Molecular characterization of virulence factors in *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Ethiopia

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Abstract

Purpose: *Staphylococcus aureus* (*S. aureus*) is the most important pathogen involved in bovine mastitis in dairy production. *S. aureus* produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. The aim of this work was to isolate and molecular characterize *S. aureus* associated with bovine subclinical mastitis in central part of Ethiopia.

Methods: A total of 265 lactating dairy cows from various dairy farms in four different geographical locations were screened by California mastitis test (CMT) for bovine subclinical mastitis. One-hundred thirty CMT positive milk samples were collected and transported to laboratory. Different biochemical tests and polymerase chain reaction (PCR) were used for the identification of *S. aureus* isolates. Finally, PCR was performed for molecular detection of virulence genes.

Results: From total of 265 lactating dairy cows screened, 49% (n=130) were positive for bovine subclinical mastitis. One-hundred thirty mastitic milk samples were subjected to bacterial culturing, one hundred (76%) *S. aureus* isolates were identified based on phenotypic characters. Sixty-eight confirmed *S. aureus* isolates were obtained using PCR. The confirmed *S. aureus* isolates were tested for six virulence genes (*tsst*-1, *hlb, eta*, *sea, clf*A and *ica*D) using PCR. Of the six virulence genes screened from all the isolates, only two (*clf*A and *eta*) were detected in the isolates. Out of 68 isolates, 25% and 22% were possessed the *eta* and *clf*A genes, respectively.

Conclusion: Presence of *Staphylococcus aureus* having virulence genes (*eta* and *clf*A) revealed that mastitis is a major concern nowadays affecting animal health, milk quality, and yield. Further genomic study of these isolates will provide broad new insights on virulence.

Background

Mastitis is considered to be the most frequent and most costly production diseases in dairy herds of developed and developing countries including Ethiopia. Mastitis is an inflammatory response of the teat canal as a result of bacterial infection (Song et al., 2020). *Staphylococcus aureus* (*S. aureus*isone of the most recognized pathogen causing many serious diseases in humans and animals worldwide, and is the most common causative agent of clinical and subclinical bovine mastitis (Ote et al., 2011).

Mastitis caused by *S. aureus* is the result of production of several virulence factors that can contribute in different ways of pathogenesis (Vaughn et al., 2020). Virulence factors of *S. aureus* can be grouped broadly into two major classes which include surface localized structural components that serve as virulence factors and secreted virulence factors, which together help this pathogen to evade the host's defenses and colonize mammary glands (Diep and Otto, 2008). Some of surface localized structural components that serve as virulence factors include membrane-bound factors (collagen binding protein, fibrinogen binding protein, elastin binding protein and penicillin binding protein), cell wall-bound factors (protein A, β-Lactamase and protease) and cell surface associated factors (capsule and slime) (Diep and

Otto, 2008). Some of the known secretory virulence factors are toxins (staphylococcal enterotoxins, toxic shock syndrome toxin 1, hemolysins and exfoliatin), enzymes (coagulase, staphylokinase, DNAase, phosphatase, lipase and phospholipase). In addition to specific virulence factor, *S. aureus* also possesses different mechanisms or traits such as biofilm formation, adhesion to and invasion into mammary epithelial cells and formation of small colony variant (SCV) that enable this pathogen to resist host defense mechanisms. Some of these toxins are known to function as superantigens that cause increased immunological reactivity in the host (Rollin et al., 2015; Bobbo et al., 2017).

The differences in pathogenicity of *S. aureus* strains could result from geographical distribution and from host-and tissue-related characteristics (van Leeuwen et al., 2005). The numbers and combination of virulence genes may be important contributions to pathogenic potential of *S. aureus* strains (Zecconi et al., 2006). High number of *S. aureus* genotypes present in bovine herds worldwide has been studied to develop better strategies of treating mastitis (Kot et al., 2016). The identification and characterization of virulence factors of *S. aureus* causing bovine mastitis will enhance our understanding of the pathogenesis of intramammary infection (Zecconi et al., 2006). In addition, the antibiogram of *S. aureus* needs to be studied which would indicate the pattern of resistance to various antibacterials contributing to their virulence properties (Graveland et al., 2011). These may in turn contribute to the development of methods to minimize the production losses due to mastitis. Further, the study of evolution of strain-specific transmission and virulence characteristics including antibiotic resistance in *S. aureus* isolated from bovine mastitis may help us to understand mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination (Yu et al., 2012).

