

Original Research Article

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Screening and Characterization *Staphylococcus aureus* from Goat Milk

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ABSTRACT

The study focused on the prevalence of *Staphylococcus aureus* and resistant strains in goat milk samples. In this study, 100 individual goat raw milk samples were collected. A total of 39 (39%) isolates were recovered. Strains identified as *Staphylococcus aureus* were further analysed by the polymerase chain reaction method for detection of the *mecA* gene. Among the 39 isolates 28 (71.7%) samples showed positive results for coagulase test, 31 (79.4%) were of biofilm producers and 21 (53.8%) samples positive for *mecA* gene. Isolates showed maximum sensitivity as followed; Cefoxitin (83.2%), Cephalothin (72%), Kanamycin (76.3%), Choramphenicol (75.8%), Erythromycin (71.55%) and Gentamicin (70.8%) & resistance against Nalidixic acid (85.8%), Polymixin B (82.6%) and Penicillin G (70.5%). Vancomycin (36.7%) resistant isolates also note worth in present study. Isolates of *S. aureus* revealed the pathogenicity and resistance against the existing antibiotics. Study shows that goat milk also having equal chances of transmission to consumers via contaminated milk consumption. Hence, to improve the quality of milk and to prevent *S. aureus* contamination more hygienic measures should be implemented from point of production to consumption.

Keywords

Goat milk, *Staphylococcus aureus*, Toxi-infection, Antibiotic resistance, Biofilm, *mecA* gene, Vancomycin

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Introduction

Milk and milk products are good source of protein easily accessible in the world. The increasing world population increasing in one side automatically increase the demand for the milk production. Finding the new source to meet the demand is a challenging work. Nowadays consumption of goat milk also increased in order to meet the demand. Goat

milk is the most complete food, highly nutritious that it can actually serve as a substitute for a meal. It is also preferred for its low fat content and its capability to neutralize the acids and toxins present in the body (Alferez *et al.*, 2001). Hygienic collection, handling and storage of goat milk globally people recognize the factors involved in the food poisoning. Food borne toxi-infection causes serious health problems to the

consumers among these, *Staphylococcus aureus* leading with maximum possibilities and along with emerging of various antibiotic resistant (Golding *et al.*, 2010). Despite all the measures taken in livestock breeding, the incidence of *S. aureus* persists in the environment, in milk, on the animal's body, and also in humans (Harrison *et al.*, 2014). It was shown that animals can spread resistant strains not only in humans but also in raw food materials intended for further processing and for consumption (Trncikova *et al.*, 2010; Loncaric *et al.*, 2013; Shockava and Janstova 2014). *S. aureus* leading overall cause of health care associated infections globally and as more patients are treated outside the hospital setting is an increasing concern in the community (Stenheim *et al.*, 2010).

In general many anti – Staphylococcal drugs including Tetracycline, Fluoroquinolones, Linezolid and Daptomycin but they quickly lose their therapeutic value due to the ability of the bacterium to develop effective mechanisms to confront these agents (Navratna *et al.*, 2010). Vancomycin considered the last choice for the resistant isolates and many authors are continuously reporting the presence of Vancomycin resistant isolates (Teshome *et al.*, 2016). Moreover MRSA may also resist Vancomycin (Cui *et al.*, 2000) and this clearly shows the threat in community. *S. aureus* is a well-known pathogen living in a wide variety of environments (Ciccio *et al.*, 2015). It also has the inherent ability to form biofilms on biotic and abiotic surfaces (Mirani *et al.*, 2013).

Even though many studies have been reported very frequently on *S. aureus* (Kumar *et al.*, 2010., Bharathy *et al.*, 2015) studies from goat milk and public health significances are not included in our country. Our present study was aimed to screen the samples for the prevalence of *S. aureus* with the main focus on public health significance.

Materials and Methods

Study population and sampling technique

A total of 100 individual raw goat milk samples were collected from randomly selected lactating goats (non-descript) in livestock production. Selected goats were kept under traditional management system of rearing. Goat milk samples collected using sterile container. All the chemicals, primers, reagents and culture media used in this study were procured from HiMedia Pvt. Ltd Mumbai and PCR mixture procured from Gene Technologies, Chennai. Required equipment and glassware items used in this study were hygienically prepared as per standard procedures.

