheese (n=15) and cake made from milk (n=4).

Isolation, enumeration and molecular identification. The isolation, enumeration and molecular identification of the isolates were performed by the methods detailed previously *(53)*. Briefly, Baird-Parker agar (Oxoid, England) supplemented with egg-yolk tellurite (Meck, Germany) was the media used for

isolation and enumeration. Conventional biochemical tests were used for identification, however, final confirmation to species level was performed by sequencing the 16S rRNA gene *(10)*. DNA was extracted by GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as per the manufacturer's instruction.

Multiplex PCR for detection of enterotoxin genes. A modified multiplex PCR was used for detection of enterotoxin genes from the *S. aureus* isolates *(32)*. The method tests 9 enterotoxin genes (*sea, seb, sec, sed, see, seg, seh, sei, sej),* the *tsst-1* and 16S rRNA of the isolates in two independent multiplex PCR reactions mixtures. Primers for *sed, see, seg, sei* and *tsst-1* were combined in reaction mixture 1 and primers for *sea, seb, sec, seh, sej* and 16S rRNA were combined in reaction mixture 2. The primers used are listed in Table 1. They were supplied by Invitrogen (Life Technologies, USA).

Each multiplex PCR reaction was performed with final reaction volume of 50 μl. It was comprised of 45 μl of reaction mixture containing final concentration of 1x AmpliTaq buffer, 4 mM $MgCl_2$, 2 U of AmpliTaq Gold polymerase (all from Applied Biosystems, USA), 400 μM dNTP, (BioLabs, USA) and 300 nM each SE primer and 60 nM 16S r RNA primers. Finally, 5 µl of DNA (10 ng/µl) was added to the mixture.

The PCR amplification was performed in a C1000 ™ Thermal Cycler (BIO RAD laboratories, USA) which was adjusted to initial denaturation at 95 ^{O}C for 10 min. followed by 15 cycles of 95 ^{O}C for 1 min, 68 ^{O}C for 45 s, 72 ^{O}C for 1 min, 20 cycles of 95 ^{O}C for 1 min, 64 ^{O}C for 45 s 72 ^{O}C for 1 min and a final extension at 72 ^{O}C for 10 min. Ten microliters of the PCR product was resolved by electrophoresis in a 2.5 % agarose gel at 100 V for 100 min. For comparison the GeneRuler 50 bp ladder (Thermo Scientific) was used. The DNA products were visualized on UV trans illuminator Gel Doc™ documentation system (BioRad, USA).

Control strains. Six *S. aureus* strains obtained from the European Union reference laboratory for coagulase positive staphylococci (Anses, Maison Alfort, France) (Table 2) were used as positive control and MilliQ water as a negative control.

***spa* typing of enterotoxigenic *S. aureus* isolates**. Amplification of the region X of the *spa* (protein A) gene was performed using primers spa -1113f (5′AAAGACGATCCTTCGGTGAGC-3′) and *spa* -1514r (5′-CAGCAGTAGTGCCGTTTGCTT-3′) *(43)*. PCR was performed in 40 µL final volume containing 1X of buffer HF, 0.4 µM of each primer, 200 µM of dNTPs and 0.02 U/µL of iProof™ taq polymerase (Biorad, Hercules, CA, USA). The PCR reaction for amplification of the DNA were: initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec and elongation at 72°C for 20 sec. Final elongation was performed at 72°C for 10 min. The PCR was purified using 1X of Agencourt AMPure XP beads (Beckman Coulter, Inc, Brea, CA, USA) according to manufacture instruction and submitted to GATC-biotech AG (GATC-biotech AG, Konstanz, Germany) for sequencing. Sequences were processed using Geneious V7 *(25)* and the *spa* types were determined using DNAgear *(2)*. Minimum spanning trees were prepared using BioNumerix software as described by *(46)*.

Data analysis. The chi-square test from Epi Info version 7.1.3.10 was used to explore statistically significant differences in the distribution of enterotoxigenic *S. aureus* among raw milk and milk products as well as among different localities. P values < 0.05 were considered as significant.

RESULTS

Distribution of *S. aureus* in milk and milk products. Out of the total 549 milk and milk product samples, 160 (29.1%) samples were shown to contain *S. aureus.*

Detection of SE encoding genes by Multiplex PCR. Among the 160 *S. aureus* dairy isolates, 82 (51%) were found to harbor staphylococcus enterotoxin genes (*se*). Nine types of *se* genes were identified, comprising *sea* (n=12), *seb* (n=3) *sec* (n=3), *sed* (n=4), *seg* (n=49*), seh* (n=2), *sei* (n=40), *sej* (n=1) *tsst-1* (n=24). The most frequent gene identified was *seg*, followed by *sei* and *tsst-1*. The least frequently identified were *seh* and *sej* and *see* was not detected. Among the 82 enterotoxigenic strains, 22 (26.8%) isolates harbored the classical *se* genes (*sea*, *seb, sec* and *sed*) either alone or in combination with the newly identified *se* genes (*seg, seh, sei, and sej*).

Among the 82 enterotoxigenic strains, 38 (46.3%) harbored one type, 34 (41.5%) two types and 10 (12.2%) more than 2 types of enterotoxin genes. Accordingly, from the total enterotoxigenic strains 18 *se* genotypes were identified. The highest gene association was observed between *sei* and *seg* (Table 3).

SE encoding genes and type of samples. Among the 82 enterotoxigenic *S. aureus* strains, 69.5% (57/82) were identified from raw bulk milk and 30.5% (25/82) from milk products. Chi-square analysis indicated that there is statistically significant difference in the prevalence of enterotoxigenic *S. aureus* between raw bulk milk and milk products ($P < 0.05$). Odds ratio (OR= 1.85, 95% CI 0.9706- 3.548) analysis showed that consumption of raw milk is 1.8 times more likely to contain enterotoxigenic *S. aureus* than consumption of a milk product. The total number of specimens collected, the type and number of enterotoxigenic genes identified is presented in Table 3.

SE encoding genes and the level/count of *S. aureus* in the food. Out of the 82 milk and milk product samples which were positive for one or more *se* genes, 32.9% and 67.1% of the original food samples contained > 5 log CFU ml $^{-1}$ and 3 – 5 log CFU ml $^{-1}$ of *S. aureus* counts, respectively (Table 4). Of the 27 samples with > 5 log CFU ml $^{-1}$ of *S. aureus*, 18 were raw bulk milk.

Enterotoxigenic *S. aureus* **strain distribution in different localities.** The distribution of the different *se* genes in different localities of the project area is presented in Table 5. There is statistically significant difference ($P < 0.05$) in the overall distribution of enterotoxigenic *S. aureus* among the five sampling localities (the other not included because of under representation) being higher in Adigudome and Wukro localities.

***spa* typing**. Twenty-two different *spa* types and 3 novel *spa* sequences, (not present in the Ridom data base) were identified. Of the total 25 *spa* types 13 (52%) were from raw milk, 3 (12%) from products and 9 (36%) from both samples (Table 6). Only 36 % of the identified *spa* types were found in both milk and product samples, whereas the remaining 64% were either in milk or in the product. Figure 1 shows the relatedness of the *S. aureus* isolates according to their *spa* types, and colored according to their toxin gene profile. No clear clustering between *spa* type and toxin genes is observed. The size of the circles represent the number of isolates within the *spa* type and the distance between the circles represent the genetic relatedness. The more they are nearer to each other, the more they have genetic relatedness. The t458 (18.3%, n=15), t314 (20.7%, n=17) and t6218 (9.6 %, n=8) (shown as multi-segmented large circles) were the most common *spa* types identified and found harboring 7, 6 and 4 different toxin profiles, respectively.

The aforementioned three most common *spa* types were frequently distributed in three out of the eight localities of the studied area (Table 6). The t314 *spa type* was more frequent in Shireendaselase (n=12, 71%), the t458 in Mekelle (n=9, 60%) and the t6218 in Wukro (n=4, 50%) localities of the studied area.

DISCUSSION

The characterization of enterotoxine genes from *S. aureus* of dairy isolates has not been previously documented in Tigray region, Northern Ethiopia. However, a similar study conducted in Central Ethiopia reported a prevalence of 25.8% enterotoxigenic *S. aureus* in bulk milk samples *(12)*, which is lower than in the current study. In Italy, 53% of the isolates from milk and milk products *(8)* and in Norway, 52.5% of

bovine bulk milk samples *(23)* were reported to harbor enterotoxigenic *S. aureus* which are comparable to our observation. However, in Sweden, 70% of *S. aureus* isolates from cheese, made from raw milk, were found to harbor one or more enterotoxin genes *(45)*.

Of the 82 milk and milk samples that contained enterotoxigenic *S. aureus*, 33 % showed *S. aureus* exceeding 5 log CFU ml $^{-1}$, which is considered to be a critical level for toxin production *(15)*. Moreover, 67% of samples containing 3-5 log CFU ml^{-1} *S. aureus* could also constitute a SFP risk if the food is exposed to ambient temperature during transport and storage thus allowing the bacteria to grow. The expression of SE is highly regulated by growth phase, environmental conditions and a complex network of regulatory genes, among which the accessary gene regulator (*agr*) plays a major role. The *agr* is activated by a quorum sensing system when cell numbers reach a critical mass *(47)*. Once the SE is produced in the food, it retains its biological activity, as it is thermostable and resistant to low pH, freezing, and drying, conditions that can easily destroy the bacterial cells that produced the toxin *(6, 17, 24)*.

In the present study, from the total number of identified enterotoxin genes 27% were genes that encode classical SEs. It is reported elsewhere that around 95% of SFP are caused by classical SE *(52)*. Among the classical SE, SEA is the most common involved in SFP worldwide, followed by SED, SEB and SEC *(4, 20, 21, 26, 30)*. However, in terms of severity, SEB results in more intense symptoms of SFP than SEA.

Among the total classical types of *se* genes, 81% were found in association with the newly identified *se* genes. In line with our observation, another study also reported the association of 64% of the classical *se* with the newly identified *se* genes *(36)*.

In the current study, the newly identified *se* genes including *tsst-1* accounted for 73% of the total identified genes. Other researchers have also noted the occurrence of a significant number of the newly identified *se* genes in dairy products and other foodstuffs *(8, 27, 34)*. From the new type of SE, SEH is reported to produce significant amount of SE that could lead to SFP *(40)*. For example, SEH was responsible for SFP

outbreaks in Norway after consumption of mashed potato made with added raw milk *(22)* and in Japan after consumption of reconstituted milk, in the latter case in combination with SEA *(19)*.

The *seg* gene was found most frequently in association with *sei*. Similar to this finding, many researchers showed also that *seg* was found linked with *sei. (8, 27, 40)* and were carried by a genomic island-borne structure called the enterotoxin gene cluster (*egc)*. This genetic structure is reported to carry additional *sem*, *sen* and *seo* genes *(55)* and it has been hypothesized that the *egc* cluster may act as reservoir and nursery for other SE genes other than the aforementioned primary *egc* related genes *(4)*.

Differences in the distribution of *se* genes were observed between the five sampling localities ($P < 0.05$), which may be indicative of the spread of certain *S. aureus* types within a specific geographical area *(22)*. The majority of the SE encoding genes are carried on mobile genetic elements (MGE) such as plasmids and prophages and spread among isolates at high frequency by a horizontal gene transfer mechanism *(35)*. This characteristic may enable the bacteria to modify its capacity to cause diseases and contribute to its evolution *(4)*.

The high prevalence and numbers of enterotoxigenic *S. aureus* in raw milk, compared to dairy products, may have two important practical implications. Firstly, in areas where raw milk consumption is a common practice *(33)* there would be high probability of SFP episodes. Secondly, although molecular epidemiological study may verify the assumption, one of the major sources of contamination to the raw bulk milk could be mastitic cows. A cow with clinical or subclinical staphylococcal mastitis can excrete *S. aureus* in numbers up to log 8 CFU ml^{-1} *(42)*.

The *tsst-1* gene was found in 29% (24/82) of the isolates. This toxin is responsible for toxic shock syndrome, which is characterized by high fever, a diffuse erythematous rash, desquamation of skin, hypotension, and involvement of three or more organ system failures *(41)*. Multiplication of *S. aureus* in a localized infected area of the body produces TSST-1 which enters the vascular system and exerts these generalized symptoms

(37). Similar to our study, combined with other enterotoxigenic genes, a *tsst* gene prevalence of 25.6 % was reported from milk of cows with mastitis *(50)*.

spa typing was used to assess the genetic background of the enterotoxigenic *S. aureus* isolated from milk and dairy products. This molecular typing method is recommended for national and international surveillance as well as for the analysis of short term local epidemiological studies due to its major advantage of being easy to conduct and easy to interpret and exchange the results *(51)*. Besides, it was reported that *spa* typing performed better than multilocus enzyme electrophoresis (MLEE), PFGE, and *coa* typing in degree of agreement with the microarray at various phylogenetic depth *(29)*.

The *spa* typing identified 22 *spa* types and 3 novel *spa* sequences from the 82 enterotoxigenic *S. aureus* thus revealing a wide genetic diversity of the isolates. The 18 *se* genotypes were found distributed evenly in all *spa* types without showing specific pattern. This may indicate that risk of SFP due to specific *se* genotype having specific genetic background may not be apparent.

The genetic similarity between the milk and milk products isolates, in terms of identical *spa* types, were only observed in 9 (36%) *spa* types that may suggest that both sample groups may have, among other reasons, small common source of contaminations. According to the *spa* typing results, the dominant genetic characteristic documented among the two sample groups was genetic diversity rather than genetic relatedness as they differed in 64% of the identified *spa* types. Moreover, higher intra milk *spa* type variation was also documented. And hence, all these observations may probably indicate that there may be many source of contaminations in the milk value chain. The X region of the *spa* gene, which is composed many 24 –bp variable tandem repeats, shows high polymorphism due to deletion, point mutation and duplication of the repeats. Variation in type or number of the repeats generates different *spa* types *(49)*. The t314, t458 and t6218 were the most common *spa* types among the isolates harboring the majority of the *se* genes identified and were also at higher prevalence in three of the eight localities of the studied areas. The generation of such molecular typing information is of paramount important for population based epidemiological, disease outbreak and phylogenetic studies of *S. aureus* in the studied area. A recent study

conducted in USA reported that *spa* typing showed comparable performance with multilocus sequence typing (MLST) and suggested for use in macro epidemiology and evolutionary studies of *S. aureus* given its lower implementation cost *(39)*.

