Identification and characterization of *Staphylococcus aureus* isolated from bovine mastitis milk in Northern Germany

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LIST OF ABBREVIATIONS

AE  Elution Buffer
AFLP  Amplified Fragment Length Polymorphism
arcC  Carbamate Kinase Gene
aroE  Shikimate Dehydro-Genase Gene
ATCC  American Type Culture Collection
AW  Wash Buffer
B3  Outer Backward Primer
BIP  Backward Inner Primer
bp  Base Pair
BTSCC  Bulk Tank Somatic Cell Count
°C  Temperature - Celsius
CC  Clonal Complex
CFU  Colony-Forming Unit
clf  Clumping Factor (A and B) Gene
CLSI  Clinical And Laboratory Standards Institute
CNS  Coagulase-Negative Staphylococci
coa  Coagulase Gene
CPS  Coagulase-Positive Staphylococci
DMF  Dimethylformamide
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic Acid
ETA  Exfoliative Toxins A
ETB  Exfoliative Toxins B
F3  Outer Forward Primer
FDR  Fluorescent Detection Reagent
FIP  Forward Inner Primer
FnBPs  Fibronectin binding proteins
g  Gravity
LIST OF ABBREVIATIONS

*glpF*  Glycerol Kinase Gene

*gmk*  Guanylate Kinase Gene

HNB  Hydroxynaphthol Blue

IMI  Intramammary Infection

L  Liter

LAMP  Loop Mediated Isothermal Amplification Assay

Loop B  Backward Loop Primer

Loop F  Forward Loop Primer

*mecA*  Methicillin Resistance *Staphylococcus aureus* Gene

mg  Milligrams

µL  Microliter

MLST  Multilocus Sequence Typing

MLVA  Multiple-Locus Variable-Number Tandem-Repeat Method

µM  Micromole

MRSA  Methicillin-resistant *Staphylococcus aureus*

MSCRAMMs  Microbial Surface Components Recognizing Adhesive Matrix Molecules

MSSA  Methicillin-Sensitive *Staphylococcus aureus*

NASBA  Nucleic Acid-Based Amplification

NIRD  National Institute for Research in Dairying

NMC  National Mastitis Council

NPV  Negative Predictive Values

*nuc*  Thermonuclease gene

PCR  Polymerase Chain Reaction

PFGE  Pulsed-Field Gel Electrophoresis

pg  Picogram

PMN  Polymorphonuclear Neutrophils

pmol  Picomole

PPV  Positive Predictive Values

*pta*  Phosphate Acetyltrans-ferase Gene
### LIST OF ABBREVIATIONS

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<tr>
<td>PTSAgs</td>
<td>Pyrogenic Toxin Superantigens</td>
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<td>PVL</td>
<td>Panton-Valentine Leukocidin</td>
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<tr>
<td><em>S. agalactiae</em></td>
<td><em>Streptococcus agalactiae</em></td>
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<td><em>S. intermedius</em></td>
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<tr>
<td>SCC</td>
<td>Somatic Cell Count</td>
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<td>SCCmec</td>
<td>Staphylococcal Cassette Chromosome <em>mec</em></td>
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<td>SE</td>
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<td>SEIU</td>
<td>Staphylococcal Enterotoxin-Like U</td>
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\begin{itemize}
\item *selu* Staphylococcal Enterotoxin U Gene
\item SEM Staphylococcal Enterotoxin M
\item *sem* Staphylococcal Enterotoxin M Gene
\item SEN Staphylococcal Enterotoxin N
\item *sen* Staphylococcal Enterotoxin N Gene
\item SEO Staphylococcal Enterotoxin O
\item *seo* Staphylococcal Enterotoxin O Gene
\item SEP Staphylococcal Enterotoxin P
\item *sep* Staphylococcal Enterotoxin P Gene
\item SEQ Staphylococcal Enterotoxin Q
\item *seq* Staphylococcal Enterotoxin Q Gene
\item SER Staphylococcal Enterotoxin R
\item *ser* Staphylococcal Enterotoxin-Like R Gene
\item *spa* *Staphylococcus* Protein A Gene
\item ST Sequence Type
\item *t* Type
\item TBE Tris/Borate/EDTA Buffer
\item *tpi_* Triosephosphate Isomerase Gene
\item TSST Toxic Shock Syndrome Toxin
\item *tst* Toxic Shock Syndrome Toxin Gene
\item UV Ultraviolet
\item *yqiL* Acetylcoenzyme A Ace-Tyrltransferase gene
\end{itemize}
Mastitis is an inflammation of the mammary gland. Bovine mastitis is a major economic disease affecting one-fourth or more of the bovine intramammary gland or udder, and can affect the health of cows. Bovine mastitis is characterized by a wide range of physical and chemical changes in the milk and also pathological changes in the udder tissues of the animals (O.M. RADOSTITS et al. 2000). Mastitis is one of the most prevalent endemic diseases and the most frequent one in dairy herds worldwide (MILLER et al. 1993; HALASA et al. 2007). The most significant clinical signs of bovine mastitis comprise swelling, heat, redness and pain in the udder. Furthermore, the major changes observed in milk affected by dairy mastitis are clots, discolouration, and the presence of a large number of leukocytes.

There are many types of bacterial pathogens that cause mastitis in dairy cows. The bacterial pathogens of mastitis can be classified into two groups. The first group is made up of contagious pathogens, which include \textit{Staphylococcus aureus}, \textit{Streptococcus agalactiae}, and \textit{Mycoplasma bovis}. The second group comprises environmental pathogens (\textit{Streptococcus uberis} and \textit{Streptococcus dysgalactiae}), environmental coliforms (Gram-negative bacteria \textit{Klebsiella} spp., \textit{Escherichia coli}, \textit{Citrobacter} spp., \textit{Enterobacter} spp., \textit{Enterobacter faecalis} and \textit{Enterobacter faecium}), and other gram-negative bacteria such as \textit{Serratia}, \textit{Pseudomonas} and \textit{Proteus} (O.M. RADOSTITS et al. 2000). There are more than 20 types of pathogens that can cause bovine mastitis, among which \textit{S. aureus} is the primary causative agent (YAO 2002).

\textit{Staphylococcus (S.) aureus} is considered as one of the most important aetiological agents to cause clinical and mainly subclinical mastitis in dairy herds (TAPONEN and PYORALA 2009). \textit{S. aureus} causes huge economic losses in the dairy industry due to the inferior quality and decreased the quantity of milk as well as the cost of treatment (KEEFE 2012b). \textit{S. aureus} is the most contagious and commonly isolated pathogen of bovine mastitis worldwide (HAFTU et al. 2012; KEANE et al. 2013). Additionally, \textit{S. aureus} is an important food-borne pathogen and the main cause of food poisoning cases and outbreaks in the world (HENNEKENNE et al. 2010). Research in the past few decades has shown that many food-borne poisoning outbreaks were caused by staphylococcal food poisoning, which is still reported as the third-most prevalent cause for food-borne illnesses worldwide (ZHANG et
INTRODUCTION

al. 1998). *S. aureus* produces more than 30 different types of virulence factors, which contribute in different ways to cause infection (OTE et al. 2011). *S. aureus* can produce various types of exotoxins, such as exfoliative toxins A (ETA) and exfoliative toxins B (ETB), toxic shock syndrome toxin-1 (TSST-1), Panton-Valentine leukocidin (PVL), and staphylococcal enterotoxins (SEs) (FRANCIS et al. 2005). SEs are the major causes of staphylococcal food poisoning in humans (BALABAN and RASOOLY 2000). Milk and dairy products are regarded as the primary sources that are reportedly associated with staphylococcal enterotoxin that causes food poisoning (AYDIN et al. 2011).

The detection and the identification of pathogens in milk are important for the definitive diagnosis of intramammary infections in dairy cows, and this is also essential for disease prevention and control. Conventional identification methods of *S. aureus* in milk based on bacterial culture include the examination of gram stain, colony morphology and hemolysis type on blood agar, coagulase reaction, and other biochemical tests. Molecular identification methods are an accurate and rapid method for identifying *S. aureus* isolated from bovine mastitis milk and they also make the final decisions regarding an appropriate antimicrobial therapy (JONAS et al. 2002). There are a number of molecular identification methods such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). In the past few decades, with the benefit of high sensitivity and specificity, PCR has been used for the rapid identification of various pathogens (JONAS et al. 2002; HULETSKY et al. 2004). PCR has been used for detecting the specific gene of the causative agent in clinical microbiology laboratories (KOSKINEN et al. 2009; TAPONEN et al. 2009). The PCR method is one of the most common but significant methods used for the detection of pathogens in bovine mastitis (KOSKINEN et al. 2009). Additionally, the PCR method is based on analysing pure microbial cultures (SPANOVA et al. 2000), but is limited by PCR inhibitors present in biological and food samples, which reduce the detection sensitivity or produce false-negative results (AL-SOUD and RADSTROM 2001). Furthermore, there is no other method that is more rapid, more sensitive, inexpensive and less laborious for detecting *S. aureus* in diagnostic laboratories.

All the isothermal nucleic acid amplification methods based on the simple heating device have been developed to give the optimal amplification for the rapid and sensitive detection of
a target nucleic acid. These include nucleic acid-based amplification (NASBA), LAMP, and ramification amplification (COMPTON 1991; NOTOMI et al. 2000; D. Y. ZHANG et al. 2001). The LAMP is a specific gene amplification, which is amplified with high specificity and efficiency under isothermal conditions (NOTOMI et al. 2000). The LAMP assay for detecting specific genes is characterized by using four to six specific primers to recognize six to eight different regions of the target gene. The LAMP reaction procedure occurs at a fixed temperature (60–65°C) and the duration of the reaction takes less than 60 minutes (NOTOMI et al. 2000; NAGAMINE et al. 2001; NAGAMINE et al. 2002). In addition, all the amplification steps in the LAMP assay are performed in one reaction tube under isothermal conditions.

The present study was designed to identify and characterize *S. aureus* that was isolated from bovine mastitis milk from various locations in Northern Germany by molecular and conventional identification methods, including LAMP, PCR, cultural, biochemical, and antimicrobial susceptibility tests and other phenotypic characteristics of the *S. aureus*. For this a LAMP assay was developed and validated by detecting the specific gene *nuc* for identifying *S. aureus*. Moreover, the genotypic characteristics of the isolates were performed using MLST and *spa* analysis.
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2.1 Loop-Mediated Isothermal Amplification (LAMP)

The detection and identification of the causative agent of bovine mastitis is important for the final diagnosis of intramammary infections in dairy cow herds and also essential for the prevention and control of the disease. The conventional identification methods of *S. aureus* in bovine mastitis milk based on bacterial culture include the examination of gram stain, colony morphology and hemolysis type on blood agar after incubation for 24 hours at 37°C, catalase reaction, coagulase reaction and other biochemical tests (HOGAN et al. 1999). However, the conventional identification methods are less reliable and time-consuming in confirming the identity of the etiological agent.

Molecular identification techniques such as PCR, real-time PCR, and multiplex PCR are molecular methods involving direct DNA detection that come with the advantage of absolutely indicating the presence or absence of the target pathogens (TAKEUCHI et al. 1997; MAEDA et al. 2006). The PCR method is most commonly used for identifying the aetiology agent by analysing small quantities of DNA of the pathogens (MULLIS and FALOONA 1987; NOTOMI et al. 2000; GILL and GHAEMI 2008). However, the PCR-based technique requires high-precision equipment for amplifying the target sequence on DNA and also post-PCR analysis of the amplified DNA as derived from the conventional method. Furthermore, PCR requires an accurate temperature control and a rapid thermocycler between 55°C and 95°C. Real-time PCR can amplify the target sequence of DNA within a short time without requiring gel electrophoresis to imagine the amplified DNA (WALKER 2002). Real-time PCR has several advantages, including sensitivity, quantitative aspects, specificity and minimum contamination. However, real-time PCR requires trained personnel, expensive and complex instruments, reagents and special probes (PARIDA et al. 2004).

Recently, Notomi et al. developed a novel molecular diagnostic method for DNA amplification called the loop-mediated isothermal amplification (LAMP), which can amplify DNA with high efficiency, specificity and more rapidly under isothermal conditions over a range of 60–65°C (NOTOMI et al. 2000). Furthermore, LAMP has been used in *Bst* DNA polymerase to create loop structures that facilitate exponential sequence-specific amplification
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(HARA-KUDO et al. 2005). Unlike conventional PCR, a denatured template is not required for DNA amplification and there is also no need for a thermocycler (NAGAMINE et al. 2001). Also, DNA amplification can be carried out in a standard heat block or water bath (TOMLINSON and BOONHAM 2008). The LAMP reaction can amplify and detect a specific gene by incubating the reaction at a suitable temperature, and it is carried out in a single reaction tube as well (NOTOMI et al. 2000).

The LAMP assay has shown precise results in medical science for detecting bacterial pathogens in samples from both humans and animals (IWAMOTO et al. 2003; SAVAN et al. 2004; SONG et al. 2005; DUKES et al. 2006; EN et al. 2008; WASTLING et al. 2010). The LAMP assay has been developed for the rapid identification of different bacteria, including *Arcanobacterium pluranimalium* (ABDULMAWJOOD et al. 2015), *Escherichia coli* O157: H7 (MARUYAMA et al. 2003), *Campylobacter jejuni* and *Campylobacter coli* (YAMAZAKI et al. 2009), *Mycobacterium tuberculosis* (IWAMOTO et al. 2003), and *Vibrio parahaemolyticus* (L. WANG et al. 2011). Recently, the LAMP assay was used to detect the aetiology of bovine mastitis, including *S. aureus* (LIM et al. 2013; X. R. WANG et al. 2015) and *Streptococcus. agalactiae* (KIMURA et al. 2013). Furthermore, the LAMP assay has been used to identify Ostrich meat (ABDULMAWJOOD et al. 2014).

The LAMP assay requires a DNA polymerase and four to six primers to recognize six to eight distinct sequences on the target DNA under isothermal amplification. The primers consist of Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Outer Primer (F3) and Backward Outer Primer (B3) (NOTOMI et al. 2000). Additionally, the reaction time of the LAMP assay for identifying pathogens is reduced by using loop primers (NAGAMINE et al. 2002; LIM et al. 2013). Therefore, the LAMP assay is a very sensitive, easy and time-saving method that is successfully applied in different fields to detect pathogens (L. CHEN et al. 2011; TANNER et al. 2012). To detect amplicons, several methods are used to detect the amplicon DNA of the specific pathogens. The most common method for detecting amplicons is agarose gel electrophoresis, with the gel stained with a placing agent such as ethidium bromide. The turbidity method is a visual inspection for detecting the LAMP reactions (MORI et al. 2001; MORI et al. 2004). The LAMP-amplified product can be visualized by using intercalating dyes, including picoGreen, SYBR Green, ethidium bromide and
Hydroxynaphthol Blue (PARIDA et al. 2008). The other methods use devices depending on the end-point detection or the real-time monitoring of LAMP reactions coupled with electrochemical detection (HSIEH et al. 2012; SAFAVIEH et al. 2014), absorbance detection (FANG et al. 2010) and fluorescence detectors (Zhou et al. 2014).

### 2.2 LAMP Primers Design

The principle of the LAMP primers is dependent on the auto-cycling strand displacement DNA synthesis simplified by Bst (\emph{S. aureus}) DNA polymerase. There are four to six types of primers that are used to recognize the six to eight distinct regions of the target gene. The types of LAMP primers are denoted as forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3) and backward outer primer (B3) (Notomi et al. 2000). The loop primers help to increase the acceleration rate of the amplification reaction by binding to extra sites that are not accessed by the inner primers (Nagamine et al. 2002).

![LAMP Primers Diagram](image)

Figure 1: Schematic diagrams showing the position of LAMP primers in the target DNA (Notomi et al. 2000).

FIP contains the F1c district at the 5’ end, which is complementary to the F1 district and the F2 district (at the 3’ end) that is complementary to the F2c district of the target DNA.

The F3 primer consists of the F3 district that is complementary to the F3c district.
BIP contains the B2 district at the 3’ end that is complementary to the B2c district and the B1c district that is complementary to the sequence of B1 district at the 5’end.

The B3 primer contains the only B3 district that is complementary to the B3c district.

There are two other primers named loop primer forward (LPF) and loop primer backward (LPB) that contain a sequence complementary to the single-stranded loop region. These primers are used to increase the amplification rate.

2.3 Principles of LAMP Primers

The LAMP amplification reaction technique comprises three steps: production of the starting material, cycling amplification, and elongation as well as recycling (NOTOMI et al. 2000). When the DNA template (the target gene) is incubated along with primers, DNA polymerase enzyme and other reagents at a constant temperature, the reaction takes place as follows (Figure 2): The forward inner primer FIP anneals to start the first strand synthesis of the target sequence (step 1), by the Bst DNA polymerase beginning from the 3’ end of the F2 district of the FIP (step 2). When comparing LAMP with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The outer primer F3 anneals to the F3c district in the target and initiates strand displacement DNA synthesis, on the outside of the FIP on the target DNA and initiates strand displacement DNA synthesis by which the FIP-linked complementary strand is released (step 3). A double strand is made from the DNA strand produced from the F3 primer and the DNA template strand (step 4). The outer primer FIP-linked complementary strand is released as a single strand because of the dislocation by the DNA strand synthesized from the F3 primer. This released single strand forms a stem-loop structure at the 5’ end because of the complementary F1c and F1 regions (step 5).

The backward inner primer BIP begins DNA synthesis, the B3 primer separating these recently synthesized DNA strands. Starting from the 3’ end, BIP anneals to the DNA strand produced by the FIP-linked complementary strand so that the synthesis of complementary DNA takes place. During the DNA synthesis, the DNA reverts from a loop structure into a linear structure. The B3 primer hybridizes to the BIP’s outside, and then through the action activity of the Bst DNA polymerase and beginning at the 3’ end, the DNA synthesized from the BIP is dislocated and released as a single strand before the DNA synthesis from the
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primer. The B3 primer strand dislocates the DNA synthesis, and the DNA reverts back from a loop structure to a linear structure (step 6). A double-stranded linear DNA is formed at this step (step 7).

At every end complementary sequence as the same strand (e.g. Fl, Flc and B1, Blc) forms a structure with stem-loops, which resemble the shape of a dumbbell. The formation of the dumbbell-like product is essential for LAMP to confirm isothermal amplification because the loop structures are constantly of a single strand, and can be hybridized by the B1 and B2 regions, or between the Fl and F2 regions (step 8). After forming a dumbbell-like structure, the amplification cycle in the LAMP method starts. At this point, Loop Primer Forward (LPF) and Loop Primer Backward (LPB) bind with their complementary sequences (F2c and B2 district, respectively). At every cycle of amplification, there is an increase in the number and the size of the present products. The final product is a mixture of stem-loop DNA with distinct stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted duplication of the target sequence in the same strand (NOTOMI et al. 2000). Seeing the animation of the LAMP principle helps to understand the amplification procedure (animation link: http://loopamp.eiken.co.jp/e/lamp/anim.html).
Figure 2: Technique of loop-mediated isothermal amplification (Source: Eike Chemical Co. Ltd.)
2.4 Detection Methods for LAMP Amplification Products

Various methods have been developed to detect the LAMP product.

2.4.1 Agarose Gel Electrophoresis

The gel electrophoresis has been one of the most common molecular biology techniques used to detect the classical PCR and LAMP amplification during the past decade, and the gel stained by an intercalating agent such as ethidium bromide to obtain the characteristic pattern defined by the number of target sequence copies (TOMLINSON and BOONHAM 2008). Under UV lighting, the LAMP reaction appears to have a ladder-like pattern, which is the different length stem-loop product of the LAMP reaction. Each lane appears as a ladder-like band due to the combination of variously sized dumbbell-shaped DNA molecules (Figure 3).

Figure 3: Analysis of the LAMP reaction by agarose gel electrophoresis. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control strain ATCC 6538, Lanes 2–7 represent positive isolates, and Lane 8 is a non-template control. Lanes M are DNA Marker 50 bp ladder (Biozym Diagnostic).
2.4.2 Turbidity

The positive LAMP reaction can be detected by depending on the monitor with the increased turbidity in the reaction tube, which can be measured by a turbidimeter (NOTOMI et al. 2000; YOSHIDA et al. 2005; PARIDA et al. 2008). The turbidity is derived from the precipitation of magnesium pyrophosphate generated as a by-product, and this correlates with the amount of DNA amplified. One study (PARIDA et al. 2008) reported that to observe the turbidity in the form of a white precipitate, monitor, the DNA yield in more than four µg is required.

The LAMP assay has the ability to amplify the target sequence with high efficiency, specificity and sensitivity compared to other molecular methods. Therefore, several investigators have developed different methods for the rapid detection of the LAMP reaction. To detect the LAMP reaction, fluorescent detection reagent (FDR) is added to the LAMP reaction mixture before beginning the amplification in order to allow the product to be immediately visualized under UV illumination and to minimize contamination. One study (TOMITA et al. 2008) developed a colorimetric method by using Calcein (fluorescence metal indicator), which is added to the pre-reaction solution in conjunction with manganese ions.

![Positive and Negative LAMP Products](image)

Figure 4: Visual detection of LAMP products detected by using turbidity observation. Turbidity increases in the positive reaction due to the generation of magnesium pyrophosphate (X. ZHANG et al. 2010).
2.4.3 Calcein and MnCl₂

Calcein is one of the methods used to detect positive LAMP reaction. Calcein is an FDR and as well-known as fluorenon. During the LAMP reaction, calcein can be used as a Ca²⁺ or Mg²⁺ indicator because its fluorescence is immediately sensitive to these ions only at strongly alkaline pH (X. ZHANG et al. 2012). Calcein is dissoluble in dimethyl sulfoxide (DMSO) and also in dimethylformamide (DMF). Pyrophosphate ions are produced through the LAMP reaction. They connect with and remove manganese from the calcein, which results in detectable bright fluorescence under UV light, which indicates the presence of the target genes (IMAI et al. 2007; YODA et al. 2007).

Figure 5: Detection of the LAMP reaction using a fluorescent metal indicator (Calcein) (TOMITA et al. 2008).

2.4.4 Real-Time Fluorometer

Genie® II is a sophisticated device that enables sensitive detection of bacteria and viruses at a molecular level. This powerful and extremely flexible platform allows isothermal amplification of DNA and RNA to take place in a compact and portable device. The
instrument is supported by specially-designed tubes and highly efficient reagents to promote ease-of-use and ultra-rapid detection capability, offering a complete solution to many nucleic acid detection requirements. All the results of the DNA amplification observe reactions as a signal and also identify specific amplification which is the accurate temperature control allows post-amplification analysis of products appear as annealing curves.

Figure 6: The LAMP amplicons were detected by using real-time fluorometer Genie® II (Source: OptiGene company, UK)

2.5 Detection of Pathogens in Bovine Mastitis

Several types of bacteria causing intramammary infection in dairy herds have been successfully detected by LAMP assay. The *S. aureus* isolates have been examined to determine gene *nuc*, which is the specific gene for *S. aureus* (MADISON and BASELSKI 1983; BRAKSTAD et al. 1992). Various studies have used LAMP assays to detect *S. aureus* isolates, which include the determination of gene *femA* and *mecA* of *S. aureus* isolated from food isolates (XU et al. 2012), gene *femA* from clinical and food samples (ZHAO et al. 2013), gene *spa* and *arcC* from spiked blood specimens (LIM et al. 2013), gene *spa* and *mecA* from positive blood culture bottle (MISAWA et al. 2007), and gene *orfX* from clinical isolates
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(SU et al. 2014). Rapid identification assays based on LAMP assay are already available for a wide variety of bacteria, including *Arcanobacterium pluranimalium* (ABDULMAWJOOD et al. 2015), *Campylobacter jejuni* and *Campylobacter coli* (YAMAZAKI et al. 2009) *Mycobacterium tuberculosis* (IWAMOTO et al. 2003), *Vibrio parahaemolyticus* (L. WANG et al. 2011) and *S. agalactiae* (KIMURA et al. 2013).

2.6 Features of the LAMP Assay

The LAMP assay possesses several important characteristics that make it a globally preferred technique for determining pathogens. The LAMP reaction needed to use the single type of enzyme and the amplification can be carried out under isothermal conditions (PARIDA et al. 2008). To determine the target sequence gene, it is required to incubate the mixture of sample, primers, and Bst DNA polymerase at a constant temperature (NOTOMI et al. 2000). The LAMP assay’s fundamental characteristic is that the amplification of the specific sequence of the target gene is more specific, more sensitive and highly efficient. The number of primers using the LAMP assay is four to six primers, to recognize six to eight distinct regions on the target gene being at a fixed temperature without needing to use initial denaturation of the template DNA requiring thermal cycling (NAGAMINE et al. 2001). One of the important characteristics of the LAMP assay is that significant DNA amplification can be obtained within a short period. The amplification of DNA can be determined by using several methods, such as agarose gel electrophoresis (TOMLINSON and BOONHAM 2008), turbidity (MORI et al. 2001; MORI et al. 2004), and fluorescence detection methods (TOMITA et al. 2008).

Several methods have been used to detect the amplification of DNA by intercalating dyes, such as SYBR Green I (IWAMOTO et al. 2003), ethidium bromide (PHAM et al. 2005), Picon Green (DUKES et al. 2006), and Hydroxynaphthol Blue (HNB) (GOTO et al. 2009). The positive reaction tubes can be detected by using a conventional UV illuminator or by fluorescence microscopy (MORI et al. 2006). The other feature of the LAMP amplification technique is that the LAMP reaction has the ability to tolerate some inhibitory material such as culture media and some biological material, which can affect the efficiency of PCR (KANEKO et al. 2005). The LAMP assay requires a small quantity of DNA to amplify, and it is also only minimally affected by the different components of the clinical samples as compared to PCR (NAGAMINE et al. 2001).
Staphylococcus aureus

2.7.1 History and Taxonomy of S. aureus

The genus Staphylococcus can be found in different habitats such as human, animal and plant. Staphylococci are important in the dairy industry and also as pathogens of animals and humans. Staphylococci were first found and cultured by Robert Koch (1843–1910) and Louis Pasteur (1822–1895), but Scottish surgeon Sir Alexander Ogston (1844–1929) was the first investigator to publish detailed studies of S. aureus a few years later (OGSTON 1880, 1882). Under the microscope, he saw clusters of round organisms and demonstrated that S. aureus was the major causative agent of abscesses (OGSTON 1880). Ogston gave the Latin name Staphylococcus to this family, which has the ability to form the pus bacteria based on its appearance under the microscope. Shortly after Ogston’s discovery, S. aureus was isolated and also grown by the German surgeon Anton J. Rosenbach (1842–1923), who categorized the first taxonomic classification of the genus Staphylococcus, splitting it on the basis of its colony colour in two ways, S. aureus and S. albus (ROSENBACH 1884; KLOOS 1980). According to (EVANS et al. 1955), the differentiation of Staphylococcus from Micrococcus was based on the use of the glucose fermentation behaviour in a standardized oxidation-fermentation test (O/F test). In the 1960s, depending on the DNA information to differentiate between staphylococci and micrococci, it was demonstrated that the ratio of the content of the guanine and cytosine at the staphylococcal DNA amounted to 30–39% and in the micrococal DNA to 63–73% (SILVESTRI and HILL 1965; ROSYPAL et al. 1966).