However, at present few reports has been reported about the occurrence of virulence factors among *S. aureus* isolated from milk of cows suffering from mastitis but not identified by molecular technique in central part of Ethiopia. Furthermore, there is a literature dearth on the prevalence and genetic characterization of virulence determinants in *S. aureus* in Ethiopia. As to our knowledge, most of the researches in Ethiopia were done in association with prevalence of bovine mastitis cases and its associated risk factors (Abera et al., 2010; Tesfaye et al., 2010) but molecular data on *S. aureus* causing bovine mastitis in remain scarce. Therefore, the aim of this work was to isolate *S. aureus* associated with bovine subclinical mastitis and study of molecular characterization of virulence factors in that isolates in central part of Ethiopia.

Methods

Samples and study population

Two-hundred and sixty-five milk samples were collected from lactating dairy cows that showed subclinical mastitis symptoms. Milk samples were collected from intensive production system across different geographical locations (Adaberga, Ambo, Bishoftu and Holeta) in central part of Ethiopia since November 2018 to June 2019. Milk samples were collected and proceeded as described in previous study

(Patel et al., 2017). Briefly, udders were wiped with 70% ethyl alcohol and few drops of milk were discarded initially. Simultaneously, CMT was executed on the site and on the basis of CMT score samples were collected (Bhatt et al., 2011; Patel et al., 2017). The study areas were purposively selected based on the agro-ecological differences and abundance of dairy farm milk sheds. The farms included in this study were involved in the production of milk for self-consumption and supplier to milk cooperative.

Bacterial isolation and identification

Milk samples were evaluated for mastitis-causing bacteria by bacteriological culture and biochemical tests following the National Mastitis Council Guidelines (Oliver et al., 2004). Briefly, 100µL of milk sample was inoculated onto nutrient broth media (Merck, Germany) with 5% sheep blood (Becton Dickinson Microbiology System, Cockeysville) and incubated at 37°C for 24 hr. Plates were evaluated for bacterial growth, colony morphology and hemolysis after 24 hr. Each pure colony was differentiated by Gram-stain. Followed by catalase test. Catalase positive cocci were considered *Staphylococcus species* and further confirmed by polymerase chain reaction (PCR), and tube coagulase test using rabbit plasma (NVI, Bishoftu, Ethiopia) to differentiate *S. aureus* from coagulase-negative *Staphylococcus species*. The resulting culture was used for bacterial DNA extraction and the remaining overnight culture of *S. aureus* isolate in tryptic soy broth (TSB) (BHI, Merck, Germany) was mixed with equal volume of sterile 85% Glycerol and stored in a -80°C freezer for further molecular work.

Bacterial DNA extraction

Staphylococcus aureus bacteria were sub-cultured on nutrient broth media (NB, Merck, Germany) and incubated at 37°C for 24 hr. Genomic DNA of all phenotypically positive *S. aureus* isolates was extracted from the culture using the Zymo Research Fungal and Bacterial Genomic DNA MiniPrepTM kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. Purity, quality and quantity of extracted DNA were measured using Nanodrop device (NanoDrop, Thermo Scientific, USA), gel electrophoresis and spectrophotometer. The extracted genomic DNA was stored at -20°C until next use.

Molecular confirmation of *S. aureus* and detection of virulence gene

Polymerase chain reaction (PCR) was used to amplify the *16SrRNA* gene fragment of *S. aureus* isolates according to previously described protocol [17] using EdvoCycler™ PCR machine (Edvotek, Inc, Bethesda). Also, all isolates were tested by PCR for the presence of the staphylococcal enterotoxin A (*sea*), exfoliative toxin A (*eta*), beta hemolysin toxin (*hlb*), clamping factor A (*clfa*), intercellular adhesion D (*icaD*) and toxic shock syndrome toxin-1 (*tsst-1*) according to previously described protocol [18-21]. Primers used for the PCR amplification were synthesized by Sigma-Aldrich (Bonn, Germany) and master mix synthesized by BioBasic company (BioBasic, Canada). The primers used for molecular identification of different virulence-associated genes are indicated in Table 1. Lyophilized primers for the target genes were reconstituted using DNase-RNase free sterile water to obtain 1000µM stock solutions. All primers were stored at -20°C and then finally diluted to working concentration of 10µM. PCR was carried out in a total volume of 25µl containing 12.5µl of 1X *Taq* PCR Master Mix (Bio Basic, Canada), 1µl of forward