One reported limitation of *spa* typing method is that 1-2 % of strains may be designated as "non-typeable" by this method if there is a rearrangement in the IgG region of the gene where the forward *spa* primer is located *(56)*.

In conclusion, this study is the first report from Tigray region of Northern Ethiopia, that has documented the high occurrence of enterotoxigenic *S. aureus* having 18 genotypes in dairy isolates. Moreover, 22 *spa* types and 3 novel *spa* sequences were also identified. The high prevalence of enterotoxigenic *S. aureus* with diversified genetic background in milk and milk products may impose considerable SFP risk to consumers. Control of mastitis at farm level, pasteurization of milk before consumption as well as educating farmers via agricultural extension network, to maintain hygienic environment during traditional milk processing as well as not to consume raw milk are important public health measures to reduce the risk. Educating of food handlers in the preparation and storage of food and the provision of cooling facilities in the milk value chain are necessary measures to reduce the risk of SFP. Using the output of this study as a springboard, molecular epidemiological studies aimed at tracing source of enterotoxigenic *S. aureus* in order to facilitate control intervention can be useful. Furthermore, research on production of SE in real food matrices to further characterize the risk, and also basic research on the role of the newly identified SE/SEI in causation of SFP are recommended.

Conflict of interest: The authors have no conflict of interest to declare.

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Figure legends

FIGURE 1. Minimum spanning tree of 25 *spa* types according to the *se* genotypes of the 82 enterotoxigenic *S. aureus* strains. The size of the circles represent the number of isolates within the *spa* type and the distance between the circles represent the genetic relatedness. The more they are nearer to each other, the more they have genetic relatedness. The colors inside the circle represent the types of toxin genes inside.

TABLE 1**.** List of primers sequences used for detection of pyrogenic exotoxins by multiplex PCR

Primer [a]	Primer sequence (5' – 3')	Amplified product size (bp)	GeneBank accession no.
sea forw.	GCA GGG AAC AGC TTT AGG C	521	M18970
sea rev.	GTT CTG TAG AAG TAT GAA ACA CG		
seb-sec forw	ACA TGT AAT TTT GAT ATT CGC ACT G	667	M11118 (*seb)*
seb rev.	TGC AGG CAT CAT GTC ATA CCA		
sec forw	CTT GTA TGT ATG GAG GAA TAA CAA	284	X05815 (*sec 1*) AY450554 (*sec* 2) X51661 (*sec* 3)
sec rev	TGC AGG CAT CAT ATC ATA CCA		
sed forw	GTG GTG AAA TAG ATA GGA CTG C	385	M28521
sed rev.	ATA TGA AGG TGC TCT GTG G		
see forw	TAC CAA TTA ACT TGT GGA TAG AC	171	M21319
see rev.	CTC TTT GCA CCT TAC CGC		
seg forw	CGT CTC CAC CTG TTG AAG G	328	AF064773
seg rev.	CCA AGT GAT TGT CTA TTG TCG		
seh forw	CAA CTG CTG ATT TAG CTC AG	359	U11702
seh rev.	GTC GAA TGA GTA ATC TCT AGG		
sei forw	CAA CTC GAA TTT TCA ACA GGT ACC	466	AF064774
sei rev.	CAG GCA GTC CAT CTC CTG		
sej forw	CAT CAG AAC TGT TGT TCC GCT AG	142	AF053140
sej rev.	CTG AAT TTT ACC ATC AAA GGT AC		
tsst-1 forw	GCT TGC GAC AAC TGC TAC AG	559	J02615
tsst-1 rev.	TGG ATC CGT CAT TCA TTG TTA T		
16S rRNA forw	GTA GGT GGC AAG CGT TAT CC	228	X68417
16S rRNA rev	CGC ACA TCA GCG TCA G		

[a] forw, forward; rev, reverse Source: Løvseth et al., 2004 *(32)*

TABLE 2. Reference *S. aureus* strains used as positive control for encoding enterotoxigenic genes

Strain	Genes
07CEB 167 STA	*sea, sec, seh*
46A (FRI326)	*see*
09CEB89 STA	*seb*
R5010 (PK) VI 50757	*sei, sed, seg*
3169 (PK) VI 50767	*tsst-1 , sed*
R5010 (PK) VI 50757	*sej*

Source: Anses, Maison Alfort, France

TABLE 3. The 18 *se* genotypes identified from 82 isolates of enterotoxigenic *S. aureus* and their distribution in the different samples types

	Sample types							
	Raw milk	Butter milk	Butter	Sour milk	Cheese	Ethiopian cottage cheese	Cake	Total (%)
Samples collected	297	64	58	97	15	14	4	549
S. aureus positive (%)	100 (33.7)	28 (43.7)	10 (17.2)	16 (16.5)	1 (26.7)	3 (21.4)	2 (50)	160 (29.1)
se negative	43	18	5	7	-	3	2	78
se positive (%)	57 (57)	10 (35.7)	5 (50)	9 (56.3)	1 (100)	-	-	82 (51)
sec	3	-	-	-	-	-	-	3
sed	1	-	-	-	-	-	-	1
seg	12	2	.	-	-	-	-	14
sei	7	-	-	-	-	-	-	7
tsst-1	7	1	1	3	1	-	-	13
sea, tsst-1	3	-	-	-	-	-	-	3
sea, sei	1	1	-	-	-	-	-	2
seb, seg	1	-	-	-	-	-	-	1
sed, seg	1	-	-	-	-	-	-	1
seg, tsst-1	2	-	1	1	-	-	-	4
seg, sei	10	5	2	4	-	-	-	21
sei, tsst-1	2	-	.	-	-	-	-	2
sea, seg, sei	2	1	-	1	-	-	-	4
sea, seh, tsst-1	1	-	1	-	-	-	-	2
seb, seg, sei	1	-	-	-	-	-	-	1
sed, seg, sei,	1	-	-	-	-	-	-	1
sea, seb, seg, sei	1							1
sed, seg, sei, sej	1	-	-	-	-	-	-	1

TABLE 4. Grouping of enterotoxigenic *S. aureus* based on the level of the *S. aureus* in the original food

S. aureus count (log CFU ml^{-1})	No of samples positive for 1 or/and > 1 type of *se* genes (%)	Type and distribution of *se* genes								
		sea	*seb*	*sec*	*sed*	*seg*	*seh*	*sei*	*sej*	*tsst-1*
3 – 5	55 (67.1)	7	2	2	2	36	2	26	1	14
>5	27 (32.9)	5	1	1	2	13	-	14	-	10
Total	82	12	3	3	4	49	2	40	1	24

TABLE 5. The distribution of staphylococcal enterotoxin *(se*) genes of *S. aureus* in respect to the different sampling localities

Localities	Total samples collected	Number of samples positive for *S. aureus*	Number of *S. aureus* isolates positive for *se* (%)	Type and number* of *se* identified								
				sea	*seb*	*sec*	*sed*	*seg*	*seh*	*sei*	*sej*	*tsst -1*
Mekelle	147	46	18 (39)	2	1	-	2	7	-	8	1	7
Shire	139	60	24 (40)	1	1	2	-	17	-	4	-	8
Adigudome	52	16	15 (93.6)	3	-	-	-	9	1	11	-	4
Adigrate	43	9	5 (55.5)	1	.	-	1	2	-	1	-	2
Wukro	60	15	11 (73.3)	3	1	-	1	6	1	9	-	2
Hagreselame	21	3	2 (67)	-	-	1	-	1	-	1	-	-
Abi-Adi	34	3	2 (67)	-	-	-	-	2	-	1	-	1
Maichew	53	8	5 (62.5)	2	-	-	-	5	-	5	-	-
Total	549	160	82(51)	12	3	3	4	49	2	40	1	24

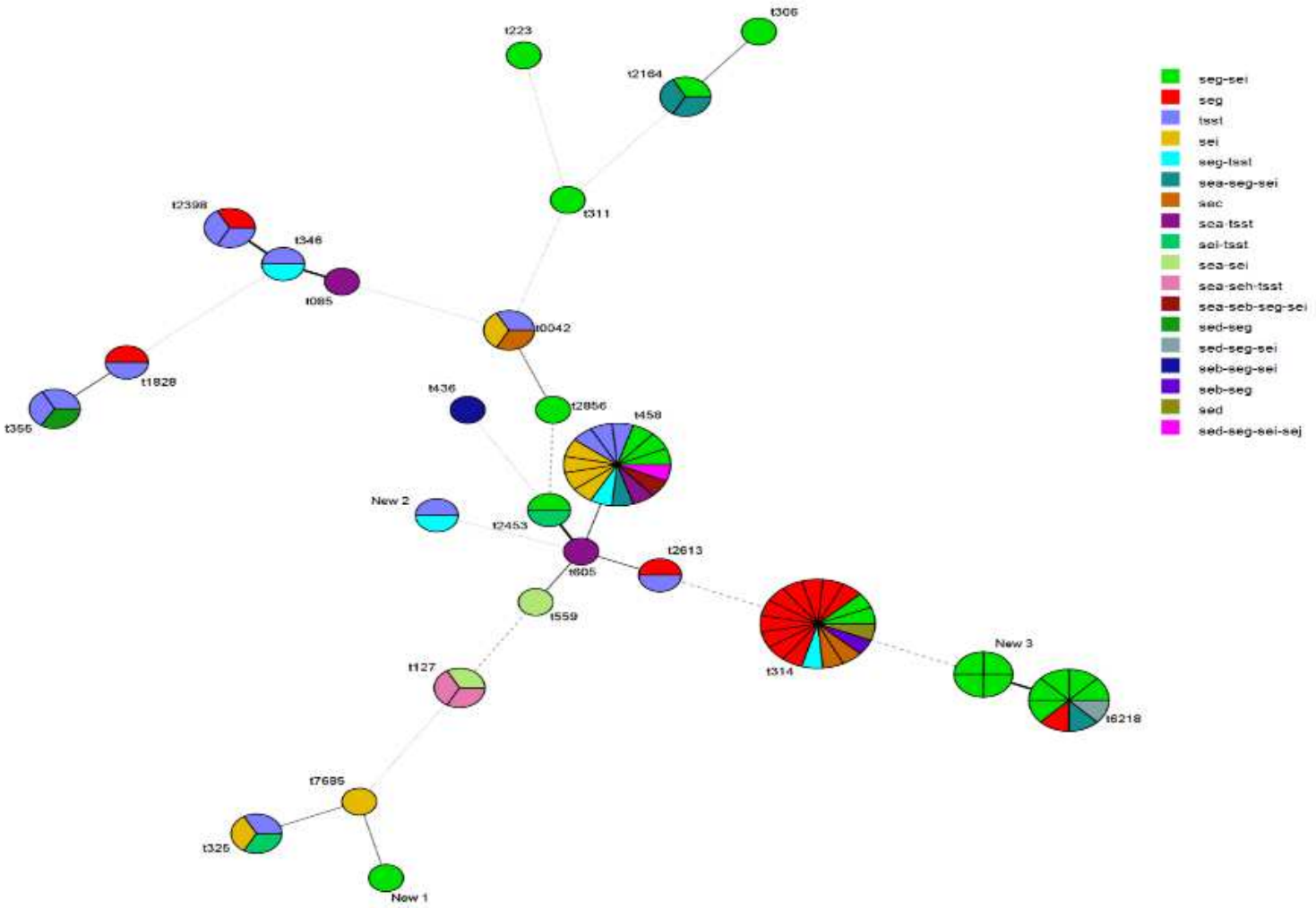
**se* alone or in combination with other *se*, Chi-square value = 19.82, df = 4, p value= 0.0005, df= 4

Table 6. The identified *spa* types in respect to number of isolates within, sample type and their geographical distribution in the studied area

spa types	Isolates N (%)	Sample type		Studied cities/towns							
		Milk	Product	Mekelle	Shire-endaselasse	Adi-gudome	Adi-grate	Wukro	Hagre-selame	Adi-Adi	Mai-chew
t042	3 (3.7)	3	-	1	1	-	-	1	-	-	-
t085	1 (1.2)	1	-	-	-	-	1	-	-	-	-
t127	3 (3.7)	1	2	-	-	2	-	1	-	-	-
t1828	2 (2.4)	1	1	-	-	2	-	-	-	-	-
t2164	3 (3.7)	-	3	-	-	-	-	-	-	-	3
t223	1 (1.2)	1	-	-	1	-	-	-	-	-	-
t2398	3 (3.7)	2	1	2	1	-	-	-	-	-	-
t2453	2 (2.4)	2	-	-	-	2	-	-	-	-	-
t2613	2 (2.4)	2	-	-	2	-	-	-	-	-	-
t2856	1 (1.2)	1	-	-	-	-	-	-	-	-	1
t306	1 (1.2)	-	1	-	-	1	-	-	-	-	-
t311	1 (1.2)	-	1	-	-	1	-	-	-	-	-
t314	17 (20.7)	14	3	2	12	1	-	-	1	-	1
t325	3 (3.7)	3	-	-	2	-	-	1	-	-	-
t346	2 (2.4)	1	1	2	-	-	-	-	-	-	-
t355	3 (3.7)	2	1	-	2	-	1	-	-	-	-
t436	1 (1.2)	1	-	-	1	-	-	-	-	-	-
t458	15 (18.3)	11	4	9	1	2	-	2	-	1	-
t559	1 (1.2)	1	-	-	-	-	-	1	-	-	-
t605	1 (1.2)	1	-	1	-	-	-	-	-	-	-
t6218	8 (9.8)	4	4	-	1	2	1	4	-	-	-
t7685	1 (1.2)	1	-	-	1	-	-	-	-	-	-
New 1	1 (1.2)	1	-	-	-	-	-	-	-	1	-
New 2	2 (2.4)	2	-	-	1	-	1	-	-	-	-
New 3	4 (4.9)	1	3	-	-	2	-	1	1	-	-
Total (%)	82 (100)	57 (69.5)	25 (30.5)	17 (31.7)	26 (31.7)	15 (18.2)	4 (4.9)	11 (13.4)	2 (2.4)	2 (2.4)	5 (6)