S. aureus is a gram-positive coccus with an average diameter of 0.8 to 1 µm. S. aureus can be arranged in pairs, tetrads, or more often grouped into irregular clusters or bunches like grapes (BLOBEL and SCHLIESSER 1994). The characterization of the S. aureus colonies is circular, convex, smooth, non-pigmented, regular edges and haemolytic (MARKEY et al. 2013). S. aureus is a coagulase-positive, catalase-positive, usually oxidase-negative, facultative anaerobic coccus, non-motile, and non-sporulating that belongs to the family Staphylococcaceae. S. aureus has been distinguished from different staphylococcal species by gold colony pigmentation, fermentation of mannitol and trehalose, production of heat-stable thermonuclease and also the production of coagulase. According to its ability to ferment mannitol, the species is termed S. aureus after the yellowish colour of the colony.
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(from the Latin aurum or ‘gold’). In 1930, the tube coagulase test was introduced, and it was used for the first time for differentiating *S. aureus* from other less virulent staphylococci (CHAPMAN et al. 1934). Based on the tube coagulase test, *Staphylococcus* spp. can be divided into two groups as per the presence or absence of the coagulase enzyme, i.e. coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci. In addition, the genus *Staphylococcus* consists of 52 species and 28 subspecies (EUZÉBY 2004). The demarcation between the species in the genus *Staphylococcus* depends on the structural features of the cell wall as well as the physiological, biochemical and molecular characteristics (BLOBEL and SCHLIESSE 1994). In recent decades, modern molecular biological techniques such as PCR (LIM et al. 2013), real-time PCR (MAKGOTLHO et al. 2009), and more recently, LAMP (NOTOMI et al. 2000) have been used to identify and differentiate *S. aureus*. Several molecular methods are used to detect the epidemiological and biological genotypic characteristics of *S. aureus*, for example multiple-locus variable-number tandem-repeat (MLVA), pulsed-field gel electrophoresis (PFGE), X region of protein A gene (*spa*) typing, multi-locus sequence typing (MLST), and amplified fragment length polymorphism (AFLP) (GRABER et al. 2009).

In 1928, Sir Alexander Fleming (1881–1955) discovered that *S. aureus* could not grow in the presence of the mould *Penicillium notatum* (A. FLEMING 2001). Ten years later, microbiologists had started to purify penicillin as well as to produce a sufficiently large amount of penicillin for use in treatment trials. Penicillin was first used in animal experiments in Oxford, England in 1939, and the results were published in the *Lancet* the following year (CHAIN et al. 1993). After that, penicillin was used to treat infected humans (ABRAHAM et al. 1992). Before the introduction of penicillin, the mortality rate of the *S. aureus* bacteraemia had exceeded 80% (SKINNER and KEEFER 1941). Later, penicillin was used regularly in various cases of therapy of infectious diseases. In 1959, methicillin, a form of penicillin, was developed by Beecham (DUTFIELD 2009). Methicillin is a narrow-spectrum β-lactam antibiotic of the penicillin class. Methicillin is the major type of antibiotics used to treat *S. aureus* infections worldwide. Since its first report in 1961 from the United Kingdom, it has been demonstrated that *S. aureus* has the ability to resist methicillin (JEVONS 1961). The term methicillin-resistant *Staphylococcus aureus* (MRSA) is used to refer to any strain of *S. aureus* that has developed, through the process of natural selection, resistance to beta-lactam
antibiotics, which include the penicillin forms of methicillin, dicloxacillin, nafcillin, oxacillin, etc., and cephalosporins (HARBARTH 2006; SCHITO 2006; TACCONELLI 2008). The first report in 1975 showed that MRSA was isolated from bovine sources (DEVRIESE and HOMMEZ 1975).

2.7.2 Epidemiology and Characteristics of Bovine Staphylococcus aureus

2.7.2.1 Prevalence and Significance

S. aureus is one of the major frequently isolated pathogens that cause mastitis in bovines and other mammals worldwide (HALASA et al. 2007; BOSS et al. 2011; HOGEVEEN et al. 2011), and the most common contagious organism isolated from raw milk (PICCININI et al. 2003; OLDE RIEKERINK et al. 2006). The percentage of the prevalence of S. aureus mastitis among different countries varies as per the hygienic practices implemented with regard to dairy herds. The prevalence of S. aureus mastitis was 70% in Hungary (PELES et al. 2007), 43% in USA at herd level (LOMBARD et al. 2008), 21.8% in Germany (TENHAGEN et al. 2006), 21.3% in Sweden (ERICSSON UNNERSTAD et al. 2009), 30% in Mexico (MIRANDA-MORALES et al. 2008), 21% in Great Britain (WILSON and RICHARDS 1980), 11% in Norway (BAKKEN 1981), 10% in Denmark (AARESTRUP et al. 1995), and 5.6% in Korea (MOON et al. 2007). In South Africa, the prevalence of S. aureus had increased from 8.1% in 2002 to 15.4% in 2006 (PETZER et al. 2009), while in Finland it decreased slightly from 11.1% in 1995 to 10% in 2001 (PITKALA et al. 2004).

S. aureus mainly causes subclinical and clinical mastitis, which are very difficult to cure (BOSS et al. 2011). In the dairy industry, S. aureus mastitis is the most common disease that leads to significant economic losses (SEEGERS et al. 2003). S. aureus leads to economic losses due to several reasons, including the inferior quality and low quantity of milk produced, premature slaughter, veterinary and treatment costs, and loss of genetic potential (SEEGERS et al. 2003; HOGEVEEN and ØSTERÅS 2005; MCDOUGALL et al. 2009; KEEFE 2012a). The economic impact of bovine mastitis varies according to the prevalence of S. aureus in dairy herds, the types of mastitis and the age of the cows (GRÖHN et al. 2004; HOGEVEEN and ØSTERÅS 2005). Previous studies considered mastitis to be the most costly disease in dairy production (DEGRAVES and FETROW 1993; KOSSAIBATI and
ESSLEMONT 1997; SEEGERS et al. 2003). Economically, mastitis has led to huge losses for farmers by way of both direct costs and indirect costs (KOSSAIBATI and ESSLEMONT 1997). Direct costs include veterinary treatment costs (DEGRAVES and FETROW 1993; BERRY et al. 2004), increased labour requirements (MILLER et al. 1993; LESCOURRET and COULON 1994) and discarded milk (during treatment) (MORIN et al. 1993; ALLORE and ERB 1998). Indirect costs include reduced milk yield and quality, which are regarded as being the main factors of economic losses due to both clinical and subclinical mastitis (PYORALA 2002; SEEGERS et al. 2003), higher culling and replacement rates leading to loss of genetic potential (DEGRAVES and FETROW 1993; ALLORE et al. 1998; BERRY et al. 2004), and death. Mastitis leads to a lowering of the nutritional value due to a decrease of casein, which is the major agent in cheese manufacturing (S. OLIVER and CALVINHO 1995).

Mastitis is one of the most common and economically significant diseases for milk producers because of its negative effects on several important aspects regarding cows and dairy products (KOSSAIBATI and ESSLEMONT 1997). The annual losses caused by mastitis have been estimated to be around $35 billion worldwide (WELLS et al. 1998). In the USA, the economic losses due to mastitis in the dairy industry were approximately $1.3 billion in 1979 (BLOSSER 1979) and $2 billion in 1988 (MILES et al. 1992). Several studies have shown that mastitis-related economic losses over dairy herds ranged from $161.8 to $344.1 per lactating cow/year (MORIN et al. 1993) and from $65 to $182 per cow/year (HUIJPS et al. 2008). The resultant mastitis costs varied in measure as well as in value. Several studies showed the cost of mastitis per lactation ranged from $138 to $1,169 (HEIKKILA et al. 2012) and $38 for each cow per lactation (MUNGUBE et al. 2005). The economic cost per case of mastitis metric varied greatly from $16.43/case to $572.2/case (SWINKELS et al. 2005; HAGNESTAM-NIELSEN and OSTERGAARD 2009). The financial costs per cow per year were $71 (BAR et al. 2008) and $95 (HULTGREN and SVENSSON 2009).

The total economic costs for mastitis comprises two important components: production losses and control expenditures (MCINERNEY et al. 1992). The occurrence of mastitis in dairy cows led to increasing losses in milk production and a change in the milk composition due to the increased somatic cell count (SCC) in milk (HARMON 1994; KEHRLI and SHUSTER
In Scotland, the total losses from dairy herds infected with mastitis in 1996 were estimated to be £140/cow/year (YALCIN 2000), while the losses from the increased Bulk Tank Somatic Cell Count (BTSCC) in herds due to the subclinical mastitis were around £100/cow/year (YALCIN et al. 1999). In the USA, the losses due to subclinical mastitis, which included a high somatic cell count, reduced milk production, and higher replacement costs, were estimated to be around $960 million (WELLS et al. 1998). In addition, the total financial losses caused by dairy mastitis with an average BTSCC of 200,000–399,999 cells/mL amounted to $108.00 per cow and $295.2 per cow for herds with an average BTSCC of 400,000, while in the dairy industry, the total losses based on BTSCC were approximately $1 billion (OTT et al. 1999).

*S. aureus* is one the most common food-borne pathogens and the major cause of food poisoning cases worldwide because of the production of various types of exotoxins such as staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST) (DINGES et al. 2000). Staphylococcal food poisoning is the third-most prevalent cause of food-borne illnesses (S. ZHANG et al. 1998). In recent years, several reports from different regions of the world have shown that MRSA was detected in cattle herds. MRSA can contaminate bovine milk and dairy products, which are good reservoirs of MRSA, and become the principal causative agents of the human MRSA infection (PEREIRA et al. 2009). It was recently reported that MRSA can be transmitted between humans and cows (JUHÁSZ-KASZANYITZKY et al. 2007).

2.7.2.2 Reservoirs and Transmission

*S. aureus* is most commonly prevalent as a contagious organism in dairy herds in developed countries during the entire lactating and non-lactating periods. The interior bovine gland is the most important reservoir for *S. aureus* (SEARS and MCCARTHY 2003). The skin of the udder and teat are also very important extramammary reservoirs of *S. aureus* causing bovine mastitis (J. R. ROBERSON et al. 1994; JØRGENSEN et al. 2005; S. P. OLIVER et al. 2005). Skin contaminated with *S. aureus* is at a higher risk of growing and multiplying *S. aureus*, thereby causing mastitis, than without skin colonization (SEARS and MCCARTHY 2003). The previous study revealed that *S. aureus* isolated from the milk and the udder skin have similar genetic information (HAVERI et al. 2008). Additionally, these bacteria have been
isolated from different areas of the body including nose, lips, vagina and rectum of cows (DAVIDSON 1961; NEMEGHAIRE et al. 2014). Besides dairy herds, *S. aureus* has also been isolated from non-dairy livestock such as veal calves (GRAVELAND et al. 2010), poultry (NEMATI et al. 2008), horses (VAN DEN EEDE et al. 2009) and pigs (FERBER 2010).

*S. aureus* is generally described as one of the most important contagious mastitis pathogens in dairy cattle because it is able to survive and multiply in extramammary sites more than other mastitis pathogens (HOGEVEEN et al. 2011). In addition, *S. aureus* has been isolated from extramammary sites that are in direct and indirect contact with the mammary glands such as housing infrastructure, feedstuffs, skin of milking personnel, insects, non-bovine animals, milking equipment, farm equipment, bedding material, on the floor, in dust and humans that can play an important role in spreading *S. aureus* infections (MATOS et al. 1991; J. R. ROBERSON et al. 1994; ANDERSON et al. 2012; PICCININI et al. 2012). (CAPURRO et al. 2010; MORK et al. 2012) showed that *S. aureus* isolated from the groin, vagina, muzzle, skin wounds, hock skin, water, feed and bedding lead to transmission of *S. aureus* between body sites and environment sites, and also among the dairy herds or from the cows to the calves via the ventilation or by feeding of milk containing *S. aureus*.

Apart from animals, *S. aureus* is also a major pathogen in humans. *S. aureus* infections cause an increase in the rates of morbidity and mortality (SHEAGREN 1984). Furthermore, it is responsible for a multitude of human infections worldwide (LOWY 1998). Previous studies have shown that *S. aureus* is found in skin and soft tissue infections (DELEO et al. 2010; MITHOE et al. 2012), foreign body infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, sepsis and bloodstream infections (H. CHEN et al. 2010; DAVID and DAUM 2010). *S. aureus* has been found in humans having direct contact with infected animals such as farmers (HUIJSDENS et al. 2006), veterinarians (MOODLEY et al. 2008), human contact with meat and people tending the animals (VAN CLEEF et al. 2010; PETON and LE LOIR 2013).

There is a relationship between the incidence of mastitis and season of the year, the age of the infected cows, the pre- and post-partum, and the stage of lactation (TRINIDAD et al. 1989; FOX et al. 1995). Furthermore, several studies have shown that the types and the number of
microorganisms differed as per the management practices in and the environment of the examined dairies (BATRA et al. 1977; CARROLL and JASPER 1978; PEARSON and MACKIE 1979; FAULL et al. 1983). The prevalence of \textit{S. aureus} is influenced by the season of the year (FOX and HANCOCK 1989), in that prevalence increases during acute cold weather as compared to the other seasons. Additionally, several studies revealed that the prevalence of \textit{S. aureus} in dairy herds significantly increased in winter (SHATHELE 2009; SENTITULA et al. 2012). With reference to age, the peak incidence of infection was found among a herd’s older animals, which are more likely to be chronically infected with \textit{S. aureus} and are more difficult to cure than their younger counterparts (BARKEMA et al. 2006; DE VLIEGHER et al. 2012). At parturition, the number of mastitis is relatively increased by \textit{S. aureus} (FOX et al. 1995). The percentage of \textit{S. aureus} isolates is lower among first-lactation cows at the farm while it is high among adult cows (CICCONI-HOGAN et al. 2013). There are several reasons for the prevalence of \textit{S. aureus}, including environmental and management aspects, which contribute to the failure of treatment and eradication of pathogens from dairy herds because of the use of unsuitable antibiotics as well as microbial resistance to the antibiotics (WATTS and SALMON 1997; WHITE 1999).

\textit{S. aureus} is considered a clonal organism with its population being composed of groups of genetically related strains with a common ancestor. In the past decade, many modern genetic methods of determination have been used to identify different \textit{S. aureus} genotypes epidemiologically and biologically, and also to differentiate among the types of \textit{S. aureus} by sharing and analysing a substantial database of genotypes (GRABER et al. 2009). Furthermore, various \textit{S. aureus} groups have been characterized by detecting the virulence factors and by large variations in the presence of these virulence genes (ZECONI et al. 2006; AYDIN et al. 2011). In addition, these genes exist on mobile genetic elements, including prophages, plasmids, and transposons (NOVICK 2003; OMOE et al. 2003; ONO et al. 2008). Several studies have previously described the diversity of genotype characterizations of \textit{S. aureus} strains isolated from bovine mastitis (HATA et al. 2010; KAMALELDIN et al. 2010; VEH et al. 2015). Several studies have shown that there are different types of \textit{S. aureus} strains isolated from the same region and source of samples based on the genetic analysis of these strains, and also often with one or a few predominant strains (KAPUR et al. 1995; FITZGERALD et al. 1997; BUZZOLA et al. 2001;
SOMMERHAUSER et al. 2003; RABELLO et al. 2007). The last mentioned studies demonstrated a close genetic relationship between *S. aureus* isolated from bovine mastitis and their prominence in humans, suggesting a human-to-bovine jump (RESCH et al. 2013). Milk and dairy products are still a source of *S. aureus*, and also the major methods that transfer the *S. aureus* isolates from animals to humans (HARAN et al. 2012; KREAUSUKON et al. 2012).

2.7.2.3 Clinical Signs of Mastitis and Outcome

Mastitis is an inflammation of the intramammary gland due to physical, chemical and microbiological changes that lead to an increase in the number of somatic cells in milk and pathological changes in the mammary tissue (International Dairy Federation 1987). Bovine mastitis can be divided according to the clinical signs of clinical mastitis and subclinical mastitis (BARKEMA et al. 2006). Subclinical mastitis is the most commonly prevalent type of mastitis and one of the most dangerous types in dairy herds because it shows no clinical signs and no change in milk appearance (BUSATO et al. 2000). Subclinical mastitis causes high economic losses, possibly accounting for 70–80% of the total losses by reducing the quality of milk due to an increase in the SCC in milk and a decrease in milk production (PHILPOT and NICKERSON 1991). The only method used to detect subclinical mastitis is mostly based on milk SCC (ANONYMOUS 1999). Threshold levels for SCC that have been proposed to distinguish between affected and non-affected quarters range from $200 \times 10^3$ to $500 \times 10^3$ cells/ml (DOHOO and MEEK 1982; BROLUND 1985). Unfortunately, SCC in milk may remain below these threshold limits and the infection is often chronic when detected. Clinical mastitis occurs when the inflammatory response is strong enough to display visible abnormal changes in the milk (clots, flakes), the udder (swelling, heat, redness as well as milk abnormalities), or the cow (off feed or fever).

2.7.2.4 Antimicrobial Resistance of Bovine *Staphylococcus aureus*

*S. aureus* shows varied amounts of resistance to a wide range of antibiotics and disinfectants (BJORLAND et al. 2001). The differences in *S. aureus* resistance to antibiotics depend on geographical regions, the type of samples and the genetic characteristics of the isolates (VINTOV et al. 2003). In addition, the isolates demonstrate resistance to almost all types of
β-lactam antibiotics, which are still being frequently used in the treatment of mastitis (SAWANT et al. 2005). Previous studies have shown that resistance to antibiotics among S. aureus isolated from bovine mastitis in the United States and European countries was lower (S. P. OLIVER and MURINDA 2012). In the United States, bovine mastitis S. aureus showed the resistance of 49.6% to penicillin or ampicillin, 1.1% to gentamicin and 8.5% to tetracycline in the period between 1994 and 2000 (ERSKINE et al. 2002). The proportion of penicillin-resistant S. aureus isolates in Norway and Sweden was 10% (NORM-VET 2005). The high resistance of S. aureus to antibiotics has been blamed on the indiscriminate use of antibiotics, inappropriate handling of infected animals without regular veterinary observation, and the lack of adherence to the veterinary guidelines of treating animals (GAO et al. 2012). Consequently, S. aureus isolates were frequently resistant to antibiotic therapy, resulting in a low cure rate for mastitis.

In recent years, MRSA is frequently found in livestock. In the 1970s, the first reports on MRSA recorded individual isolates from cases of mastitis in dairy cows (DEVRIESE et al. 1972). MRSA has acquired one of the many staphylococcal cassette chromosome mec elements (SCCmec) (KATAYAMA et al. 2000), possessing a gene (mecA) encoding a penicillin binding protein (PBP 2a) with low affinity for β-lactam antibiotics (UTSUI and YOKOTA 1985). In 2007, there were reports of the transmission of MRSA between dairy cows and the person milking them (JUHÁSZ-KASZANYITZKY et al. 2007). The presence of MRSA in bovine milk and the environment poses a potential risk to veterinarians, farm workers and farm animals exposed to contaminated cattle (LEE 2003). MRSA has been isolated from other species of domestic animals such as veal calves (GRAVELAND et al. 2010), poultry (NEMATI et al. 2008) and horses (VAN DEN EEDE et al. 2009). The recent emergence of vancomycin-resistant S. aureus strains in human medicine has made the control of S. aureus infections increasingly difficult (JONES 2008).

2.7.2.5 Control of Staphylococcus aureus

To control contagious mastitis, experts on the infection had developed the ‘five-point plan’ decades ago, and it was applied worldwide (NEAVE et al. 1969). These elements are post-milking teat disinfection, total dry cow therapy, appropriate therapy of clinical cases during lactation, proper maintenance of the milking machinery, and the culling of chronically
infected cows. The main purpose of the programme was to reduce *S. aureus* and *Streptococcus (S.) agalactiae* isolated from cattle herds. The five-point plan has considerably decreased *S. agalactiae* mastitis, but it has less effect on *S. aureus* mastitis. For monitoring bovine mastitis, it is important to detect the major cause of mastitis in order to prevent the prevalence and distribution of pathogens and also the pathogen-specific risks associated with this disease (OLDE RIEKERINK et al. 2010). The main risk factors causing *S. aureus* mastitis in dairy herds include over-milking, poor teat-end condition, epidermal wounds, higher parity, infected rear quarters and additional quarters of the same cow or herd being infected with *S. aureus* (DUFOUR et al. 2012).

The scientific methods are important to control and prevent the prevalence of the mastitis among the dairy herds. Water or sanitizing solution and drying with individual towels is essential for washing and cleaning the udder, and it is advisable to use disinfectants so as to prevent the spread of pathogens between cows (SEARS and MCCARTHY 2003). During the pre- and post-milking stages, the cow milkers should wear disposable gloves and sanitize the equipment before each use to prevent the spread of the pathogens among the bovine herd (J. ROBERSON 1999). After milking, the teat should be dipped in a solution of disinfectant to reduce new infections by *S. aureus* and *Streptococcus* as well as to prevent the transfer of infection from one cow to another (VESTWEBER 1994). In addition, dipping the quarters before and after milking helps to decrease the rate of new infections as compared to dipping the quarters only after milking (VESTWEBER 1994).

The main method used for the control of bovine mastitis is antibiotic therapy. The major goal of cow therapy using antibiotics is to prevent the broad prevalence and transmission between the cows in the same herd and also among herds in the same regions. In the US and some other countries, it is recommended to treat all udder quarters during bovine mastitis (National Mastitis Council 1996 1996). On the other hand, antibiotics are used in some countries such as Norway, Finland and Switzerland to treat the infection depending on the identification of the udder pathogen (PITKALA et al. 2004; OSTERAS et al. 2006). The other method used to prevent or reduce the prevalence of mastitis in a herd is by culling the infected cows (O. M. RADOSTITS et al. 1994). In addition, segregating the infected cattle from healthy cattle also helps to reduce the prevalence of pathogens among herds (VESTWEBER 1994).
National Mastitis Council (NMC) and the National Institute for Research in Dairying (NIRD) recommended the culling of cows infected with chronic mastitis in order to prevent and control the prevalence of infection between the animals. The infected cows are major sources of infection for the other cows in a herd because the *S. aureus* may reside inside the udder on the teat skin. In spite of the limited success of dry cow treatment for chronic infections, culling is regarded as the most reliable method for removing pathogens and reducing exposure of other cows to infection.

2.7.2.6 Entry into and Attachment of *S. aureus* to the Mammary Gland

Intramammary infection (IMI) occurs during the entry of a sufficient number of *S. aureus* bacteria through the teat canal into the mammary gland that leads to the development of natural IMI. Previous studies have shown that quantities as small as 10 CFU are able to cause infection in an experimental model, in which bacteria were infused directly into the teat duct, but the infective dose of *S. aureus* in bovine IMI is not known precisely (REITER et al. 1970; POSTLE et al. 1978). *S. aureus* entering the mammary gland is exposed to the first line of defence mechanisms, which are the physical barriers of the teat canal. Between one period of milking and another, the ends of the teats’ apex are tightly constricted and they close rapidly. The teat canal is covered by keratin material that inhibits bacterial growth, although heifers sometimes harbour *S. aureus* in this tissue (TRINIDAD et al. 1990).

*S. aureus* infection in the bovine mammary gland can be divided into three stages: entry into and attachment of *S. aureus* bacteria to the mammary gland, the interaction between the bovine immune system, evasion of immune defence, survival and tissue invasion. *S. aureus* may infect the bovine udder, but this depends on certain conditions as for any infection: the initial number of bacteria, the microbe’s access to the target tissue, the strain’s virulence and the host’s immunity (PROJAN and NOVICK 1997). The first step for colonization and intramammary infection is the attachment of *S. aureus* to host cells or extracellular matrix molecules. The adherence to these substances is thought to occur due to non-specific physicochemical mechanisms and because of specific bacterial host cell binding (KLUYTMANS et al. 1997). After that, the *S. aureus* are exposed to the force of flushing milk, and they resist this until they can accomplish improved adherence to the epithelial cells lining the ductless and alveoli of the mammary gland (FROST et al. 1977). *S. aureus* is better
than most other bacterial species in adhering to bovine mammary epithelial cells, and the presence of milk enhances this adherence as well (MAMO and FROMAN 1994). *S. aureus* adheres particularly well to the cells of the upper part of the mammary gland (FROST et al. 1977). *S. aureus* is also able to adhere to fat globules, which allows dissemination to the upper part of the gland by floating (SANDHOLM et al. 1989).

Bruises and injuries on the teat increase the risk of their colonization by *S. aureus*. Epithelial damage showed that the underlying subepithelial components e.g. fibrinogen and collagen—allow staphylococcal surface proteins to mediate adherence to the host cell matrix (PATTI et al. 1994). *S. aureus* secretes different types of exotoxins, which can also be involved in epithelial damage (MAMO et al. 1988). Callusing of the teat canal prevents tight closing, and extremely callused teats are more susceptible to *S. aureus* mastitis (ZADOKS et al. 2001).

One study (MYLLYS et al. 1994) revealed that teat canal colonization, teat canal infection, and IMI were more widespread in quarters in which the teat orifice epithelium was experimentally abraded compared to the controlled quarters. Many studies have shown that very large numbers of viable staphylococci are needed to cause cutaneous or subcutaneous infection in animal experiments, unless a foreign body is present (NOBLE 1965; BUNCE et al. 1992).

2.7.2.7 Virulence Factors of *S. aureus* Involved in Infection

Ever since the use of antibiotics in treating *S. aureus* mastitis, there have been cases in which bovine mastitis did not respond to treatment. Therefore, the development of control measures must be based on an understanding of the virulence factors involved in the invasion and possibly the protection of the mammary gland. The virulence factor is a substance that produces a pathogenic effect, when it is purified to homogeneity and introduced into a test animal. *S. aureus* possesses several types of virulence factors that are produced out of the bacterial cytoplasm, allowing for survival within or on a host organism in a non-symbiotic manner (PROJAN and NOVICK 1997). In addition, *S. aureus* is able to produce numerous putative virulence factors that help *S. aureus* to adhere to eukaryotic membranes, resist phagocytosis, lyse eukaryotic cells and trigger the production of a cascade of host immunomodulating molecules. Many studies have reported that *S. aureus* bovine mastitis can
produce several types of staphylococcal enterotoxin. Genes encoding these toxins have been the most studied (ZSCHOCK et al. 2004; FUEYO et al. 2005; SRINIVASAN et al. 2006).

*S. aureus* is able to produce several adhesins or microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which have been shown to mediate attachment to different cell surface proteins such as collagen, fibrinogen, fibronectin, elastin, bone sialoprotein, laminin and thrombospondin (FOSTER and HOOK 1998). *S. aureus* can colonize tissues and initiate infection by ligating to these proteins (BROUILLETTE et al. 2003). Fibronectin binding proteins (FnBPs) have been confirmed to be involved in cell invasion and to mediate adhesion to platelets via fibronectin and fibrinogen (DZIEWANOWSKA et al. 1999; HEILMANN et al. 2004). *S. aureus* is able to invade eukaryotic cells and phagocytosing cells, and antibiotics are unable to reach the intracellular bacteria (BALWIT et al. 1994).

*S. aureus* is able to adhere to the polymer surface of the plastic material, to form microcolonies, and to produce extracellular slime or glycocalyx, which produces a biofilm covering the bacteria (DONLAN and COSTERTON 2002). FnBPs are required for adhesion to fibronectin-coated surfaces e.g. those in medical devices whereas double mutants appear to be unable to adhere (GREENE et al. 1995). In addition, FnBPs have recently been established to mediate biofilm formation (O'NEILL et al. 2008).

The virulence factors of *S. aureus* are divided into several categories—surface-associated factors, degradative enzymes, and superantigen toxins. The main factors play an important role in causing an infection that includes hemolysins, leukocidins, SAgs, nucleases, proteases and lipases.