primer and 1µl of reverse primer, 3µl of DNA template and 7.5µl sterile nuclease free water. The cyclic polymerase chain reaction conditions of the different primer sets are described on Table 2**.** PCR products were run on a 1% agarose (w/v) gel using electrophoresis, stained with gel red (Merck, Darmstadt, Germany) at 120 volts for 1hr and visualized under UV light using a BioDoc-itTM imaging system (Cambridge, UK). We used GeneRuler 100bp Plus DNA Ladder (Bioneer).

Statistical analysis

The data generated from the study was arranged, coded and entered to excel spread sheet (Micro oft® office excels 2010) and subjected to statistical analysis. The prevalence to every test was calculated as the number of positive cattle divided by the number of examined cases within the specified period. The Pearson Chi-square test ($\chi 2$) was applied to determine existence of any association between sampling areas and virulence associated genes using SPSS software *version* 22.0. The significance level was set at P-value (0.05) and 95% confidence level. In all cases, 95% confidence level and p-value less than 0.05 was consider as statistical significance.

Table 1: Description of the primers used for molecular identification of different virulence-associated genes detection in *S. aureus* isolates

Target gene	Primer name and its sequence (5′→3′)	Amplicon size (in bp)	Reference
16SrRNA *Staph. aureus* specific	Sau234_F: CGATTCCCTTAGTAGCGGCG Sau1501_R: CCAATCGCACGCTTCGCCTA	1267	(Riffon et al., 2001)
sea	SEA_F: TTGGAAACGGTTAAAACGAA SEA_R: GAACCTTCCCATCAAAAACA	120	(Mehrotra et al., 2000)
tsst-1	TSST_F: ATGGCAGCATCAGCTTGATA TSST_R: TTTCCAATAACCACCCGTTT	350	(Mehrotra et al., 2000)
eta	ETA_F: CGCTGCGGACATTCCTACATGG ETA_R: TACATGCCCGCCACTTGCTTGT	676	(Li et al., 2018a)
hlb	HLB_F: GTGCACTTACTGACAATAGTGC HLB_R: GTTGATGAGTAGCTACCTTCAGT	309	(Li et al., 2018a)
*clf*A	CLFA_F: GCAAAATCCAGCACAACAGGAAACGA CLFA_R: CTTGATCTCCAGCCATAATTG GTGG	638	(Kumar et al., 2009)
icaD	ICAD_F: AAGCCAGACAGAGGCAATATCCA ICAD_R: AGTACAAACAAACTCATCCATCCGA	249	(Greco et al., 2008)

NB: *Se*a = Staphylococcal enterotoxin a; *tsst-1=* Toxic shock syndrome toxin one; *et*a = Exfoliative toxin A; *hlb =* Beta hemolysin toxin; *clf*A = clumping factorA; *icaD =* Intracellular adhesive toxin

Table 2: Cyclic polymerase chain reaction conditions of the different primer sets

Target genes	Initial denaturation	Amplification (35 cycles)			Final extension
		Denaturation	Annealing	Extension	
16SrRNA *S. aureus* specific	94°C / 5 min	94°C / 30 sec	55 °C / 30 sec	72°C /45 sec	72°C / 5 min
*se*a	95°C / 10 min	94°C / 2 min	55 °C / 2 min	72°C / 1 min	72°C / 1 min
tsst-1	95°C / 10 min	94°C /2 min	55 °C / 2 min	72°C / 1 min	72°C / 1 min
*et*a	94°C / 5 min	94°C / 30 sec	57 °C / 30 sec	72°C / 45 sec	72°C / 10 min
hlb	94°C / 5 min	94°C / 30 sec	58 °C / 30 sec	72°C / 20 sec	72°C / 10 min
*clf*A	94°C / 10 min	94°C / 10 min	55 °C / 1 min	72°C / 1 min	72°C / 10 min
icaD	94°C / 10 min	94°C / 30 sec	53 °C / 30 sec	72°C /30 sec	72°C / 10 min