58 **Figure 1**

59



PAPER III

Multidrug resistance including borderline-oxacillin resistance and *spa* typing of *Staphylococcus aureus* from milk and milk products of Tigray region, Northern Ethiopia

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Abstract

Antimicrobial resistance is one of the greatest global public health threats and *Staphylococcus (S.) aureus* has marked ability to become resistant to antimicrobials. The objectives of this study were to assess the antimicrobial resistance profile of *S. aureus* from dairy isolates to the commonly used human and veterinary antibiotics, to verify the present or absence of methicillin-resistant *S. aureus* (MRSA) and, in addition, to assess the genetic background of the resistance strains by *spa* typing. One hundred sixty genetically confirmed *S. aureus* isolates from bulk milk and milk products were subjected to 12 types of antibiotics for susceptibility. The Kirby-Bauer disk diffusion was used for phenotypic susceptibility tests and PCR was used for detection of *mecA* and *mecC* genes. Out of these 160 isolates, 137 (86%) isolates showed resistance to one or more types of antibiotics of which 61 (45%) were multidrug resistant (MDR) showing 35 types of resistance patterns. Of the total MDR strains, 61% showed resistance to five or more classes of antibiotics. MDR strains were more frequent in milk (69%) than products (30%). Thirty-two (23%) strains were also borderline oxacillin-resistant *S. aureus* (BORSA), showing oxacillin-resistance phenotypically but with neither *mecA* nor the *mecC* gene carriage. However, they were susceptible to β- lactam inhibitor (amoxycllin/clavulanic acid). The most common resistance was observed for penicillin G (69.4), followed by streptomycin (53.1%) and erythromycin (41.9%). The least common resistance was for ciprofloxacin (4.3%). The *spa* typing of MDR *S. aureus* and BORSA showed 20 *spa* types and 3 novel *spa* sequences, thus indicating a high genetic diversity among the resistant strains. There was no observed pattern or correlation between *spa* types and resistance profiles. Of the total *spa*

types, 12 were identified from milk, 4 from products and only 7 from both samples, indicating that the source of contaminations may be diversified. The spa types t314, t458 and t6218 were the most common and were widely distributed in three localities of the studied area. The study showed a wide spread of MDR *S. aureus* and BORSA with diversified genetic background in dairy food which is of public health concern.

Key words: *S. aureus*, Multidrug resistance (MDR), BORSA, Dairy products, *spa* typing, Ethiopia

Introduction

Staphylococcus (S.) aureus is an ubiquitous organism and may be found in humans, animals and the environment. *S. aureus* causes many health problems in humans and animals due to its toxin-mediated virulence factors, invasiveness and propensity for antibiotic resistance (Ortega et al., 2010). Besides staphylococcal food poisoning and toxic shock syndrome (TSS), which are mediated by exotoxins, the bacterium is responsible for various skin and superficial infections (furuncles, boils) and may also infect internal organs resulting in pneumonia, osteomyelitis and endocarditis (Dinges et al., 2000; Liu et al., 2011). In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) presents a serious health problem due to its resistance to antibiotic treatment. In animals, the bacterium causes subclinical mastitis in dairy cows. This significantly reduces milk production and has also public health importance as *S. aureus* may be transferred to consumers via milk or milk products (Akineden et al., 2011).

Humans and animals are reservoirs of *S. aureus* on their skin and mucosal membranes (Hennekinne et al., 2011). According to Kluytmans and Wertheim (2005), around 10-35% and 20-75% of humans are persistent and intermittent carriers of *S. aureus* , respectively. *S. aureus* carriers are at high risk of infection and could also act as source of infection for others. The main mode of transmission is usually skin-to skin contact but contaminated objects and surfaces may also have a role (Seo and Bohach, 2007).

Antimicrobial resistance is an ever-increasing global public health threat. The WHO 2014 annual report indicated that resistance to common bacteria has reached an alarming level in many parts of the world indicating that many of the available treatment options for common infections in some settings are becoming ineffective (WHO, 2014).

Globally, widely-accepted causes of antibiotic resistance are the over-use and misuse of antibiotics. In developing countries, the situation is escalating since besides the increasing use of antibiotics and readily availability of the antibiotics without prescription, the poor sanitation condition around premises aid in the spread of resistance strains (Levy and Marshall, 2004).

One of the biggest issues in antimicrobial resistance is the use of antibiotics in animals. There is a continuous debate on the association between antimicrobial use in the production of food animals and the emergence of resistant organisms in human populations. In veterinary medicine, antibiotics are given for treatment of contagious and infectious animal diseases including clinical and subclinical mastitis. However, in addition, antibiotics are widely administrated at sub-therapeutic doses to animals as a growth promoter. This practice favors emergence and propagation of large number of resistance genes (Aarestrup, 2005; Marshall and Levy, 2011).

S. aureus is notorious for its ability to become resistant to antibiotics and the development of multidrug resistance in *S. aureus* is a global problem. *S. aureus* can develop drug resistance more readily because of its ability to produce an exopolysaccharide barrier and also their location in the body in micro-abscesses that limit the penetration of the drug (Jeljaszewicz et al., 2000). Of all the resistance traits *S. aureus* has acquired since the introduction of antimicrobial chemotherapy in the 1930s, methicillin resistance is clinically the most important, since a single genetic element confers resistance to the β-lactam antibiotics, which are the most commonly prescribed class of antimicrobials and which include penicillins, cephalosporins and carbapenems (Grundmann et al., 2006).

MRSA is currently a major health problem among hospital patients and health workers. It is an endemic problem globally except in Scandinavian countries and in The Netherlands, where it is controlled by a "search and destroy" strategy (Kluytmans, 2010). In addition to this, a community-associated MRSA is currently emerging which is epidemiologically linked to food animals. The first livestock-associated MRSA was isolated from the milk of a mastitic cow (Devriese et al., 1997) and since then the prevalence of livestock associated MRSA in food animals has steadily increased. There are many reports showing the transfer of antibiotic-resistant microorganisms from food animals to humans. Resistance genes and resistance patterns observed in food animals have been identified in humans, particularly those who have been in contact with the animals, such as veterinarians and farm attendants (Aarestrup et al., 2000; Lee, 2003). Verkade and Kluytmans (2014) reported that MRSA type CC398 is spread extensively in food

114 animals and also in people in close contact with these animals. Direct contact with animals, environmental
115 contamination, as well as eating or handling contaminated food are the main routes by which livestock-
116 associated MRSA could be transferred to humans. Surveillance studies also indicated that the epidemiology
117 of the MRSA has changed in the last 15 years and it has become an emerging organism in livestock settings
118 around the world including Europe, Asia and Northern America (Smith and Pearson, 2011).

119 The methicillin-resistance trait of *S. aureus* is attributed to the penicillin-binding proteins (PBP) 2a protein,
120 which is encoded by chromosomal *mecA* gene (Deurenberg and Stobberingh, 2008a). The presence of *mecA*
121 has been the "golden standard" for detection of MRSA. However recently a novel *mecC* (mecA $_{LGA251}$) gene,
122 which has 70 % nucleotide homology with the conventional *mecA*, has been investigated from MRSA of
123 bovine and human isolates which typically have a livestock lineage; CC130, CC705 and ST 425 (García-
124 Álvarez et al., 2011). The epidemiology of the isolates was associated with rural areas and livestock settings
125 (Petersen et al., 2013).

126 Some *S. aureus* strains have neither *mecA* nor *mecC* gene but nevertheless show resistance to methicillin
127 and oxacillin. McDougal and Thornsberry (1986) have reported the presence of such *S. aureus* strains,
128 which exhibit total resistance to penicillin and partial resistance to oxacillin, methicillin and cepahlospores
129 and yet do not have the *mecA* gene. They named them as borderline oxacillin-resistant *Staphylococcus*
130 *aureus* (BORSA). According to Brakstad and Mæland (1997), the main mechanism by which these strains
131 develop resistance to methicillin/oxacillin is due to hyper-production of β-lactamase enzyme.

132 It is important to differentiate heterogeneous MRSA from BORSA, in that the former has *mecA* gene but
133 does not express the gene during growth and hence is phenotypically susceptible to oxacillin. On the other
134 hand, BORSA do not have the *mecA* gene but showed full or intermediate oxacillin resistance
135 characteristics (Coyle, 2005). There are reports that indicate the occurrence of BORSA in food and clinical
136 isolates. In Portugal, out of 56 phenotypically oxacillin-resistant *S. aureus* of food isolates, all were *mecA*
137 negative except one (0.68%) (Pereira et al., 2009). In Tunisia, Maalej et al. (2012) reported the identification
138 of 23 BORSA from clinical isolates.

Molecular typing of *S. aureus* is important for many reasons. From a molecular epidemiology perspective, typing of *S. aureus* is important in order to trace the source and understand transmission during epidemiological disease outbreak investigations and surveillance studies. Besides, it is important to follow the phylogenetic evolution of the strains and understand their genetic relatedness or diversity (Koreen et al., 2004; Stefani and Agodi, 2000). There are two main molecular typing methods: "band-based" and "sequence-based" The sequence-based method like that of *spa* typing is preferable, because data is exchangeable among researchers and institutions (Deurenberg and Stobberingh, 2008b). *spa* typing depends on the polymorphic X-region of the protein A gene (*spa*) which is composed mainly of a number of repeats of 24 bp in length. The region shows polymorphism due to point mutation, deletions and duplication of the repeats (Kahl et al., 2005; Shopsin et al., 1999). It became a popular and preferable method for local short-term epidemiological studies because of its comparable performance with pulse-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) methods and yet rapid and low cost (O`Hara et al., 2016; Strommenger et al., 2008). The PFGE, MLST, staphylococcal cassette chromosome (SCC)*mec* (International Working Group, 2009) and *spa* typing are widely employed for characterization of *S. aureus* to understand the resistance pattern and molecular epidemiology in dairy and other foodstuffs (Feltrin et al., 2016; Haran et al., 2011; Vossenkuhl et al., 2014).

There is a major gap in knowledge about the magnitude of antibiotic resistance problem globally and such information is needed to guide urgent public health action (WHO, 2014). In light of this, there is no documented data on resistance profile of *S. aureus* and MRSA in food value chain, particularly in food of animal origin in Northern Ethiopia. The status of antibiotic resistance profiles of *S. aureus* including community associated MRSA in food of animal origin such as milk and milk products is not studied. Except very few veterinary medicine-related preliminary antimicrobial resistance studies focusing on treatment of mastitis there is, to date, not sufficient data on the antimicrobial resistance profile of *S. aureus* including MRSA from bulk raw milk and dairy products in the Tigray region, Northern Ethiopia.

Hence, the objectives of this study were: i) to assess the antimicrobial resistance profile of *S. aureus* isolated from milk and milk products to the commonly-used antibiotics in veterinary and human medicine in the project area ii) to verify the presence or absence of MRSA in the isolates. iii) to assess the genetic background of multidrug resistant strains using *spa* typing.

Material and methods

Bulk milk and dairy product samples

A total of 549 bulk raw cows milk and milk products were collected from the Tigray region, Northern Ethiopia, from August 2012 to May 2014. The samples comprised raw bulk milk (n=297), buttermilk (n=64), butter (n=58), sour milk (spontaneously fermented milk, n=97), Ethiopian cottage cheese (n=14), cheese (n=15) and cake made from milk (n=4). They were collected from eight purposely-selected cities/towns of the region, namely Mekelle (n=147), Shireendaselase (n=139), Wurko (n=60), Adigudome (n=52), Hagreselame (n=21), Maichew (n=53), Adigrate (n=43) and Abi-adi (n=34). The sampling points included dairy farms, milk collection centers, milk and milk product vendors, restaurants and cafeterias.

Isolation and molecular identification of S. aureus

The isolation and molecular identification of the isolates to species level were performed as described by Tarekgne et al. (2015). Briefly, Baird-Parker agar (Oxoid, England) supplement with egg-yolk tellurite (Merck, Germany) was used for isolation and conventional biochemical tests were employed for identification. Final confirmation to species level was carried out by sequencing the 16S rRNA gene (Clarridge, 2004). DNA was extracted by GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed on isolates of *S. aureus* from milk and milk products using the disc diffusion according to the Bauer and Kirby method (Coyle, 2005) using Muller-Hinton agar (Oxoid, CM0405B, UK). The isolates were tested for 12 antibiotics which had been reported as being commonly in use in the project area, confirmed by a pharmaceutical wholesaler and veterinary team of the region. Accordingly, the following antibiotics were tested, obtained from Oxoid and Remel (UK): Penicillin G (10 units), Oxacillin (1μg), Ceftiofur (30 μg), Vancomycin (*30* μg), Amoxicillin/Clavulanic acid (30 μg), Gentamicin (*10* μg), Streptomycin (10 μg), Tetracycline (30 μg), Erythromycin (*15* μg), Ciprofloxacin (*30* μg), Norfloxacin (5 μg), and. Trimethoprim-Sulfamethoxazole (*25* μg). The procedures recommended by National Committee on Clinical Laboratory Standards (NCCLS, 2005) for antimicrobial susceptibility testing was followed. Accordingly, each *S. aureus* isolate was grown on tryptic soya agar (Remel, UK) for 18 hours. From the fresh growth of each isolate, 2-5 colonies were taken to prepare a suspension in a saline water and the turbidity was adjusted to 0.5 McFarland standard using a spectrophotometer (HACH-Company, USA). A petri dish of Muller-Hinton agar was inoculated with the colony suspension using a fresh sterile cotton tipped swab. To each inoculated plate, 4 antibiotics discs were applied using a disc dispenser (Oxoid). The plates were incubated at 37 $^{\circ}$C for 16- 18 hours (24 hours for Oxacillin) and the zone of inhibition was measured and compared to standards as per the manufacturer's recommendation. Results were recorded as susceptible, intermediate and resistant according to the size of the zone of growth inhibition.