Primary enrichment and isolation

One ml of milk sample diluted with 9 ml of sterile Staphylococcal broth and enriched for 24 hrs at 37^o C. Baird parker agar was used for the selective isolation with 2% egg yolk emulsion. A loopful of inoculation from enrichment were streaked onto Baird Parker agar and incubated for 24- 48 hrs for the presence selective typical presumptive black colonies surrounded by white halo. Plates with selective colonies were further purified on Muller Hinton agar. After purification, colonies were further streaked onto 5% sheep blood agar for the assessment of ability of isolates to produce hemolysis.

Primary and secondary identification of *Staphylococcus aureus*

Specific colonies initially smeared subjected for the Grams staining and smear shown positive, spherical cells arranged in irregular clusters resembling to bunch of grapes were further subjected for primary and secondary biochemical procedures as per Cowan and Steel's, 1993. Confirmed colonies were

subjected for Tube coagulase test as per (Bharathy *et al.*, 2015). Details of the tests given in table no. 2 and 3

Preservation and revival of isolates

A loopful of the isolated organism was added to the sterile DMSO-glycerol broth vials and mixed well in the vortex mixture. The vials were then labeled and stored at -40°C . The isolates were revived once in three months. The vials stored at -40°C was thawed and streaked on Nutrient agar and incubated at 37°C overnight. The isolated organism was also streaked onto MH slants in screw capped test tubes and incubated at 37°C for 24 hours. Once the growth was obtained the slants were stored at 4°C

Detection of *mecA* gene by PCR technique

The reference strain of *Staphylococcus aureus* was obtained from Institute of Microbial Technology, Chandigar. PCR assay was performed using the DNA amplification instrument Mastercycler gradient (Eppendorf, Germany) to identify MRSA strains. Single colonies were collected from Muller hington agar upon overnight incubation at 37°C . DNA was extracted as per the manufacturer's instructions given in DNA Extraction Kit (Gene Technologies, Chennai). The *mecA* specific primer pairs used for the amplification of 533 base pair fragment are Forward, 5'- AAAATCGATGGTAAAG GTTGGC- 3' and Reverse, 5'- AGTTCTGG AGTACCGGTTTGC- 3' (Buhlmann *et al.*, 2008). A 25 μl reaction mixture was prepared in 0.2 ml thin walled PCR tubes.

The reaction mixture consists of the following: Template DNA - 5 μl , Primers (*rfpB*) - 20 pmol each primer, Master mix - 12.5 μl and Triple distilled water to 5.5 μl . Amplification was carried out in an automated thermal cycler (Eppendorf

Mastercycler, Germany) according to the following programme. Initial Denaturation was at 94°C for 3 minutes followed by 30 cycles of Denaturation at 94°C for 30 seconds, Annealing at 60°C for 30 seconds, Extension at 72°C for 30 seconds and Final Extension at 72°C for 3 minutes.

Biofilm formation assay (Slime Production Assay)

Qualitatively, biofilm formation among *S. aureus* isolates were assessed using method as described by (Jyothi *et al.*, 2018) with little modification. Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved. The isolates were inoculated and incubated aerobically for 24 to 48 hours. The ability of the isolates to produce bio-films was indicated by black colonies with a dry crystalline consistency. The results were recorded with positive and negative control.

Antimicrobial susceptibility testing

All isolates were tested for 16 antimicrobial drugs. List of the antibiotics were used in this study showed in table no. 1. Isolates were sub cultured on nutrient agar plates incubated for 24 hrs at 37°C . Colonies were picked from the agar plates, and suspended in normal saline (0.85% w/v), and the density of the suspension was adjusted to 0.5 McFarland standard.

The bacterial suspension was spread on the Mueller Hinton agar plates using a sterile swab stick, allowed to dry, and impregnated with antibiotic disk. Plates were incubated at 37°C for 24 hrs. Diameters of the zones of inhibition were measured and interpreted, as susceptible, intermediate or resistant according to the Clinical Laboratory Standard Institute guidelines (CLSI, 2006).