Staphylococcal toxins can be classified into pyrogenic toxin superantigens (PTSAsgs), exfoliative toxins, leukocidins, and other toxins. The group of PTSAsgs consists of staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST) (LINA et al. 2004). As many as 21 staphylococcal enterotoxins are known so far, and these are grouped into staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SER, SES, SET, SEK, SEL, SEM, SEN, SEO, SEP, and SEQ) and enterotoxin-like SEIJ, SEIU and SEIV (HENNEKINNE et al. 2010). SAgs possess at least three biological properties: pyrogenicity,
super-antigenicity (DINGES et al. 2000), and they can also enhance the lethality of rabbits to endotoxin shock up to 100,000 fold in experimental models. Meanwhile, TSST-1 and endotoxin are administered to test animals (SCHLIEVERT 1982). SAgs have the ability to bind to the outer surface of the major histocompatibility complex (MHC) class II proteins and outside the site in T-cell receptors, to which antigens can bind normally. Furthermore, the mechanism of normal antigen presentation is bypassed and consequently up to 30% of the T-cells are activated instead of the normal activation of 0.1% T-cells (FLEISCHER and SCHREZENMEIER 1988). Several studies have shown that this leads to a massive and uncontrolled release of cytokines, resulting in a capillary leak, hypovolemic shock, and multi-organ failure, which is similar to toxic shock syndrome (DINGES et al. 2000; ALOUF and MULLER-ALOUF 2003). SAgs can produce several inflammatory mediators, including tumour necrosis factor-α, nitric oxide, interleukin-6 and IL-10, which stimulate macrophages and monocytes. In addition, the macrophages become cytolytic in the presence of interferon-γ (S. D. FLEMING et al. 1991). SAgs are considered to facilitate S. aureus to evade host defence systems and are thus suggested to contribute both to acute and chronic bovine mastitis. SAgs may help S. aureus to persist in mucosal membranes (FERENS and BOHACH 2000). SEC affected the bovine immune system by increasing the proliferation of CD8+ T-cells, resulting in suppression of CD4+ T-cells. (FERENS et al. 1998).

*S. aureus* cells express surface proteins that encourage attachment to host proteins such as laminin and fibronectin, which form the extracellular matrix of epithelial and endothelial surfaces. The pathogen adhesion mechanism works through clumping factors (clf) A and B; these are fibrinogen-binding proteins that contribute to initiating infection (ZECCONI and SCALI 2013). Protein A is a surface protein that is originally found in the cell wall of the bacterium *S. aureus*, and it is also encoded by the *spa* gene. Previous studies have revealed that protein A interacts with several host factors, including immunoglobulins G, A, E, tumour necrosis factors and platelets attenuating opsonization and phagocytosis (PETERSON et al. 1977; GOMEZ et al. 2004). Binding of IgM-associated with B cells by protein A promotes apoptosis, a mechanism that leads to the death of host cells such as polymorphonuclear neutrophils (PMN) and macrophages (GOODYEAR and SILVERMAN 2004). Coagulase is one of the extracellular enzymes that prompt the polymerization of fibrinogen to fibrin as well
as its production by the majority of *S. aureus* strains. Furthermore, distinct from coagulase, the fibrinogen-binding protein (EFB) is an intracellular protein that specifically encourages the binding to fibrinogen (BODEN and FLOCK 1989). In addition, coagulase binds a protein to produce a molecular complex with a thrombin-like activity, which converts fibrinogen to fibrin around the infection site.

The second extracellular enzyme produced by *S. aureus* is nuclease. In the past decade, *S. aureus* has been found to possess a thermostable nuclease activity enzyme that is highly conserved among clinical isolates, and it has also been used as a marker for direct detection of *S. aureus* (LAGACÉ-WIENS et al. 2007). The enzyme is called by different names, such as a micrococcal nuclease, thermonuclease, deoxyribonuclease and DNase, and hereafter, we will refer to the enzyme as nuclease or Nuc. Due to its ease of purification (FUCHS et al. 1967), the Nuc protein became a favourite subject of study for enzymologists and crystallographers, leading to numerous kinetic, protein folding and structural researches (CUATRECASAS et al. 1967).

### 2.8 Typing of Bovine *S. aureus*

Several novel molecular methods are used to characterize *S. aureus* genotypically with different epidemiological and biological properties such as different virulence and pathogenicity factors for different strains (GRABER et al. 2009). Typing of *S. aureus* is very important for determining the sources of infection, the routes of transmission in disease outbreaks, and the presence of strains of different virulence. In addition, it is important to know the characteristics of the isolates both phenotypically and genotypically (KLEIN et al. 2012). In recent times, several genotypic methods have been developed and they have superseded several traditional phenotypic methods. Genotyping is often concordant with suitable phenotypic data, such as profiles for antimicrobial resistance.

#### 2.8.1 Phenotypic Methods

The detection and identification of *S. aureus* in bovine mastitis are regarded as being the definitive diagnosis of intramammary infections, and also provide crucial information for the prevention and control of this disease. The traditional methods for identifying intramammary
S. aureus are based on a series of microbiological tools, i.e. coagulase test, catalase reaction, colony morphology, and hemolysis on blood agar after overnight incubation (OLIVER et al. 2004). This method can be useful for the initial screening of coagulase production which is an important property used by clinical microbiology laboratories to identify S. aureus isolated from bovine mastitis (GOH et al. 1992). In addition, the traditional method was used to detect S. aureus by means of the antibiotics susceptibility test, which revealed multi-resistant strains or strains resistant to a given, important antibiotic such as penicillin. The selective and differential media have been used to identify S. aureus in bovine mastitis. Mannitol salt agar is one of the most common selective and differential media used in diagnostic laboratories due to its ability to specifically detect S. aureus within a short period of time. (BACHOON and DUSTMAN 2008).

In biotyping, S. aureus isolates are characterized and differentiated on the basis of their cultural and biochemical properties. The resolution power of biotyping is relatively low, and non-typable isolates are frequent. S. aureus capsular types isolated from bovine mastitis have been differentiated by using serotyping methods (SORDELLI et al. 2000). Non-typable isolates are common (TOLLERSRUD et al. 2000). In phage-typing, the sensitivity of staphylococcal isolates to a standard collection of bacteriophages is tested. Thorne and Wallmark (THORNE and WALLMARK 1960) were probably the first to use phage typing in epidemiological studies on S. aureus mastitis. The importance of the hands of milkers and milking unit liners as fomites, and teat skin as a reservoir of S. aureus IMI were used in phage typing to differentiate between isolates (FOX et al. 1991). Phage-typing is no longer used due to lack of discrimination, a large number of non-typable strains, changing phage patterns of a given strain over time, and complex technical requirements.

2.8.2 Genotypic Methods

In recent reports, PCR has emerged as one of the most commonly used methods to detect mastitis pathogens in a sensitive and specific manner (TAPONEN et al. 2009). In addition, many different types of novel molecular methods are described to identify different S. aureus genotypes (GRABER et al. 2009) e.g. MLVA, PFGE, typing the X region of protein A gene (spa), MLST and AFLP. Typing the X region of protein A gene (spa) and MLST have been widely applied to differentiate types of S. aureus by sharing and analysing a substantial
PFGE of fragmented *S. aureus* DNA is a widely used method. In PFGE, the bacterial genome is cleaved with a rare-cutting enzyme (mostly Sma1) to 10–30 large fragments ranging in size from 10 to 800 kb. Sufficient fragment separation is obtained by electrophoresis, in which the current changes polarity in pulses. PFGE, currently regarded as the gold standard method, is highly reproducible and discriminatory, and coordinated inter-laboratory surveillance has enabled the development of standardized protocols (VAN BELKUM et al. 1998). PFGE has been widely applied in typing bovine *S. aureus* (RABELLO et al. 2007). The major disadvantage of PFGE lies in the subjective interpretation of fragmentation patterns, and the comparison of the results from different laboratories has proven difficult. PFGE is also technically difficult (STRUELENS et al. 2009).

*Spa* typing utilizes polymorphism in the variable region of the gene encoding *Staphylococcus aureus* protein A (*spa*). The *spa* gene possesses a repetitive region called the short sequence repeat (SSR), which consists of a variable number of 21 to 27 bp nucleotide repeats. *Spa* typing was performed with the specific primers mentioned previously (HARMSEN et al. 2003), and the numeric *spa*-repeats and *spa* type codes were determined using the Ridom SpaServer website (www.spaserver.ridom.de).

The aim of MLST is to provide an accurate, and highly discriminating typing system that can be used for *S. aureus* and also most bacteria as well as some other organisms. MLST has the advantage of generating results, which can be easily compared between laboratories. It is envisaged that this approach is particularly helpful for the typing of bacterial pathogens. MLST is dependent on the sequencing of seven housekeeping genes in the *S. aureus* genome. It is highly unlikely that two unrelated isolates would have identical allelic profiles at all of the seven loci by chance (ENRIGHT et al. 2000). A sequence type (ST) number is assigned to those isolates that are identical by MLST and closely related STs are grouped into clonal clusters (CCs). The founder and CCs of each ST are determined by using the enhanced version of based upon Related Sequence Types (eBURST) (FEIL et al. 2004). A phylogenetic tree can be constructed using the ClustalW website (http://clustalw.ddbj. Nig.ac.jp/top-e.html), based on the 3,198 bp of seven target loci sequence, which were concatenated in the
order *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*. Isolates with the same MLST profile may have descended from a common ancestor that existed years ago (ENRIGHT et al. 2000). MLVA is a method used to perform molecular typing of particular microorganisms. It utilizes the naturally occurring variation in the number of tandems repeated DNA sequences found in many different loci in the genome of a variety of organisms. MLVA is a high-throughput method increasingly utilized in place of PFGE (STRUELENS et al. 2009). MLVA is also widely used to assess the molecular fingerprint of micro-organisms such as bacteria. There is no standardized nomenclature and multiple schemes are currently used.
3 AIMS OF THE STUDY

1. To develop and evaluate the loop-mediated isothermal amplification assay (LAMP) by using six primers to detect *S. aureus* based on the specific gene *nuc*.

2. To identify the phenotypic and genotypic characteristics of *S. aureus* mastitis isolates by using the conventional and the molecular methods.

3. To investigate antimicrobial resistance of *S. aureus* isolated from bovine mastitis using antimicrobial susceptibility test and also to determine the specific gene *mecA* for MRSA.

4. To estimate the relationship between the *S. aureus* isolates in the present study with the *S. aureus* isolates from a human and with *S. aureus* isolates from different European countries.
4 MATERIALS AND METHODS

4.1 Development of a Molecular Identification Method for the Detection of S. aureus

4.1.1 Bacterial Isolates

All S. aureus isolates (n = 70) used in the present study were isolated from bovine mastitis milk from August 2001 to March 2014 from different regions of Northern Germany. These were obtained from routine mastitis diagnostics conducted at the Institute of Food Quality and Food Safety, University of Veterinary Medicine, Foundation, Hannover, Germany.

4.1.2 Reference Strains

The following strains were used in the present study: ATCC 6538 reference strain S. aureus was used as a positive control to determine the nuc gene. For exclusivity testing, 21 non-S. aureus strains were used, which were obtained from the institute’s strains collection (Table 1). S. aureus (MRSA) strain (120/14) was kindly provided by Dr Jochen Schulz, Institute of Animal Hygiene, Animal Welfare and Ethology, University of Veterinary Medicine Hannover, Foundation. Toxin-producing S. aureus reference cultures 619/93 (SEA), 62/92 (SEB), 1229/93 (SEC), 1634/93 (SED), FRI 918/93 (SEE), 161/93 (TSST-1), Ly 990055 (SEG + SEI), Ly 990552 (SEH), and 2724 (SEJ) were kindly provided by Dr Ömer Alkineden, Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig-University Giessen, Germany.
Table 1: Strains used for inclusivity and exclusivity testing of the *S. aureus* LAMP assay based on the *nuc* gene

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain number/origin</th>
<th>Detection time min: sec</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
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<td><em>S. aureus</em></td>
<td>Bovine mastitis isolates n = 70</td>
<td>05:55</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em></td>
<td>ATCC 6538</td>
<td>07:30</td>
</tr>
<tr>
<td>3</td>
<td><em>S. aureus</em> (MRSA)</td>
<td>(120/14)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>S. aureus</em></td>
<td>619/93 (SEA)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>S. aureus</em></td>
<td>62/92 (SEB)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>S. aureus</em></td>
<td>1229/93 (SEC)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>S. aureus</em></td>
<td>1634/93 (SED)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>S. aureus</em></td>
<td>FRI 918/93 (SEE)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>S. aureus</em></td>
<td>161/93 (TSST-1)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>S. aureus</em></td>
<td>Ly 990055 (SEG + SEI)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>S. aureus</em></td>
<td>Ly 990552 (SEH)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>S. aureus</em></td>
<td>2724 (SEJ)</td>
<td></td>
</tr>
</tbody>
</table>

**Non-*S. aureus* strains**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain number/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Acinetobacter baumannii</em></td>
</tr>
<tr>
<td>2</td>
<td><em>Arcanobacterium phocae</em></td>
</tr>
<tr>
<td>3</td>
<td><em>Arcanobacterium hippocoleae</em></td>
</tr>
<tr>
<td>4</td>
<td><em>Arcanobacterium canis</em></td>
</tr>
<tr>
<td>5</td>
<td><em>Arcanobacterium pluranimalium</em></td>
</tr>
<tr>
<td>6</td>
<td><em>Clostridium perfringens A</em></td>
</tr>
<tr>
<td>7</td>
<td><em>Clostridium perfringens E</em></td>
</tr>
<tr>
<td>8</td>
<td>Coagulase-negative staphylococci</td>
</tr>
<tr>
<td>9</td>
<td><em>Enterococcus faecalis</em></td>
</tr>
<tr>
<td>10</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>11</td>
<td><em>Klebsiella pneumoniae</em></td>
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<tr>
<td>12</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>13</td>
<td><em>Salmonella enterica</em></td>
</tr>
<tr>
<td>14</td>
<td><em>Streptococcus uberis</em></td>
</tr>
<tr>
<td>15</td>
<td><em>Streptococcus agalactiae</em></td>
</tr>
<tr>
<td>16</td>
<td><em>Streptococcus dysgalactiae</em></td>
</tr>
<tr>
<td>17</td>
<td><em>Trueperella pyogenes</em></td>
</tr>
<tr>
<td>18</td>
<td><em>Trueperella bialowiezense</em></td>
</tr>
<tr>
<td>19</td>
<td><em>S. epidermidis</em></td>
</tr>
<tr>
<td>20</td>
<td><em>S. intermedius</em></td>
</tr>
<tr>
<td>21</td>
<td><em>S. hyicus</em></td>
</tr>
</tbody>
</table>

* Institutes’ collection strains
MATERIALS AND METHODS

4.1.3 Extraction and Preparation of Bacterial DNA

According to the manufacturer’s instructions on using the Gram-positive protocol, the total chromosomal DNA of *S. aureus* isolates was extracted by DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The isolates were cultured on the blood agar and incubated at 37°C for 24 hours. 5–10 colonies were suspended in 180 µL of lysis buffer containing 20 mg µL⁻¹ lysozyme and 3 µl lysostaphin (1 mg µL⁻¹, Sigma-Aldrich Chemie, Steinheim, Germany) was added and then incubated for 30 minutes at 37°C. After incubation, 20 µL proteinase K (20 mg mL⁻¹) and 200 µL buffer AL (Qiagen) were added and mixed by vortexing, then incubated at 56°C for 30 minutes. Later, 200 µL of ethanol was added to the sample. The mixture was transferred to the Mini Spin column and centrifuged at 6,200 × g for 1 minute. 500 µL of wash buffer (AW1) was added to the Mini Spin column and centrifuged at 6200 × g for 1 minute. 500 µL wash buffer (AW2) was added to the Mini Spin column and centrifuged at 17,000 × g for 3 minutes. The Mini Spin column was placed in clean 1.5 mL microcentrifuge tubes. Then 200 µL elution buffer AE was added and centrifuged at 6,200 × g for 1 minute. The DNA concentration was estimated by using NanoDrop 2000c (PeQlab, Erlangen, Germany). The DNA yield was stored at -20°C until further use.

4.1.4 Designs of Oligonucleotide Primers for LAMP Assay

The development of LAMP primers was based on the sequence of the *nuc* gene (accession number DQ399678.1) published in the National Center for Biotechnology (NCBI) GenBank, USA (Figures 7). According to Tables 2, the six LAMP primers were designed using LAMP Designer software, ver. 1.10 (PREMIER Biosoft, USA) and included the outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), forward loop primer (LoopF) and backward loop primer (LoopB). The primers were synthesized by Eurofins MWG Operon (Eurofins MWG Operon, Ebersberg, Germany).

The set of LAMP primers for *S. aureus* designed by using LAMP Designer software based on the sequence of the *nuc* gene for the detection of *S. aureus*, amplifies an approximate 694 bp fragment (Figure 7). Based on these specifications, the size of the amplicon region used to generate oligonucleotide primers ranged from 217 to 574 bp.
Figure 7: The positions of the six LAMP primers designed. The outer forward primer (F3) and the outer backward primer (B3) of the *nuc* gene are indicated in yellow colour. The forward loop primer (Loop F) and the backward loop primer (Loop B) are indicated in blue colour, while the forward inner primer (FIP) (F2+F1c) is indicated by green colour and the backward inner primer (BIP) (B2+B1c) is indicated by red colour. The used nucleotide sequence of the *nuc* gene of *S. aureus* was published by the National Center for Biotechnology (NCBI) GenBank, USA under the accession number DQ 399678.
MATERIALS AND METHODS

Table 2: Oligonucleotide primer sequence of LAMP and their positions for detecting the nuc gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Position on DQ 399678</th>
<th>Primer length</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>GAAGTGGTTCTGAAGATCCAA</td>
<td>218</td>
<td>21 bp</td>
<td>55.9°C</td>
</tr>
<tr>
<td>B3</td>
<td>CCAAGCCTTGACGACTAA</td>
<td>574</td>
<td>19 bp</td>
<td>54.5°C</td>
</tr>
<tr>
<td>FIP (F1c+F2)</td>
<td>AGGATGCTTTTGGTCAGGTGTCG ATTGATGGTGATACGGTTA</td>
<td>42 bp</td>
<td>71.4°C</td>
<td></td>
</tr>
<tr>
<td>BIP (B1c+B2)</td>
<td>AATATGGTCCTGAAGCAAGTGTC AATATGGTCCTGAAGCAAGTGTC</td>
<td>40 bp</td>
<td>72.5°C</td>
<td></td>
</tr>
<tr>
<td>Loop F</td>
<td>TGTCATTGGTTGACCTTTGTAC</td>
<td>348</td>
<td>22 bp</td>
<td>56.5°C</td>
</tr>
<tr>
<td>Loop B</td>
<td>GAAGTCGAGTTTGACAAAGGTGC</td>
<td>466</td>
<td>22 bp</td>
<td>58.4°C</td>
</tr>
<tr>
<td>F2</td>
<td>CGATTGATGGTGATACGGTTA</td>
<td>299</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1c</td>
<td>AGGATGCTTTTGGTCAGGTGTC</td>
<td>390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>GCTAAGCCACGTCCATAT</td>
<td>518</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1c</td>
<td>AATATGGTCCTGAAGCAAGTGTC</td>
<td>407</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1.5 LAMP Reaction

The LAMP reaction was carried out in the Integrated Cap (Amplex Diagnostics GmbH, Giessen, Germany) with a total volume of 25 µL (Table 3) containing 0.5 µL of each primer F3 and B3 (25 pmol µL⁻¹) equivalent to 0.5 µM final concentration, 2.0 µL of each primer FIP and BIP (25 pmol µL⁻¹) equivalent to 2 µM final concentration and 1.0 µL of each primer LoopF and LoopB (25 pmol µL⁻¹) equivalent to 1 µM final concentration. The amount of Isothermal Master Mix Iso-001 (OptiGene, UK) needed for one reaction was 13 µL. Finally, 5 µL of isolated DNA was added as a template and amplification was performed at 65°C for 30 minutes. The temperature of the melting curve analysis was 98–80°C ramping at 0.05°C per second in the real-time fluorometer (Genie II®) (OptiGene Limited) according to the manufacturer's instructions.
Table 3: Composition of the LAMP reaction mixture used for detecting nuc gene

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
<th>Require vol/reaction µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2× Master Mix</td>
<td>13 µL</td>
<td></td>
<td>13 µL</td>
</tr>
<tr>
<td>Primer F3 (25 pmol/µL)</td>
<td>0.5 µL</td>
<td>0.5 µM</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Primer B3 (25 pmol/µL)</td>
<td>0.5 µL</td>
<td>0.5 µM</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Primer Loop F (25 pmol/µL)</td>
<td>1.0 µL</td>
<td>1.0 µM</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Primer Loop B (25 pmol/µL)</td>
<td>1.0 µL</td>
<td>1.0 µM</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Primer FIP (25 pmol/µL)</td>
<td>2.0 µL</td>
<td>2.0 µM</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>Primer BIP (25 pmol/µL)</td>
<td>2.0 µL</td>
<td>2.0 µM</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>Template DNA (52 ng/ µL⁻¹)</td>
<td>5.0 µL</td>
<td></td>
<td>5.0 µL</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>25 µL</td>
<td></td>
<td>25 µL</td>
</tr>
</tbody>
</table>

4.1.6 Additional Detections of LAMP Products

The LAMP amplicons were also detected by gel electrophoresis of 10 µL of the amplicon in a 2% peqGOLD Universal agarose gel (PeQlab), with Tris/Borate/EDTA buffer (TBE, pH 8.3). A molecular marker for 50 bp or 100 bp (Biozym Diagnostic, Germany) was used.

4.1.7 S. aureus Isolates Identity Confirmation

S. aureus isolates were confirmed with conventional PCR method using species-specific primers for the detection of nuc genes. The total volume of PCR reaction mixtures was 30 µL⁻¹ that consisted of 1 µL⁻¹ primer 1 (10 pmol µL⁻¹), 1 µL⁻¹ primer 2 (10 pmol µL⁻¹) (Eurofins Genomics), 15 µL⁻¹ of 2 × Red Y: Gold Mix Master containing (1 unit GoldStar DNA polymerase, 200 µM dNTPs, 1.5 µM MgCl₂, 20 µM [NH₄]₂SO₄, 75 µM Tris—HCl [pH 8.8 at 25°C], 0.01% [v/v] Tween 20™ and Red Dye Loading Buffer) (Eurogentec Deutschland, Cologne, Germany) and 10.5 µL⁻¹ Nuclease-Free Water (Qiagen). Finally, 2.5 µL⁻¹ DNA templates of S. aureus were added to each reaction tube. The PCR conditions consisted of initial denaturation at 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 40 seconds and a final elongation at 72°C for 7 minutes. The PCR products were visualized by using electrophoresis of 10 µL of the amplicon in a 2% peqGOLD Universal agarose gel.
4.1.8 Analytical Sensitivity of the LAMP Assay and Limit of Detection (LOD)

Serially diluted DNA from the *S. aureus* reference strain was used for determining the analytical sensitivity of the LAMP reaction. The amplicons were detected with the real-time fluorometer. Serial dilutions of *S. aureus* DNA (10^{-1}-10^{-8}) were prepared using Tris buffer (TE, pH 8.0). The limit of detection (LOD) was estimated by spiking milk (10 ml) with serial dilutions (10^{-1}-10^{-8}) of the *S. aureus* reference strain freshly cultured overnight at 37°C in blood agar. The concentration of the initial suspension was estimated by using the colony-forming unit (CFU). All the spiked milk samples were centrifuged at 3700 × g for 45 minutes. The supernatant, including the cream layer, was discarded. The pellets were resuspended in 180 ml lysis buffer, and the DNA was later isolated as mentioned above.

4.1.9 Positive and Negative Predictive Values (PPV and NPV)

The PPV and NPV are regarded as descriptions of the performance of a newly developed diagnostic test. The calculation of PPV and NPV was based on the final diagnosis for each tested *S. aureus* isolate. The diagnosis was based on the microbiological results that included morphological colony features, haemolytic characteristics, catalase test, clumping factor (Oxoid, Altrincham, England), coagulase tests using rabbit plasma (tube method) (Becton, Dickinson, Heidelberg, Germany) and biochemical tests (API Staph identification system) (bio-Mérieux, S.A., Marcy l’Etoile, France). The PCR was additionally performed to confirm the presence of the *nuc* gene in all the isolates of this study.

The PPV was calculated by the formula:

\[
PPV = \frac{(\text{Number of true positive})}{(\text{Number of true positive} + \text{number of false positive})} \times 100
\]

The NPV was calculated by the formula:

\[
NPV = \frac{(\text{Number of true negative})}{(\text{Number of true negative} + \text{number of false negative})} \times 100
\]
The accuracy (AC) was calculated by the formula:

\[
AC = \frac{\text{Number of true positive}}{\text{Number of true positive} + \text{number of false positive}} \times 100
\]

### 4.2 Conventional Identification and Phenotypic Characterization of \textit{S. aureus} Isolates

All \textit{S. aureus} isolates were cultured on blood agar (Oxoid, Wesel, Germany) and incubated for 24 hours at 37°C. The isolation of \textit{S. aureus} from milk samples was conducted by using the method proposed by the National Mastitis Council (NMC) recommendations (BOSS et al., 2011). Isolates grown on blood agar plates were identified tentatively according to the morphological colony features, haemolytic characteristics, the catalase test, staphaurex latex agglutination test (clumping factor and protein A) (Oxoid) and coagulase test using rabbit plasma (Becton).

The further identification of the isolates was performed by using the commercial API-32 Staph biochemical identification system, according to the manufacturer’s instructions. Briefly, a few colonies from overnight culture on blood agar were transferred to a tube containing 2 ml of suspension medium. This suspension medium was compared with the turbidity control (McFarland Standard 0.5). After preparation, the strips were incubated aerobically at 37°C for 24 hours.

### 4.3 Antimicrobial Susceptibility Testing by Microdilution Test

Susceptibility to 11 types of antibiotics was examined in 96-well round-bottom U plates. The types of antibiotics used in the test were amoxicillin (AMC), ampicillin (AMP), cefazolin (CEZ), cefoperazone (CPZ), cefquinome (CEQ), erythromycin (ERY), gentamicin (GEN), tetracycline (TET), oxacillin (OXA), penicillin (PEN), and pirlimycin (PIR) (Merlin, Bornheim-Hersel, Germany). The walls of the plates were inoculated with Mueller-Hinton broth cultured with the isolates. The plates were incubated at 37°C for 24 hours, as recommended by the manufacturer and by the Clinical and Laboratory Standards Institute.
(CLSI) guide M31–A3 (CLSI, 2014). After 24 hours, the plates were studied by the naked eye.

4.4 Molecular Characterization of *S. aureus* Isolates Using DNA-Based Methods

4.4.1 Detection of Genes Encoding Virulence Factors Using Polymerase Chain Reaction

The presence of the genes encoding staphylococcal virulence factors, which include thermonuclease (*nuc*), clumping factor (*clfA*), clumping factor (*clfB*), Methicillin Resistant *S. aureus* (MRSA) (*mecA*) and coagulase (*coa*), was investigated by PCR. In addition, PCR was also used to amplify the genes encoding staphylococcal enterotoxins, i.e. SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), SEE (*see*), SEG (*seg*), SEH (*seh*), SEI (*sei*), SEIJ (*selj*), SEM (*sem*), SEN (*sen*), SEO (*seo*), SEP (*sep*), SEQ (*seq*), SER (*ser*), SEIU (*selu*) and TSST (*tst*). The primers and the references used for detection of genes are listed in Table 5.