Detection of mecA or mecC genes/Search for MRSA

For phenotypically oxacillin-resistant *S. aureus* strains, a conventional PCR was performed to detect the presence of either *mecA* or *mecC* genes, as the presence of *mecA* or *mecC* has been the "golden standard" for detection of MRSA.

For *mecA* gene detection the utilized primers were: 5'-CTCAGGTACTGCTATCCACC-3' (upstream) and 5'-CCTTGGTATATCTTCACC-3' (Downstream) described by Hedin and Løfdahl (1993) with expected 449 bp product and for *mecC* gene the primers 5'-GAAAAAAAGGCTTAGAACGCCTC-3'

(mecA$_{LGA251}$MultiFP) and 5'GAAGATCTTTTCCGTTTTCAGC-3' (mecA $_{LG251}$ MultiRP) recommended by Stegger et al. (*2012*) with expected PCR product of 138 bp. The PCR was performed with final volume of 20 µ*l* that contain 10 x PCR buffer, 25mM of dNTP, 0.4 µM of each primer, and Taq Polymerase and 1 µ*l* of genomic DNA. The program was adjusted to run at 95 °C for 15 min, and 30 cycles of 95 °C for 25 sec., 56 °C for 30 sec. 72 °C for 30 sec. and finally 72 °C for 5 min.

Control strain

A penicillin-susceptible (ATCC 25923), penicillin-resistant but oxacillin-susceptible (ATCC 29213) and oxacillin-resistant (ATCC 43300) *S. aureus* strains were used as controls in the antimicrobial susceptibility test. The *S. aureus* strains 2012-50-2037 and ATCC4330 were use as positive controls for detection of *mecC* gene and *mecA* gene, respectively.

spa typing of the MDR *S. aureus* isolates. Amplification of the region X of the *spa* (protein A) gene was performed using primers spa -1113f (5′AAAGACGATCCTTCGGTGAGC-3′) and *spa* -1514r (5′-CAGCAGTAGTGCCGTTTGCTT-3′) (Ridom, 2004). PCR was performed in 40 µL final volume containing 1X of buffer HF, 0.4 µM of each primer, 200 µM of dNTPs and 0.02 U/µL of iProof™ taq polymerase (Biorad, Hercules, CA, USA). The PCR reaction for amplification of the DNA was: initial denaturation at 98 ºC for 30 sec, followed by 35 cycles of denaturation at 98 ºC for 15 sec, annealing at 60 ºC for 30 sec and elongation at 72 ºC for 20 sec. Final elongation was performed at 72 ºC for 10 min. The PCR was purified using 1X of Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA) according to manufacturers instruction and submitted to GATC-biotech AG (GATC-biotech AG, Konstanz, Germany) for sequencing. Sequences were processed using Geneious V7 (Kearse et al., 2012) and the *spa* types were determined using DNAgear (AL-Tam et al., 2012). Minimum spanning trees were prepared using BioNumerix software as described by (Roussel et al., 2015).

Statistical analysis: Microsoft Excel (Windows version 8.1 version) was utilized for descriptive analysis of the data and to generate the box plot that show the prevalence of antibiotic resistance in the studied area. Epi info™ version 7.1.3.10 (CDC, Atlanta, USA) was utilized to calculate rates with confidence intervals (CI) which were required for the box plot.

Results

S. aureus was isolated from 160 (29.1%) of the 549 milk and milk product samples: raw milk (n=100), butter milk (n = 28), butter (n = 10), sour milk (n = 16), cheese (n = 1), Ethiopian cottage cheese (n = 3) and cake made from milk (n=2).

Antimicrobial susceptibility test

The 160 *S. aureus* food isolates were tested for 12 antibiotics and 137 (86%) strains showed resistance to one or more types of antimicrobial agents. Among these 137 resistant strains, 22 (16%) were resistant to one type of antibiotic, 54 (16%) to two types/classes of antibiotics and 61 (45%) to three or more classes of antibiotics. Among the antimicrobial agents tested, the resistance most frequently observed was for penicillin G (69.4%) followed by streptomycin (53.1%), and erythromycin (41.9%). Less frequent resistance was observed for ciprofloxacin (4.3%) and amoxicillin/clavulanic acid (4.4%). Oxacillin resistance was observed in 32 (20%) *S. aureus* isolates, among which 28 (17.5%, 28/160) were fully resistant and 4 (2.5%, 4/160) showed intermediate resistance (Table 1). According to the description by Wendlandt et al. (2015) the 61 (45%) *S. aureus* were considered as multidrug resistant (MDR) because they showed resistance to three or more classes of antibiotics. From the 61 MDR strains, 35 types of resistance patterns were observed, of which 24 (39%) showed resistance to 3 - 4 classes of antibiotics and 37 (61%) to 5 or more classes of antibiotics (Table 2). Among the 61 MDR *S. aureus* isolates, 69% (42/61) were from bulk milk and 31 % (19/61) from milk products (Table 3).

Detection of mecA and mecC genes

To confirm the presence of MRSA, DNA from the 32 strains of phenotypically intermediate/full oxacillin-resistant *S. aureus* was subjected to PCR for detection of either *mecA* or *MecC* genes. None of the strains were positive for these genes. However, analysis of the phenotypic results of each isolate to each antibiotic revealed that of the 32 strains of intermediate/fully oxacillin resistant *S. aureus*, 28 (87%) were fully susceptible to amoxycllin/clavulanic acid. Such strains of *S. aureus* are named as borderline oxacillin-resistant *Staphylococcus aureus* (BORSA). According to McDougal and Thornsberry (1986), BORSA exhibits resistance to lactam group antibiotics such as oxacillin and methicillin by an extrinsic non-*mecA* mediated mechanism. BORSA develop intermediate or full resistance to oxacillin due to mainly high production of β-lactamase that could partially hydrolysis the lactam group.

The distribution of the 32 BORSA in milk and milk product samples and in the different localities of the studied areas is presented in Table 4. Among the total 32 BORSA isolates, 91 % (29/32) were with MDR characteristics.

Antimicrobial resistance profile in different samples

Among the 137 *S. aureus* strains that exhibited resistance to one or more antimicrobial agents, 86 (62.8%) were from raw bulk milk, 14 (10.2%) from sour milk, 25 (18.2%) from butter milk, 9 (6.6%) from butter, 1 (0.7%) from Ethiopian cottage cheese, 1 (0.7%) from cheese, 1 (0.7%) from cake made from milk.

Antimicrobial susceptibility profile in different localities

The distribution of antibiotic resistance in strains from different localities of the studied area is presented in Figure 1. Generally, the percent prevalence of antibiotic resistance of the *S. aureus* in the studies localities is high. It ranged from 61% in Mekelle to 100% in Shireendaselasse.

Spa typing

Spa typing of MDR isolates: spa typing of the 61 MDR *S. aureus* isolates identified 20 *spa* types and 3 novel *spa* sequences (not available in the Ridom data base). Table 3 presents the distribution of the 23 *spa*

types and sequences in milk and milk product samples as well as in different localities of the studied area. The most common *spa* types identified were t314 (25%, n=15), t458 (18%, n=11) and t6218 (7%, n=4) . Out of the 23 *spa* types, only 7 *spa* types (t127, t1828, t2613, t314, t355, t458, and t6218) were common to both bulk milk and products. The remaining 16 *spa* types were found distributed either in bulk milk (n=12) or milk products (n= 4) samples. The minimum spanning tree (Figure 2) shows the genetic relatedness of the identified *spa* types to each other and distribution of antibiotic resistance pattern (the 35 identified resistance pattern) on each *spa* type. The distance between the circles showed the genetic relatedness. The nearer they are to each other, the greater their genetic relatedness. For example, the most common *spa* type, t314, is genetically more related to *spa* sequence t3 and t2613 than to t355 and t186, which are at far distance. The color inside the circles showed the different antibiotics profiles. There is no clear pattern or relationship between *spa* types and antibiotics resistance patterns. The size of the circles represent the number of isolates within the *spa* types.

The most common *spa* types, t314, t458 and t6218 were more prevalent in Shireendaselase (n=11), Mekelle (n=7) and Wukro (n=3) of the studied area, respectively.

spa typing of BORSA: The identified *spa* types in BORSA strains, their distribution in the samples and in the studies areas is presented in Table 4. Totally 16 *spa* types and 3 spa sequences were identified. The t458, t2613 and t314 were the common *spa* types identified and distributed mainly in Mekelle and Shireendaselasse localities. Out of the total identified BORSA, 63% were from bulk milk and 37 % from products.

Discussion

The present study showed that 86 % of *S. aureus* from dairy food were resistant to different antibiotics, out of which 45 % were MDR having 35 different resistance patterns with diversified genetic background. This may impose public health risk to the consumers as antimicrobial resistance could transfer to humans via the food chain. The transfer of antimicrobial resistance could occur through antibiotic residues in food, through

the transfer of resistance food-borne pathogens, or through ingestion of resistant strains of the original food microflora and then resistance transfer to pathogenic microorganisms (Khan et al., 2000; Kruse and Sørum, 1994; Mayrhofer et al., 2004; Pesavento et al., 2007). A MDR *S. aureus* in a dairy food may be ingested and colonize in the intestinal tract of the consumer, particularly of immunocompromised people (Vesterlund et al., 2006). The resistance genes may further transfer to the commensal gut microflora via horizontal gene transfer. The majority of genes responsible for antibiotic resistance are carried by mobile genetic elements (MGE) and transfer at high frequency between isolates is common (Levy and Marshall, 2004; Lindsay, 2013). This condition constitutes a high risk as commensal intestinal bacteria could act as a reservoir of resistance genes for pathogenic bacteria. Furthermore, when the number of resistant bacteria in the intestine is high, they may disseminate to the outside via feces and contaminate the environment (Andremont, 2015; van den Bogaard and Stobberingh, 2000). Stiefel and Donskey (2004) reported that there is a strong evidence that colonization with resistant bacteria such as *S. aureus* plays an important role in the dissemination of resistance to other bacterial populations.

It is well documented that *S. aureus* causes a wide range of serious diseases in humans that range from sepsis and abscess formation to endocarditis and toxic shock syndrome (Astiz and Rackow, 1998; Ferry et al., 2005; Libman and Arbeit, 1984; Lowy, 1998). With this risk, the emergence of multidrug resistant *S. aureus* may lead to increased morbidity and mortality due to therapeutic failure. From a veterinary medicine perspective, the emergence of MDR *S. aureus* may have significant therapeutic impact, as the bacteria is responsible for many animal diseases including clinical and subclinical mastitis. Mastitis is the major health problem in the majority of dairy farms of the country including in the studied area (Alemu et al., 2014; Duguma et al., 2014; Tolosa et al., 2015). Failure of treatment leads to high economical losses and other public health consequences.

Literature on resistance profiles of *S. aureus* from dairy foods generally in Ethiopia and specifically in the studied area is scant. However, a recent antibiotic resistance study of *S. aureus* from milk and milk products in Central Ethiopia reported that 57 % of the isolates were resistant to more than one type of antibiotic and

the highest resistance was documented for penicillin (83%) and the lowest for amoxicillin/clavulanic acid (3%) (Tigabu et al., 2015). Another study on antimicrobial susceptibility of *S. aureus* from cow milk samples reported 45% MDR *S. aureus* strains and the highest resistance was documented for penicillin G (92.2%) followed by tetracycline (66.7%) (Mekuria et al., 2013). On the other hand, hospital-associated MRSA prevalence was estimated at 55 % in Ethiopia (Falagas et al., 2013).

Likewise, in other countries, there are many reports that show the emergence of antibiotic resistant *S. aureus* from milk and dairy products. In Italy, 68.8% of *S. aureus* isolates from dairy and other foods were found resistant to at least one type of antibiotics (Normanno et al., 2007). Sasidharan et al. (2011) reported the present of antibiotic resistance *S. aureus* in three dairy food products and pointed out a need for a preventive strategy as antibiotic resistance could be transferred to human consumers. In South Africa 59% of *S. aureus* isolates from bulk milk were MDR (Akindolire et al., 2015). A recent study conducted in Iran (Jamali et al., 2015), reported also that 5.8% and 2.1% of the *S. aureus* isolates from milk and dairy products were resistant to gentamicin and streptomycin, respectively. Differences in the prevalence of antibiotic resistance, as reported in the above studies, may be attributed by many factors among which the frequency and manner of antibiotics use as well as the sanitation condition prevailing in the study areas may influence the development of resistant strains in a given geographical area (Levy and Marshall, 2004).