Results and Discussion

Staphylococcus aureus causes wide variety of diseases in humans and animals, ranging in severity from a mild skin infection to more severe diseases, such as pneumonia and septicaemia (Fagundas *et al.*, 2010). Milk is an important vehicle for human infection of food borne pathogens, but the number of contamination is usually low and unnoticed. It is necessary to use selective enrichment media which allow the small number organisms to grow, that may otherwise be killed by the toxic effects of different enrichment media. Enrichment normally helps to multiply or resuscitate, that have been sub lethally damaged. In present study *Staphylococcus* broth was used to assist the selective isolation. Baired Parker agar used for the selective plating and typical black colonies surrounded by white halo were identified as a presumptive of *S. aureus* (Singh and Prakash, 2008).

Out of 100 samples 39 samples were showed presumptive results for *Staphylococcus aureus* isolates on Baired parker agar which further subjected for primary and secondary biochemical tests and confirmed as *S. aureus* (Colony morphology, Gram staining and biochemical tests). According to our study overall showed less prevalence than previous studies on cow milk conducted by Lingadurai and Velladurai 61% in Madurai in 2010 and by Sadashiv and Kaliwal 53.7% in North Karnataka in 2013. This variation in prevalence of *Staphylococcus aureus* in raw milk may be due to the number samples collected and methods used for isolation. In general goat milk has fewer tendencies to contaminate and handling also may not be very frequent as like cow milk this may also contribute to the overall prevalence in present study. Worldwide several studies suggest that *S. aureus* isolation rates in milk can vary from 13.5% to 64.7%.

Among the 39 isolates, 21 was showed positive for tube coagulase test, it considered less than a study from Andhra Pradesh (Bharathy *et al.*, 2015), who reported 65.5% in a total of 40 samples. The differences in the results might be due sample size and types of sample included in the study. This all the positive isolates were induced hemolysis on 5% sheep blood agar. This is agreed by (Teshome *et al.*, 2016), who actually isolated the hemolysis induced colonies from raw milk samples in Ethiopia. This hemolysis property indicates the pathogenicity of the isolates.

DNA was extracted from all the presumptive positive isolates for *Staphylococcus aureus* by culture. PCR targeting *mecA* gene for *Staphylococcus aureus* was carried out and the results showed that out of 21/39 for *mecA* gene were positive. Out of the total 36 confirmed isolates, 6 (16.6%) isolates were confirmed to be MRSA when subjected to PCR amplification using specific primers for *mecA* gene (Hamid *et al.*, 2017). Presence of more isolates in the present study might be due to frequent use antibiotics in goats than other food animals. Our results also in accordance with the results of various authors who have also reported the presence MRSA by using PCR based *mecA* gene amplification which confirmed more than 99% of MRSA isolates (Hata *et al.*, 2010).

Among the 39 isolates of *S. aureus*, 32 isolates were produced biofilm colonies on congo red agar methods. Taj *et al.*, (2012) assessed the clinical isolates of *S. aureus* for the production of biofilm using different methods. Out of 115 *S. aureus* isolates, screening on Congo Red Agar showed biofilm formation in four (3.47%) strains. The CRA method is fast, reproducible, and presents an advantage: the colonies remain viable in the medium for further analysis. Therefore the method was chosen in an attempt to improve its ability to identify

biofilm production in *Staphylococcus* strains by making changes in the formula and adjusting different physical parameters. The method was easy to carry out and the results are usually based on the colony color produced, which ranges from red for non-bio film producing strains to black for biofilm-producing strains. Our results are in accordance with the results of (Arciola *et al.*, 2002) who also contribute same advantages for this method.

MRSA strains are often resistant to antimicrobials other than beta lactams of which many members are widely used in both human and veterinary medicine (Pinho *et al.*, 2001). Studies show that susceptibility patterns of *Staphylococcus aureus* to antimicrobial agent have varied worldwide, but isolates were usually susceptible to Kanamycin, Ciprofloxacin, Vancomycin and Gentamicin (Alian *et al.*, 2012; Daka and Yihdego, 2012).