Results

Isolation and identification ofS. aureus**isolates**

In this study, of the 265 lactating dairy cows screened, 130 (49%) were positive for bovine mastitis based on CMT. One-hundred and thirty mastitic milk samples were subjected to bacterial culturing, 100 (76%) *S. aureus* isolates were identified based on the morphological and biochemical characters. From a total of 100 phenotypically positive *S. aureus* isolates, 68(68%) of them were confirmed *S. aureus* isolates through PCR amplification. The presence of *16SrRNA* gene (1267bp) was confirmed by PCR in *S. aureus*-positive isolates (Fig. 1).

Prevalence of virulence genes inS. aureus

All 68 PCR confirmed *S. aureus* isolates were tested for six virulence genes including *tsst*-1, *hlb, eta*, *sea, clf*A and *ica*D using PCR amplification. Of the six virulence genes screened from all the isolates, only two (*clf*A and *eta*) were detected. The isolates for the current study were obtained from mastitic bovine milk samples representing four geographical locations (Adaberga, Ambo, Bishoftu and Holeta) in the central parts of Ethiopia. Out of 68 isolates, 17 (25%), 15 (22%) and 6 (8.8%) isolates were possessed *eta, clf*A and combination of *eta* and *clf*A genes, respectively. The large proportion of these isolates which harbor *eta* and *clf*A genes were obtained from Holeta (46%, 7/15) and Adaberga (52%, 9/17), respectively. The prevalence of virulence gene was not statistically significant between different sampling areas (X^2 = 1.239; P = 0.744). The prevalence rates of the virulence genes were depicted in Fig. 3 below. The expected PCR product sizes obtained from these PCR products were 638 and 676bp for *clf*A and *eta*, respectively (Fig. 2).

Discussion

Staphylococcus aureus is one of the major cause of mastitis that leads to reduction of milk production in dairy cattle (Krishnamoorthy et al., 2017). The control of bovine mastitis is vital not only in Ethiopia but also in the world. Therefore, it is essential to investigate the pathogens using molecular techniques as vibrant components to control intra-mammary infections. In dairy industry the mastitis can be reduced by identification of exact pathogenesis and virulent factors present in infectious microorganisms. The molecular typing of infectious agents is known to be essential part of infection control strategies and is crucial to track and spread of contagious infections from one region to others or among different herds. Consequently, it is crucial to examine the mastitis causing bacteria using molecular methods as forceful tools to control IMI. Because *S. aureus* is the most commonly contagious mastitis pathogens worldwide, it is important to reveal virulence factors of these agents to develop effective control strategies against mastitis caused by this pathogen (Khan et al., 2013). In addition, an effective vaccine against IMI is not available, therefore prevention and control of mastitis needs detection of the principal antigenic determinants for the strategy and progress of more proficient vaccines against mastitis causing bacteria, especially *S. aureus*.

A number of studies have been conducted in Ethiopia on the prevalence of *S. aureus* in bovine milk (Abera et al., 2012; Mekonnen et al., 2017). Most of these researches focused on the importance of this pathogen as a cause of clinical and subclinical mastitis, however, its virulence determinants have not been well addressed. To our knowledge, there is no reliable information on molecular data of virulence genes in S. *aureus* from mastitic bovine milk samples in Ethiopia. Epidemiological studies indicates that *S. aureus* strains agents of milk produce a group of virulence factor and it is believed that there is a relationship between severity of infection and the virulence factors produced by *S. aureus* (Almaw et al., 2008). Hence, in this study, the prevalence of certain virulence genes such as *sea*, *eta*, *hlb*, *clf*A, *icaD* and *tsst-1* for *S. aureus* was evaluated.