In the present study, 32 phenotypically oxacillin-resistant *S. aureus* carried neither *mecA* nor *mecC* genes which implies that their oxacillin resistance characteristic is not mediated by the conventional PBP 2a, and hence they could not be considered as MRSA. However, all oxacillin-resistant *S. aureus* strains were susceptible to amoxicillin/clavulanic acid, which is β-lactamase inhibitor. Based on these observations, these strains can be considered as BORSA. According to McDougal and Thornsberry (1986) BORSA exhibit resistance to lactam group antibiotics such as oxacillin and methicillin by an extrinsic non-*mecA* mediated mechanism. BORSA develop intermediate or full resistance to oxacillin and other beta lactams such as cephalothin and methicillin due to a high production of β-lactamase that could partially hydrolyse the lactams group (Cavalieri et al., 2005; McDougal and Thornsberry, 1986). Unlike MRSA, when oxacillin

352 is administered in combination with β-lactamase inhibitors, the BORSA become fully susceptible to
353 oxacillin in agar-diffusion testing. This shows that lactamase inhibitors are helpful to differentiate BORSA
354 from MRSA (Chang et al., 1995; Liu and Lewis, 1992; McDougal and Thornsberry, 1986; Montanari et al.,
355 1990; Sierra-Mader et al., 1988). In addition to the hyper-production of β-lactamase, other mechanisms for
356 the phenotypic oxacillin-resistant characteristic of BORSA have been also identified. A plasmid-mediated
357 methicillinase production (Montanari et al., 1996) and modification of PBP that does not bind oxacillin
358 efficiently due to amino acid substitutions in the transpeptidase domain (Nadarajah et al., 2006; Tomasz et
359 al., 1989) were also documented as additional mechanisms for the phenotypic character of BORSA. In the
360 present study, out of the 32 oxacillin-resistant *S. aureus*, 4 (8%) were not susceptible to amoxillin/clavulanic
361 acid. This may suggest that the mechanism by which these strains develop oxacillin-resistance may not be
362 related with hyper-production of β-lactamase but with one or a combination of the aforementioned
363 mechanisms of action. Whatsoever, BORSA is one form of oxacillin resistance pattern. Leahy et al. (2011)
364 suggested that BORSA have developed as a result of antibiotic pressure. As MRSA developed *mecA* gene
365 to resist the antibiotic pressure, BORSA also developed the above-described mechanism to counteract
366 antibiotic pressure (Brakstad and Amæland, 1997).

367 Similar to our study, other researchers including in Ethiopia have reported the presence of phenotypically
368 oxacillin-resistant *S. aureus* without *mecA* gene carriage in dairy foods. Wang et al. (2015) reported that
369 out of 34 phenotypically considered MRSA strains, 28 *S. aureus* strains were without *mecA* gene. They
370 described them as predominant strains in Chinese dairy farms. In South Africa, from 92 phenotypically
371 oxacillin- resistant *S. aureus* from milk isolates, 80% were negative to PBP2a by PBP2a latex agglutination
372 test (Akindolire et al., 2015). In central Ethiopia, 58 phenotypically cefoxitin resistant *S. aureus* from milk
373 isolates were negative for *mecA* gene (Tigabu et al., 2015).

374 From a clinical perspective, BORSA are important strains that are commonly reported in hospital settings.
375 Two human skin disease outbreaks caused by BORSA strains have been documented from Denmark.
376 Molecular typing of the BORSA by pulsed field gel electrophoresis (PFGE) and *spa* typing showed no

genetic relatedness among the lineage (Balslev et al., 2005; Thomsen et al., 2006). As choice of treatment to BORSA depends on its identification, correct laboratory identification of BORSA from the clinical cases is very important (Leahy et al., 2011; Maalej et al., 2012).

Antibiotic resistant *S. aureus* in dairy products may be from two sources; either from the mastitic cow or from a human carrier involved in dairy food production, transportation or processing. *S. aureus* is reported as a common causative agent of clinical and subclinical mastitis and hence prolonged or inappropriate use of the antibiotics for treatment of mastitis my lead to the development of multidrug resistance. Moreover, it is well-documented that humans are carriers of *S. aureus* and a major source of food contaminants (Kluytmans and Wertheim, 2005; Seo and Bohach, 2007). There are reports in Ethiopia and elsewhere that showed that humans carry MDR *S. aureus*. Among school children and other community members from Southwest Ethiopia, 23% (39/169) *S. aureus* from nasal isolates were MDR (Kejela and Bacha, 2013). Mekuria et al. (2013) also reported 33% MDR *S. aureus* isolates from nasal swab of farm workers in Addis Abeba, Ethiopia. In Botswana, out of the 200 food handlers examined, 57% were carriers of MDR *S. aureus* showing resistance to 9 types of antimicrobial agents at different frequencies, including vancomycin (Loeto et al., 2007). Furthermore, contamination of animals by human MRSA carriers has been also documented in a veterinary teaching hospital in USA (Seguin et al., 1999b).

In light of the above facts, the prolonged and misuse of antibiotics are the major problem in developing resistance strains in a given geographical location (Levy and Marshall, 2004). With this perspective, there are many misuse practices of antibiotics in the studied area. The use of antibiotics without prescription and administration of the incorrect dose for the wrong number days by unauthorized personnel is common. The availability of illegal veterinary drugs on the open market encourages farmers to treat their sick animals by themselves, particularly in the Shireendaselasse area where the prevalence of MDR is relatively higher (Dr Abrhame, personal communication, 2016). All these are major misuse practices that increases the prevailing antibiotic resistance problem of the studied area, and this should be addressed. Nevertheless, although the

use of antibiotics as a growth promoter is one cause of development of resistance strains globally, this is not widely practice in the studied area.

spa typing of the MDR *S. aureus* isolates from milk and milk product generated 20 *spa* types and 3 novel *spa* sequences, thus showing the genetic diversity of the isolates. Knowledge of the wide distribution of the common *spa* types, in Shireendaselase (t314), Mekelle (t458) and Wukro (t6218) is important for further molecular understanding of the source, transmission and other epidemiological significant factors of MDR *S. aureus* strains. Such data are of paramount importance with respect to proposals of antibiotics control interventions. From a macro-epidemiological perspective, the documentation of such data is also important in order to understand the phylogenetic lineage of the MDR *S. aureus* isolates from milk and milk products in these geographical areas. In Ghana for example, similar to our report, the *spa* types t314, t311and t355 were identified from MDR *S. aureus* clinical isolates and *spa* type t314 was the most common. (Egyir et al., 2014). The *spa* types t314, t127 and t325 were also reported from five African countries, Cameroon, Niger, Senegal, Madagascar and Morocco but in methicillin susceptible *S. aureus* (MSSA) isolates (Breurec et al., 2011). The *spa* type t127 which is the 5th common *spa* in this study, was registered as one of the top ten-registered clones in the global frequency of *S. aureus* on the Radom *spa* data base server (Ridom, 2004).

The minimum spanning tree analysis of the *spa* types versus the resistance profile of the MDR isolates indicated that there is no specific pattern or relationship between *spa* types and resistance pattern. This showed that risk of specific MDR *S. aureus* attributed by specific *spa* type is not apparent.

According to the *spa* typing results, genetic diversity was observed between bulk milk and milk products. Out of the total 23 *spa* types, only 7 *spa* types were common to both types of samples while the 16 *spa* types were different. This may indicate that the sources of MDR *S. aureus* contamination to bulk milk and milk products may be different.

In contrast to the wide application of *spa* tying for epidemiological investigations, some limitation of the method has been reported such as least discriminatory power (Robinson and Enright, 2004) and some

strains may be designated as “non-typeable” by the method (Votintseva et al., 2014). Moreover, some suggested also that as *spa* typing depends on a single locus, it is preferable to use the method in combination with other virulent gene markers such as SCC*mec* or *se* genes (Hallin et al., 2007).

To conclude, MDR and BORSA *S. aureus* isolates with diversified genetic backgrounds are widely distributed in bulk cows milk and dairy products of the studied areas and this is of public health concern as antibiotic resistance could be transferred to humans through food chain. Furthermore, from a human and veterinary medicine perspective, therapeutic failure due to resistant strains is inevitable. Therefore, measures should be implemented to control the current scenario through education of the community and regulatory action of the government. Such measures should include: educating the community to use antibiotics for human and animal infections only after prescribed by the authorized professional, keeping personal hygiene of personnel involving dairy food production and preparation as well as control of mastitis at farm level.

A regular antibiotic resistance-monitoring program could expose the magnitude of the problem and also intervene with control measures on time when needed. Molecular epidemiological studies to characterize the resistant strains and lineages not only enhance our understanding of the source, spread, and transmission of the resistance genes but also give scientific grounds to propose long-term strategies for the control of antimicrobial resistance at a regional and national level.

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778 Table 1. Susceptibility test results of 160 *S. aureus* dairy isolates to different antimicrobial agents

	Type of the antimicrobial agents	**Number of strains (%)**		
		S	**I**	**R**
1	Penicillin G (P)	49 (30.6)	-	111 (69.**4)**
2	Oxacillin (OX)	114 (71.3)	4 (2.5)	28 (17.5)
3	Ceftiofur (EFT)	124 (77.5)	11 (6.9)	25 (15.6)
4	Vancomycin (VA)	152 (95)	-	8 (5)
5	Amoxicillin/Clavulanic acid (AMC)	153 (95.6)	-	7 (4.4)
6	Gentamicin (CN)	118 (73.7)	2 (1.3)	40 (25)
7	Streptomycin (S)	21 (15)	54 (32)	85 (53)
8	Tetracycline (TE)	115 (71.9)	-	45 (28.1)
9	Erythromycin (E)	56 (35.6)	36 (22.5)	67 (41.9)
9	Ciprofloxacin (CIP)	75 (46.9)	78 (48.8)	7 (4.3)
10	Norfloxacin (NOR)	81 (50.6)	54 (33.8)	25 (15.6)
11	Sulphamethoxazole/Trimethoprim (SXT)	130 (81.2)	12 (7.5)	18 (11.3)

779 S= susceptible, I= Intermediate R= Resistant

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782 Table 2. The observed 35 antibiotic resistance patterns in the 61MDR strains of *S. aureus* isolated from milk and milk products

	Antibiotic resistance pattern	Number of isolates		Antibiotic resistance pattern	Number of isolates
1	P-E-CIP	2	**19**	P-OX-EFT-VA-AMC/CLA-CN-S-TE-E-CIP-NOR	2
2	P-E-NOR	4	**20**	P-OX-EFT-VA-AMC/CLA-CN-S-TE-E-NOR	1
3	P-E-CIP-NOR	4	**21**	P-OX-EFT-VA-CN-S-TE-E-CIP-NOR	2
4	P-E-CIP-NOR-SXT	1	**22**	O-OX-EFT-VA-CN-S-TE-E-CIP-NOR-SXT	1
5	P-NOR-SXT	2	**23**	P-OX-S-CIP-NOR	1
6	P-OX-CN-S-TE-E-CIP-NOR	1	**24**	P-OX-TE-NOR	1
7	P-OX-CN-S-TE-SXT	1	**25**	P-S-CIP	1
8	P-OX-CN-S-E-CIP-NOR	1	**26**	P-S-E	6
9	P-OX-EFT-CN-E-CIP-NOR	1	**27**	P-S-E-CIP	1
10	P-OX-CN-S-E-CIP-NOR	1	**28**	P-S-E-CIP-NOR	4
11	P-OX-EFT-CN-E-CIP-NOR-SXT	1	**29**	P-S-E-CIP-NOR-SXT	2
12	P-OX-EFT-CN-S-E	1	**30**	P-S-TE-CIP	1
13	P-OX-EFT-CN-S-E-CIP-NOR	4	**31**	P-S-TE-CIP-NOR	1
14	P-OX-EFT-CN-S-E-CIP-NOR-SXT	1	**32**	P-S-TE-E	1
15	P-OX-EFT-CN-S-TE-E-CIP-NOR	4	**33**	P-S-TE-E-SXT	1
16	P-OX-EFT-CN-S-TE-E-CIP-NOR-SXT	2	**34**	P-S-TE-SXT	1
17	P.OX-EFT-CN-S-TE-E-NOR	1	**35**	P-TE-SXT	1
18	P-OX-EFT-S-E	1			

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Table 3. The *spa* types and spa sequences identified from 61 MDR *S. aureus* isolates from milk and milk products and their distribution in the studied area

	spa ***types***	***Number of isolates (n)***	***Sample type***		***Distribution of the*** spa ***types in the studied area***							
			Bulk milk	**Milk Products**	**Mekelle**	**Shire-Endaselasse**	**Hagreselam**	**Adigudome**	**Wukro**	**Agigrate**	**Abi-adi**	**Maichew**
1	t042	2	2	--	-	-	-	-	1	1	-	-
2	t085	1	1		--	-	-	-	-	1	-	-
3	t127	2	1	1	-	-	-	1	1	-	-	-
4	t1828	2	1	1	-	-	-	2	-	-	-	-
5	t186	1	-	1	-	1	-	-	-	-	-	-
6	t2164	1	-	1	-	-	-	-	-	-	-	1
7	t223	1	1	-	-	1	-	-	-	-	-	-
8	t2453	2	2	-	-	-	-	2	-	-	-	-
9	t2613	2	1	1	-	2	-	-	-	-	-	-
10	t2856	1	1	-	-	-	-	-	-	-	-	1
11	t306	1	-	1	-	-	-	1	-	-	-	-
12	t314	15	12	3	2	11	1	1	-	-	-	-
13	t325	3	3	-	-	2	-	-	1	-	-	-
14	t346	1	1	-	1	-	-	-	-	-	-	-
15	t355	3	2	1	-	2	-	-	1	-	-	-
16	t436	1	1	-	-	1	-	-	-	-	-	-
17	t458	11	8	3	7	-	-	2	-	1	1	-
18	t605	1	1	-	1	-	-	-	-	-	-	-
19	t6218	4	1	3	-	1	-	-	3	-	-	-
20	t5725	1	1	-	-	1	-	-	-	-	-	-
21	New type 1	1	1	-	-	-	-	-	-	-	1	-
22	New type 2	1	1	-	-	1	-	-	-	-	-	-
23	New type 3	3	-	3	-	-	.1	2	-	-	-	--
	Total	61	42	19	11	23	2	11	7	3	2	2