4.4.2 PCR Reaction

The total volume of PCR reaction mixtures was 30 µL, which consisted of 1 µL of each forward primer and reverse primer (each 10 pmol µL⁻¹) (Eurofins Genomics, Ebersberg, Germany), 15 µL of 2 × Red Y Gold Mix Master (containing 1 unit GoldStar DNA polymerase, 200 µM dNTPs, 1.5 µM MgCl₂, 20 µM [NH₄]₂SO₄, 75 µM Tris–HCl [pH 8.8 at 25°C], 0.01% [v/v] Tween 20™ and Red Dye Loading Buffer) (Eurogentec Deutschland, Cologne, Germany) and 10.5 µL Nuclease-Free Water (Qiagen). Finally, 2.5 µL DNA templates of *S. aureus* were added to each reaction tube (Table 4). The amplification was performed in the Gene Amplification PCR system Thermal Cycler (Biometra, Göttingen, Germany). Each program consisted of three steps—denaturation, annealing, and extension. The temperature of each step differed according to the program. The specific gene probes were amplified by using the primers and conditions described in Table 5. A positive control and a negative control (reaction mixture without DNA template) were included in each PCR run.
Table 4: Composition of PCR reaction mixture used for detecting genes encoding virulence factors and housekeeping genes

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
<th>Require vol/reaction µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>1 µL</td>
<td>0.4 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1 µL</td>
<td>0.4 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>2×Master Mix</td>
<td>15 µL</td>
<td></td>
<td>15 µL</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>10.5 µL</td>
<td></td>
<td>10.5 µL</td>
</tr>
<tr>
<td>Template DNA (52 ng/ µL⁻¹)</td>
<td>2.5 µL</td>
<td></td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>30 µL</td>
<td></td>
<td>30 µL</td>
</tr>
</tbody>
</table>

4.4.3 Agarose Gel Electrophoresis

The DNA amplifications were detected by using gel electrophoresis and subsequent optical representation. Due to the size of the expected amplicons, two owned agarose gel were used. For this purpose, in 2% agarose gel (PeQlab) with 1×Tris–acetate–Electrophoresis Buffer (Roth, Karlsruhe, Germany), and 10.8 g L⁻¹ Tris base, 5.5 g L⁻¹ boric acid, 0.75 g L⁻¹ EDTA and deionized were melted in the microwave oven and after cooling, 44°C to about 56°C, the liquid agarose was poured into a gel mould with the adjusted comb in Flatbed forms. After solidification of the agarose, the comb was carefully removed and then the gel in a column was filled with a suitable running buffer in the gel chamber. After that, 10 µL of the PCR product was loaded on the agarose gel. Electrophoresis in the gel chambers was performed as running buffer served 1x TBE buffer. For the estimation of the molecular weight of each amplicon, the molecular weight standard markers of 100 bp (1 µg/µl) and 50 (1 µg/µl) bp (Roche or Biozym) were used. The electrophoretic separation of the reaction mixtures was carried out at 120 Voltage for two hours.

4.4.4 Ethidium Bromide Staining

After the successful completion of electrophoresis, the gel was stained with an aqueous ethidium bromide solution (5 µg/ml) for five minutes. Ethidium bromide fluorescence staining material is characterized by the ability to interact and impregnated with double-stranded DNA. The excessive staining material was removed by rinsing the gel in aqua dest for 30 minutes at room temperature. After that, the gel was neatly removed from the aqua des
and then the band was visualized by ultra violet transilluminator and photographed (INTAS, Göttingen, Germany). The fragments could be seen as bright bands on a dark background.

4.5 Genotypic Characterisation of S. aureus Isolates

4.5.1 Multi-Locus Sequence Typing (MLST)

MLST was carried out with the primers that were previously designed by Enright et al. (2000) for detecting S. aureus housekeeping genes (Table 6). These include seven housekeeping genes: carbamate kinase (arcC), shikimate dehydrogenase (aroE), glycerol kinase (glpF), guanylate kinase (gmk), phosphate acetyltransferase (pta), triosephosphate isomerase (tpi), and acetyl coenzyme A acetyltransferase (yqiL). PCR was used to amplify all the housekeeping genes, and the amplicons were sequenced by Seqlab (Hann-vogt, Göttingen, Germany). DNA sequences were assembled by using the software program BioNumerics v7.5 (Applied Maths, Sint-Martens-Latem, Belgium). All allele and sequence types (ST) were determined according to the MLST website (www.mlst.net).

The relatedness of the fingerprints between the isolates was determined by using both computer analysis (BioNumerics v7.5, Applied Maths) and visual comparison. In the present study, the isolates were collected during several years from different regions, and the genetic relatedness were determined by using the following criteria based on the different fingerprints in visual comparison: Fingerprints with one to two band shifts were considered to be closely related and clustered in groups. Fingerprints with more than three band shifts were interpreted as being genetically unrelated.

4.5.2 Protein A (spa) Typing

The spa typing was performed with specific primers, which were previously described (SHOPSIN et al. 1999) (Table 6). Staphylococcal Protein A contains a specific repeat region that was amplified; afterwards, DNA sequencing was done by PCR. All the spa-repeats and typing were assigned by using the software program BioNumerics v7.5. All numeric spa typing and spa type codes were determined according to the Ridom Spa Server website (www.spaserver.ridom.de).
Table 5: Oligonucleotide primers and PCR programs for amplification of various toxin genes of *S. aureus* and staphylococcal proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Amplicon size [bp]</th>
<th>PCR Programme*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>clfA</td>
<td>clfA-1</td>
<td>5-ATTGGCGTGGCTTCAGTGCT-3</td>
<td>288</td>
<td>I</td>
<td>(TRISTAN et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>clfA-2</td>
<td>5-CTTTACTCCTGGATGTTG-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clfB</td>
<td>clfB-1</td>
<td>5-ACATCGAATATGAGGGTAAC-3</td>
<td>203</td>
<td>I</td>
<td>(TRISTAN et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>clfB-2</td>
<td>5-CTCGCATCTTGGGATGTC-3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>coa</td>
<td>coa-1</td>
<td>5-ATAGAGATGCTTGTCACAGG-3</td>
<td>674-917</td>
<td>I</td>
<td>(QUINN et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>coa-2</td>
<td>5-GCTTCCGATTTGCTTGAC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecA</td>
<td>MEC A-1</td>
<td>5-GTGAAGATATACCAAGTGATT-3</td>
<td>147</td>
<td>I</td>
<td>(K. ZHANG et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>MEC A-2</td>
<td>5-ATGGCGTGGCTTCAGTGCT-3</td>
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<tr>
<td>nuc</td>
<td>nuc-1</td>
<td>5-CCTGAAGCAAGTGCATTTACGA-3</td>
<td>666</td>
<td>I</td>
<td>(GRABER et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>nuc-2</td>
<td>5-CTTTACTCCTGGATGTTG-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sea</td>
<td>SEA-1</td>
<td>5-AAAGTCCCGATCAATTTATGGCTA-3</td>
<td>219</td>
<td>I</td>
<td>(TSEN and CHEN 1992)</td>
</tr>
<tr>
<td></td>
<td>SEA-2</td>
<td>5-GTAAATACCAAGGGTCCAAC-3</td>
<td></td>
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<td></td>
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<tr>
<td>seb</td>
<td>SEB-1</td>
<td>5-TCGCATACAAGTGCAGG-3</td>
<td>478</td>
<td>I</td>
<td>(JOHNSON et al. 1991)</td>
</tr>
<tr>
<td></td>
<td>SEB-2</td>
<td>5-GCAAGCTCTTATGTCAG-3</td>
<td></td>
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<tr>
<td>sec</td>
<td>SEC-1</td>
<td>5-GACATAAAGCTAGAATTTG-3</td>
<td>257</td>
<td>I</td>
<td>(JOHNSON et al. 1991)</td>
</tr>
<tr>
<td></td>
<td>SEC-2</td>
<td>5-AAATCGGATACCACTATCC-3</td>
<td></td>
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<tr>
<td>sed</td>
<td>SED-1</td>
<td>5-CTAGTTTGGTATATATGCTC-3</td>
<td>317</td>
<td>II</td>
<td>(JOHNSON et al. 1991)</td>
</tr>
<tr>
<td></td>
<td>SED-2</td>
<td>5-CTATCCGATTACCACTATCC-3</td>
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<td>see</td>
<td>SEE-1</td>
<td>5-TACCAATTAACTTGTTGACAC-3</td>
<td>171</td>
<td>I</td>
<td>(PEREIRA et al. 2009)</td>
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<tr>
<td></td>
<td>SEE-2</td>
<td>5-CTTTCTGCACTTACCCG-3</td>
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<td>seg</td>
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<td>5-AAATTGCATGCTCAACCGGC-3</td>
<td>642</td>
<td>I</td>
<td>(JARRAUD et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>SEG-2</td>
<td>5-AATTTCTATAGGAAAAGGTATAGTC-3</td>
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<td>sch</td>
<td>SEH-1</td>
<td>5-CAATCAGATGCTCAACCGGC-3</td>
<td>375</td>
<td>II</td>
<td>(JARRAUD et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>SEH-2</td>
<td>5-CATCTACCAACCGGCATAC-3</td>
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<tr>
<td>sei</td>
<td>SEI-1</td>
<td>5-CTCAAGGGTGATTTGTCGG-3</td>
<td>576</td>
<td>I</td>
<td>(JARRAUD et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>SEI-2</td>
<td>5-GAATTTTACAGGCGATCAC-3</td>
<td></td>
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<td>selj</td>
<td>SELJ-1</td>
<td>5-GATCTGATGCTGTAGGCTTAG-3</td>
<td>142</td>
<td>I</td>
<td>(MONDAY and BOHACH 1999)</td>
</tr>
<tr>
<td></td>
<td>SELJ-2</td>
<td>5-GTGTTACAACTTGAGGAT-3</td>
<td></td>
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<td>sem</td>
<td>SEM-1</td>
<td>5-CTTTGCACTTACCCG-3</td>
<td>471</td>
<td>I</td>
<td>(AKINEDEN et al. 2008)</td>
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<tr>
<td></td>
<td>SEM-2</td>
<td>5-TCTTCTGCACTTACCCG-3</td>
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<td>sen</td>
<td>SEN-1</td>
<td>5-GAAGGTACGATATGCTACCCG-3</td>
<td>292</td>
<td>I</td>
<td>(AKINEDEN et al. 2008)</td>
</tr>
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<td>5-CTTTTCTGCACTTACCCG-3</td>
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<td>seo</td>
<td>SEO-1</td>
<td>5-ATCAAGGCTGGCAAGGATGAC-3</td>
<td>249</td>
<td>II</td>
<td>(AKINEDEN et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>SEO-2</td>
<td>5-ATGTTGAGCTGGCAAGGATGAC-3</td>
<td></td>
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<tr>
<td>sep</td>
<td>SEP-1</td>
<td>5-ACTGAAACTATGCTGAGG-3</td>
<td>148</td>
<td>I</td>
<td>(CHIANG et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>SEP-2</td>
<td>5-ACTGAAACTATGCTGAGG-3</td>
<td></td>
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<tr>
<td>seq</td>
<td>SEQ-1</td>
<td>5-TCAGGACTGTTTTGATACCAA-3</td>
<td>359</td>
<td>I</td>
<td>(CHIANG et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>SEQ-2</td>
<td>5-TCTTACTGAGGCTGTTTACTG-3</td>
<td></td>
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<tr>
<td>ser</td>
<td>SER-1</td>
<td>5-AGATTTTGGAATGACCCATT-3</td>
<td>123</td>
<td>I</td>
<td>(CHIANG et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>SER-2</td>
<td>5-ACCTGACAGTGTTTCGCTG-3</td>
<td></td>
<td></td>
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<tr>
<td>selu</td>
<td>SELU-1</td>
<td>5-ATTTGTGTTTTTTATCTTC-3</td>
<td>167</td>
<td>III</td>
<td>(CHIANG et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>SELU-2</td>
<td>5-GAGACTTAAAGGTATTGTG-3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>tst</td>
<td>TSS-1</td>
<td>5-GCTTCCGAGCAGCTGTCAG-3</td>
<td>559</td>
<td>I</td>
<td>(LOVSETH et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>TSS-2</td>
<td>5-GTTGATTTTTTTATCTTC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*I: 35 times (94°C – 30s, 55°C – 30s, 72°C – 30s); II: 35 times (94°C – 30s, 53°C – 30s, 72°C – 30s); III: 35 times (94°C – 30s, 51°C – 30s, 72°C – 30s).
Table 6: Sequences of primers used in the MLST and spa typing analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3)</th>
<th>Amplicon Size [bp]</th>
<th>PCR Programme*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamate kinase (arcC)</td>
<td>arcC-1</td>
<td>5'-TTGATTCACCAGCCGCTATTGCT-3</td>
<td>569</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>arcC-2</td>
<td>5'-AGGTATCTGGTTCAACAGC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shikimate dehydrogenase (aroE)</td>
<td>aroE-1</td>
<td>5'-ATCGGAAATCTTCTTCCACATTC-3</td>
<td>535</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>aroE-2</td>
<td>5'-GATTTGTTAATTAAACGGATATC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol kinase (glpF)</td>
<td>glpF-1</td>
<td>5'-CTTAGGAACTGCAATTTAATCC-3</td>
<td>575</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>glpF-2</td>
<td>5'-TGGTAAATCGCATGCTCAATTCC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanylate kinase (gmk)</td>
<td>gmk-1</td>
<td>5'-ATCGTTTTATCGGGACCATC-3</td>
<td>487</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>gmk-2</td>
<td>5'-TCATTAAACTAACTGTAATCGTA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate acetyltransferase (pta)</td>
<td>pta-1</td>
<td>5'-GTTTAAATCGTATTACCTGAAGG-3</td>
<td>574</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>pta-2</td>
<td>5'-GACCCCTTTGTGAAAAGCTTA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triosephosphate isomerase (tpi)</td>
<td>tpi-1</td>
<td>5'-TCGTTTCATTTGAACGTCGTA-3</td>
<td>470</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>tpi-2</td>
<td>5'-TTGGCACCTCTAACAATTGT-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcoenzyme A acetyltransferase (yqiL)</td>
<td>yqiL-1</td>
<td>5'-CAGCATAACGAGACCTTATG-3</td>
<td>597</td>
<td>I</td>
<td>(ENRIGHT et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>yqiL-2</td>
<td>5'-CGTGGAGGATTACTGGA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spa</td>
<td>SPA-1</td>
<td>5'-AGACGATCCTTCGTTGAAC-3</td>
<td>variable</td>
<td>II</td>
<td>(SHOPSIN et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>SPA-2</td>
<td>5'-GCTTTGTGAAAATGCTTTACTG-3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR programme: I: 35 times (94°C – 30s, 55°C – 30s, 72°C – 30s), II: 35 times (94°C – 30s, 55°C – 30s, 72°C – 30s)
5 RESULTS

5.1 Specificity of the LAMP Assay for Detecting nuc Genes

The six designed primers and the reaction conditions enabled the detection of nuc genes in all the investigated S. aureus isolates. The results of the LAMP assays revealed that the nuc gene was found in all the 70 S. aureus isolates investigated in the present study (100%). No signal was detected with any of the tested non-S. aureus strains. The results of the LAMP assays were compared to the conventional PCR results, revealing that the nuc gene was found in all S. aureus isolates (Figure 25).

The results of the LAMP reactions for detecting nuc genes were measured by using the real-time fluorometer (Figures 8) in addition to gel electrophoresis (Figures 9). The target sequences amplified by the LAMP assay showed ladder-like bands in the gel images due to the formation of stem-loop structures of amplified DNA of various stem lengths. The fluorescent signal was detected by using real-time fluorometer (Genie II®). Melting curve analysis, which is termed by Genie II® as anneal curve analysis, showed that there was no significant difference among the nuc genes of the tested S. aureus isolates. The melting temperature of the nuc gene-specific amplicon amounted to 81.9°C (± 0.3°C).
RESULTS

Figure 8: Upper curves (a): Typical amplification curves of the *S. aureus* isolates DNA for detecting the *nuc* gene in the present study. Lower curves (b): The melting curve (anneal reaction) of the same amplicons.
Figure 9: Agarose gel electrophoresis (2%) of the nuc gene LAMP reaction of *S. aureus* isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control strain ATCC 6538, Lanes 2–7 represent positive isolates, and Lane 8 is a non-template control. Lanes M are DNA Marker 50 bp ladder (Biozym Diagnostic).

### 5.2 Analytical Sensitivity of the LAMP Assay and Limit of Detection (LOD)

The DNA concentration of the serial dilution ranged from 5.2 ng µL⁻¹ (10⁻¹) to 0.052 pg µL⁻¹ (10⁻⁶). Figure 10 shows the amplification curves of the *S. aureus* DNA using the real-time fluorometer. The analytical sensitivity of the LAMP assay for detecting *S. aureus* DNA was 0.052 pg µL⁻¹, resulting in a DNA of 0.26 pg per reaction (5 µL of DNA/reaction) measurable after isothermal amplification for 21:22 (± 06:38) minutes (Table 7). The detection probability of this concentration was determined as 50% (two from four were positive). The detection probability of the other concentration was 100%.
RESULTS

Table 7: Analytical sensitivity of the LAMP assay using serial dilutions of *S. aureus* ATCC 6538 DNA

<table>
<thead>
<tr>
<th>Dilution step</th>
<th>DNA amount pg µL⁻¹</th>
<th>Detection time (min:sec)</th>
<th>Mean (Sd ±)</th>
<th>Detection probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹</td>
<td>5200</td>
<td>07:15 07:30 08:00 08:00</td>
<td>07:41 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻²</td>
<td>520</td>
<td>08:15 08:15 08:45 09:00</td>
<td>08:34 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻³</td>
<td>52</td>
<td>09:45 09:30 10:15 10:15</td>
<td>09:56 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>5.2</td>
<td>11:15 11:00 11:30 12:15</td>
<td>11:30 (00:28)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0.52</td>
<td>14:00 13:30 17:00 17:30</td>
<td>15:30 (01:46)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>0.052</td>
<td>14:45 28:00</td>
<td>21:22 (06:38)</td>
<td>50%</td>
</tr>
</tbody>
</table>

The LOD for the LAMP assay of the *S. aureus* reference strain in spiked milk was 9×10² CFU mL⁻¹ milk when isolated from 10 ml milk sample. The detection probability was 71.5% (five out of seven were positive) after a mean detection time of 22:21 (± 06:08) minutes (Table 8). The melting temperature of the DNA used for the LOD calculation in milk was 81.4°C (± 0.6°C) (Figure 10b).

Table 8: The limit of detection (LOD) of the LAMP assay by using milk samples spiked with serial dilutions of *S. aureus* ATCC 6538 reference strain

<table>
<thead>
<tr>
<th>CFU mL⁻¹ milk</th>
<th>Detection time (min:sec)</th>
<th>Mean (Sd ±)</th>
<th>Detection probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>9×10⁶</td>
<td>11:01 21:00 10:45 20:45 12:41 19:30 26:45</td>
<td>17:30 (05:39)</td>
<td>100%</td>
</tr>
<tr>
<td>9×10⁵</td>
<td>19:30 22:00 20:15 20:45 22:15 19:30 25:00</td>
<td>21:19 (01:49)</td>
<td>100%</td>
</tr>
<tr>
<td>9×10⁴</td>
<td>15:45 13:30 11:15 13:00 12:00 17:00 14:45</td>
<td>13:54 (01:54)</td>
<td>100%</td>
</tr>
<tr>
<td>9×10³</td>
<td>19:30 27:45 19:15 18:45 21:00 19:30 22:45</td>
<td>21:13 (02:57)</td>
<td>100%</td>
</tr>
<tr>
<td>9×10²</td>
<td>10:30 25:45 23:30 24:00 28:00</td>
<td>22:21 (06:08)</td>
<td>71.5%</td>
</tr>
</tbody>
</table>
Figure 10: Upper curves (a): Typical amplification curves of serially diluted *S. aureus* DNA of the reference strain. Lower curves (b): The melting curve (anneal reaction) of the same amplicons.

### 5.3 Estimation of Positive and Negative Predictive Values

The PPV and NPV for detecting the *nuc* gene from all *S. aureus* were 100% for the developed LAMP assay.
RESULTS

5.4 Microbiological Identification and Phenotypic Characterization of S. aureus Isolates

According to the guidelines of the National Mastitis Council (NMC), a total of 70 isolates were obtained from milk samples (one isolate per sample) that could be identified as S. aureus. Among these, 27 isolates (38.6%) showed alpha-hemolysis, 26 (37.1%) beta-hemolysis and 17 (24.3%) were non-haemolytic (Table 9). The staphaurex (clumping factor and protein A) test revealed that 75.7% of the isolates (n = 53) were positive, while all the investigated isolates were positive for the catalase test. The coagulase test was performed on the isolates that had shown a negative result in the staphaurex test to ensure that the isolates were S. aureus. The API 32 Staph identification system confirmed that all the isolates were S. aureus. The rate of identification of 64 isolates was ID 95% to 99.8%. The remaining isolates displayed lower rates of identification, i.e. 82%, 79%, 78% (one isolate each) and 62% (three isolates).

Table 9: Conventional identification methods for detecting the characterization of S. aureus isolates

<table>
<thead>
<tr>
<th>Test</th>
<th>Frequency of occurrence</th>
<th>Number of isolate</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>only α-hemolysis</td>
<td></td>
<td>27</td>
<td>38.6</td>
</tr>
<tr>
<td>only β-hemolysis</td>
<td></td>
<td>26</td>
<td>37.1</td>
</tr>
<tr>
<td>non-haemolytic</td>
<td></td>
<td>17</td>
<td>24.3</td>
</tr>
<tr>
<td>clumping factor</td>
<td></td>
<td>53</td>
<td>75.7</td>
</tr>
<tr>
<td>catalase test</td>
<td></td>
<td>70</td>
<td>100</td>
</tr>
</tbody>
</table>

5.5 Antimicrobial Susceptibility Testing

All the isolates were tested for their resistance to 11 antimicrobials (Table 10). Phenotypically, the majority of the isolates, i.e. 53 (75.7%), were sensitive, and only 17 (24.3%) showed any resistance to one or more antibiotics. The isolates showed a high resistance rate to penicillin and ampicillin (22.9% each). The resistance of S. aureus to pirlimycin, amoxicillin and cefoperazone were 5.7%, 2.9%, and 1.4%, respectively.
The isolates of *S. aureus* demonstrated sensitivity to most antimicrobials in varying proportions. All the *S. aureus* isolates were sensitive to cefazolin, cefquinome, tetracycline and oxacillin 70 (100%). Nearly all (69; 98.6%) the isolates were sensitive to gentamicin. The sensitivity of the isolates to amoxicillin, pirlimycin, cefoperazone, ampicillin, penicillin and erythromycin was 68 (97.1%), 66 (94.3%), 66 (94.3%), 54 (77.1%), 54 (77.1%) and four (5.7%), respectively. The *S. aureus* isolates appeared to be intermediate to erythromycin 66 (94.3%). The intermediate of the other isolates to cefoperazone and gentamicin could be found in three (4.3%) and in one (1.4%) of the strains, respectively.

### Table 10: Antimicrobial resistance pattern of bovine mastitis *S. aureus* isolates

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Sensitive No. (%)</th>
<th>Intermediate No. (%)</th>
<th>Resistant No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>68 (97.1)</td>
<td>0</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>54 (77.1)</td>
<td>0</td>
<td>16 (22.9)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>70 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>66 (94.3)</td>
<td>3 (4.3)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Cefquinome</td>
<td>70 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4 (5.7)</td>
<td>66 (94.3)</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>69 (98.6)</td>
<td>1 (1.4)</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>70 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>70 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>54 (77.1)</td>
<td>0</td>
<td>16 (22.9)</td>
</tr>
<tr>
<td>Pirlimycin</td>
<td>66 (94.3)</td>
<td>0</td>
<td>4 (5.7)</td>
</tr>
</tbody>
</table>

### 5.6 Multi-Locus Sequence Typing (MLST)

In the present study, 16 different sequence types (ST) were determined (Table 11). Of these, six were novel STs, which were ST2821 (n = 2), ST2823 (n = 1), ST2824 (n = 1), ST2825 (n = 2), ST2826 (n = 1) and ST2827 (n = 1). These new STs were submitted as new registrations to the MLST database. The most common frequencies of ST were ST133 (n = 20), ST504 (n = 16) and ST97 (n = 11). The less frequent STs were ST398 (n = 4), ST479 (n = 3), ST1380.
RESULTS

(n = 3) and ST151 (n = 2), while other types such as ST7 (n = 1), ST71 (n = 1) and ST464 (n = 1) were found only for one of the *S. aureus*.

As shown in Table 12, the 16 STs were grouped into eight clonal complexes (CCs). CC133, represented by ST133 and ST2821, was the most prevalent genotype (31.4%, 22/70), followed by CC151 (27.1%, 19/70) and CC97 (21.4%, 15/70), which was the most diverse clonal complex consisting of ST97, ST71, ST464, ST2824, and ST2826. The other types of clonal complexes were less prevalent genotypes: CC479 (8.6%, 6/70), CC398 (5.7%, 4/70), CC5 (2.9%, 2/70) and one each of CC8 and CC50 (1.4%, 1/70).

Table 11: The clonal complex and MLST types of *S. aureus* isolate with the housekeeping genes

<table>
<thead>
<tr>
<th>Clonal complex (n= )</th>
<th>MLST type</th>
<th>No. [%]</th>
<th>arcC</th>
<th>aroE</th>
<th>glpF</th>
<th>gmk_</th>
<th>pta_</th>
<th>tpi_</th>
<th>YqiL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC133 (22)</td>
<td>ST133</td>
<td>20 28.6</td>
<td>6</td>
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*Novel sequence types found in the present study
The *arcC* gene of the 70 *S. aureus* isolates was shown to be successfully amplified by PCR. The size of the amplicon of all the isolates was 569 bp relative to the DNA size marker (Figure 11).

Figure 11: Agarose gel electrophoresis (2%) showing the typical amplicon of the *arcC* gene product of *S. aureus* isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

Gene \textit{aroE} of \textit{S. aureus} isolates was shown to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 535 bp relative to the DNA size marker (Figure 12).

Figure 12: Agarose gel electrophoresis (2\%) showing the typical amplicon of the gene \textit{aroE} product of \textit{S. aureus} isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
Gene *glpF* of *S. aureus* isolates was discovered in the same fragment with the size 535 bp with successful amplification by PCR. The size of the amplicon of all the isolates was relative to the DNA size marker (Figure 13).

Figure 13: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *glpF* product of *S. aureus* isolates. Amplification of DNA appears as a ladder-like pattern. Lane M, Marker 100 bp ladder (Biozym Diagnostic). Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

Gene \textit{gmk\_} of \textit{S. aureus} isolates was shown to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 487 bp relative to the DNA size marker (Figure 14).

![Agarose gel electrophoresis (2%) showing the typical amplicon of the gene \textit{gmk\_} product of \textit{S. aureus} isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).](image)

Figure 14: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene \textit{gmk\_} product of \textit{S. aureus} isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
Gene $pta_\text{of } S.\text{ aureus}$ isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 574 bp relative to the DNA size marker (Figure 15).

Figure 15: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene $pta_\text{ product of } S.\text{ aureus}$ isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

Gene $tpi_\text{ }$ of $S. aureus$ isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 470 bp relative to the DNA size marker (Figure 16).

Figure 16: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene $tpi_\text{ }$ product of $S. aureus$ isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
Gene $yqiL$ of *S. aureus* isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 597 bp relative to the DNA size marker (Figure 17).