In this study, from a total of 130 CMT positive isolates, *S. aureus* was the most frequently encountered organism with an isolation rate of 76%. The predominance and primary role of *S. aureus* isolate in bovine

mastitis has also been reported in other studies (Abera et al., 2012; Demissie et al., 2018). Apart from Ethiopia, *S. aureus* has also been reported as the chief etiological agent of mastitis in cattle by many studies from African and Asian countries (Abebe et al., 2016). Though direct comparisons among studies might be difficult, but in general, the variation in the prevalence between the present and previous studies might be due to differences in detection methods, geographical location of the study sites, and differences in farm management practices in each studied farms. *S. aureus* is adapted to survive in the udder and usually establishes mild subclinical infection of long duration from which it is shed through milk serving as source of infection for other healthy cows and transmitted during the milking process (Radostits et al., 2007). Hence, the organism has been assuming apposition of major importance as a cause of bovine mastitis.

Out of 100 phenotypically positive *S. aureus* isolates, 68 % of them were confirmed *S. aureus* isolates by using PCR amplification. The finding of this study was in agreement with (Li et al., 2018b). Regardless of the isolation and identification techniques employed, the confirmation of *S. aureus* in milk using molecular highlights the need for both strict farm management practices and proper sanitary procedures to be implemented during milking operations.

The pathogenicity of *S. aureus* is closely related to presence of various virulence genes (Kot et al., 2016). In this study, six virulence factors of the pathogen were screened but only two of them were positive based on PCR amplification. Our data showed that 15 out of 68 *S. aureus* isolates carried exfoliative toxin A (*eta*) (22.05%) and 17 out of 68 *S. aureus* isolates contained *clfA* (25%) genes. Of 68 *S. aureus* examined, 32 (47.05%) were positive for one or more virulence genes. About half (52.95%) of the isolates did not contain any of the virulence genes tested. The *eta* and *clfA* were found at higher frequencies whereas *sea*, *hlb*, *icaD* and *tsst-1* were not found in all tested isolates. Five isolates harbored both *eta* and *clfA* genes. There has been no published information regarding clamping factor A (*clf*A) and exfoliative toxin A (*eta*) in the Ethiopian context. This is the first investigation regarding to these genes in Ethiopia and there is no other work on these virulence factors. This finding is different from (Srinivasan et al., 2006) who examined 78 *S. aureus* isolates from the milk of cows with mastitis for 16 enterotoxin genes and found that 73 (93.6%) of the isolates were positive for one or more enterotoxin genes from a similar area. However, (Srinivasan et al., 2006) tested for 16 enterotoxin genes whereas in this study only one enterotoxin genes were tested. This might be the reason for the low prevalence of positive isolates in this study. The presence of the clumping factor gene is considered as *Staphylococcus* species virulence gene in development and severity of mastitis in cows (Aarestrup et al., 1995). The above results suggested that *S.aureus* isolates with different genetic background have different ability to acquire mobile genetic elements such as plasmids, phages and pathogenicity islands.

Conclusions

High prevalence of virulence genes (*clf*A and *eta*) in *S.aureus* bacteria were the most important findings of our study. All of the *S. aureus* bacteria harbored *clf*A and *eta* putative virulence factors which showed that they can use as specific genetic markers for detection of pathogenic *S.aureus* bacteria in bovine

subclinical mastitis cows. Presence of virulence factors in mastitis causing *Staphylococcus aureus* is an alarming spot for veterinarians, as several sources are there for spreading of microorganisms to human being. The emergence of different antibiotic resistance and virulence in the last two decades is exerting a lot of pressure in the health sector. Detailed genomic evaluation of particular antibiotic resistant strain with virulent factors may possess a great scope to develop new disease control strategy.

Abbreviations

*clf*A = clumping factor A; CMT = California mastitis test; DNA = Deoxyribonucleic acid; *et*a = Exfoliative toxin A; *hlb =* Beta hemolysin toxin; *icaD =* Intracellular adhesive toxin D; P –value = predictive value; PCR = polymerase chain reaction; *Se*a = Staphylococcal enterotoxin a; *tsst-1 =* Toxic shock syndrome toxin one; χ2 = Pearson chi-square;

Declarations

Ethics approval and consent to participate

This study was conducted after gaining full approval by the ethical review board of the College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia. Informed written consent was taken from all participants prior to participation in this study. Also, permission from dairy farm owners/managers was obtained before collection of milk samples