Table 4. The identified 19 *spa* types and *spa* sequences of the 32 BORSA isolates from milk and milk products and their distribution in the studied area

	spa ***types***	***Number of isolates (n)***	***Sample type***		***Distribution of the*** spa ***types in the studied area***							
			Bulk milk	**Milk Products**	**Mekelle**	**Shire-Endaselasse**	**Hagreselam**	**Adigudome**	**Wukro**	**Agigrate**	**Abi-adi**	**Maichew**
1	t042	1	1	-	-	-	-	-	-	1	-	-
2	t085	1	1	-	-	-	-	-	-	1	-	-
3	t186	1	-	1	-	1	-	-	-	-	-	-
4	t2164	1	-	1	-	-	-	-	-	-	-	1
5	t223	1	1	-	-	1	-	-	-	-	-	-
6	t2453	1	1	-	-	-	-	1	-	-	-	-
7	t2613	3	1	2	-	3	-	-	-	-	-	-
8	t314	3	2	1	1	2	-	-	-	-	-	-
9	t325	1	1	-	-	1	-	-	-	-	-	-
10	t346	1	1	-	1	-	-	-	-	-	-	-
11	t355	2	1	1	-	1	-	-	-	1	-	-
12	t436	1	1	-	-	1	-	-	-	-	-	-
13	t458	8	5	3	5	1	-	1	-	-	1	-
14	t605	1	1	-	1	-	-	-	-	-	-	-
15	t6218	2	1	2	-	-	-	-	2	-	-	-
16	t5725	1	1	-	-	1	-	-	-	-	-	-
17	New type 1	1	1	-	-	-	-	-	-	-	1	-
18	New type 2	1	1	-	-	1	-	-	-	-	-	-
19	New type 3	1	1	-	-	-	-	1	-	-	-	-
	Total	32	20 (63 %)	12 (37%)	8	13	-	3	2	3	2	1

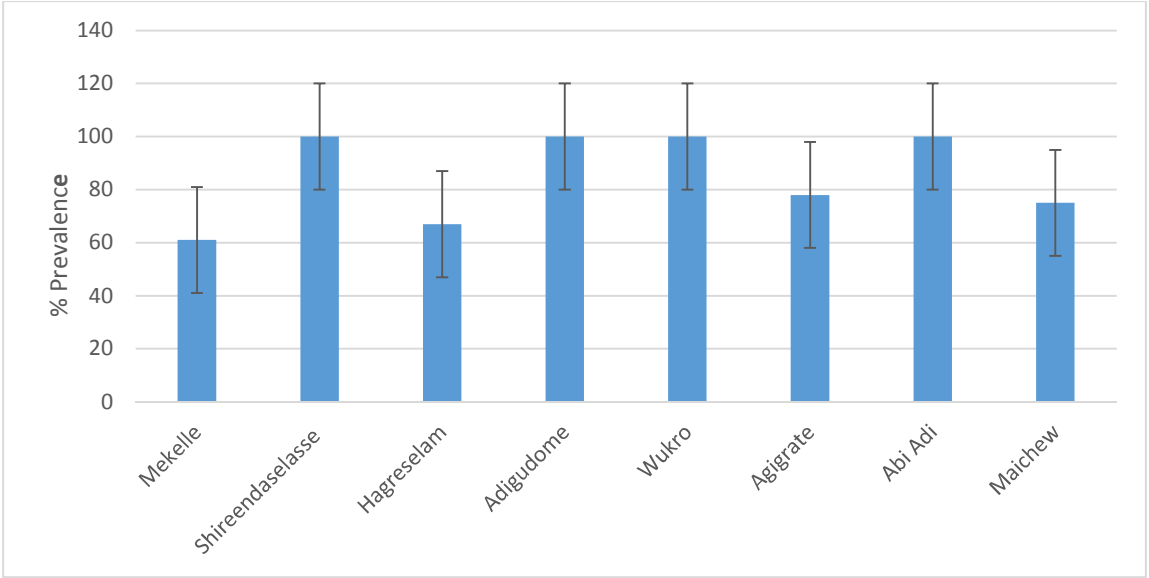

Figure 1. Box plot showing the percent resistance prevalence of *S. aureus* to 12 types of commonly used antibiotics in 8 sampling localities of the studied area. There is no statistically significant difference among the different localities, being high in all areas.

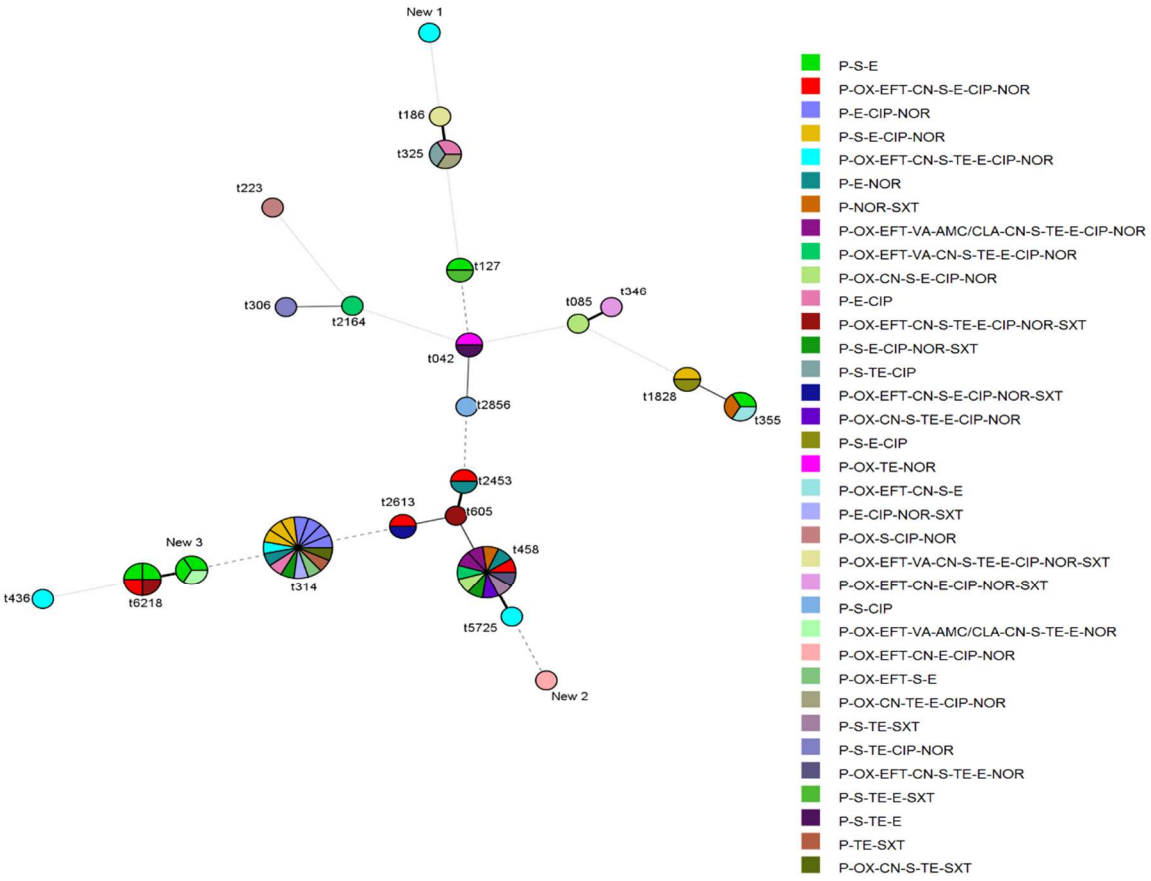

Figure 2. Minimum spanning tree (MST) of 23 *spa* types according to antibiotics resistance pattern of the 61 MDR different resistance pattern are represented by the different colors on the right side of the Figure. The size of the circles represents the number of MDR isolates within the *spa* type and the distance between the circles represent the genetic relatedness. The nearer they are to each other, the greater their genetic relatedness. The colors inside the circles represent the resistance pattern exhibited by the *spa* type

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PAPER IV

Comparison of Real-time PCR Targeting *nuc* Gene with Plate Count Method for Quantification of *Staphylococcus aureus* in Bulk Milk

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Abstract

Staphylococcal food poisoning (SFP) is caused by ingestion of staphylococcal enterotoxins (SEs) which are produced by enterotoxigenic *Staphylococcus aureus* when the cell-population exceeds 5 CFU gram^{-1} in contaminated food. As milk and dairy products are commonly associated with SFP outbreaks, monitoring the *S. aureus* level in such foodstuffs is of public health significance. The objectives of the study were to evaluate the performance of SYBR Green 1 based real-time PCR (qPCR) that targets the *nuc* gene for quantification of *S. aureus,* and to compare the assay with traditional plate count method. The qPCR was applied for quantification of *S. aureus* in artificial and 72 naturally contaminated bulk milk samples collected from Tigray region, Northern Ethiopia.

The primers were able to discriminate *S. aureus* from other species of *Staphylococcus* with a large difference in quantification cycle (Cq) (mean *S. aureus* Cq = 13.83± 0.93; other staphylococci Cq= 30.34 ± 2.65).The standard curve showed 91 % amplification efficiency and 0.98 coefficient of correlation (R^2) that indicated the linearity of the amplification. The detection and quantification limit of the assay was 1.8 copies of *nuc* gene /PCR. The precision of the assay as expressed by standard deviation was 0.12 – 0.3 for intra-assay variation and 0.29 – 0.5 for inter-assay variability.

In artificially contaminated milk, the R^2 between CFU ml^{-1} and *S. aureus* cell equivalent (SCE) ml^{-1} was 0.95, which showed that estimation of CFU ml^{-1} in raw milk by qPCR is possible. In naturally contaminated milk samples higher, statistically significant ($P < 0.05$), SCE ml $^{-1}$count by qPCR was documented than CFU ml $^{-1}$ by plate count method. A quarter of the samples which contained < 5 Log ml^{-1} *S. aureus* by plate count were found to have > 5 Log ml^{-1} by qPCR which has an implication in microbial risk assessment. The study showed that qPCR was reliable, sensitive and faster than the plate count method, which are important factors in microbial food safety risk assessment and other food poisoning related studies.

Key words: *S. aureus*, qPCR, *nuc* gene, bulk milk.

Introduction

Staphylococcal food poisoning (SFP) caused by *Staphylococcus aureus* is an important foodborne disease worldwide. *S. aureus* has many virulence factors and staphylococcus enterotoxin (SE) is one among others, which is responsible for food poisoning. Ingestion of less than 1.0 µg of SE can cause SFP. Milk and milk products are commonly associated with SFP (Cretenet et al., 2011). Enterotoxigenic *S. aureus* can produce SE to a level that can cause illness when the population exceeds 5 Log CFU g^{-1} of contaminated food (Food and Drug Adminstration, 2012).

Quantitative data of *S. aureus* in bulk milk and dairy products during production, processing and storage is of food safety concern as this information reflects the microbial quality of the food. The presence of high numbers of *S. aureus* in the product is an indicator of poor cow health and /or hygiene and hence the possibility of SE production. However, if the numbers of *S. aureus* in the food are low, it does not necessarily indicate the absence of the SE. The large bacterial populations which are prerequisite for production of SE may die during food processing, but the produced SE can survive in the milk and products as SE is thermostable and resistant to different enzymatic actions (Hennekinne et al., 2011; Loir et al., 2003).

Several countries set a microbiological criterion for presence of *S. aureus* in milk and dairy products, to be taken as microbiological standards. For example the European Commission directive 2073/2007, stated that in milk powder and whey powder, < 10 CFU g^{-1} of coagulase positive staphylococci is considered as acceptable level, 10-100 CFU g^{-1} as marginally acceptable and > 100 CFU g^{-1} as an unacceptable level (EU, 2005). The enumeration of *S. aureus* is also important for microbial risk assessment studies of SFP (Postollec et al., 2011). Moreover, in diagnosis of a SFP outbreak, enumeration of *S. aureus* is important as the finding of 5 Log CFU g^{-1} *S. aureus* in the food remnants is confirmatory for SFP outbreak (Hennekinne et al., 2011).

There are two approaches for detection and enumeration of *S. aureus* in milk and milk products; culture-dependent and culture-independent. The conventional culture dependent methods, including the conventional plate count method and most-probable numbers (MPN), are laborious. They require up to 6 days for detection, enumeration and verification of *S. aureus* from the food samples (Alarco'n et al., 2006). Real-time quantitative PCR (qPCR) is a culture independent molecular method that can detect and quantify *S. aureus* and other pathogens from different foods. Compared to the culture- based conventional methods, qPCR is reported to be fast, sensitive and more specific (Postollec et al., 2011), but requires special equipment.

For detection and enumeration of *S. aureus,* several molecular markers have been developed and tested such as the enterotoxin genes (Johnson et al., 1991), 23S rDNA (Straub et al., 1999) and *femA, fmhA* genes (Riyaz-Ul-Hassan et al., 2008). Detection of *S. aureus* based on the *nuc* gene was initially developed by Brakstad et al. (1992) and was the gene widely used as a molecular marker for *S. aureus* from clinical and food samples. However, it has been reported to form primer-dimers and non-specific products when used with SYBR Green I in qPCR assay (Hein et al., 2001b). To solve this problem, a new SYBR Green-based primer that targets the *nuc* gene has been developed, tested and reported to be effective (Alarco'n et al., 2006). As SYBR Green I is cheaper and the designed primers are reported to be appropriate, it is important to evaluate and assess this qPCR protocol, as an alternative approach to the conventional culture-based method, in the local food matrixes and local strains of the studied area.

The objectives of this study were to evaluate the performance of SYBR Green I based qPCR that targets the *nuc* gene for quantification of *S. aureus* in both artificially and naturally contaminated bulk milk from the Tigray region, Northern Ethiopia, and to compare the assay with the conventional plate count method.

Material and methods

Milk and milk product samples

A total of 310 dairy food samples were collected from the Northern part of Ethiopia with the objective of detection and enumeration of *S. aureus*. Out of the 310 samples, 120 (38.7%) were positive for *S. aureus* using plate count method. From the *S. aureus* positive samples, 72 bulk milk samples were randomly selected and subjected to qPCR assay, in order to quantify the *S. aureus*.