![Agarose gel electrophoresis (2%) showing the typical amplicon of the gene $yqiL$ product of *S. aureus* isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).](image-url)

Figure 17: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene $yqiL$ product of *S. aureus* isolates. Amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

5.7 Minimum Spanning Tree Analysis and the Relationship between S. aureus Isolates Based on MLST

From the 70 S. aureus isolates, 16 different sequence types were detected. The MLST profiles used a categorical clustering coefficient, and a minimum spanning tree was constructed to show the connection between the various MLST types (Figure 18). The minimum spanning tree revealed the presence of four varied MLST clusters that were assigned as group names. The S. aureus isolates were clustered in groups. The size of each circle indicated the number of isolates with the same sequence type. The colours of the halo surrounding the MLST types showed the types that belonged to the same group. An MLST was assigned as one group if at least five of the seven allele loci were similar. The first group comprised 26.8% of all the isolates. This group contained three different sequence types, which included ST504 (n = 16), ST151 (n = 2) and ST2823 (n = 1). The second group was composed of 31% isolates, and contained ST133 (n = 20) and ST2821 (n = 2). The third group comprised 21.1%, and consisted of five sequence types—ST97 (n = 11), ST71 (n = 1), ST464 (n = 1), ST2824 (n = 1) and ST2826 (n = 1). The fourth group comprised 8.5% isolates, including ST479 (n = 3) and ST1380 (n = 3). In the MLST, all the groups that were completely made up of all the types of antibiotics were closely related. The groups I, II and IV were sensitive to all types of antibiotics, except in the case of erythromycin where they were intermediate. The same true so for strains of group III, i.e. strains ST2824 and ST464, and two isolates (2.8%) of ST97. One isolate (1.4%) from ST97 was sensitive to all types of antibiotic. In contrast, only five isolates (7%) in ST97 were resistant to penicillin and ampicillin, while they were sensitive to the other types of antibiotics. Thus, the minimum spanning tree revealed the presence of four closely-related clusters in groups, providing the genetic relationships among the different S. aureus isolates. These groups frequently also reflect the resistance pattern towards antibiotics, but some in group III did not.
RESULTS

Figure 18: The minimum spanning tree of the *S. aureus* bovine mastitis isolates in this study (n = 70). All the *S. aureus* isolates were analyzed by MLST. The tree was constructed by using BioNumerics version 7.5 that allowed a presumptive coefficient. The clusters were created from two types within two changes of neighbouring distances.
RESULTS

5.8 Protein A (spa) Typing

The *S. aureus* isolates were grouped into 17 *spa* types (Table 12). One new *spa* type (t13769) was found. The most frequent *spa* types were t1403 (n = 21), t529 (n = 19) and t521 (n = 8). The other *spa* types were found less frequently: t528 (n = 4), t034 (n = 3), t2873 (n = 3), t586 (n = 2), t091 (n = 1), t267 (n = 1), t3297 (n = 1), t359 (n = 1), t5180 (n = 1), t519 (n = 1), t524 (n = 1), t571 (n = 1), and t5920 (n = 1).

Table 12: Frequency of *spa* types among the 70 *S. aureus* isolates investigated in this study

<table>
<thead>
<tr>
<th><em>spa</em> type</th>
<th>Frequency</th>
<th>Per cent %</th>
<th>Spa repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1403</td>
<td>21</td>
<td>30</td>
<td>03-23-24</td>
</tr>
<tr>
<td>t529</td>
<td>19</td>
<td>27.1</td>
<td>04-34</td>
</tr>
<tr>
<td>t521</td>
<td>8</td>
<td>11.4</td>
<td>07-23-12-21-17-34-34-34-33-34</td>
</tr>
<tr>
<td>t528</td>
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<td>5.7</td>
<td>04</td>
</tr>
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<td>t034</td>
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<td>3</td>
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<td>04-20-17-31-24</td>
</tr>
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</tr>
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<td>1.4</td>
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</tr>
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<td>14-12-21-17-34-34-33-34</td>
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<td>07-23-21-12-21-17-34-34-34-33-34</td>
</tr>
</tbody>
</table>

*Novel *spa* type found in the present study
The X region of the protein A gene *spa* was detected in all the isolates, where significant differences between the sizes of the amplified products were detected. This could be assigned to the corresponding number of the so-called ‘repeat’ (Table 12, Figure 19).

Figure 19: Agarose gel electrophoresis (2%) showing the typical amplicon X regions of the protein A gene *spa* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
5.9 The Relationship between S. aureus Isolates Based on spa Typing

Spa typing was regarded as a typing method for the S. aureus isolates. Spa typing was performed on the 70 S. aureus isolates. The spa types were grouped by using the spa-plugin in the BioNumerics software. All the results were displayed in the minimum spanning tree. The S. aureus isolates were clustered in groups. Each circle represents a distinct spa type. Three groups of spa types were distinguished by using the minimum spanning tree (Figure 20). Group I includes 36.6% (n = 26) of all the isolates. Group I has t529, t528, 524, and t586. Group II includes 18.3% (n = 13) of all the isolates. The spa types of group II were t521, t13769, t267, t359, t5180, and t5920. Group III includes 7% (n = 5) of all the isolates. Group III includes t34, and t571.
Figure 20: The minimum spanning tree of the *S. aureus* bovine mastitis isolates in this study (n = 70). All the *S. aureus* isolates were typed by *spa*-sequence typing. The tree was constructed by using BioNumerics version 7.5 that allowed a presumptive coefficient. The clusters were created from two types within two changes of neighbouring distances.
RESULTS

5.10 The Relationship between ST Types and spa Types

Determining the relationship between ST types and spa types revealed a relation between ST133 and spa type (t1403) for 19 (27.1%) isolates (Table 13). The correlation between ST504 and spa type (t529) was found for 16 (22.9%) isolates, the relation between ST97 and spa type (t521) was found for seven (10%) isolates. Furthermore, the correlation between ST398 and spa type (t34) was found for three (4.3%) isolates. The relationship between ST151 and spa type (t529), ST2821 and spa type (t1403), ST1380 and spa type (t2873), ST479 and spa type (t528), ST2825 and spa type (t586) were represented by two isolates (2.9%). The relation between the ST and spa types of the S. aureus isolates was found in only one isolate (1.4%). Based on the MLST (Table 13), the clonal complex CC97 was one of the most varied ones, consisting of eight spa types, namely t521, t13769, t359, t267, t5920, t524, t3297 and t5180. Some of the isolates had an identical spa type, but had differing STs (t529–ST504, t529–ST151 and t529–ST2823). Overall, every clonal complex has specific, exclusive ST and spa types. The highest congruence between a given ST and spa type occurred in CC133, but CC97 contained the highest amount of spa types.
## RESULTS

Table 13: The genotypes of *S. aureus* isolates determined by the molecular typing methods and the relationship between sequence types (ST) of MLST and Protein A (spa type) analysis and housekeeping genes information

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<th>Clonal Complex</th>
<th>MLST type</th>
<th>Spa type</th>
<th>No. of isolates</th>
<th>Percent</th>
<th>Spa repeats</th>
<th>Housekeeping genes</th>
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</tr>
</tbody>
</table>
RESULTS

5.11 The Relationship between Isolates from Bovine Mastitis and Human Disease

The MLST profiles of 118 human strains from Germany were obtained from the MLST Databank (www.mlst.net). The obtained data were compared with the MLST profiles of the mastitis isolates in this study. The relationship between all the strains was defined via the seven housekeeping genes that were sequenced to identify the ST. The clustering of MLST profiles was completed by using a categorical coefficient and the isolates were clustered in groups. A MLST was assigned to one group if at least five of the seven allele loci were similar. According to the minimum spanning tree (Figure 21), the results of the correlation between the isolates display five groups. Group I (CC8) showed a relationship with two isolates of human origin. Group II (CC5) showed a correlation with nine of the isolates from humans. Group III (CC151) correlated with two of the isolates from humans. Group IV (CC133) was related to five isolates from humans, while group V (CC97) correlated with three isolates from humans. In this way, according to the minimum spanning tree, mastitis strains were related to human strains. In some clonal complexes, this relationship was closer than in others, suggesting that the pathogen may be transmitted between these two host species.
The colour of the MLST of *S. aureus* isolates from bovine mastitis was red and that of the MLST of *S. aureus* isolated from human samples was light green.

- The MLST types are displayed as circles. The size of each circle indicates the number of isolates with the same sequence type.
- Thick solid lines connect the types that differ in a single allele locus and a thin solid line connects the types that differ in 2 allele loci.
- The colours of the halo surrounding the MLST types indicate that the types belong to the same group. An MLST was assigned to one group if at least 5 of the 7 allele loci were similar.
RESULTS

Figure 21: Minimum spanning tree of the *S. aureus* bovine mastitis isolates in this study (n = 70) combined with isolates from a human in Germany (n = 118). All the *S. aureus* isolates were analysed by MLST. The tree was constructed by using BioNumerics version 7.5 allowed a presumptive coefficient. The clusters were created from two types within two changes of neighbouring distances.

5.12 The Relationship between the *S. aureus* Bovine Mastitis Isolates in this Study and *S. aureus* Isolates from Different European Countries

The minimum spanning tree analysis was conducted for the 70 isolates of *S. aureus* in this study and 33 isolates of *S. aureus* from bovine mastitis from different European countries. The minimum spanning tree displayed the correlation between the MLST types of all the isolates as circles. The colours of the halo surrounding the MLST types indicated the types belonging to the same group. Group I (CC133) showed a correlation between the ST133 in this study with one isolate from the Netherlands. Group II (CC5) revealed a relationship between ST2825 and three isolates from England, the Netherlands, and France. Group III (CC8) displayed a relationship between ST7 and one isolate from France (Figure 22). Thus, some clonal complexes were found all over Europe, while others were detected only in Germany.
RESULTS

Figure 22: The minimum spanning tree of the *S. aureus* isolates in this study (n = 70) combined with the results of the *S. aureus* isolates from different European countries (n = 33). The tree was constructed by using BioNumerics version 7.5 which allowed a presumptive coefficient. The clusters were created from two types within two changes of neighbouring distances.

- The size of each circle indicates the number of isolates with the same sequence type.
- Thick solid lines connect the types that differ in a single allele locus and a thin solid connects the types that differ in 2 allele loci.
- The colours of the halo surrounding the MLST types indicate that the types belong to the same group.
RESULTS

5.13 Prevalence of Genes Encoding Staphylococcal Virulence Factors

All *S. aureus* isolates were examined for the presence of the genes *coa*, *clfA*, *clfB* and *nuc* (Table 14). The results revealed that all of the 70 *S. aureus* isolates possessed 70 (100%) of the genes *coa*, *clfB*, and *nuc*, whereas the *clfA* was found in 69 (98.6%) of the *S. aureus* isolates. No isolates showed the presence of the *mecA* gene.

Table 14: Presence of genes encoding staphylococcal virulence factors in *S. aureus* isolated from bovine mastitis

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of isolates</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>clfA</em></td>
<td>69</td>
<td>98.6</td>
</tr>
<tr>
<td><em>clfB</em></td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td><em>coa</em></td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td><em>nuc</em></td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td><em>mecA</em></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Gene *clfA* of the *S. aureus* isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 288 bp relative to the DNA size marker (Figure 23).

Figure 23: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *clfA* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Gene *clfB* of the *S. aureus* isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 203 bp relative to the DNA size marker (Figure 24).

Figure 24: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *clfB* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

Gene *coa* of the *S. aureus* isolates was revealed to be successfully amplified by PCR. Gene *coa* was detected in all those isolates in which case significant differences in size of the amplified products were detected (Figure 25).

![Agarose gel electrophoresis](image)

**Figure 25:** Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *coa* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
The *nuc* gene of the *S. aureus* isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 166 bp relative to the DNA size marker (Figure 26).

![Agarose gel electrophoresis](image)

Figure 26: Agarose gel electrophoresis (2%) showing the typical amplicon of the *nuc* gene product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–13 represent positive isolates, and Lane 14 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

Gene mecA of the S. aureus isolates was revealed to be successfully amplified by PCR. The size of the amplicon of all the isolates was the same fragment with a size of 147 bp relative to the DNA size marker (Figure 27).

Figure 27: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene mecA product of S. aureus isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are the DNA Marker 100 bp ladder (Biozym Diagnostic).
5.14  Prevalence of Genes Encoding Staphylococcal Enterotoxin

The prevalence of enterotoxin genes of *S. aureus* obtained by PCR revealed that 37 (52.9%) of the 70 isolates were positive for more than one of the enterotoxin genes (Table 15). 33 isolates (47.1%) did not possess any enterotoxin gene. The most frequent genes detected in the present study were *sei, sem, sen*, and *selu* in 28 (40%) isolates, and they were found among the clonal complexes CC151, CC479, CC5, and CC50. This was followed by *seg* with a total of 26 (37.1%) and it was found among CC151, CC479, and CC5. According to the results, gene *seo* was found in nine isolates (12.9%) which belonged to CC479, CC5, and CC50, The *sec, sed, selj* and *tst* genes were found in eight isolates (11.4%). The gene *ser* was found in seven isolates (10%) and *seh* and *sep* in three (4.3%) of the isolates, respectively. In this study, all the isolates were negative for *sea, seb, see*, and *seq* and the clonal complexes CC133 and CC398 were also negative for all types of staphylococcal enterotoxin genes.
Table 15: Presence of genes encoding different staphylococcal enterotoxins and toxic shock syndrome toxin in *S. aureus* isolated from bovine mastitis

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of isolates</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>seb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sec</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>sed</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>see</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>seg</td>
<td>26</td>
<td>37.1</td>
</tr>
<tr>
<td>seh</td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>sei</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>selj</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>sem</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>sen</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>seo</td>
<td>9</td>
<td>12.9</td>
</tr>
<tr>
<td>sep</td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>seq</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ser</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>selu</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>tst</td>
<td>8</td>
<td>11.4</td>
</tr>
</tbody>
</table>

The *S. aureus* isolates were divided into nine different enterotoxigenic profiles on the basis of the presence of different enterotoxin genes among the *S. aureus* lineages (Table 16), suggesting a horizontal transfer of toxin genes among the *S. aureus* isolates. The number of *S. aureus* isolates that most frequently showed enterotoxigenic profile I (*seg, sei, sem, sen* and *selu*) was 11 (15.7%). Seven (10%) of the isolates had enterotoxigenic profile II (*sec, seg, sei, sem, sen, selu*, and *tst*). Enterotoxigenic profile III (*sed, selj, and ser*) was found in seven (10%) isolates. *S. aureus* isolates with enterotoxigenic profile IV (*seg, sei, sem, sen, seo, and selu*) numbered six (8.6%), whereas two (2.3%) *S. aureus* isolates showed enterotoxigenic
profile V (seg, seh, sei, sem, sen, seo, sep, and selu). Enterotoxigenic profiles VI to IX were found in one (1.1%) isolate. None of the S. aureus isolates had a single enterotoxin gene.

Some enterotoxin genes are known to be grouped either as a gene cluster or organized as an operon. In this study, as shown in Table 16, the most prevalent toxin gene cluster was enterotoxigenic profile I, followed by enterotoxigenic profile II, which were found in CC151. In addition, enterotoxigenic profile VII was also found in CC151. Enterotoxigenic profiles III and VI were found in isolates belonging to CC79. Enterotoxigenic profile IV was only found in CC479. Enterotoxigenic profile V was found solely in isolates belonging to CC5. The gene group seh + sep (profile VIII) was found in only one isolate that belonged to CC8. Enterotoxigenic profile IX was found in CC50. S. aureus isolates belonging to CC133 and CC398 did not possess any of the staphylococcal enterotoxin genes.

Table 16: Enterotoxin gene profile of the S. aureus isolates (n = 70) from bovine mastitis milk

<table>
<thead>
<tr>
<th>Toxin genes profile</th>
<th>Staphylococcus enterotoxin</th>
<th>n</th>
<th>Isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>seg + sei + sem + sen + selu</td>
<td>11</td>
<td></td>
<td>15.7</td>
</tr>
<tr>
<td>II</td>
<td>sec + seg + sei + sem + sen + selu + tst</td>
<td>7</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>sed + self + ser</td>
<td>7</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>seg + sei + sem + sen + seo + selu</td>
<td>6</td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td>V</td>
<td>seg + seh + sei + sem + sen + seo + sep + selu</td>
<td>2</td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>VI</td>
<td>sed + self</td>
<td>1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>VII</td>
<td>sec + sei + sem + sen + selu + tst</td>
<td>1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>VIII</td>
<td>seh + sep</td>
<td>1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>IX</td>
<td>sei + sem + sen + seo + selu</td>
<td>1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>0</td>
<td>No enterotoxin gene</td>
<td>33</td>
<td></td>
<td>47.1</td>
</tr>
</tbody>
</table>
RESULTS

A species-specific part of gene *sea* of *S. aureus* could successfully be amplified for the *S. aureus* reference strain by using the primer pair SEA-1 and SEA-2 (Table 5). The specific amplicon had a size of 219 bp (Figure 28). All the investigated *S. aureus* isolates were negative.

Figure 28: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sea* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Using the primer pair SEB-1 and SEB-2 (Table 5), an *S. aureus* specific part of gene *seb* could be amplified for all *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 478 bp (Figure 29). All *S. aureus* isolates investigated were negative.

Figure 29: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *seb* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–7 represent negative isolates, and Lane 8 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
A species-specific part of gene sec of *S. aureus* could successfully be amplified for the *S. aureus* reference strain by using the primer pair SEC-1 and SEC-2 (Table 5). The specific amplicon had a size of 257 bp (Figure 30). Eight of the investigated *S. aureus* isolates were positive, while the rest were negative.

Figure 30: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene sec product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Using the primer pair SED-1 and SED-2 (Table 5), an *S. aureus* specific part of gene *see* could be amplified for all the *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 317 bp (Figure 31). Eight of the *S. aureus* isolates investigated were positive, while the others were negative.

![Figure 31: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sed* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).](image-url)
A species-specific part of gene see of *S. aureus* could successfully be amplified for the *S. aureus* reference strain by using the primer pair SEE-1 and SEE-2 (Table 5). The specific amplicon had a size of 171 bp (Figure 32). All the investigated *S. aureus* isolates were negative.

Figure 32: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene see product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Using the primer pair SEG-1 and SEG-2 (Table 5), an *S. aureus* specific part of gene *seg* could be amplified for all the *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 642 bp (Figure 33). Among the *S. aureus* isolates investigated, \( n = 26 \) were positive, while the others were negative.

![Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *seg* product of *S. aureus* isolates.](image)

Figure 33: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *seg* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
A species-specific part of gene seh of *S. aureus* could successfully be amplified for the *S. aureus* reference strain by using the primer pair SEH-1 and SEH-2 (Table 5). The specific amplicon had a size of 375 bp. Three of the *S. aureus* isolates investigated were positive. Gene seh analysis of the *S. aureus* isolates yielded two types of fragment sizes (Figure 34).

Figure 34: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene seh product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–4 represent positive isolates, Lanes 5-9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

Using the primer pair SEI-1 and SEI-2 (Table 5), an *S. aureus* specific part of gene *sei* could be amplified for all the *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 576 bp (Figure 35). Of the *S. aureus* isolates under investigation, \( n = 28 \) were positive, while the rest were negative.

Figure 35: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sei* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

A species-specific part of gene selj of *S. aureus* could be successfully amplified for the *S. aureus* reference strain by using the primer pair SEJ-1 and SEJ-2 (Table 5). The specific amplicon had a size of 142 bp (Figure 36). Eight of the *S. aureus* isolates under investigation were positive, while the others were negative.

Figure 36: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene selj product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Using the primer pair SEM-1 and SEM-2 (Table 5), an *S. aureus* specific part of gene *sem* could be amplified for all the *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 471 bp (Figure 37). Among the investigated *S. aureus* isolates, $n = 28$ were positive, while the others were negative.

Figure 37: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sem* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
A species-specific part of gene *sen* of *S. aureus* could be successfully amplified for the *S. aureus* reference strain by using the primer pair SEN-1 and SEN-2 (Table 5). The specific amplicon had a size of 292 bp (Figure 38). Of the investigated *S. aureus* isolates, \( n = 28 \) were positive, while the others were negative.

Figure 38: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sen* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

Using the primer pair SEO-1 and SEO-2 (Table 5), an *S. aureus* specific part of gene *seo* could be amplified for all the *S. aureus* isolates as well as the reference strain. The specific amplicon had a size of 249 bp (Figure 39). Nine of the *S. aureus* isolates investigated were positive, while the rest were negative.

Figure 39: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *seo* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent positive isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
RESULTS

A species-specific part of gene *sep* of *S. aureus* could be successfully amplified for the *S. aureus* reference strain by using the primer pair SEP-1 and SEP-2 (Table 5). The specific amplicon had a size of 148 bp (Figure 40). Three of the *S. aureus* isolates investigated were positive and the others were negative.

Figure 40: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *sep* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2, 3 and 4 represent positive isolates, Lanes 5–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
Using the primer pair SEQ-1 and SEQ-2 (Table 5), an *S. aureus* specific part of gene *seq* could be amplified for all the *S. aureus* isolates and also the reference strain. The specific amplicon had a size of 359 bp (Figure 41). All the *S. aureus* isolates investigated were negative.

Figure 41: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *seq* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2-9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

A species-specific part of gene *ser* of *S. aureus* could be successfully amplified for the *S. aureus* reference strain by using the primer pair SER-1 and SER-2 (Table 5). The specific amplicon had a size of 123 bp (Figure 42). Seven of the *S. aureus* isolates investigated were positive, while the others were negative.

Figure 42: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *ser* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–8 represent positive isolates, and Lane 9 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
Using the primer pair SEIU-1 and SEIU-2 (Table 5), an *S. aureus* specific part of gene *selu* could be amplified for all the *S. aureus* isolates as well as the reference strain. The specific amplicon had a size of 167 bp an addition band (about 220 bp) was always observed above the specific band (Figure 43). Of the *S. aureus* isolates investigated, 28 were positive, while the rest were negative.

Figure 43: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *selu* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Roche Diagnostic).
RESULTS

A species-specific part of gene *tst* of *S. aureus* could be successfully amplified for the *S. aureus* reference strain by using the primer pair TSST-1 and TSST-2 (Table 5). The specific amplicon had a size of 559 bp (Figure 44). Eight of the *S. aureus* isolates investigated were positive, while the others were negative.

![Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *tst* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).](image)

Figure 44: Agarose gel electrophoresis (2%) showing the typical amplicon of the gene *tst* product of *S. aureus* isolates. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–9 represent negative isolates, and Lane 10 is a non-template control. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic).
6 DISCUSSION

6.1 Loop Mediated Isothermal Amplification Assay

*S. aureus* is considered to be the main cause of mastitis in dairy cows worldwide and cases are usually subclinical and difficult to treat (TAPONEN & PYORALA, 2009). In addition, *S. aureus* is major pathogen causing food poisoning by producing several staphylococcal enterotoxins (LONCAREVIC et al., 2005). Therefore, a rapid and highly accurate detection of the pathogen is necessary for suitable treatment, preventing it from spreading and the reduction of risk factors caused by this organism (TENG et al., 2007). At present, several methods are available for identifying *S. aureus*. The identification of *S. aureus* by traditional methods that include culture and biochemical identification and immunological detection usually requires 24 hours. The PCR method is sensitive, accurate and faster. The identification of *S. aureus* by the PCR method takes 4–5 hours, but it requires expensive equipment such as a thermocycler, electrophoresis set and gel documentation system.

The LAMP assay is a relatively new DNA amplification technique. This method uses four or six primers that can recognize six to eight distinct sequences of the target gene. The complete reaction takes place by using *Bst* DNA polymerase, DNA template and reaction buffers at a constant temperature. The target sequence can be amplified with high efficiency, rapidity, and specificity under isothermal conditions (NOTOMI et al., 2000). In this study, a LAMP assay was developed and validated. This was achieved by using six primers for recognizing eight distinct regions of the target gene that helped to facilitate the detection of very small quantities of the target DNA compared to other studies that used four primers to detect the same target *nuc* gene (HANAKI et al., 2011; NAWATTANAPAIBOON et al., 2015). One study (NAGAMINE et al., 2002) demonstrated the use of an additional primer, termed ‘loop primer’, in the LAMP assay that led to the accelerated identification of *S. aureus* and higher specificity. In addition, the LAMP reaction was carried out under isothermal conditions at 65°C and a positive result was observed after seven to 22 minutes. In accordance with previous findings, this indicates that it is important to use the loop primers in the assay to achieve an accurate result within a short time.

The amplification products were detected by using a real-time fluorometer that gives a positive signal as amplification curve. Previous studies detected the amplified gene with the
naked eye, either as turbidity in the form of a white precipitate or through a colour change employing a fluorescent intercalating dye (SYBR green I) (NOTOMI et al., 2000). In the present study, the LAMP assay had the advantage of allowing a positive result to be viewed as a curve through the LAMP real-time fluorometer screen. The real-time fluorometer could be used to develop a quantitative assay. Additionally, the DNA amplification could already be followed during the still active reaction.

The analytical sensitivity of the developed LAMP assay for detecting S. aureus DNA amounted to 0.052 pg\(^{-1}\), which was less than the analytical sensitivity mentioned by (LIM et al., 2013; X. R. WANG et al., 2015) that amounted to 2.5 ng \(\mu\text{L}^{-1}\) and 0.147 pg \(\mu\text{L}^{-1}\), respectively. This difference is attributed to the use of two additional primers in our study to detect the \textit{mec} gene compared to the two previous studies that used only four primers. One previous study (NAGAMINE et al., 2002) recommended the use of additional primers (loop primers) to accelerate the LAMP reaction and to increase sensitivity. The LOD for the developed LAMP assay was \(9\times10^2\) CFU mL\(^{-1}\) milk. These LOD results are comparable to those of Tie et al. (2012) and Lim et al. (2013), who also showed the same limit of detection. In the present study, however, the results of the LOD were more sensitive than the LOD of the used PCR that was \(1\times10^4\) (TIE et al., 2012) and \(10^3\) (LIM et al., 2013), respectively. The results for the analytical sensitivity and LOD obtained in the present study revealed that the developed LAMP assay was more sensitive than those developed in the previous studies (TIE et al., 2012; LIM et al., 2013) and more sensitive compared to the PCR method. (H. MAEDA et al., 2005) and (MIYAGAWA et al., 2008) confirmed that the sensitivity of LAMP assays was equivalent or higher compared to the PCR methods.

The LAMP assay complemented with the use of a real-time fluorometer (Genie II\textsuperscript{®}) has several advantages that qualify it as one of the most common methods in the world for detecting pathogens. The combination of an isothermal reaction together with a real-time fluorometer enables the observation of the positive result during amplification directly on the optical screen. The LAMP assay is a promising modern method for the rapid identification of S. aureus and various types of bacteria. The use of specific loop primers for detecting S. aureus can facilitate a reduction in the necessary amplification time to less than 30 minutes. The equipment used was a small, portable instrument (Genie II\textsuperscript{®}). Therefore, it can be
transported to any place or room in a diagnostic laboratory. In addition, the instrument used comes with rechargeable battery and it can therefore be used in the field as well.

6.2 Identification of *S. aureus* isolates

In the present study, phenotypic and genotypic methods were performed to identify 70 *S. aureus* isolates from bovine mastitis milk. The results obtained by phenotypic determination were totally in concordance with the results of the genotypic identification. According to the results from the biochemical reactions and also on the basis of the presence of the *nuc* and *coa* genes, all the isolates were identified as *S. aureus*.

6.3 The Antimicrobial Susceptibility Testing

Phenotypically, 70 (24.3%) isolates were resistant to one or more antibiotics, and 53 (75.7%) isolates were sensitive. The proportion of resistant isolates in this study was close to the results of (HANA MUFTAH, 2011), who also reported that 21% of the tested *S. aureus* isolates were resistant to at least one antibiotic. Our findings were lower than those of (SHI et al., 2010), who reported that 98.3% of the tested isolates were resistant to at least one antibiotic.

The isolates showed a high resistance rate to penicillin and ampicillin, a result that tallied with those presented by other investigations e.g., Kamaleldin et al. (2010) and Mordmuang and Voravuthikunchai (2015) reported that *S. aureus* isolates were seen to be highly resistant to penicillin and ampicillin. In fact, *S. aureus* isolates show a wide difference in their resistance to antibiotics due to various factors like geographic regions, the type and the genetic characteristics of the isolates (VINTOV et al., 2003). The high resistance of *S. aureus* to antibiotics is blamed on the results of the indiscriminate use of antibiotics, the inappropriate handling of infected animals without regular veterinary observation, and ignoring the veterinarian’s medical advice in treating the animals (GAO et al., 2012). As a consequence, *S. aureus* isolates were frequently resistant to antibiotic therapy, leading to low cure rate for mastitis.