Results of Table1 antibiotic susceptibility test against *Staphylococcus aureus* showed high resistance to Nalidixic acid(85%) followed by Polymixin B(82.6%), Penicillin G(70.5%). The high per cent of antimicrobial resistance exhibited to Nalidixic acid, Polymixin B and Penicillin G. In this study, line with the findings of who reported 87.2% resistance to Penicillin in Ethiopia, 64.3% of resistance to Penicillin G (Daka and Yihdego, 2012) in Hawassa area of Ethiopia and 80% resistance to penicillin which is reported in Sweden by Landin (2016) but in other hand it is in contrast to findings observed by Adesiyun (1994) who reported 23% of resistance to Penicillin G in West India and (Alian *et al.*, 2012) who reported 17.4% of resistance to Penicillin G in Iran. Penicillin very commonly used antibiotics in ruminants this might be reason for the resistant isolates from goat milk. The probable explanation to the presence of antibiotic resistant is

indiscriminate and repeated usage in animal and human health facilities. The present study has demonstrated the existence and alarming of Vancomycin resistance isolates and this put further threat to the public.

The antimicrobial susceptibility results in present study is contrast with the results (Alamin *et al.*, 2013) obtained in where *S. aureus* isolates were found to be sensitive for the tested antibiotics in the following percentage: Amoxicillin (66.7%), Gentamicin (88.8%), Ciprofloxacin (77.8%), Tetracycline (77.8%). In the (Mekuria *et al.*, 2013) study *S. aureus* isolates were sensitive to Vancomycin (88.2%) and Trimethoprim-Sulfamethoxazole (66.7%). In present study Vancomycin showed only 51.5% sensitivity against the isolates and indicates the increased prevalence of resistance. Tofaily *et al.*, (2011) showed in their findings that *S. aureus* isolates were sensitive to Chloramphenicol (83.3%), Doxycycline (100%), Erythromycin (16.6%) which has no similarity with the present study. Present isolates showed 71.55% sensitive to Erythromycin, which shows that not very frequent used in the treatment purpose in animals. In present study all the isolates were showed 72%, 83.2 % and 76.3 % sensitivity to Cephalothin, Cefoxitin and Kanamycin compound which is agreed by (Teshmore *et al.*, 2016), who also reported the maximum sensitivity in goat milk isolates.

In conclusion present study samples were collected from healthy goats and screened for the species specific *S. aureus*. Study clearly shows the presence of bacterial contamination and number of possible isolates also high compared to other findings on same research. Upon the characterization of *S. aureus* indicates the presence of pathogenicity by means of coagulase, hemolysis and biofilm positive isolates which represents the public health threat. Presence of MRSA produced isolates actually a matter of concern and it

needs strict hygienic procedure during handling, storage and transportation. Irrational use of antibiotics in animals has

played a significant role in the emergence of resistance isolates.

Table.1 Antimicrobial susceptibility pattern of *S. aureus* isolated from goat milk samples

Antibiotics	% Resistance	% Intermediate	% Sensitive
Penicillin G(10 units)	70.5	0	29.5
Amoxicillin(25 mcg)	48.5	0	51.5
Cephalothin(30 mcg)	20.8	7.2	72
Cefoxitin(30 mcg)	10.5	6.3	83.2
Gentamicin(10 mcg)	8.7	20.5	70.8
Kanamycin(30 mcg)	15.5	8.2	76.3
Nalidixic acid(30 mcg)	85.8	8.2	6
Ciprofloxacin(5 mcg)	27.5	16.8	55.7
Norfloxacin(10 mcg)	20.8	10.5	68.7
Trimethoprim- Sulfamethoxazole(25 mcg)	27.5	5.8	66.7
Polymixin B(300 units)	82.6	0	17.4
Erythromycin(15 mcg)	17.2	11.25	71.55
Vancomycin(30 mcg)	36.7	11.8	51.5
Chloramphenicol(30 mcg)	18.9	5.3	75.8
Doxycycline (30 mcg)	20.5	15.8	63.7
Tetracyclin(30 mcg)	42.8	10.8	46.4

Table.2 Results for Primary Identification Tests

Test Performed	Results
Gram Staining	Positive
Coagulase	Positive
Catalase	Positive
Citrate	Positive
Gas	Positive
H₂S	Positive
Hemolysis	Positive
Indole	Positive
Motility	Positive
Methyl Red	Positive
Nitrate Reduction	Positive
Oxidative Fermentative	Positive
Oxidase	Positive
Pigment	Positive
Shape	Positive
Urease	Positive
Voges Proskauer	Positive

Table.3 Results for Secondary / Fermentation Tests

Tests Performed	Results
Arabinose	Positive
Cellobiose	Positive
DNase	Positive
Fructose	Positive
Glucose	Positive
Lactose	Positive
Mannitol	Positive
Mannose	Positive
Raffinose	Positive
Sucrose	Positive
Trehalose	Positive
Xylose	Positive