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Figures

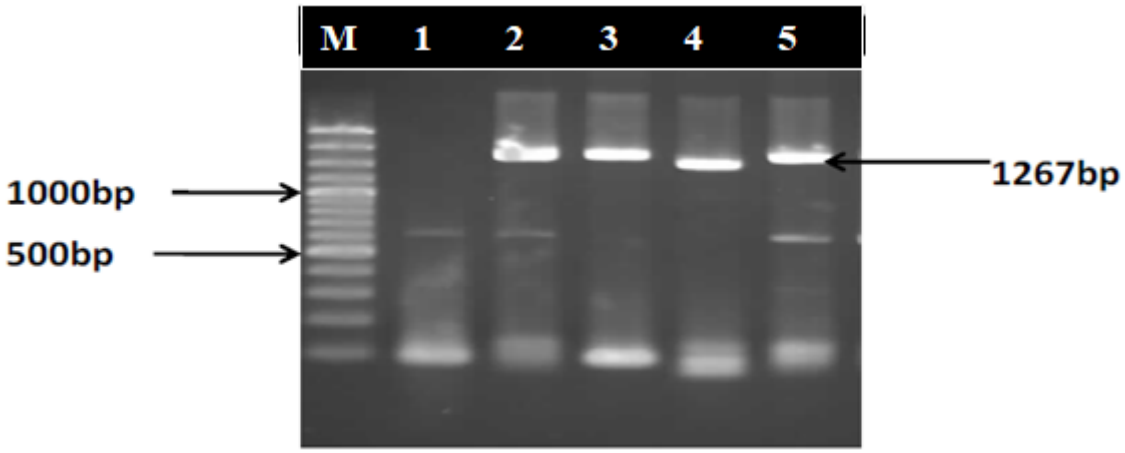

Figure 1

Amplicons of the 16Sr RNA gene of representative S. aureus with a size of 1267bp. Lane M is a 100 bp plus DNA marker (DNA ladder, BioBasic); lanes 1 to 5 are test samples

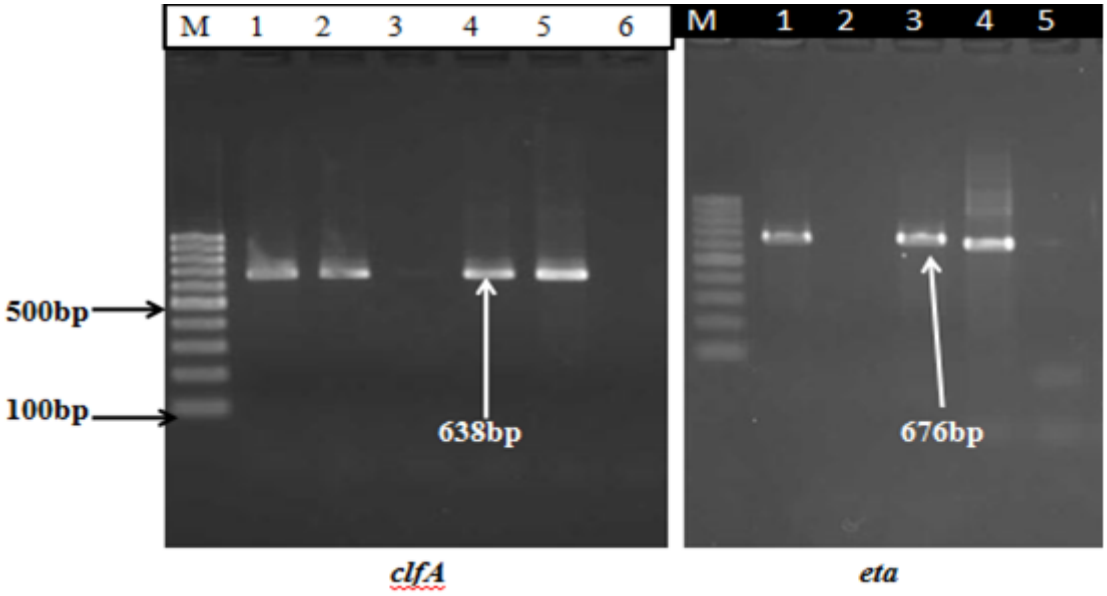

Figure 2

Agarose gel electrophoresis of PCR amplicon of clfA and eta genes of representative S. aureus isolates. Lane M is a 1000-bp DNA marker (DNA ladder, Bio Basic); lanes 1 to 6 are test samples.