Phenotypically, all the *S. aureus* isolates were shown to be sensitive to methicillin. This was confirmed with genotypic tests, which revealed that none of the tested isolates carried gene
DISCUSSION

Gene mecA encodes for the Penicillin Binding Protein 2A that is referred as methicillin resistance (K. ZHANG et al., 2008). The results of the antimicrobial susceptibility testing in this study were in agreement with other reports that showed a high prevalence of methicillin-sensitive *S. aureus* (MSSA) in milk and dairy product samples (KAMALELDIN et al., 2010) (HARAN et al., 2012). Moreover, other studies (NORMANNO et al. 2007); (NEMEGHAIRE et al. 2014; VAN DUIJKEREN et al. 2014) reported similar results concerning the presence of *S. aureus* carrying gene mecA from bovine mastitis.

6.4 Multi-Locus Sequence Typing (MLST)

Genotypically, among the eight CCs identified in our study, CC133, CC151 and CC97 were the most predominant. CC133 and CC151 accounted for 58.6% of the 70 isolates examined. This was in agreement with the studies of (GUINANE et al., 2010; SCHLOTTER et al., 2012). Guinane et al. (2010) reported that the majority of ruminant-associated sequence types (STs) belonged to CC133, CC151 and CC97, while (SCHLOTTER et al., 2012) showed that CC151 and CC133 were the dominant lineage in bovine milk strains in Germany. CC133 was also present in relatively developed regions like Europe, Australasia and the Americas (SMITH et al. 2014). However, CC133 was not only found in bovine mastitis milk isolates but also in *S. aureus* obtained from other types of animals; for example, in small ruminants (GUINANE et al., 2010; ERIKSSON et al., 2013) and in ungulates (GHARSA et al., 2012). No evidence of CC133 causing human infections has been reported till date. In contrast, CC151 isolates were predominantly associated with cows and were not previously detected in humans (GUINANE et al., 2008). CC97 was also predominant, while other clonal complexes (CC479, CC398, CC5, CC8 and CC50) were only present in smaller numbers. CC97 was the dominant lineage in bovine mastitis in Denmark (HASMAN et al., 2010), and in Switzerland and Germany (MONECKE et al., 2007), in the United States, Chile and the United Kingdom (SMITH et al., 2005). Although CC97 *S. aureus* isolates have been commonly isolated from bovines, they have also been detected among human and porcine hosts (SMYTH et al., 2009; BATTISTI et al., 2010). All the isolates identified in our study belonging to this clonal complex were methicillin-susceptible *S. aureus* (MSSA) (Table 5). Our findings were in agreement with previous studies showing a higher prevalence of MSSA in bovine milk samples (HARAN et al., 2012; OIKONOMOU et al., 2012; SILVA et al., 2013). CC398 is of
major importance in the context of livestock and are found in different species, including poultry, horses, swine and cattle ((MONECKE et al., 2007; NEMATI et al., 2008; KADLEC et al., 2009). The MRSA strains of this CC have recently gained a lot of attention worldwide, especially the strains that are isolated from pigs and livestock (HUIJSDENS et al., 2006; LEWIS et al., 2008; NEMATI et al., 2008). According to (PRICE et al., 2012), MRSA CC398 appears to have originated from human MSSA CC398, which acquired methicillin resistance in livestock. Our study revealed that all the S. aureus isolates belonging to CC398 were MSSA. Therefore, antimicrobial selection associated with food animal production decreases the potential for the presence of MSSA CC398 in food. The difference in clonal complex frequencies as compared to other studies and the significant variations in frequencies with regard to herd location confirm that the genetic characteristics of S. aureus may be related to different geographical regions (COSTA et al., 2012).

An MLST analysis is necessary for generating results that are readily comparable between laboratories (OLIVE & BEAN, 1999). The present study determined 16 different STs, including six novel types. The novel types reported for the first time indicated the evolutionary emergence of unique clones in different regions, whose importance needs to be established. ST133, ST504, and ST97 are the most common frequency of the sequence types that cause bovine mastitis. Previous studies have referred to ST133 as being one of the most frequent types in small ruminants (GUINANE et al., 2010; PORRERO et al., 2012). In addition, ST133 was also found in other types of animals, including ungulates, rodents and carnivores such as lions and cats (ESPINOSA-GONGORA et al., 2012; SASAKI et al., 2012), in red deer in Southern Spain (P. GOMEZ et al., 2015), in pigs (GOMEZ-SANZ et al., 2010) and wild boars in Germany (MEEMKEN et al., 2013). ST504 was only found in S. aureus isolated from bovine mastitis (http://www.mlst.net). Earlier studies revealed the presence of ST97 in S. aureus isolated from bovine mastitis milk in the Netherlands (KOZYTNSKA et al., 2010), Northwestern China (LI et al., 2015b), Denmark (HASMAN et al., 2010) and Japan (HATA et al., 2010). In China, a study showed that ST97 may be found in milk and beef, indicating that ST97 correlated with cattle (M. SONG et al., 2015). Interestingly, ST97 has previously been described as being present in low frequency in humans (FEIL et al., 2003), in Spain (LOZANO et al., 2011), the United Kingdom (SUNG et al., 2008) and Turkey (BOZDOGAN et al., 2013). Several studies have demonstrated that
DISCUSSION

Healthy pets, goats, and cows could be reservoirs and an epidemiological source of *S. aureus* that may infect humans (ERIKSSON et al., 2013; GHARSA et al., 2015).

6.5 Spa Typing

The 70 *S. aureus* isolates were grouped into 18 *spa* types, including the newly identified *spa* type t13769. The most frequently prevalent *spa* types were t1403, t529 and t521. Several studies have reported the similarities among the diversity of *spa* types of the *S. aureus* isolates from different kinds of food, animals and humans. In the present study, the *spa* type t1403 was found to be most frequent. However, this *spa* type was already reported in bovine mastitis in Germany (JOHLER et al., 2011) and Sweden (Smyth et al., 2009), as well as from slaughterhouse cattle in the Netherlands (VAN DUIJKEREN et al., 2014), and Denmark (HASMAN et al., 2010). In addition, *spa* type t1403 was detected in the *S. aureus* isolated from sheep in the slaughterhouse (ERIKSSON et al., 2013). Recent studies in Germany, Japan, Switzerland, and India have revealed that *spa* type t529 is the predominant type of *S. aureus* isolated in bovine milk (MONECKE et al., 2007; HATA et al., 2010) and in dairy cattle (HATA et al., 2010; KAMALELDIN et al., 2010; JOHLER et al., 2011; VAN DUIJKEREN et al., 2014; VEH et al., 2015). On the contrary, other studies have described t529 as solely occurring in bovine milk cheese in Switzerland (HUMMERJOHANN et al. 2014) and in swine in Japan (ASAI et al., 2012). Other investigations have revealed that *spa* type t521 could be found in *S. aureus* isolates obtained from bovine mastitis in Canada (KAMALELDIN et al., 2010), China (LI et al., 2015a), Denmark and the Netherlands (HASMAN et al., 2010), and Japan (HATA et al., 2010).

6.6 The Relationship between MLST Types and Spa Types

There is a great diversity concerning the discriminatory ability of *spa* typing as compared to MLST typing and there is a close relationship between ST types and *spa* types (ERIKSSON et al., 2013). The combination of ST and *spa* types has been proven to be useful. In many types, which show instances of these methods, *spa* typing has been used to identify the most common ancestral lineages. In this study, ST133-t1403, ST504-t529, and ST97-t521 (Table 5) were the most common STs and *spa* types that were identified in the investigated isolates of *S. aureus*. Nineteen of the ST133 isolates belonged to *spa* type t1403, whereas one ST133
isolate belonged to type t528. ST133-t1403 was found in bovine mastitis milk from Sweden, Germany and Denmark (SMYTH et al., 2009; HASMAN et al., 2010; JOHLER et al., 2011). In this study, 16 of the ST504 isolates belonged to the spa type t529, which was already reported in \textit{S. aureus} isolates in bovine mastitis samples from Switzerland (www.mlst.net). The overall ST97 was split into 11 spa types; seven were t521, one was novel, one t359, one t5920 and one t267. The results of this study agreed with other studies that had detected ST97-t521, ST97-t267, and ST97-t359 in \textit{S. aureus} isolated from cows and bovine mastitis (HASMAN et al., 2010; HATA et al., 2010) as well as from humans in Brazil (www.spaserver.ridom.de). The isolates belonging to ST97-t359 and ST97-t267 were also found in fresh beef (M. SONG et al., 2015), in chickens and chicken meat as well as in pork meat in Poland (KRUPA et al., 2015) as well as in isolates from humans in Spain (ROBERTS et al., 2011; MENEGOTTO et al., 2012). Both the ST151 isolates belonged to spa type t529. Our results agreed with other studies that had detected ST151-t529 in \textit{S. aureus} isolated from bovine milk (SMYTH et al., 2009; HASMAN et al., 2010; JOHLER et al., 2011).

6.7 Minimum Spanning Tree

Several studies have identified the presence of host specific genotypes of \textit{S. aureus} (van Leeuwen et al., 2003; Smyth et al., 2009). The minimum spanning tree indicates the existence of \textit{S. aureus} isolates belonging to numerous clonal complexes of closely related genotypes or lineages within the species. Our findings showed the existence of a relationship between the strains from animals and from humans. The majority of bovine mastitis milk-associated sequence types belonged to the four clonal complexes CC133, CC151, CC97, CC8, and CC5 which showed that they were more closely related to the sequence types of human origin. The results of this study were consistent with previously published data that found a similarity of genotypes between \textit{S. aureus} isolates from human and animal hosts. Recent studies have demonstrated a close genetic relationship between \textit{S. aureus} isolated from milk resp. dairy products and humans. RESCH et al. (2013) analysed CC8 strains which are found frequently among animals and human beings. They found a high degree of relation between bovine and human strains. In fact, they established three subgroups in which two contained human and bovine strains alike, and only one was made up entirely by bovine strains. This suggests the possibility of a reciprocal contamination between humans and cattle as postulated by
SAKWINSKA et al. (2011). The present study supports this assumption basically. However, the ST and spa types presented by RESCH et al. (2013) did not match those found in this investigation.

Being so, a special focus should be laid on avoiding the transmission of \textit{S. aureus} between man and dairy cow. Since the pathogen is not enteric, the consumption of raw dairy products is of minor importance (KREAUSUKON et al., 2012). Instead, hygienic milking should be emphasised. Regarding MRSA strains, HARAN et al. (2012) recommended a “careful monitoring of the resistance status” (p. 694), since the development of resistances is a highly dynamic process.

### 6.8 Staphylococcal Enterotoxin Genes

\textit{S. aureus} isolates belonging to the clonal complexes CC151, CC79, CC479, CC5, CC8 and CC50 carried various types of staphylococcal enterotoxin genes, while \textit{S. aureus} isolates belonging to CC133 and CC398 carried no staphylococcal enterotoxins genes. Several studies have revealed that there are significant geographical differences in the existence of enterotoxin genes in \textit{S. aureus} isolated from bovine mastitis (DA SILVA et al., 2005; ZSCHOCK et al., 2005). The testing of milk samples is important for determining the types of staphylococcal enterotoxins prevalent in milk and dairy products, and they are important for public health as well (ZADOKS et al. 2011). The existence of enterotoxigenic \textit{S. aureus} isolated from cow’s milk is important because it is a potential source of staphylococcal food poisoning. This is valuable information concerning the milk produced in various farms catering to the dairy industry and food industry in northern Germany. In the present study, the most frequent genes were sei, sem, sen, and selu (40%) and seg (37.1%). The results of the study were in agreement with previous studies (SRINIVASAN et al., 2006; HWANG et al., 2010; IKAWATY et al., 2010) that reported the highest prevalence rate of seg, sei, sem, and sen genes in \textit{S. aureus} isolated from bovine mastitis milk. The present study showed that none of the \textit{S. aureus} isolates possessed the genes sea, seb, see, and seq. This is in agreement with previous studies reporting that the enterotoxin genes seb, see, and seq could never be recovered from bovine milk (SRINIVASAN et al., 2006; OTE et al., 2011; HUMMERJOHANN et al., 2014). Many former studies have revealed that in some strains of \textit{S. aureus}, the gene seh is responsible for many cases of food poisoning and also for an
outbreak of staphylococcal food poisoning among humans (IKEDA et al., 2005; JORGENSEN et al., 2005). The various types of enterotoxin genes of *S. aureus* isolated in this study as well as in other studies were attributed to the differences in geographical regions (KLEIN et al., 2012), and the wide variety of the primers utilized, types of samples, the source of samples and environments (X. WANG et al., 2012). The toxic shock syndrome toxin (*tst*) gene combined with other types of enterotoxin genes in *S. aureus* isolates from bovine mastitis milk was also reported (SRINIVASAN et al., 2006).
7 SUMMARY

Omar Hashim Sheet

Identification and characterization of Staphylococcus aureus isolated from bovine mastitis milk in Northern Germany

The LAMP (Loop-Mediated Isothermal Amplification) assay is regarded as one of the most efficient methods in detecting human and animal pathogens, because it can amplify DNA with high efficiency, specificity, and more quickly under isothermal conditions. S. aureus is one of the most important pathogens affecting humans and animals alike. In dairy cattle, mastitis caused by S. aureus represents an important economical factor, and herd management regarding udder health depends on quick and correct pathogen identification. The present study consisted of two main parts: First, development and validation of a new LAMP assay for detecting S. aureus, and second, phenotypical and genotypical characterization of S. aureus from milk mastitis.

Loop-mediated isothermal amplification is a powerful, innovative gene amplification method which is regarded as an easy to perform, rapid diagnostic tool with more specificity and more sensitivity for detecting and identifying pathogens. In the present study, a LAMP assay based on gene nuc to identify S. aureus was developed and validated. The specificity of the LAMP assay was confirmed by using 70 S. aureus isolates collected from bovine mastitis milk samples during the period from August 2001 to March 2014 in different regions of Northern Germany and 21 non-S. aureus strains. The results of the LAMP assays enabled the detection of gene nuc in all investigated S. aureus isolates (100%), while no signal was detected with any of the non-S. aureus strains tested. The analytical sensitivity of the developed LAMP assay amounted to 0.26 pg of S. aureus DNA per reaction. The limit of detection evaluated with milk spiked with S. aureus was \(9 \times 10^2\) CFU mL\(^{-1}\). The developed LAMP assay is a rapid detection method for identifying S. aureus with high specificity and sensitivity. The use of a real-time fluorometer enabled S. aureus to be identified in less than two hours including the DNA preparation. Therefore, this assay is a rapid, flexible and simple tool for identifying S. aureus isolates.
To understand the molecular ecology of *S. aureus*, the present study compared phenotypical and genotypical characteristics of 70 *S. aureus* isolates. All *S. aureus* isolates were characterised phenotypically, as well as for their genetic diversity using multilocus sequence typing (MLST), staphylococcal protein A (*spa*) typing, and for the presence of virulence genes encoding 16 staphylococcal enterotoxins (*sea*-*selu*), toxic shock syndrome toxin (*tst*), thermonuclease (*nuc*), clumping factor (*clfA* and *clfB*), coagulase (*coa*), and the methicillin resistance gene *mecA*. Additionally, the present study was carried out to show a relationship between *S. aureus* isolates in this study with the profile of 118 human strains isolated in Germany and the profile of 33 strains of *S. aureus* from bovine mastitis from different European countries.

The staphaurex (clumping factor and protein A) test revealed that 75.7% of the isolates were positive, while all investigated isolates were positive for the catalase test. The API 32 Staph identification system confirmed that all isolates were *S. aureus*. According to the antimicrobial susceptibility testing, all *S. aureus* investigated isolates were methicillin sensitive (MSSA), whereas the isolates showed a high resistance rate to penicillin and ampicillin.

By means of MLST analysis, sixteen different sequence types (ST) were determined. From these, six were novel STs which were designated as ST2821, ST2823, ST2824, ST2825, ST2826, and ST2827. A total of 16 sequence types were grouped into eight clonal complexes (CCs). Seventeen different *spa* types were identified. One new *spa* type (t13769) was found. The prevalence of enterotoxin genes of *S. aureus* obtained by PCR revealed that most of the isolates were positive for more than one of the enterotoxin genes, while other isolates did not possess any enterotoxin genes. In addition, the *S. aureus* isolates displayed nine different enterotoxigenic profiles. The majority of bovine mastitis milk-associated sequence types belonged to the clonal complexes CC5, CC97, CC133 and CC151 which showed closely related genotypes or lineages with sequence types of human origin. The genotypical characterization of *S. aureus* isolated from bovine mastitis milk by MLST and *spa* typing showed that the infections in cow herds of different regions in Northern Germany were caused by numerous clones related to those previously circulating in various regions of the world. The identification of novel STs and novel *spa* types in some isolates highlights all the
more the importance of this type of study in cows, since there is growing evidence of a direct transmission of *S. aureus* between dairy cows and humans. The minimum spanning trees developed in this study support this evidence, providing new possibilities of linking human and animal strains from an epidemiological point of view.
ZUSAMMENFASSUNG

Omar Hashim Sheet

Identifizierung und Charakterisierung von *Staphylococcus aureus* Isolaten aus boviner Mastitismilch aus Norddeutschland


ZUSAMMENFASSUNG

Real-Time-Fluorometer ermöglicht eine Identifizierung von *S. aureus* in weniger als zwei Stunden (einschließlich der DNA-Extraktion). Deshalb ist diese Methode eine schnelle, flexible und einfache Methode, um *S. aureus* nachzuweisen.

Um die molekulare Ökologie von *S. aureus* zu verstehen, wurden in dieser Studie die phänotypischen und genotypischen Merkmale von 70 *S. aureus*-Isolaten verglichen. Alle *S. aureus*-Isolate wurden phänotypisiert. Außerdem wurde ihre genetische Vielfalt anhand der „multilocus sequence typing“-Methode (MLST) und der „Staphylokokken Protein (spa) typing“-Methode charakterisiert. Für die Anwesenheit von virulenten Genen wurden 16 Staphylokokken-Enterotoxine (sea-selu), das „Toxic-Shock-Syndrom-Toxin“ (tst), die Thermonuklease (nuc), der Clumping-Faktor (clfA und clfB), die Koagulase und das Methicillin-resistente-Gen mecA kodiert. Außerdem wurde die vorliegende Studie durchgeführt, um die Beziehungs zwischen *S. aureus*-Isolaten mit dem Profil von 118 menschlichen *S. aureus* Stämmen in Deutschland und dem Profil von 33 *S. aureus*-Stämmen von boviner Mastitis aus verschiedenen europäischen Ländern festzustellen.

Der „Staphaurex-Test“ (Clumping Faktor und Protein A) zeigte, dass 75,7% der Isolate positiv waren. Weiterhin reagierten alle untersuchten Isolate im Katalase-Test positiv. Das „API 32 Staph identifikation system“ bestätigte, dass alle Isolate auch *S. aureus* waren. Gemäß dem „antimicrobial susceptibility–Test“ waren alle untersuchten *S. aureus*-Isolate sensitiv gegenüber Methicillin, wohingegen die Isolate eine hohe Resistenz gegenüber Penicillin und Ampicillin zeigten.

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Development and validation of a loop mediated isothermal amplification (LAMP) assay for the detection of Staphylococcus aureus in bovine mastitis milk samples

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10.1 Publication

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Abstract

Staphylococcus (S.) aureus is one of the most important animal pathogens causing bovine mastitis. Also, it is a major human pathogen that may produce a variety of toxins which cause staphylococcal food poisoning. In the present study a LAMP assay based on gene nac to identify S. aureus was developed and validated. The specificity of the LAMP assay was confirmed by using 70 S. aureus isolates and 21 non-S. aureus strains. The optimal temperature-time combination to amplify gene nac successfully was 65 °C and 30 min. The analytical sensitivity of the developed LAMP assay was 0.26 µg of S. aureus DNA per reaction. The limit of detection evaluated with milk spiked with S. aureus was 9 × 10^6 CFU/ml. The final results of this assay were available within less than 2 h. The present study showed that the LAMP assay based on gene nac appeared to be rapid and simple. It could also be used to identify S. aureus isolates from mastitis milk of dairy cows.

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3. Introduction

Staphylococcus (S.) aureus is one of the major pathogens causing mastitis in bovines and other mammals. By decreasing the quality and quantity of milk production, it leads to large losses in the dairy industry [1,2]. In addition, S. aureus is regarded as being one of the most important foodborne pathogens. It is responsible for causing food poisoning cases and outbreaks worldwide due to the production of a variety of virulence factors including staphylococcal enterotoxin (SEs) and toxic shock syndrome toxin (TSST) [3]. Milk and dairy products are well known as common vehicles of staphylococcal food poisoning [4]. The gene nac is specific for S. aureus and molecular methods for detecting this gene allowed a rapid identification of S. aureus in clinical samples [5-6].

For detecting S. aureus, several methods including manual methods and automated systems are used [7]. The traditional methodology used for diagnosing bovine mastitis caused by S. aureus includes microbiological culture, followed by biochemical identification and antimicrobial susceptibility testing. However, it may take more than 48-72 h to produce a final result [8]. To accelerate identification processes, it is important to find methods characterizing bacteria associated with mastitis more rapidly and more accurately. Molecular methods like conventional gel-based polymerase chain reaction (PCR) [9] and real-time PCR [10] have been used for rapid identification of most pathogens, also including S. aureus.

The loop mediated isothermal amplification (LAMP) assay was first published by Notomi et al. (2000). It is a method to amplify DNA with high specificity and sensitivity and can be conducted using isothermal conditions with temperature ranges from 60 to 75 °C within 1 h [9,12]. Essentially, the LAMP methodology needs fewer (9), B3, F3, P3, and BIP or six oligonucleotide primers (F3, B3, P3, BIP, Loop F, and Loop B) to recognize six to eight distinct regions on the target gene. At present, LAMP assays are widely used for detecting viral, bacterial and parasitic pathogens [13,14]. Furthermore, the LAMP assay has been used to identify extrich meat [15]. Bacterial identification assays based on LAMP technology are already available for a wide variety of bacteria, including Arthrobacter plurimusculus [16], Campylobacter jejuni and Campylo-

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10 APPENDIX

APPENDIX
S. aureus [9,20] and S. pyogenes [21].

The aim of the present study was to design and to optimize a LAMP assay for detecting S. aureus based on gene nuc using a highly specific oligonucleotide primer set. The sensitivity was also determined using spiked milk samples. Furthermore, a comparison of a published PCR method for detecting gene nuc of S. aureus was carried out [22].

2. Material and methods

2.1. Bacterial strains

All S. aureus isolates (n = 70) used in the present study were isolated from bovine mastitis milk in different regions of Northern Germany. These isolates were obtained from routine mastitis diagnosticians conducted at the Institute of Food Quality and Food Safety, University of Veterinary Medicine, Foundation, Hannover, Germany. The reference strain S. aureus ATCC 6538 was used as a positive control. For the exclusivity testing 21 non-S. aureus strains used were obtained from the institute's strain collection (Table 1).

2.2. DNA extraction and template preparation

All S. aureus strains were cultured for 24 h at 37 °C on sheep blood agar (Oxoid Deutschland GmbH, Wesel, Germany). DNA of S. aureus and non S. aureus was isolated with the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions using the protocol for Gram-positive bacteria. According to this protocol, three colonies of freshly cultured bacteria were lysed for 30 min at 37 °C in 180 µl of lysis buffer (20 mg ml⁻¹ lysozyme) complemented with lyostaphin (2 µl 1 mg ml⁻¹) (Sigma–Aldrich Chemie, Steinheim, Germany). Proteinase K 25 µl (20 mg ml⁻¹) and AL buffer 200 µl (QIAGEN) were added and the suspension mixed by vortexing. Following incubation at 56 °C for 30 min, 200 µl ethanol was added to each sample. The mixture was transferred to the DNeasy Mini spin column and centrifuged at 6200g for 1 min. The DNA in the spin column was washed using 500 µl of buffer AW1 (QIAGEN) and centrifuged at 6200g for 1 min. AWG buffer (500 µl) (QIAGEN) was added and centrifuged at 17,000g for 3 min. The spin column was positioned in a clean 1.5 ml microcentrifuge tube. Elution was done with 200 µl buffer AE (QIAGEN). The DNA concentration was measured using a NanoDrop 2000c (PeQLab, Erlangen, Germany) and the DNA was stored at −20 °C until further use.

2.3. Design of oligonucleotide primers for LAMP assay

The development of LAMP primers was based on the sequence of gene nuc published in the National Center for Biotechnology Information (NCBI) GenBank USA under accession number DQ919678.1. The six LAMP primers were designed using LAMP Designer software, ver. 1.10 (PREMIER Biosoft, USA) and included the outer forward primer (nuc–F2), outer backward primer (nuc–B2), inner forward primer (nuc–R2), inner backward primer (nuc–R1), forward loop primer (nuc–LoopL) and backward loop primer (nuc–LoopR). As confirmation method for the presence of gene nuc, a published PCR was used [22]. Table 2 contains the LAMP and the PCR primers used in this study. The primers were synthesized by Eurofins MWG Operon (Eurofins MWG Operon, Ebersberg, Germany).

2.4. LAMP reaction

The LAMP reaction was carried out in the Integrated Cap (Amplix Diagnostics GmbH, Gießen, Germany) with a total volume of 25 µl containing 0.5 µl of each primer F3 and B3 (25 pmol µl⁻¹) equivalent to 0.5 µM final concentration, 2.0 µl of each primer FIP and BIP (25 pmol µl⁻¹) equivalent to 2 µM final concentration and 1.0 µl of each primer LoopL and LoopR (25 pmol µl⁻¹) equivalent to 1 µM final concentration. The amount of Isothermal Master Mix iso-001 (OptiGene, UK) needed for one reaction was 13 µl. Finally, 5 µl of isolated DNA was added as a template and amplification was performed at 65 °C for 30 min. The temperature of the melting curve analysis was 80–98 °C ramping at 0.05 °C per sec in the real-time fluorometer (Genie IP™) (OptiGene Limited) according to the manufacturer's instructions.