Fig.1 Prevalence of *Staphylococcus aureus*

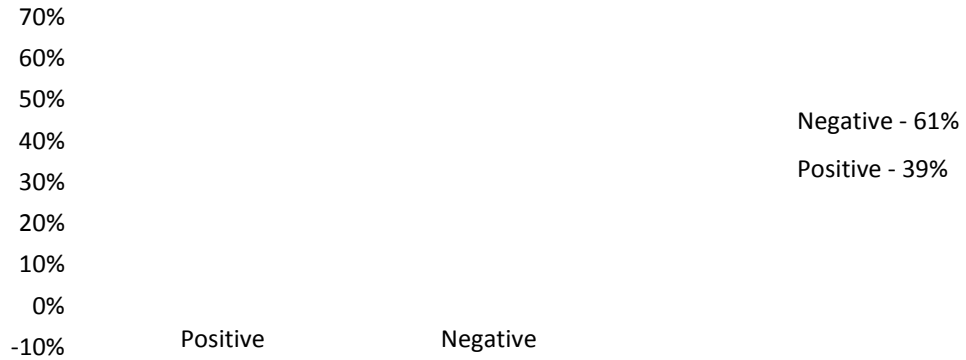


Fig.2 Prevalence of Coagulase positive and *mecA* gene isolates

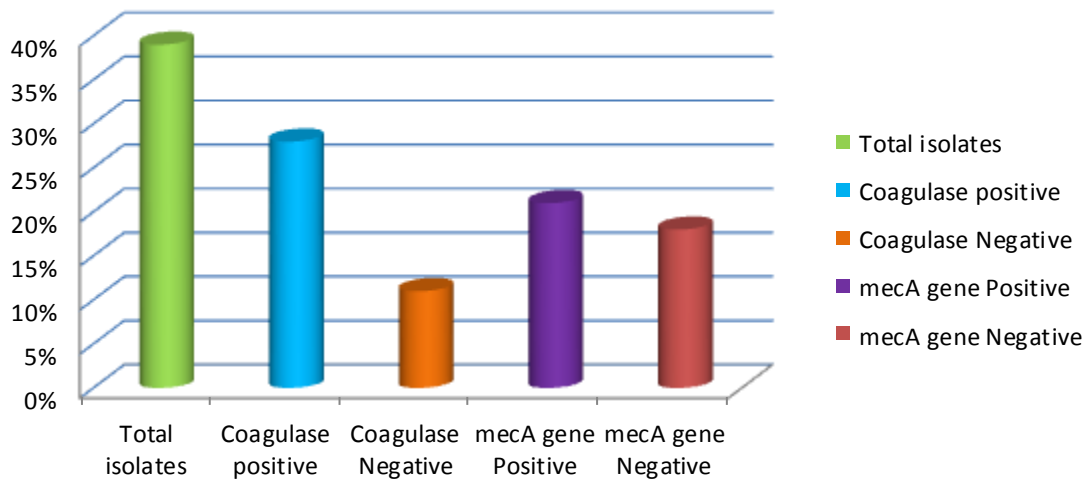
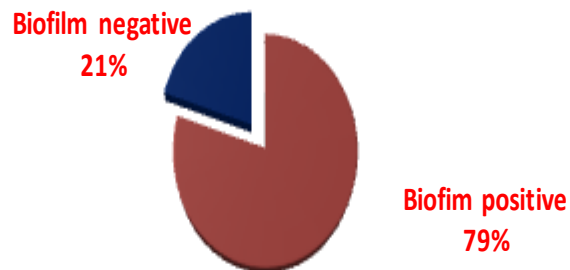


Fig.3 Biofilm induced isolates



Hence, there is an urgent need for more strict and hygienic preventive measures to reduce the *S. aureus* contamination. The progressive increase in drug resistant isolates has revealed the need for the comprehensive and systematic integration of healthcare management systems. Further epidemiological studies need to be conducted to monitor the pathogens in order to get good correlation. Also, proper hygiene by both farmers and milk vendors suggested to follow at the time of handling, this will improve quality of health in society and reduce the cost of treatment and management of MRSA and emergence of Vancomycin resistant.

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