The isolation, identification and enumeration of *S. aureus* using the plate count method was performed as detailed in Tarekgne et al. (2015). Briefly, Baird-Parker agar (Oxoid, England) supplemented with egg yolk tellurite (Merck, Germany) was used for isolation and enumerations of *S. aureus*. Final identification of the isolates to species level was done by sequencing the 16S r RNA gene. After the conventional microbiological work, samples were frozen at – 20 $^{\circ}$C for subsequent molecular qPCR assay.

The qPCR assay

Bacterial isolates and strains used in the study: The *S. aureus* strain MSSA 476 (accession no. BX571857) purchased from National Veterinary Institute, Debre-Zeit, Ethiopia, was used as reference strain for the study. This strain was used for artificial contamination of milk, preparation of a standard curve, and in all cases as positive control.

For the specificity study, strains representing 12 species of *Staphylococcus*, including *S. aureus*, which had been previously isolated from dairy products of the Tigray region were used. *S. aureus* (n=15), *S. epidermidis* (n= 20), *S. warneri* (n=3), *S. cohnii* (n=10), *S. hemolyticus* (n=6), *S. carnosus* (n=2), *S. sciuri* (n=5), *S. hominis* (n=3), *S. devriesei (n=1), S. chromogenes* (n=1), *S. saprophyticus* (n=1) and *S. caprae* (n=1). All were confirmed to species level by 16S rRNA gene sequencing.

Artificial contamination of raw milk

Raw milk from a healthy cow was collected and confirmed to be free from *S. aureus* by conventional microbiological and molecular methods. The milk was then inoculated with the reference *S. aureus* strain

and subjected to microbiological and molecular analysis as per the method described by Hein et al. (2005) with some modifications. Briefly, the reference *S. aureus* strain was cultured in BHI broth (Oxoid, England) at 37 °C for 15 hr. From this broth, tenfold dilutions ranging from 10^{-1} to 10^{-5} were prepared in peptone water (Sigma-Aldrich, Switzerland). From each dilution, 100 μl was inoculated in duplicate onto Baird-Parker agar (Oxoid) supplement with egg yolk tellurite (Merck, Germany) and incubated at 37 °C for 48 hours. The Log CFU ml^{-1} of each dilution was then calculated. From each dilutions of the culture, 100 μl was added to each 900 μl of raw milk. The inoculated raw milk samples were subjected to DNA extraction in triplicate and the *nuc* gene copy number was measured twice in each sample by qPCR.

DNA extraction from pure cultures of S. aureus and other Staphylococcus species

DNA was extracted from overnight cultures of *S. aureus* and other species of *Staphylococcus* in BHI using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction with some modification. One ml of the broth was centrifuged at 16 000 *x g* for 2 min. and the pellet was washed twice with 0.9% NaCl. The content was homogenized with 200 μl of lysosome solution and incubated at 37 °C for 90 min. To this suspension, 20 μl of RNAase and Proteinase K was added followed by 200 μl of lysis solution C and incubated at 55 °C for 60 min. The lysate was homogenized with 200 μl of ethanol to precipitate the DNA and transferred into a new binding column and centrifuged at 4000 x g for 1 min. The content was washed with washing solution I. Finally, the column was put into a new 2 ml tube and the DNA was harvested using 2x 50 µl elution buffer solution. The concentration and quality of the DNA was determined by NanoDrop ND-2000 spectrophotometer (Thermo Fischer Scientific inc., Waltham, MA, USA). The genomic DNA was stored at -20 °C until use.

DNA extraction from artificially and naturally contaminated milk samples

Each frozen milk sample was defrosted at room temperature and thoroughly mixed to obtain a homogenous suspension. Four ml of milk was added to 6 ml of sterile 2 % w/v sodium citrate in a 15 ml Eppendorf tube

and centrifuged for 5 minutes at 16 x g at 4 °C. As much of the fat layer as possible was removed using a sterile swab tip and the supernatant was transferred into a new 10 ml Eppendorf tube and pelleted at 4500 x g for 15 minutes at 4 °C and then transferred into 1.5 ml Eppendorf tube. The content was washed twice with 1 ml 2% w/v sodium citrate solution by centrifuging at 16000 *x g* for 2 min. at 4 °C and subjected to DNA extraction with GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as described above.

Primers for quantitative PCR assay (qPCR)

Primers (F372/R465) that targeted the *nuc* gene of *S. aureus* designed by Alarco'n et al. (2006) for qPCR, were used. The oligonucleotide sequences of the primers were verified by BLAST algorithm in Genbank.

F 5`TGTAGTTTCAAGTCTAAGTAGCTCAGCAA 3`, (F372) and

R 5`TGCACTATATACTGTTGGTCTTCAGAA 3 ` (R465). The expected amplicon size was 94bp which was verified by electrophoresis in 2% agarose gel.

Specificity study

To assess the specificity of the primers, the DNA of the isolates was standardized to 5 ng/µl concentration and subjected in duplicate to the qPCR assay using the same equipment and reaction conditions as previously described. Milli-Q water was used as negative control. The mean Cq and melting temperature (TM) with standard deviation was calculated.

Quantification Standard for qPCR

DNA was extracted from 1 ml of the pure culture of *S. aureus* using the procedures described above. After quantification of DNA concentration by Qubit® 2.0 Fluorometer assay (Invitrogen, USA), tenfold dilutions with milli-Q water, containing 1.8×10^1 to 1.8×10^5 copies of *nuc* gene were prepared assuming that 1 ng of DNA equals 6×10^5 times the entire genome and that the *nuc* gene is a single-copy gene (Hein et al.,

2001a). Each dilution was subjected to qPCR assay in triplicate. The slope (s) of the standard curve was used for calculation of the PCR efficiency (E) using the equation $E = 10^{-1/s} - 1$.

Detection limit

Three tenfold dilutions with milli-Q water containing 1.8 copies of *nuc* gene (0.000003 ng/µl), 1.8×10^{1} copies of *nuc* gene (0.00003 ng/µl) and 1.8×10^{2} copies of *nuc* gene (0.0003 ng/µl) were prepared from the reference *S. aureus* genomic DNA extracted from pure culture, as well as from artificially inoculated milk. The genomic DNA was quantified by Qubit® 2.0 Fluorometer assay (Invitrogen, USA). Each dilution, from both of the two sources, was subjected to qPCR assay in ten replicates. The number of positive signals exhibited in each dilution, as well as mean and standard deviation of the quantification cycle (Cq) were documented. For the dilution that was considered to be the limit of detection, the relative standard deviation (RSD) of the DNA measurement of the ten replicates was calculated.

Repeatability (Intra-assay) and reproducibility (Inter-assay)

To assess the precision of qPCR, three dilutions with Milli-Q water, containing 1.8×10^{4} copies of *nuc* gene (0.03 ng/µl), 1.8×10^{5} copies of *nuc* gene (0.3 ng/µl) and 1.8×10^{6} copies of *nuc* gene (3 ng/µl) were prepared by Qubit® 2.0 Fluorometer assay (Invitrogen, USA). Ten replicates of each of the three dilutions were subjected to qPCR to determine intra-assay variance (repeatability) whereas three independent assays were performed to document the inter-assay variability (reproducibility) of the qPCR. For all runs the mean Cq and standard deviation (SD) were calculated.

Quantitative qPCR amplification conditions

All samples were quantified by qPCR in duplicate. The qPCR was run with a final volume of 20 *µl.* The LightCycler® 480 SYBR Green I Master mix (Roche, Mannheim, Germany) containing FastStart Taq DNA polymerase, reaction buffer, d NTP mix, SYBR Green I dye and $MgCl_2$ was utilized. Further, 300 nM of each primer and 2 *µl* of genomic DNA was added. PCR-grade water (Roche, Mannheim, Germany) was used to adjust the final reaction volume. The reference *S. aureus* (positive control) and PCR-grade H_2O

(negative control) were included in all qPCR runs. All amplification was conducted in LightCycler ® 480 Instrument (Roche, Mannheim, Germany) in 96 well plates and sealed with sealing foil (Roche, Mannheim, Germany).

The LightCycler ® 480 Instrument was programmed as follows. Pre-incubation of 1 cycle at 95 $^{\circ}$C for 5 min. Amplification of 35 cycles with denaturation at 95 $^{\circ}$C for 15 sec, annealing at 60 ^{0}C for 1 min. and elongation at 72 $^{\circ}$C for 5 sec. Melting curve of 1 cycle at 95 ^{0}C for 5 sec. 65 $^{\circ}$C for 1 min. and continuous at 97 $^{\circ}$C and cooling at 40 $^{\circ}$C for 10 sec.

Quantitative qPCR results were expressed as the increase in fluorescence signals of the reporter dye, SYBR Green I, detected and visualized by the LCS480 1.5.1.62 software (Roche).

At the end of each run, the melting curves generated by the LCS480 1.5.1.62 software were analyzed in order to determine the melting point of the amplicons.

The absolute quantification method was applied in this assay. This method depends on comparison of the Cq value of the samples with the standard curve generated from amplification of the reference *S. aureus* as described previously. In every qPCR a new standard curve was included.

Data analysis: Microsoft Excel for Windows (MS office version 8.1 version) was used as database. All counts were transformed into log value before further analysis. Descriptive analysis of the data was conducted with the Microsoft Excel program. The student's paired t-test from The Unscrambler X version X 10.3 software were used to compare the plate count with the qPCR quantification data. Before analysis, all the data were checked for possible outliers. P-value < 0.05 was considered as statistically significant.

Results

Standard Curve: A typical standard curve generated from tenfold dilution of 1.8 X 10^{1} to 1.8 ^{5} copies of the *nuc* gene is shown in Fig 1. It has a slope of -3.354, which gives an amplification efficiency of 91.4%. The

coefficient of correlation between Cq value and copy number was calculated as 0.98, showing the linearity of the quantification.

Specificity study: The specificity of the primers that targeted the *nuc* gene were studied, with an optimized qPCR reaction on *S. aureus* and other *Staphylococcus* spp. of dairy isolates. The result showed that the overall mean Cq value for *S. aureus* was 13.83 ± 0.93 (13.08 ± 0.18 for the reference strain) and for the other 11 *Staphylococci* species the overall mean Cq value was 30.34 ± 2.65. The mean Cq value for each species including *S. aureus* is presented in Fig 2. The mean melting temperatures of the amplicon for *S. aureus* was 77.49 ± 0.04 ^{O}C while for other *Staphylococci* species was 77.71 ± 0.33 ^{O}C.

The limit of detection (LOD): There was no difference in the LOD between the pure culture and milk extracted DNA. Out of the 10 replicates (in both sources) of 1.8 x 10^2 copies of *nuc* gene, 10 showed positive signal with mean Cq value of 29.09 ±, 0.22 1. Of the 10 replicates containing 1.8 x 10^1 copies, 10 revealed also positive signal with mean Cq value of 31.98 ± 0.47. However, in the dilution containing 1.8 copies only 6 showed positive signal from pure culture and four from the milk with mean Cq value of 33.23 ± 0.14. Therefore, the detection limit of this qPCR assay was 18 copies of *nuc* gene per PCR reaction both with and without matrix effect. It was also possible to quantify the 10 replicates of the 18 copies/qPCR with the assay. Hence, 18 copies of *nuc* gene /qPCR was not only the detection limit but also a quantification limit of the assay. Taking the final re-suspended DNA from 4 ml of milk as 60-80 µl and 2 µl added into the reaction assay, the detection limit of the assay can be estimated as 135-180 SCE ml^{-1}.

Repeatability (Intra-assay) and Reproducibility (Inter-assay)

The repeatability (intra-assay) and reproducibility (inter-assay) of the qPCR assay as expressed by standard deviation (SD) of Cq is presented in Table 1. The SD ranges from 0.12 to 0.3 for intra assay and 0.29 to 0.59 for inter-assay (all < 1.0 SD variation), which were in acceptable range. This showed that precision of the qPCR assay was good.

Artificial contamination of milk

Staphylococcus aureus in artificially contaminated milk (10^{-1} to 10^{-5} dilutions) was quantified by qPCR (Log SCE ml^{-1}) and plate count (Log CFU ml^{-1}) method. The obtained result indicated that the coefficient of correlation between the SCE ml^{-1} and CFU ml^{-1} was 0.9578.

Naturally contaminated milk

Plate count method: The mean *S. aureus* count in the 72 naturally contaminated bulk milk samples by plate count method was 4.8 Log CFU ml^{-1}.

Quantification of S. aureus in the milk samples by qPCR

All samples (n=72) counted by plate count method were quantified by the qPCR assay. The mean *S. aureus* quantified by qPCR in the 72 naturally contaminated bulk milk was 5.59 Log SCE ml^{-1}. The 3.8 Log SCE ml^{-1} was the minimum count and 8.6 Log SCE ml^{-1} was the maximum count documented by the qPCR assay in the 72 bulk milk samples. The median quantification value was 5.5 Log SCE ml^{-1}.

Comparison of plate count data (CFU ml^{-1}*) and qPCR (SCE* ml^{-1}*) data of naturally contaminated raw milk.*

Comparison of the quantification results by paired student's t-test revealed that there is statistically significant difference ($P < 0.05$) between the two methods being approximately 0.5 log unit higher in qPCR assay than the plate count method. The coefficient of correlation between the two count results was 0.81. The scatter plot of Log SCE ml^{-1} and Log CFU ml^{-1} of the samples is presented in Fig. 3.

Discussion

There is no single standardized criteria and protocol to run qPCR for all types of foods and pathogens due to difference in food matrixes and nature of the pathogens as this requires different sample preparation, as well as different reaction and amplification component (Alarco'n et al., 2006). With this perspective, it is

important to evaluate the performance of the qPCR protocol, which was relevant and appropriate elsewhere, in local food matrixes and strains.