Table 1: Strains used for inclusivity and exclusivity testing of the S. aureus LAMP assay based on gene nuc.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains number/strain</th>
<th>Detection time (min)</th>
<th>Melting temperature (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. aureus</td>
<td>Bovine mastitis isolate 60-70</td>
<td>05:35</td>
</tr>
<tr>
<td>2</td>
<td>S. aureus</td>
<td>ATCC 6538</td>
<td>07:30</td>
</tr>
<tr>
<td>Non-S. aureus strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S. epidermidis</td>
<td>DSM 1798</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S. intermedius</td>
<td>DSM 2073</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>S. hyicus</td>
<td>DSM 01935</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Aerococcus haemolyticus</td>
<td>DSM 30088</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Arsenobacter phycophaga</td>
<td>DSM 00002</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Arsenobacter acidiphilum</td>
<td>DSM 15539</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Arsenobacter ohlinii</td>
<td>DSM 13463</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Gluconobacter suboxydans A</td>
<td>CCMG 47273</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Gluconobacter suboxydans B</td>
<td>CCMG 1795F</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Dysgonomonas roseus</td>
<td>DSM 0115</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Butyrivibrio fibrisolvens</td>
<td>DSM 13591</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Escherichia coli</td>
<td>DSM 0008</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Klebsiella pneumoniae</td>
<td>NCTC 13405</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Pseudomonas aeruginosa</td>
<td>DSM 0390</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Staphylococcus aureus</td>
<td>DSM 01937</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Streptococcus albus</td>
<td>13/15°</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Streptococcus agalactiae</td>
<td>13/15°</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Streptococcus dysgalactiae</td>
<td>13/15°</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Streptococcus salivarius</td>
<td>13/15°</td>
<td></td>
</tr>
</tbody>
</table>

* Institute strain. Please cite this article in press as: O.H. Sleet, et al., Development and validation of a loop mediated isothermal amplification (LAMP) assay for the detection of Staphylococcus aureus in bovine mastitis milk samples, Molecular and Cellular Probes (2016), http://dx.doi.org/10.1016/j.mcp.2016.08.001.
APPENDIX

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Table 2
Oligonucleotide primer sequences of LAMP assay and PCRs for amplification of gene muc used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Primer length</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ruc−3</td>
<td>5′-CAA GTG TGT TCT AAC ATC CAA-3′</td>
<td>21 bp</td>
<td>55.9 °C</td>
</tr>
<tr>
<td>ruc−5</td>
<td>5′-GCC CGG CAC CGC TCA-3′</td>
<td>19 bp</td>
<td>54.5 °C</td>
</tr>
<tr>
<td>ruc−BP</td>
<td>5′-AGG ATC TCT TCT TCT TCT CGA TGC TGC TCA TGC TCA-3′</td>
<td>42 bp</td>
<td>71.4 °C</td>
</tr>
<tr>
<td>ruc−cin</td>
<td>5′-ATC TCT CTC AGC AGC GCC TGG TAT GAC AGG TAC AAA-3′</td>
<td>40 bp</td>
<td>72.5 °C</td>
</tr>
<tr>
<td>ruc−cin'</td>
<td>5′-TCT GCA TCT CAT TGG TGC TAC AAC TAC-3′</td>
<td>22 bp</td>
<td>56.5 °C</td>
</tr>
<tr>
<td>ruc−cinBP</td>
<td>5′-CAC GAC TTA TGC AAA GCA CTA C-3′</td>
<td>22 bp</td>
<td>58.4 °C</td>
</tr>
<tr>
<td>PCR</td>
<td>5′-CA CTC GGA AAT GTC AGG AAG A-3′</td>
<td>22 bp</td>
<td>66.4 °C</td>
</tr>
<tr>
<td>muc−1</td>
<td>5′-GTA TGG GCA CCT CCA AAA-3′</td>
<td>23 bp</td>
<td>69.7 °C</td>
</tr>
</tbody>
</table>

2.5. Additional detection of LAMP products

The LAMP amplicons were also detected by electrophoresis of 10 μL of the amplicon in a 2% agarose gel with a molecular marker for 50 bp or 100 bp (Biozym Diagnostic, Germany) was used.

2.6. Analytical sensitivity of the LAMP assay and limit of detection (LOD)

Serially diluted DNA from the S. aureus reference strain was used for determining the analytical sensitivity of the LAMP reaction. The sensitivity was detected with the real-time fluorometer. Serial dilutions of S. aureus DNA (10−1 to 10−9) were prepared using Tris buffer (TE, pH 8.0). The LOD was estimated by spiking milk (10 ml) with serial dilutions (10−1 to 10−9) of the S. aureus reference strain. The concentration of the initial suspension was estimated using the colony-forming unit (CFU). All spiked milk samples were centrifuged at 3700g for 45 min. The supernatant, including the cream layer, was discarded. The pellets were resuspended in 180 ml lysis buffer and afterward the DNA was isolated as mentioned above.

2.7. Positive and negative predictive values (PPV and NPV)

The PPV and NPV are regarded as a description of the performance of a newly developed diagnostic test. The calculation of PPV and NPV was based on the final diagnosis for each S. aureus isolate tested. The diagnosis was based on the microbiological results which included morphological colony features, hemolytic characteristics, catalase test, clumping factor (Oxford, Attrinchan, England), coagulase tests using rabbit plasma (tube method) (Becton, Dickinson, Heidelberg, Germany) and biochemical tests (API Staph identification system). The PCR was additionally performed to confirm the presence of gene muc in all isolates of this study.

The PPV was calculated by the formula:

$$PPV = \frac{\text{(number of true positive)}}{\text{(number of true positive + number of false positive)}} \times 100$$

The NPV was calculated by the formula:

$$NPV = \frac{\text{(number of true negative)}}{\text{(number of true negative + number of false negative)}} \times 100$$

The accuracy (AC) was calculated by the formula:

$$AC = \frac{\text{(number of true positive + number of false positive)}}{\text{100}}$$

3. Results

3.1. Specificity of the LAMP assay for detecting gene muc

The six designed primers and the reaction conditions allowed to detect gene muc in all investigated S. aureus isolates (100%). No signal was detected with any of the non-S. aureus strains tested. The results of the LAMP assays were compared to the conventional PCR results which also revealed that gene muc was found in all S. aureus isolates.

The results of LAMP reactions for detecting gene muc were measured by using the real-time fluorometer (Fig. 1) and by gel electrophoresis. The target sequences amplified by the LAMP assay showed ladder-like bands in the gel images due to the formation of stem-loop structures of amplified DNA of various stem lengths. Melting curve analysis, which is termed by Gene II™ as anneal curve analysis, showed that there was no significant difference among gene muc of the S. aureus isolates tested. The melting temperature of gene muc specific amplicon amounted to 81.4 °C (±0.26 °C).

3.2. Analytical sensitivity of the LAMP assay and limit of detection (LOD)

The DNA concentration of the serial dilutions ranged from 5.2 ng μL−1 (10−9) to 0.052 pg μL−1 (10−6). Fig. 1 shows the amplification curves of the S. aureus DNA using the real-time fluorometer. The analytical sensitivity of the LAMP assay for detecting S. aureus DNA was 0.052 pg μL−1, resulting in an LOD of 0.26 pg per reaction (5 μL of DNA/reaction) measurable after isothermal amplification for 21,22 (±0.68 min) (Table 2). The detection probability of this concentration was determined as 50% (two from four were positive). The melting temperature of the S. aureus DNA used for LOD calculations was 53.2 °C (±0.21 °C). The LOD for the LAMP assay of the S. aureus reference strain in spiked milk was 9 × 10−6 CFU ml−1 milk when isolated from 10 ml milk sample. The detection probability was 71.5% (five out of seven were positive) after a mean detection time of 22.21 (±0.68) min (Table 4). The melting temperature of the DNA used for LOD calculation was 81.40 °C (±0.06 °C) (Fig. 1b).

3.3. Estimation of positive and negative predictive values

PPV and NPV for detecting gene muc from all S. aureus were both

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**Table 3**

Analytical sensitivity of the LAMP assay using serial dilutions of *S. aureus* ATCC 6538 DNA.

<table>
<thead>
<tr>
<th>Dilution step</th>
<th>DNA amount pg µl⁻¹</th>
<th>Run 1 (min)</th>
<th>Run 2 (min)</th>
<th>Run 3 (min)</th>
<th>Run 4 (min)</th>
<th>Mean (SD ±)</th>
<th>Detection probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁴</td>
<td>5380</td>
<td>07:15</td>
<td>07:30</td>
<td>08:00</td>
<td>08:00</td>
<td>07:41 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻³</td>
<td>520</td>
<td>09:15</td>
<td>09:15</td>
<td>08:45</td>
<td>08:45</td>
<td>09:05 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻²</td>
<td>52</td>
<td>09:45</td>
<td>09:30</td>
<td>10:15</td>
<td>10:15</td>
<td>09:55 (00:19)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>5.2</td>
<td>11:15</td>
<td>11:00</td>
<td>11:50</td>
<td>12:15</td>
<td>11:30 (00:28)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁻⁰</td>
<td>0.52</td>
<td>14:00</td>
<td>13:30</td>
<td>17:00</td>
<td>17:30</td>
<td>15:30 (01:46)</td>
<td>100%</td>
</tr>
<tr>
<td>10⁰</td>
<td>0.02</td>
<td>14:45</td>
<td>28:00</td>
<td>ND</td>
<td>ND</td>
<td>21:02 (02:30)</td>
<td>50%</td>
</tr>
</tbody>
</table>

*ND: Not detected.*

**Table 4**

Limit of detection (LOD) of the LAMP assay using milk samples spiked with serial dilutions of *S. aureus* ATCC 6538 reference strain.

<table>
<thead>
<tr>
<th>CFU ml⁻¹ milk</th>
<th>Detection time (min)</th>
<th>Mean (SD ±)</th>
<th>Detection probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>run 1</td>
<td>run 2</td>
<td>run 3</td>
</tr>
<tr>
<td>9 x 10⁶</td>
<td>11:01</td>
<td>21:00</td>
<td>15:45</td>
</tr>
<tr>
<td>9 x 10⁵</td>
<td>19:39</td>
<td>22:00</td>
<td>20:15</td>
</tr>
<tr>
<td>9 x 10⁴</td>
<td>15:45</td>
<td>16:30</td>
<td>11:15</td>
</tr>
<tr>
<td>9 x 10³</td>
<td>19:30</td>
<td>27:45</td>
<td>10:15</td>
</tr>
<tr>
<td>9 x 10²</td>
<td>10:30</td>
<td>25:45</td>
<td>23:30</td>
</tr>
</tbody>
</table>

*ND: Not detected.*

---

*Please cite this article as: O.H. Sheel, et al., Development and validation of a loop mediated isothermal amplification (LAMP) assay for the detection of *Staphylococcus aureus* in bovine mastitis milk samples, Molecular and Cellular Probes (2016). http://dx.doi.org/10.1016/j.mcp.2016.08.001"
100% for the developed LAMP assay. The accuracy of the new developed LAMP assay was compared to the result of previous publication PCR [9,20] was also 100%.

4. Discussion

S. aureus is considered to be the main cause of mastitis in dairy cows and is also the major pathogen causing food poisoning by producing several staphylococcal enterotoxins. Therefore, a rapid and highly accurate detection of the pathogen is necessary for suitable treatment, prevention of spreading and the reduction of risk factors caused by this organism. Currently, there are several methods available for identifying S. aureus. The identification of S. aureus by traditional methods which include culture and biochemical identification and immune methods requires 24 h. The PCR method is sensitive, accurate, and already faster. To identify S. aureus by means of the PCR method, this takes 4–6 h. This method requires expensive equipment such as a thermocycler, electrophoresis set, and gel documentation system.

The LAMP assay is a relatively new DNA amplification technique. This method uses four or six primers which can recognize six to eight distinct sequences of the target gene. The complete reaction occurs using the Integrated Cap containing hot DNA polymerase, DNA template and reaction buffers at a constant temperature. The target sequence can be amplified with high efficiency, rapidity, and specificity under isothermal conditions. In this study, the results of the LAMP assay were specific. This was achieved by using six primers for recognizing eight distinct regions of the target gene that helps to facilitate the detection of very minute quantities of target DNA compared to other studies that used four primers to detect the same target gene nucle [23,24]. One study [25] demonstrated the use of additional primer, termed “loop primer” in the LAMP assay that led to the accelerated identification of S. aureus and higher specificity. In addition, the LAMP reaction is carried out under isothermal conditions at 65 °C, and a positive result was observed after seven to 22 min. In accordance with previous findings, this indicates that it is important to use the loop primers in the assay to achieve an accurate result in a short time.

The amplification products were detected by using the real-time fluorometer that gives a positive signal as an amplification curve. Previous studies detected the amplified gene with the naked eye, either as turbidity in the form of a white precipitate or through a colour change employing a fluorescent intercalating dye (SYBR green I) [11]. In the present study, however, this LAMP assay had the advantage of allowing a positive result to be viewed as a curve through the LAMP real-time fluorometer screen. The real-time fluorometer could be used to develop a quantitative assay. Additionally, the DNA amplification could be followed during the still active reaction.

In the present study, the analytical sensitivity of the LAMP assay for detecting S. aureus DNA amounted to 0.652 pg -1 which was less than the analytical sensitivity mentioned by Lim et al. [9] and Wang et al. [20] that amounted to 2.5 ng ml -1 and 0.147 pg ml -1, respectively. This difference is attributed to the use of two additional primers in the present study to detect gene nucle compared to these previous studies which used only four primers. One previous study [25] recommended the use of additional primers (loop primers) to accelerate the LAMP reaction and to increase the sensitivity. The LOD for the LAMP assay was 9 × 104 CFU ml -1 milk. These results for LOD are comparable to those of Kolde et al. [12] and Haraj-Kiou et al. [13] who also obtained the same limit of detection. However, in the present study, the results of the LOD were more sensitive than the LOD of the used PCR that was 1 × 106 [26] and 108 [11] respectively. The results for analytical sensitivity and LOD obtained in the present study revealed that the developed LAMP assay was more sensitive than those developed in previous studies [9,20], and more sensitive compared to the PCR. Maeda et al. [27] and Miyagawa et al. [28] confirmed that the sensitivity of LAMP assays was equivalent or higher compared to PCR methods.

The LAMP assay complemented with the use of a real-time fluorometer (Genie II) has several advantages qualifying it as one of the most common methods for detecting pathogens worldwide. The combination of an isothermal reaction together with a real-time fluorometer enables the possibility to observe the positive result directly through the amplification on the optical screen. The LAMP assay is a promising method for the rapid identification of S. aureus and various types of bacteria. The use of specific loop primers for detecting S. aureus can facilitate a reduction in the necessary amplification time to less than 30 min. The equipment used included thermal cycling, Genie II and PCR equipment.

5. Conclusion

The developed LAMP assay is a rapid detection method for identifying S. aureus with high specificity and sensitivity. The use of a real-time fluorometer enabled an identification of S. aureus less than 2 h including the DNA preparation. Therefore, this assay is a rapid, flexible and simple tool for identifying S. aureus isolates.

Funding

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no conflicts of interest.

Informed consent

Not applicable.

References

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10.2 Manuscript

Phenotypic and Genotypic Characterization of Staphylococcus aureus Isolated from Bovine Mastitis Milk from Northern Germany

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Abstract

Background: There are many types of bacterial pathogens causing mastitis in dairy herds. *Staphylococcus (S.) aureus* is the most important causative agent of contagious intramammary infections in dairy cattle. In addition, *S. aureus* is an important food-borne pathogen and the main cause of food poisoning cases and outbreaks worldwide. *S. aureus* can be detected and identified by using the conventional and molecular identification methods. These identification methods are need to laboratories, time-consuming and high experience.

Results: To understand the molecular ecology of *S. aureus*, the present study compared phenotypic and genotypic characteristics of 70 *S. aureus* isolates from bovine mastitis milk samples collected during the period from August 2001 to March 2014 in different regions of Northern Germany. All *S. aureus* isolates were characterized phenotypically, as well as for their genetic diversity using multilocus sequence typing (MLST), staphylococcal protein A (spa) typing and the presence of virulence genes encoding 16 staphylococcal enterotoxins (sea-sek), toxic shock syndrome toxin (tsst), thermonuclease (tnu), clumping factor (clfA and clfB), coagulase (coa) and the methicillin resistance gene *mecA*. A total of 16 sequence types were grouped into eight clonal complexes (CCs). 17 spa types were identified. This included six novel sequence types and one novel spa type. The genotype CC133 (ST133-t1403) was predominant, constituting 27.14% of the isolates. In addition, the *S. aureus* isolates displayed nine different enterotoxigenic profiles. All *S. aureus* were methicillin sensitive (MSSA). The majority of bovine mastitis milk-associated sequence types belonged to the clonal complex CC5, CC97, CC133 and CC151 and showed closely related genotypes or lineages with sequence types of human origin.

Conclusion: The current study provided new information on phenotypic and genotypic traits of *S. aureus* isolates from bovine mastitis. The results of the present study showed the existence of a relationship between mastitis isolates of this study with human isolates. This might help to understand the distribution of *S. aureus* isolates and also help to control *S. aureus* infections in cattle herds.
Key words: *Staphylococcus aureus*, mastitis milk, staphylococcal enterotoxins, MLST and staphylococcal protein A (spa)

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Background

*Staphylococcus (S.) aureus* is one of the most important etiological agents of contagious clinical and subclinical mastitis in dairy herds [1]. The bacterium causes significant economic losses in the dairy industry by affecting both the quality and quantity of milk produced, premature slaughter, veterinary and treatment costs [2]. The interior bovine gland is one of the most important reservoirs for *S. aureus* [3]. However, it has also been found on extramammary sites such as the teat skin, teat orifice, feed stuff, skin of milking personnel, insects, non-bovine animals, milking equipment, farm equipment [4, 6].

Besides the mere detection and identification of *S. aureus* isolated from bovine mastitis, it is important to know characteristics of the isolates both phenotypically and genotypically [7]. Traditional methods for identifying intramammary *S. aureus* are based on a series of microbiological tools, i.e. coagulase test, catalase reaction, colony morphology and hemolysis on blood agar after overnight incubation [8]. In recent reports, polymerase chain reaction (PCR) has become one of the most common methods used to detect mastitis pathogens in a sensitive and specific way [9]. In addition, many different types of novel molecular methods were described to identify different *S. aureus* genotypes [10], e.g. Multiple Locus Variable-Number Tandem Repeat (MLVA), Pulsed-Field Gel Electrophoresis (PFGE), typing the X region of protein A gene (*spa*), Multi-locus Sequence Typing (MLST) and Amplified Fragment length polymorphism (AFLP). Typing the X region of protein A gene (*spa*) and MLST have been widely applied to differentiate types of *S. aureus* by sharing and analyzing a substantial database of genotypes. Furthermore, these methods are supported by many online resources (www.mlst.net and www.spaerver.ridom.de) [11].

In recent years, several reports from different regions of the world have shown that Methicillin-resistant *Staphylococcus aureus* (MRSA) could also be detected in bovine mastitis and dairy products which are considered to be the primary source for human MRSA infection [12]. The reports from the European Food Safety Authority in 2009 indicated that the presence of MRSA was increasing in food-producing animals and recommended that the detection and quantification of MRSA should be performed on food and the environment [13]. *S. aureus* causes poisoning via many different types of
food contaminated by one or more of staphylococcal enterotoxins worldwide [14]. In addition to the enterotoxins, *S. aureus* produces more than 30 different types of virulence factors which can contribute in various ways to causing infection [15]. These factors are divided into several categories, i.e. surface-associated factors, degradative enzymes and superantigen toxins. In term, staphylococcal toxins can be classified into pyrogenic toxin superantigens (PTSAs), exfoliative toxins, leukocidins and other toxins. The group of (PTSAs) consists of staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST) [16]. At present there are 21 staphylococcal enterotoxins known so far, these being grouped into staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEL, SER, SES, SET, SEK, SEL, SEM, SEN, SEO, SEP, SEQ and enterotoxin-like SEU, SEI and SEIV) [17].

The aim of the present study was to characterize *S. aureus* isolated from bovine mastitis in Northern Germany by determining phenotypic and genotypic properties and also to examine the potential correlation of genes encoding toxins with *S. aureus* genotypes. In addition, the relationship between the *S. aureus* isolates of the present study and the profiles of *S. aureus* isolates from a human (obtained from MLST-database) were compared.

Methods

Isolation, identification and phenotypic characterization of the *S. aureus* isolates

All *S. aureus* isolates (n = 70) used in the present study were isolated from bovine mastitis milk during the period from August 2001 to March 2014 in different regions of Northern Germany. The isolates were obtained from routine mastitis diagnosis conducted at the Institute of Food Quality and Food Safety, University of Veterinary Medicine, Foundation, Hannover, Germany. The isolation of *S. aureus* from milk samples was conducted using the method proposed by the National Mastitis Council (NMC) recommendations [1]. Isolates grown on blood agar plates were identified tentatively according to morphological colony features, hemolytic characteristics, the catalase test, staphaurex latex agglutination test (clumping factor and protein A) (Oxoid, Altrincham, England) and coagulase test using rabbit plasma (Becton, Dickinson, Heidelberg, Germany).
The further identification of the isolates was performed using the commercial API-32 Staph biochemical identification system (bio-Mérieux, S.A., Marcy l’Etoile, France), according to the manufacturer's instructions. Briefly, few colonies from overnight culture on blood agar were transferred to a tube containing 2 ml of suspension medium. This suspension medium was compared with the turbidity control (McFarland Standard 0.5). After preparation, the strips were incubated aerobically at 37°C for 24 hours.

**Antimicrobial susceptibility testing**

Susceptibility to eleven types of antibiotics was examined in 96-well round-bottom U plates. The types of antibiotics used in the test were amoxicillin (AMC), ampicillin (AMP), cefazolin (CEZ), cefoperazone (CPZ), cefquinome (CEQ), ceftriaxone (CRY), gentamicin (GEN), tetracycline (TET), oxacillin (OXA), penicillin (PEN) and pirlimycin (PIR) (Merlin, Böhringer-Hersel, Germany). The wells of the plates were inoculated with Mueller-Hinton broth cultured with the isolates. The plates were incubated at 37°C for 24 hours, as recommended by the manufacturer and by the Clinical and Laboratory Standards Institute (CLSI) guide M31–A3 [18]. After 24 hours, the plates were read by the naked eye.

**Extraction of chromosomal DNA**

According to the manufacturer's instructions using the Gram-positive protocol, the total chromosomal DNA of *S. aureus* isolates was extracted by DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

The isolates were cultured on the blood agar and incubated at 37°C for 24 h. 5-10 colonies were suspended in 180 µL of lysis buffer containing 20 mg µL⁻¹ lysozyme and 3 µl lysostaphin (1 mg µL⁻¹, Sigma-Aldrich Chemie, Steinheim, Germany) was added and then incubated for 30 minutes at 37°C.

After incubation, 20 µL protease K (20 mg mL⁻¹) and 200 µL buffer AL (Qiagen) were added and mixed by vortexing, then incubated at 56°C for 30 minutes. Afterwards 200 µL ethanol were added to the sample. The mixture was transferred to the Mini Spin column and centrifuged at 6200 × g for 1 min. 500 µL of wash buffer (AW1) were added to the Mini Spin column and centrifuged at 6200 × g for 1 min. 500 µL wash buffer (AW2) were added to the Mini Spin column and centrifuged at 17,000 × g for 3 min. The Mini Spin column was placed in clean 1.5 mL microcentrifuge tubes. Then 200 µL
elution buffer AE were added and centrifuged at 6200 × g for 1 min. The DNA concentration was estimated by using NanoDrop 2000c (PeQlab, Erlangen, Germany). The DNA yield was stored at 20°C until further use.

**PCR Amplification of genes encoding staphylococcal virulence factors**

The presence of the genes encoding staphylococcal virulence factors which include thermolysin (mec), clumping factor (clf), clumping factor (clfB), coagulase (coa) and mecA was investigated by PCR. The primers and the references are shown in Table 1.

PCR was also used to amplify the genes encoding staphylococcal enterotoxins, i.e. SEA (sea), SEB (seb), SEC (sec), SEE (sec), SEG (seg), SEH (seh), SEI (sei), SEL (sel), SEM (sem), SEN (sen), SEQ (seg)

PCR was also used to amplify the genes encoding staphylococcal enterotoxins, i.e. SEA (sea), SEB (seb), SEC (sec), SEE (sec), SEG (seg), SEH (seh), SEI (sei), SEL (sel), SEM (sem), SEN (sen), SEQ (seg) and TSST-1 (tsa). The primers and the references are shown in Table 1. The total volume of PCR reaction mixtures was 30 µL, this consisting of 1 µL of each forward primer and reverse primer (each 10 pmol µL⁻¹) (Eurofins Genomics, Ebersberg, Germany), 15 µL of 2 × Red Y Gold Mix Master containing 1 unit GoldStar DNA polymerase, 200 µM dNTPs, 1.5 µM MgCl₂, 20 µM [NH₄]₂SO₄, 75 µM Tris – HCl (pH 8.8 at 25°C), 0.01% [v/v] Tween 20, and Red Dye Loading Buffer (Eurogentec Deutschland, Cologne, Germany) and 10.5 µL Nuclease-Free Water (Qiagen). Finally, 2.5 µL DNA template of S. aureus was added to each reaction tube. The amplification was performed in the Gene Amplification PCR system Thermal Cycler (Biometra, Göttingen, Germany) and the conditions for the amplification were used as previously described (Table 1). The PCR products were determined by gel electrophoresis of 10 µL of the reaction product in 2% agarose gel (PeqLab) with 1×Tris – acetate – Electrophoresis Buffer (Roth, Karlsruhe, Germany), (10.8 g L⁻¹ Tris base, 5.5 g L⁻¹ boric acid, 0.75 g L⁻¹ EDTA) and 100 – bp DNA Ladder (Roche, Berlin, Germany) or Biorbym, Hildesheim, Germany) as molecular marker. S. aureus strains producing SEA (619/93), SEB (62/92), SEC (1229/93), SED (1644/93), SEE (FRI 918), TSST-1 (161/93), SEG/SEI (Ly 990555) and SEH (Ly 990552), SEI (2724) were collected from the strain collection of Dairy Sciences, Institute of Veterinary Food Science, Justus- Liebig-University Giessen, Germany. The S. aureus strain (120/14) was used as positive control to determine gene mecA, this was obtained from the Institute of Animal Hygiene, Animal Welfare and
Ethology, University of Veterinary Medicine Hannover, Foundation, Bündneweg 17, 30559 Hannover, Germany. *S. aureus* ATCC 6538, used as a positive control to determine gene *mec* and the other virulence genes was collected from the institute’s strains collection.

**Multi-locus Sequence Typing (MLST)**

MLST was carried out with primers that had been previously designed by Enright et al. (2000) for detecting *S. aureus* housekeeping genes (Table 2). These include seven housekeeping genes, i.e., carbamate kinase (arcC), shikimate dehydrogenase (aroE), glyceral kinase (gpiF), guanylate kinase (gmk), phosphate acetyltransferase (pta), triosephosphate isomerase (tpi) and acetyl coenzyme A acetyltransferase (yqiL). PCR was used to amplify all housekeeping genes and the amplicons were sequenced by Seqlab (Hann-Vogt, Göttingen, Germany). DNA sequences were assembled by using the software program BioNumerics v7.5 (Applied Maths, Sint-Maartens-Latem, Belgium). All allele and sequence types (ST) of the present study isolates and the profiles of *S. aureus* isolates from bovine mastitis and human, were determined according to the MLST website (www.mlst.net).

**Protein A (spa) typing**

The spa typing was performed with specific primers which were previously described [19] (Table 1). Staphylococcal Protein A contains a specific repeat region that was amplified; afterwards, sequenced. All the spa-repeats and typing were assigned by using the software program BioNumerics v7.5. All numeric spa typing and spa type codes were determined according to the Ridom Spa Server website (www.spaserver.ridom.de).

**Results**

**Microbiological identification and phenotypic characterization of the *S. Aureus* isolates**

According to the guidelines of the National Mastitis Council (NMC), a total of 70 isolates obtained from milk samples (each one isolate per sample) could be identified as *S. aureus*. Among these, 27 isolates (38.57%) showed alpha-hemolysis, 26 (37.14%) beta-hemolysis and 17 (24.28%) were nonhemolytic. The staphaurex (clumping factor and protein A) test revealed that 75.71% of the
isolates (n = 53) were positive while all investigated isolates were positive for the catalase test. The
coagulase test was performed on the isolates that had given a negative result in the staphaurex test to
ensure that the isolates were *S. aureus*. The API 32 Staph identification system confirmed that all
isolates were *S. aureus*. The rate of identification of 64 isolates was ID 95% to 99.8%. The remaining
isolates displayed lower rates of identification, i.e. 82%, 79%, 78% (one isolate each) and 62% (three
isolates), respectively.

**Antimicrobial susceptibility testing**

All isolates were tested for their resistance to eleven antimicrobials (Table 3). Phenotypically, the
majority of the isolates, i.e. 53 (75.7%) were sensitive, only seventeen (24.3%) showing any resistance
to one or more antibiotics. The isolates showed a high resistance rate to penicillin and ampicillin
(22.85% each). The resistance of *S. aureus* to pirlimycin, amoxicillin and cefoperazone was 5.71%,
2.85% and 1.42%, respectively.