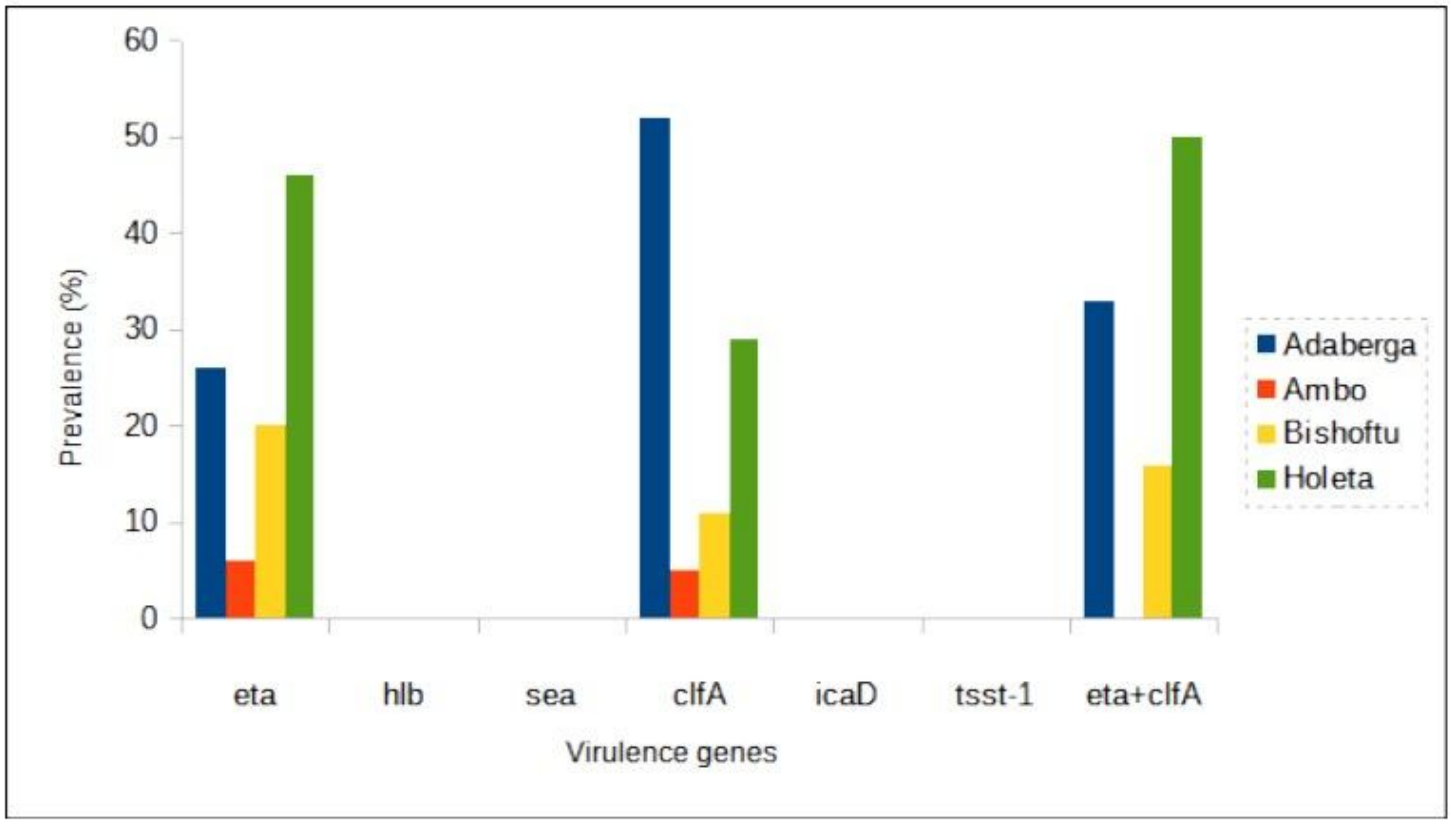

Figure 3

Prevalence of virulence genes in S. aureus isolated from bovine subclinical mastitis