In the present study, primers that targeted the *nuc* gene, having species-specific nucleotide sequences (Brakstad et al., 1992) and designed to be suitable for qPCR assay (Alarco'n et al., 2006) were employed as molecular markers for detection and quantification of the *S. aureus*. According to our results, the primers showed good specificity using SYBR Green I as the detection dye. They could differentiate *S. aureus* from other *Staphylococcus* species by the markedly lower Cq value. One limitation of the primers that we observed is that when the level of *S. aureus* in the samples is low, the Cq value becomes high and could overlap the Cq value obtained from samples containing a high level of other *Staphylococcus* species (unpublished data). To minimize this problem, the number of qPCR amplification cycles were therefore reduced from 40 to 35. However, this reduction cycle has affected the detection limit of the assay. Analysis of the melting temperature (Tm) of the amplicons indicated that, unlike the Cq value, the difference between the *S. aureus* and other *Staphylococcus* spp. is not so wide. Alarco'n et al. (2006) also reported Tm value that ranged from 76.77 – 77.3 ^{0}C for *S. aureus* and 77.9 -78.2 ^{0} C for other *Staphylococcus* spp., which is comparable with our observation.

The sensitivity or limit of detection of an assay can be defined as minimum number of copies in a sample that can be measured accurately with reasonable certainty (commonly 95%) with a given analytic procedure (Bustin et al., 2009). In the present study, the sensitivity of the qPCR both from pure culture and milk was 18 copies /PCR reaction (135 - 180 SCE ml $^{-1}$). There is no difference in the sensitivity of the assay in the DNA extracted from the pure culture and the milk. The reason may be associated with efficient DNA extraction procedure. A good DNA extraction procedure can significantly improve the sensitivity of PCR assay for detection of *S. aureus* in milk. Kim et al. (2001) improved the sensitivity of the PCR assay from 80% to 100% after improving the quality of the DNA.

Alarco'n et al. (2006) demonstrated a detection limit of 10-20 cells of *S. aureus* per PCR reaction (10^2 CFU ml-^{1}) from food by qPCR using a SYBR-Green I as detection chemistry, which is comparable with our

study. On the other hand, a detection limit of 6.5 copies of *nuc* gene /PCR reaction (78-130 SCE ml^{-1}) was reported from naturally contaminated milk (Hein et al., 2005) which is lower than the current study.

It was possible to quantify the ten replicates of the 18 copies of the *nuc* gene with the assay. Hence, 18 copies of *nuc* gene/PCR was not only the detection limit but also the quantification limit of the qPCR assay. It is reported that, besides the advantage of low cost, SYBR Green I dye increases the sensitivity of the qPCR assay (Alarco'n et al., 2006).

In our data, there was good coefficient of correlation between qPCR and plate count methods for quantification of *S. aureus* in artificial contaminated raw milk. If this is the case, according to Hein et al. (2001b), it is possible to quantify the CFU ml^{-1} of the plate count with the culture independent molecular method. Accordgly, in naturally contaminated milk samples there was statistically significant difference in *S. aureus* count between the two methods being higher in qPCR. In consistence with this observation, other research also reported higher *S. aureus* detection in qPCR than the plate count method. Hein et al. (2005) in Norway reported two Log scale higher *S. aureus* count in artificially contaminated bovine milk by qPCR than plate count method. Graber et al. (2007) also noted that the analytic sensitivity of qPCR was 507 times higher than the conventional bacteriology in detection of *S. aureus* in bovine mastitis milk. Moreover, Studer et al. (2008) clearly asserted that the qPCR *S. aureus* count were on average about 200 times higher than those agar plate count method in their longitudinal field study in milk. Postollec et al. (2011) reviewed the following possible reasons for the higher bacterial count in culture independent molecular method than culture dependent method. The presence of intact DNA from dead cells as well as the presence of viable but non-cultivable bacteria, which can only be detected by molecular method are likely reasons for the higher values in qPCR. Moreover, one colony on a plate may originate from more than one cell and, in addition, some primers involved in the molecular assay may target multi copy genes (e.g. 16S rRNA) that could increase the count.

The detection of dead cells of *S .aureus* in food is a relevant public health matter. It helps for retrospective analysis of the level of contamination starting from the production up to that point in time, which is

important for the microbial risk assessment study (Hein et al., 2005). In the present study 29% of the samples, which were considered as having < 5 Log ml^{-1} *S. aureus* count with the plate count method, were found having > 5 Log ml^{-1} count with qPCR which indicated the higher sensitivity of the qPCR assay. Samples with > 5 Log ml^{-1} count of *S. aureus* may contain SE which could stay biologically active for a long period of time as it is thermostable and resistance to enzymatic activities, although the bacteria that produced it have died (Hennekinne et al., 2011).

The current study shows the following advantages and prospects of qPCR over the conventional plate count method. qPCR can generate quantified results within a short period of time, which is important during investigation of food poisoning outbreaks. qPCR is not only much faster but also but gives quantified data of viable and dead *S. aureus* cells in the samples. From a microbial risk assessment perspective, this kind of quantified data are important to characterize and quantified the microbial risks that influence food safety (Postollec et al., 2011). In comparison with the plate count method, the level of contamination in qPCR is also minimum even better that the conventional PCR (use of gel electrophoresis) because the data are generated in real time immediately after amplification. On the other hand, the plate count method may be a preferable method as it can be carried out in any typical microbiological laboratory and the initial cost of consumables is less than the qPCR.

qPCR has been reported as an important and efficient alternative to plate count method in monitoring intra-mammary infections elsewhere (Graber et al., 2007; Studer et al., 2008). With this suggestion, in Ethiopia where clinical and subclinical mastitis is major problem in dairy cows (Duguma et al., 2014; Tolosa et al., 2015) and transport of chilled samples is a challenge, monitoring of the etiological agents with qPCR could be considered as alternative approach to tackle the problem, after further detailed study of the assay.

Furthermore, it has been reported that the SYBR Green based qPCR based on frozen samples is a rapid and reliable method for detection and quantification of *S. aureus* harboring the enterotoxin gene cluster (*egc*) in raw milk (Fusco et al., 2011). They suggested that the developed method will become increasingly important in controlling SFP in milk and milk-based products

To conclude

To our knowledge, this is the first study to assess the performance of a relevant qPCR protocol for quantification of *S. aureus* from bovine raw milk of the Tigray region and comparing the assay with the widely used plate count method. The output of our study showed that the performance of SYBR Green I based qPCR that targets the *nuc* gene, in terms of amplification efficiency, linearity of the quantification, detection limit and precision were in acceptable range. Therefore, application of qPCR assay with the above protocol for quantification of *S. aureus* in milk is relevant. It enables to exploit the inherent advantages of speed, sensitivity and reliability over the conventional plate count. These gained advantages are important factors in microbial food safety risk assessment and other food poisoning related studies.

Conflict of interest: The authors have no conflict of interest to declare.

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List of Tables

Table 1. The mean and SD of three tenfold dilution of copies of the *nuc* gene subjected to qPCR to determine the intra-assay and inter-assay variation of the assay

	Number of replicates N	1.8×10^4 copies		1.8×10^5 copies		1.8×10^6 copies	
		Mean Cq	SD	Mean Cq	SD	Mean Cq	SD
Intra-assay	10	21.8	0.30	18	0.17	14.8	0.12
Inter-assay	3	22.3	0.59	18.22	0.32	14.8	0.29

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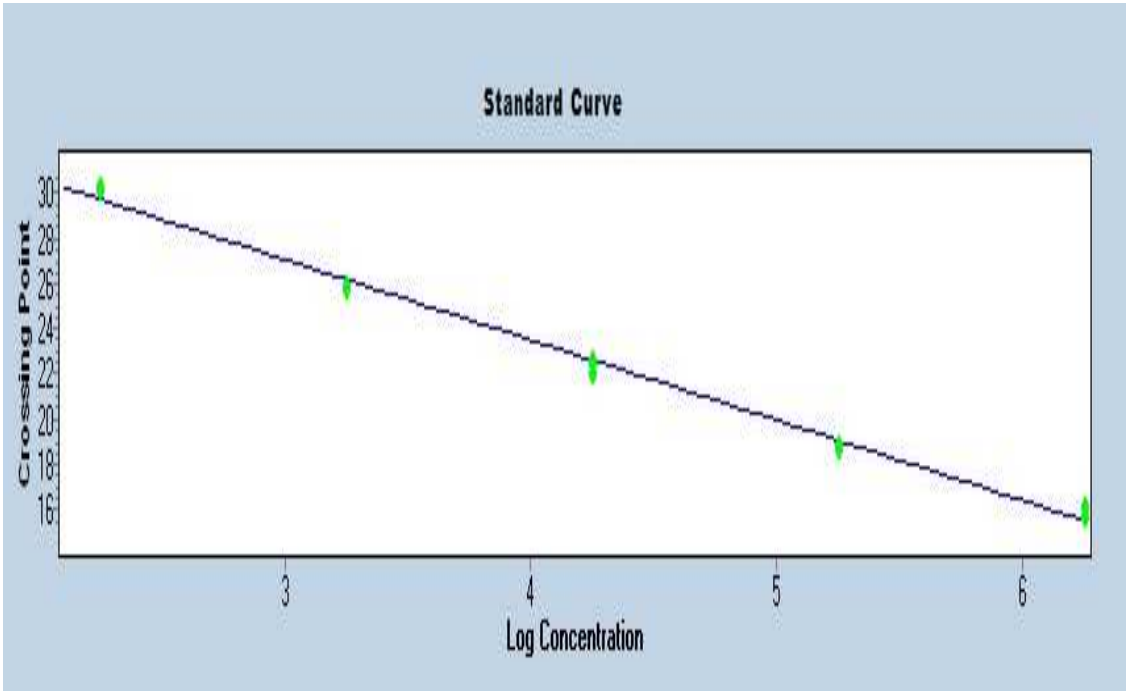
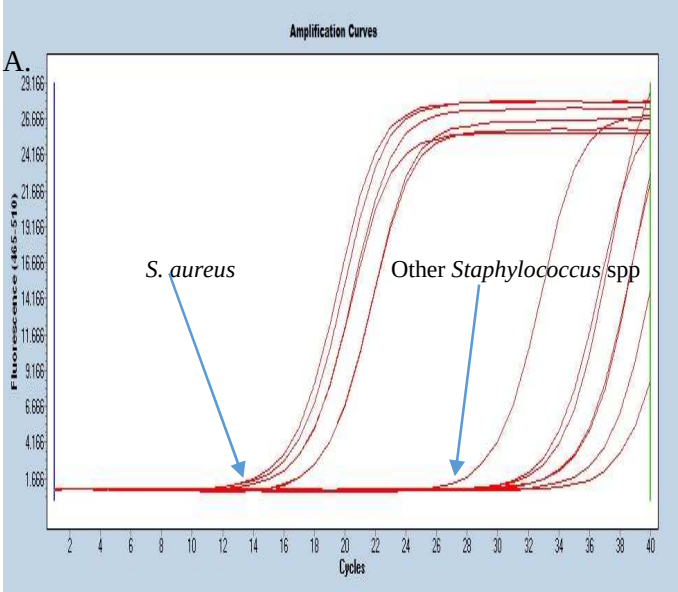


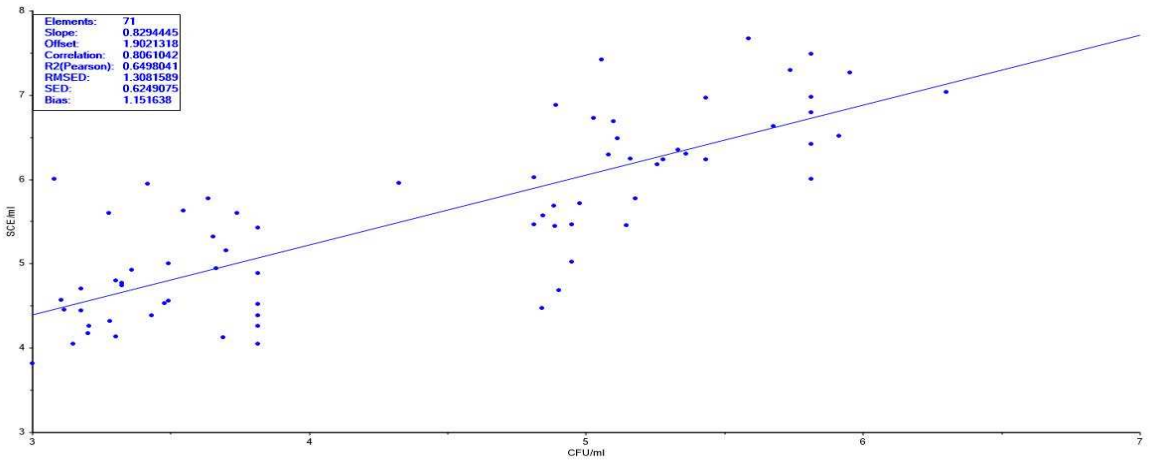
Fig.1. The standard curve generated (Efficiency: 1.914, Slope -3.354, Y/intercept 37.68, error 0.182) from serial dilution of 1.8×10^1 - 1.8×10^5 copies of *nuc* gene.

A.



B. Staphylococci species	Mean Cq
Staphylococcus aureus	13.8
Staphylococcus epidermidis	29.6
Staphylococcus cohnii	29.4
Staphylococcus hemolyticus	27
Staphylococcus carnosus	30.9
Staphylococcus sciuri	34
Staphylococcus hominis	31.9
Staphylococcus devriesei	32.6
Staphylococcus chromogenes	27.9
Staphylococcus saprophyticus	29.1
Staphylococcus warneri	26.4
Staphylococcus caprae	34.6

Fig 2. One of the qPCR runs for specificity study. A. The marked difference of Cq value of *S. aureus* from the other *Staphylococci* species in the amplification curve of the qPCR assay B. The summarized mean Cq value of *S. aureus* and other *Staphylococcus* spp.



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484 Fig.3. Correlation of the qPCR (SCE ml^{-1}) count and plate count of (CFU ml^{-1}) of *S. aureus* from 71

485 samples of naturally contaminated raw milk.