**Multi-locus sequence typing (MLST)**

In the present study, sixteen different sequence type (ST) were determined (Table 4). Of those, six
were novel STs which were designated as ST2821 (n = 2), ST2823 (n = 1), ST2824 (n = 1), ST2825 (n
= 2), ST2826 (n = 1) and ST2827 (n = 1). These new STs were submitted as new registrations to the
MLST database. The most common frequencies of ST were ST133 (n = 20), ST504 (n = 16) and ST97
(n = 11). Less frequent STs were ST398 (n = 4), ST479 (n = 3), ST1380 (n = 3) and ST151 (n = 2),
while other types as ST7 (n = 1), ST71 (n = 1) and ST464 (n = 1) were found only for one *S. aureus,
respectively.

As shown in Table 4, the sixteen STs were grouped into eight clonal complexes (CCs). CC133,
represented by ST133 and ST2821 as most prevalent genotype (31.42%, 22/70), followed by CC151
(27.14%, 19/70) and CC97 (21.42%, 15/70) which was the most diverse clonal complex, consisting of
ST97, ST71, ST464, ST2824 and ST2826. The other types of clonal complexes were less prevalent
genotypes; CC479 (8.57%, 6/70), CC398 (5.71%, 4/70), CC5 (2.85%, 2/70), CC8 (1.4%, 1/70) and
CC50 (1.4%, 1/70).
Protein A (spa) typing

The 70 *S. aureus* isolates were grouped into seventeen spa types (Table 4). One new spa type (t13769) was found. The most frequent spa types were t1403 (n = 21), t529 (n = 19) and t521 (n = 8). The other spa types were found less frequently: t528 (n = 4), t034 (n = 3), t2873 (n = 3), t586 (n = 2), t091 (n = 1), t267 (n = 1), t297 (n = 1), t559 (n = 1), t5180 (n = 1), t519 (n = 1), t524 (n = 1), t571 (n = 1), t5920 (n = 1).

The relationship between ST types and spa types

Determination of the relationship between ST types and spa types revealed a relation between ST133 and spa type t1403 for 19 (27.14%) isolates. The correlation ST504 and spa type t529 was found for sixteen (22.85%) isolates and the relation between ST97 and spa type t521 for seven (10%) isolates. Furthermore, the correlation between ST398 and spa type t034 was found for three (4.28%) isolates.

The relationship between ST151 and spa type t529, ST2821 and spa type t1403, ST1380 and spa type t2873, ST479 and spa type t528, ST2825 and spa type t586 were found for two isolates (2.85%), respectively. The remaining ST and spa type was found in only one isolate (1.41%). Based on the MLST (Table 4) the clonal complex CC97 was one of the most varied ones, consisting of eight of spa types, namely (t521, t13769, t359, t267, t5920, t524, t3297 and t5180). Some isolates had the identical spa type, but had differing STs (t529 – ST504, t529 – ST151 and t529 – ST2823).

Detection of enterotoxins encoding genes

The prevalence of enterotoxin genes of *S. aureus* obtained by PCR revealed that 37 (52.85%) of the 70 isolates were positive for more than one of the enterotoxin genes (Table 5). 33 isolates (47.14%) did not possess any enterotoxin genes. The most frequent genes detected in the present study were (sec), (seo) and (sel), which were found among the clonal complexes CC151, CC479, CC5 and CC50, followed by seg with a total of 26 strains (37.14%) which was found among clonal complexes CC151, CC479 and CC5. In addition, gene seo was found in 9 strains (12.85%) which belonged to clonal complexes CC479, CC5 and CC50, whereas, the sec, sed, sel and tss genes were found in 8 strains (11.42%), respectively. Gene ser was found in seven isolates (10%) and solh and seq in 3
(4.28%) of the isolates, respectively. All isolates were negative for seb, see, sej and seq and also the strains with the clonal complex CC133 and CC398 were negative for all types of staphylococcal enterotoxin genes.

The S. aureus isolates were subsequently divided into nine different enterotoxigenic profiles which were based on the presence of different enterotoxin genes among the S. aureus lineages (Table 6), suggesting a horizontal transfer of toxin genes among the S. aureus isolates. The S. aureus isolates most frequently showed the enterotoxigenic profile I (seg, sei, sen, sen and seln) with eleven (15.71%) isolates. Seven (10%) of the isolates had the enterotoxigenic profile II (seg, sei, sen, sen, seln and seln). The enterotoxigenic profile III (seg, sei and seln) was found in seven (10%) isolates. S. aureus isolates with the enterotoxigenic profile IV (seg, sei, sen, sen, seo and seln) was found in six (8.57%) isolates. Whereas, two (2.28%) S. aureus isolates showed the enterotoxigenic profile V (seg, sei, sen, sen, seo, sep and seln). The enterotoxigenic profiles VI to IX were found in one (1.14%) isolate, respectively. None of the S. aureus isolates had a single enterotoxin gene.

Some enterotoxin genes are known to be grouped either as a gene cluster or organized as an operon. In this study, as shown in Table 6, the most prevalent toxin gene cluster was enterotoxigenic profile I, followed by the enterotoxigenic profile II which were found in the clonal complex CC151. In addition, enterotoxigenic profiles III and VI were found in isolates belonging to CC79. Enterotoxigenic profile IV was only found in CC479. The enterotoxigenic profile V was found solely in isolates belonging to CC5. The gene group seh + sep (profile VIII) was found in only one isolate that belonged to CC8. The enterotoxigenic profile IX was found in CC50. S. aureus isolates belonging to CC133 and CC398 that did not possess any of the staphylococcal enterotoxin genes.

Prevalence of genes encoding staphylococcal virulence factors

All S. aureus isolates were examined for the presence of the genes coa, clfA, clfB and nuc (Table 5). The results revealed that all of the 70 (100%) S. aureus isolates possessed the genes coa, clfB and nuc. Whereas clfA was found in 69 (98.60%) of the S. aureus isolates. No isolates showed the presence of gene mecA.
Relationship between isolates from bovine mastitis and human disease

MLST profiles of 118 human strains from Germany were obtained from the MLST Databank (www.Mlst.Net). The data were compared with the MLST profiles of the mastitis isolates of this study. The relationship among all strains was defined via the seven housekeeping genes that were sequenced to identify the ST. Clustering of MLST profiles was completed using a categorical coefficient and the isolates were clustered in groups. MLST was assigned to one group if at least 5 of 7 allele loci were similar. According to the minimum spanning tree (Figure 1), the results of the correlation between isolates displayed five groups. Group I (CC8) showed the relationship with two isolates of human origin. Group II (CC5) showed a correlation with nine of the isolates from humans. Group III (CC151) correlated with two of the isolates from humans. Group IV (CC133) was related to five isolates from humans, while group V (CC97) correlated with three isolates from humans.

Relationship between the S. aureus bovine mastitis isolates of this study analysed with S. aureus isolates from different European countries

Minimum spanning tree analysis of 70 isolates of S. aureus of this study and 33 isolates of S. aureus from bovine mastitis from different European countries were conducted. The minimum spanning tree displayed the correlation between the MLST types of all the isolates as circles. The colors of the halo surrounding the MLST types indicated types belonging to the same group. Group I (CC133) showed the correlation between the ST133 in this study with one isolate from the Netherlands. Group II (CC5) revealed the relationship between ST2825 and three isolates isolated from England, the Netherlands and Germany. Group III (CC8) displayed a relationship between ST7 with one isolate from Germany (Figure 2).

Discussion

S. aureus is one of the major pathogens causing mastitis in bovines and other mammals worldwide [1, 20, 21] and the most common contagious organism isolated from bovine mastitis [1]. In the present study, phenotypic and genotypic methods were performed to identify and further characterize 70 S. aureus isolated from bovine mastitis milk. The results obtained by phenotypic investigations were in concordance with the results of the genotypic identification. According to the results from the
biochemical reactions and also based on the presence of genes mecA and coa all isolates were identified
as S. aureus.

Phenotypically, seventeen (24.3%) isolates were resistant to one or more antibiotics, 53 (75.7%) isolates
were sensitive. The proportion of resistant isolates of this study was close to the finding of [22] who also reported that 21% of the tested S. aureus isolates were resistant to at least one antibiotic.

However, [23] reported that 98.3% of the tested isolates were resistant to at least one antibiotic.

The isolates showed a high resistance rate to penicillin and ampicillin, these results falling with those
of other investigations. For example, [24, 25] reported that S. aureus showed a high resistance to
penicillin and ampicillin. In fact, S. aureus showed a wide difference in their resistance to antibiotics
due to the various geographic regions, type of sample and the genetic characteristics of the isolates
[26]. The high resistance of S. aureus against antibiotics has been referred to as being the result of the
indiscriminate use of antibiotics and inappropriate handling of infected animals without regular
veterinary observation and ignoring the medical advice of the veterinarian to treat animals [27]. As a
consequence, S. aureus isolates were frequently resistant to antibiotic therapy which led to low cure
rates for mastitis.

Phenotypically, all 76 S. aureus isolates of the present study were shown to be sensitive to methicillin.
This was confirmed with the genotypic tests which revealed that none of the tested isolates carried
gene mecA. Gene mecA encodes for the Penicillin Binding Protein 2A that referred methicillin
resistance [28]. The results of the antimicrobial susceptibility testing in this study were in agreement
with other reports showing a high prevalence of methicillin-resistant S. aureus (MSSA) in milk and
dairy product samples [24, 29]. However, other studies reported similar results concerning the presence
of S. aureus from bovine mastitis carrying gene mecA [30, 31].

Genotypically, among the 8 CCs identified in our study, CC133, CC151 and CC97 were predominant.
CC133 and CC151 accounted for 58.6% of the total isolates examined. This was in agreement with the
studies [32, 33], [33] reported that the majority of ruminant-associated sequence types (STs) belonged
to CC133, CC151 and CC97, while [32] mentioned that CC151 and CC133 were the dominant
lineages from bovine milk strains in Germany. CC133 was also found in relatively developed regions.
of Europe, Australasia and the Americas [34]. However, CC133 was not only found in bovine mastitis milk isolates but also in _S. aureus_ from other types of animals; for example, in small ruminants [33, 35] and in ungulates [36]. No evidence of CC133 causing human infections has been found to date.

CC151 isolates were predominantly associated with cows and were not previously detected in humans [37]. CC97 was also predominant while other clonal complexes (CC479, CC398, CC5, CC8 and CC50) were only present in a smaller number of isolates. CC97 was the dominant lineage from bovine mastitis in Denmark [38] and in Switzerland and Germany [39] and in the United States, Chile and the United Kingdom [40]. Although CC97 _S. aureus_ isolates have been commonly isolated from bovines, they have also been detected among human and porcine hosts [41, 42]. All isolates identified in our study belonging to this clonal complex were methicillin-susceptible _S. aureus_ (MSSA) (Table 4). Our findings were in agreement with other previous studies showing a higher prevalence of MSSA in bovine milk samples [29, 43, 44]. CC398 is of major importance in livestock and can be found in different animal species including poultry, horses, swine and cattle [39, 45, 46]. MRSA strains of this CC have recently received a lot of attention especially strains that had been isolated from pigs and livestock animals worldwide [46, 48]. According to [49] MRSA CC398 appears to have originated from human MSSA CC398, which acquired methicillin resistance in livestock. Our study revealed that all isolates of _S. aureus_ belonging to CC398 were MSSA. Therefore, antimicrobial selection associated with food animal production increases the potential for MSSA CC398 in food to obtain the SCCmec cassette. The difference in clonal complex frequencies detected in comparison to other studies and the significant variations in frequencies concerning the herd location confirm that _S. aureus_ genetic characteristics may be related to geographic regions [50].

MLST analysis is necessary for generating results that are readily comparable between laboratories [51]. In the present study, sixteen different STs, including six novel types, were determined. The novel types reported for the first time indicated the evolutionary emergence of unique clones in the different regions, the importance of which needs to be established. The most common frequency of the sequence types which cause bovine mastitis were ST133, ST304 and ST97. Previously studies have referred to ST133 as being one of the most frequent types in small ruminants [33, 52]. In addition, ST133 were also found in other types of animals, including ungulates, rodents and carnivores such as
ions and cats [53, 54], in red deer in Southern Spain [55], in pigs [56] and wild boars in Germany [57]. ST504 was only found in S. aureus isolated from bovine mastitis (http://www.mlstat.net). Earlier studies revealed the presence of ST97 in S. aureus isolated from bovine mastitis milk in the Netherlands [58], Northwestern China [59], Denmark [38] and Japan [60]. In China, the study showed that ST97 may be found in milk and beef, indicating that ST97 correlated with cattle [61]. Interestingly, ST97 has previously been described as being present at low frequency in humans [62], in Spain [63], the United Kingdom [64] and Turkey [65]. Several studies demonstrated that healthy pets, goats and cows could be reservoirs and a epidemiological source of S. aureus which may infect humans [35, 66].

In the present study, the 70 S. aureus isolates were grouped into eighteen spa types, including the newly identified spa type t13769. The most frequent prevalence of the spa types was t1403, t529 and t521. Several studies showed the similarity between the diversity of spa types of S. aureus isolates from different kind of food, animals and humans. In the present study, the spa type t1403 was most frequent. However, this spa type has already been reported in bovine mastitis in Germany [67] and Sweden [41] and from cattle in the slaughterhouses in the Netherlands [30] and Denmark [38]. In addition, spa type t1403 was detected in S. aureus isolated from sheep in the slaughterhouse [35]. Recent studies in Germany, Japan, Switzerland and India revealed that spa type t529 is the predominant type of S. aureus isolated from bovine milk [39, 60] and in dairy cattle [24, 30, 60, 67, 68]. On the contrary, other studies described t529 as only occurring in bovine milk cheese in Switzerland [69] and in swine in Japan [70]. Other investigations revealed that spa type t521 could be found in S. aureus isolates obtained from bovine mastitis in Canada [24], China [71], Denmark and the Netherlands [38] and Japan [60].

There is a great diversity concerning the discriminatory ability of spa typing compared to MLST typing and there is a close relationship between ST types and spa types [35]. The combination of ST and spa types has been shown to be useful. In many typing experiments, spa typing has been used to identify most common ancestor lineages. In this study, the most common STs and spa types identified were ST133-t1403, ST504-t529 and ST97-t521 (Table 4). Nineteen of the ST133 isolates belonged to
spa type t1403, whereas one ST133 isolate belonged to type t528. ST133-t1403 was found in bovine mastitis milk in Sweden, Germany and Denmark [38, 41, 67]. In this study, 16 of the ST504 isolates belonged to spa type t529 which was already reported in *S. aureus* isolates from bovine mastitis in Switzerland (www. Mlst. Net). All ST97 were split into eleven spa types; seven were t521, one was novel, one t359, one t5920 and one t267. The results of this study were in agreement with other studies which detected ST97-t521, ST97-t267 and ST97-t359 in *S. aureus* isolated from cows and bovine mastitis [38, 60] as well as in humans in Brazil (www. sparserver. ridom.de). Isolates belonging to ST97-t359 and ST97-t267 could also be found in fresh beef [61], in chickens and chicken meat and also in pork meat in Poland [72] as well as in isolates from humans in Spain [73, 74]. Both ST151 isolates belonged to spa type t529. Our results were in agreement with other studies which detected ST151-t529 in *S. aureus* isolated from bovine milk [38, 41, 67].

Several studies have identified the presence of host specific genotypes of *S. aureus* [41, 75]. The minimum spanning tree indicates the existence of *S. aureus* isolates belonging to numerous clonal complexes of closely related genotypes or lineages within the species. Our findings showed the existence of a relationship between strains from animals and from humans (Figure 1). The majority of bovine mastitis milk-associated sequence types belonged to the four clonal complexes, which include CC133, CC151, CC97 and CCS which showed a close relation to sequence types of human origin. The results of this study were consistent with previously published data that found a similarity of genotypes between *S. aureus* isolates from humans and animals hosts. However, recent studies demonstrated a close genetic relationship between MSSA isolated from milk and dairy products and the prominent human CC8, suggesting a human-to-bovine jump [76].

*S. aureus* isolates belonging to CC151, CC79, CC479, CC5, CC8 and CC50 carried various types of staphylococcal enterotoxin genes, while *S. aureus* belonging to CC133 and CC398 carried no staphylococcal enterotoxins genes. [77] revealed that there is a significant geographic difference in the existence of enterotoxin genes in *S. aureus* isolated from bovine mastitis. The tests of milk samples are important for determining the types of staphylococcal enterotoxins prevalent in milk and dairy products and also, they are important for public health [78]. The existence of enterotoxigenic *S. aureus*
isolated from cow’s milk of the present study is importance as a potential source of staphylococcal food poisoning. This is a valuable information concerning the milk produced on various farms for the dairy and food industry in northern Germany. In the present study, the most frequent genes were sel, sem, sen and selu (40%), respectively and seq (37.14%). The results of the study were in agreement with previous studies [79-81] that reported the highest prevalence rate of seq, sel, sem and sen genes in S. aureus isolated from bovine mastitis milk. The present study showed that none of the S. aureus isolates possessed the genes sea, seb, see and seq. This is in agreement with previous studies which reported that the enterotoxin genes seb, see and seq could never be recovered in bovine milk [15, 69, 81]. While, gene seb in some strains of S. aureus was responsible for cases of food poisoning and also for a staphylococcal food poisoning outbreak in humans [82, 83]. The various types of enterotoxin genes of S. aureus isolated in this study and other studies were attributed to the difference in the geographic region [7], the variation in utilized primers, types of samples, the source of samples and environments [84]. The toxic shock syndrome toxin (tsa) gene combined with other types of enterotoxin genes in S. aureus isolated from bovine mastitis milk has also been reported by [81].

The S. aureus isolates of the present study were divided into nine groups based on the existence of different enterotoxin genes with or without tsa. A relationship between S. aureus genotype and toxin gene profile was found in all 70 isolates of the present study study. The genotypes CC151, CC79, CC479, CC5, CC8 and CC50 were revealed to carry more toxin genes, while CC133 and CC398 were found to possess no toxin genes, CC151, CC479 and CC5 possessed a high content of toxic genes compared to other types of CC.

Conclusion

S. aureus is one of the most important pathogens in humans and livestock. Milk and dairy products regarded the primary sources of S. aureus which might cause human food poisoning. The genotypic characterization of S. aureus isolated from bovine mastitis milk by MLST and spa typing showed that the infections in cow herds of different regions in Northern Germany were caused by numerous clones related to those previously circulating in various regions of the world. The identification of novel STs and novel spa type in some isolates highlights even
more the importance of this type of study in cows. The minimum spanning tree indicates the existence of a relationship between strains from animals and from humans. Additional studies are important for a better understanding of the epidemiology and the various distribution of the geographic location of *S. aureus* in cow herds.

**Declaration section:**

**Availability of Data and Materials:** All information data were included in the manuscript.

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of Interest:** The authors declare no conflicts of interest.

**Funding:** Authors did not receive any research grants.

**Authors’ contributions:**

OS, AA, and GK were involved in the conception and design of the study.

OS and AA conducted the molecular investigations (DNA isolation, PCR assays and data interpretation).

OS, NG, OA and AA were involved in drafting of the manuscript.

GK, AA NG and OA performed the critical revision of the manuscript and gave important intellectual advice for final approval.

**Abbreviations**

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<th>Description</th>
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<tr>
<td><em>S. aureus</em></td>
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<td>nuc</td>
<td>Thromonuclease gene</td>
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<td>cff</td>
<td>Clumping factor (A and B) gene</td>
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<td>cco</td>
<td>Coagulase gene</td>
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<td>mecA</td>
<td>Methicillin resistance <em>Staphylococcus aureus</em> gene</td>
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<td>Pulsed-Field Gel Electrophoresis method</td>
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<td>Clinical and Laboratory Standards Institute</td>
</tr>
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</tr>
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</tr>
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### Table 1: Oligonucleotide primers and PCR programs for amplification of various genes of *S. aureus*

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<th>PCR Program</th>
<th>Reference</th>
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*Note: Times refer to temperature ranges for the PCR amplification.*
Table 2: Sequences of primers used in the MLST and spa types analysis

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<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Amplicon Size [bp]</th>
<th>Reference</th>
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<td>Triosephosphate isomerase (tpi)</td>
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PCR program: 35 cycles (94°C - 30s, 55°C - 30s, 72°C - 30s)
Table 3: Antimicrobial resistance pattern of bovine mastitis *S. aureus* isolates

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<th>Antimicrobial</th>
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<td>3 (4.28)</td>
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<td>4 (5.71)</td>
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Table 4: The genotypes of *S. aureus* isolates determined by the molecular typing methods and relationship between sequence types (ST) of MLST and Protein A (*spa* type) analysis and housekeeping genes information

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<th>Spa type</th>
<th>No. of isolates</th>
<th>Percent</th>
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<td>ST470</td>
<td>t287</td>
<td>1</td>
<td>1.42</td>
<td>04-20-17-34-24</td>
<td>52-79-54-18-56-32-65</td>
</tr>
<tr>
<td></td>
<td>ST1380</td>
<td>t287</td>
<td>2</td>
<td>2.85</td>
<td>04-20-17-34-24</td>
<td>169-79-54-18-56-32-65</td>
</tr>
<tr>
<td></td>
<td>ST1380</td>
<td>t528</td>
<td>1</td>
<td>1.42</td>
<td>04</td>
<td>169-79-54-18-56-32-65</td>
</tr>
<tr>
<td>CC479</td>
<td>ST398</td>
<td>t14</td>
<td>3</td>
<td>4.28</td>
<td>08-16-20-25-02-25-24-24-25</td>
<td>03-35-19-02-20-26-39</td>
</tr>
<tr>
<td></td>
<td>ST398</td>
<td>t571</td>
<td>1</td>
<td>1.42</td>
<td>08-16-20-25-02-25-24-25</td>
<td>03-35-19-02-20-26-39</td>
</tr>
<tr>
<td></td>
<td>ST7</td>
<td>t91</td>
<td>1</td>
<td>1.42</td>
<td>07-23-21-17-34-34-34-33-34</td>
<td>05-04-61-04-04-06-03</td>
</tr>
<tr>
<td>CC5</td>
<td>ST2825</td>
<td>t506</td>
<td>2</td>
<td>2.85</td>
<td>26-34</td>
<td>01-04-61-04-12-01-326</td>
</tr>
<tr>
<td></td>
<td>ST2827</td>
<td>t519</td>
<td>1</td>
<td>1.42</td>
<td>04-20-17-25</td>
<td>317-16-53-02-14-13-15</td>
</tr>
</tbody>
</table>

*Note: Novel sequence types and *spa* type found in the present study*
Table 5: Presence of genes encoding different staphylococcal enterotoxins, toxic shock syndrome toxin and staphylococcal proteins in *S. aureus* isolated from bovine mastitis

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>clfA</td>
<td>69 (98.60%)</td>
</tr>
<tr>
<td>clfB</td>
<td>70 (100%)</td>
</tr>
<tr>
<td>coa</td>
<td>70 (100%)</td>
</tr>
<tr>
<td>meCA</td>
<td>0</td>
</tr>
<tr>
<td>muc</td>
<td>70 (100%)</td>
</tr>
<tr>
<td>sea</td>
<td>0</td>
</tr>
<tr>
<td>seb</td>
<td>0</td>
</tr>
<tr>
<td>sec</td>
<td>8 (11.42%)</td>
</tr>
<tr>
<td>sed</td>
<td>8 (11.42%)</td>
</tr>
<tr>
<td>see</td>
<td>0</td>
</tr>
<tr>
<td>seg</td>
<td>26 (37.14%)</td>
</tr>
<tr>
<td>seh</td>
<td>3 (4.28%)</td>
</tr>
<tr>
<td>sei</td>
<td>28 (40%)</td>
</tr>
<tr>
<td>selj</td>
<td>8 (11.42%)</td>
</tr>
<tr>
<td>scm</td>
<td>28 (40%)</td>
</tr>
<tr>
<td>sen</td>
<td>28 (40%)</td>
</tr>
<tr>
<td>seo</td>
<td>9 (12.85%)</td>
</tr>
<tr>
<td>scp</td>
<td>3 (4.28%)</td>
</tr>
<tr>
<td>seq</td>
<td>0</td>
</tr>
<tr>
<td>ser</td>
<td>7 (10%)</td>
</tr>
<tr>
<td>selu</td>
<td>28 (40%)</td>
</tr>
<tr>
<td>tst</td>
<td>8 (11.42%)</td>
</tr>
</tbody>
</table>
Table 6: Enterotoxin gene profile of the *S. aureus* isolates (n=70) from bovine mastitis milk.

<table>
<thead>
<tr>
<th>Toxin genes profile</th>
<th>Staphylococcus enterotoxin</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>seg + sei + sem + sen + selu</em></td>
<td>11</td>
</tr>
<tr>
<td>II</td>
<td><em>sec + seg + sei + sem + sen + selu + tal</em></td>
<td>7</td>
</tr>
<tr>
<td>III</td>
<td><em>sed + self + ser</em></td>
<td>7</td>
</tr>
<tr>
<td>IV</td>
<td><em>seg + sei + sem + sen + selu</em></td>
<td>6</td>
</tr>
<tr>
<td>V</td>
<td><em>seg + sel + sei + sem + sen + selu</em></td>
<td>2</td>
</tr>
<tr>
<td>VI</td>
<td><em>sed + self</em></td>
<td>1</td>
</tr>
<tr>
<td>VII</td>
<td><em>sec + sel + sem + sen + selu + tal</em></td>
<td>1</td>
</tr>
<tr>
<td>VIII</td>
<td><em>seh + sep</em></td>
<td>1</td>
</tr>
<tr>
<td>IX</td>
<td><em>sei + sem + sen + selu</em></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>No enterotoxin gene</td>
<td>33</td>
</tr>
</tbody>
</table>


APPENDIX


APPENDIX


47. Huijsdens XW, van Dijk BJ, Slijk B, van Santen-Verheuvel MG, Heck ME, Pruister GN, 

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Staphylococcus aureus isolates from food-poisoning cases in Taiwan. Int J Food Microbiol 

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characterization of methicillin-resistant and methicillin-susceptible clones of 
The color of the MLST of S. aureus isolates from bovine mastitis was red while that of the MLST of S. aureus isolated from the human was light green.

- The MLST types are displayed as circles. The size of each circle indicates the number of isolates with the same sequence type.
- Thick solid lines connect types that differ in a single allele locus and a thin solid line connects types that differ in 2 allele loci.
- The colors of the halo surrounding the MLST types indicate types belonging to the same group. MLST was assigned to one group if at least 5 of 7 allele loci were similar.
Figure 1: Minimum spanning tree of the *S. aureus* bovine mastitis isolates in this study (n = 70) combined with isolates from a human in Germany (n = 119). All the *S. aureus* isolates were analyzed by MLST. The tree was constructed by using BioNumerics version 7.5 which allowed a presumptive coefficient. The clusters were created from two types within two changes of neighboring distance.

Figure 2: Minimum spanning tree of the *S. aureus* isolates in this study (n = 70) combined with the results of the *S. aureus* isolates from different European countries (n = 33). The tree was constructed using BioNumerics version 7.5 which allowed a presumptive coefficient. The clusters were created from two types within two changes of neighboring distance.
ACKNOWLEDGEMENT

11 ACKNOWLEDGEMENT

After an intense period of four years, today is the day: writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period. I would like to express my deepest appreciation to all those who provided me the possibility to complete this report. Firstly, I would like to express my sincere gratitude to my supervisor Professor Dr. Günter Klein for the continuous support of my doctoral study and related research, for his patience, motivation, and immense knowledge.

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