The Thai Journal of Veterinary Medicine

Volume 49
Issue 4 *December, 2019*

Article 7

12-1-2019

Molecular analysis of Staphylococcus aureus isolated from infected dairy goats

Muhammad Asghar Qazi

Shahid Sherzada

Abdul Wajid

Sonia Iqbal

Usman Atique

See next page for additional authors

Molecular analysis of Staphylococcus aureus isolated from infected dairy goats

Authors

Muhammad Asghar Qazi, Shahid Sherzada, Abdul Wajid, Sonia Iqbal, Usman Atique, Rabia Bibi, Ayesha Maqbool, Saeed Akram Khan, Akhtar Ali, Tanveer Hussain, and Masroor Elahi Babar

Original Article

Molecular analysis of *Staphylococcus aureus* isolated from infected dairy goats

Muhammad Asghar Qazi[1] Shahid Sherzada[1,2] Abdul Wajid[1] Sonia Iqbal[2]

Usman Atique[3] Rabia Bibi[1] Ayesha Maqbool[1] Saeed Akram Khan[2]

Akhtar Ali[1] Tanveer Hussain[1] Masroor Elahi Babar[1*]

Abstract

Staphylococcus aureus is the leading cause of mastitis in dairy goats and is globally recognized as a significant mastitis-causing pathogen in dairy animals. Previous studies have established it as the causative agent of various human disorders, including food poisoning and *Staphylococcal* enterotoxins. Furthermore, poor sanitary conditions suit infection by this bacterium. This study determined the occurrence of *S. aureus* in infected dairy goats by molecular analysis. A hundred raw milk samples were collected from two infected dairy goat breeds (Beetal = 71; Teddy = 29) and cultured on blood agar media. The strain identified as *S. aureus* by morphological method (Gram staining), biochemical tests (catalase and coagulase) and further identified through molecular method using the *16SrRNA* gene. Overall, 58 (Beetal = 45; Teddy = 13) out of 100 raw milk samples (58%) were found to be positive for *S. aureus*. Further, ten samples of fresh milk positive for other microbial species including *S. hominis, S. capitis and S. lentus* were isolated. The *16SrRNA* gene was sequenced of fifteen *S. aureus* isolates representing various geographical regions. Phylogenetic analysis was performed based on the *16SrRNA* gene. In conclusion, *S. aureus* was more prevalent in the raw milk samples of the infected goats and acted as an etiologic agent of mastitis in the dairy goats. Hence, more measures that are hygienic should be implemented to improve milk quality and to prevent *S. aureus* contamination.

Keywords: *16SrRNA*; Biochemical tests; Goat milk; Mastitis; *S. aureus*

[1]*Virtual University of Pakistan, Lahore-Pakistan.*
[2]*Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore-Pakistan*
[3]*Department of Biosciences and Biotechnology, Chungnam National University, South Korea.*
***Correspondence**: masroor.ellahi@vu.edu.pk* (M.E. Babar)

Introduction

Staphylococcus aureus is a gram-positive organism and exists in the form of cocci. These bacteria primarily live in the nose, skin and respiratory tract. In animals, it mostly infects the mammary glands and can result in mastitis and alter milk quality. *S. aureus* is a nosocomial pathogen in humans while in cattle.it is the causative agent of mastitis (Abed and Hamim, 2015). The *Staphylococcus* genus comprises 52 species and 28 subspecies. The infectious strains *of S. aureus* are recognized as a leading cause of mortality in animals and farmers on a global scale (Haran *et al.,* 2011; Abo-Shama, 2014).

Mastitis is the inflammation of mammary glands that changes the physical and chemical properties of milk; therefore, pathological changes in udder tissue, quality and quantity of milk are expected. The causative agents of the disease can be microorganisms like bacteria, algae and yeasts. Bovine mastitis is prevalent in dairy cattle worldwide as inflammation depends upon clinical, subclinical and chronic conditions. The intensity of pathogenicity is contingent upon causative agents, immunological vigor and the lactation state. Clinical mastitis is visible in abnormal milk or the glands, while in subclinical mastitis, the affected animals produce less milk than their potential (Chu *et al.,* 2012; Abed and Hamim, 2015).

Catalase and coagulase-positive tests are mainly linked with *S. aureus* strains. *S. aureus* shows its presence during unhygienic conditions in dairy herds. Antibiotics treat the diseases of goats and other animals. Therefore, the isolates of *S. aureus* that are resistant to antibiotics have become a serious challenge to human and cattle health (Chandrasekaran *et al.,* 2014). Mastitis is an etiologic agent of goats and economically affects dairy farms (Cremonesi *et al.,* 2005). The presence of *S. aureus* infection in dairy farms is estimated to be up to 90%. Antimicrobial agents used in bulk to enhance food production have also increased the resistance of zoonotic bacterial pathogens. That is why antibiotic therapy is used for infection treatment, including subclinical mastitis in dairy animals (Cremonesi *et al.,* 2005; Ceniti *et al.,* 2017). The occurrence of mastitis reduces the quality of milk. *S. aureus* is contagious, and its pathogenicity makes it an essential agent of caprine mastitis. Therefore, the identification of specific genes associated with mastitis can lead to new developments in studies involving disease resistance in ruminants. The genomic studies of domestic animals determined that goats are closely related to bovine species (Daaloul-Jedidi *et al.,* 2016).

The presence of *S. aureus* in milk represents a severe public health problem because some strains produce extracellular protein toxins such as *Staphylococcal* enterotoxins (Elnasri *et al.,* 2016; Daaloul-Jedidi *et al.,* 2016). Furthermore, *S. aureus* leads to life-threatening toxic shock syndrome in humans. This organism can persist for a long time in the host without any symptoms. The ability of *S. aureus* to cause disease is due to virulence factors such as toxins, cell surface-associated adhesions, and secreted exo-proteins (Gopal and Divya, 2017). The intra-mammary infections of *S. aureus* can be controlled by washing udders pre-milking and drying them with cloths post-milking. The main reservoir of *S. aureus* in dairy herds is the infected skin of the udder and teats. Vectors responsible for the transmission of this bacterium are the hands of milking staff and the dirty cloths used to dry the udder during milking practices (Haran *et al.,* 2011).

By considering the compelling details, the principal aim of this study was to determine the occurrence rate of *S. aureus* using goat milk samples employing molecular techniques, identification of variations in the *16SrRNA* gene sequence of *S. aureus* and genetic diversity by constructing a phylogenetic tree.

Materials and Methods

Milk Samples Collection: A total of 100 raw milk samples were collected aseptically from two famous lactating goat breeds (Beetal, n = 71 and Teddy, n = 29) from seven towns (Kahuta, Kotli-Sattian, Kallar-Syedan, Rawal city, Potohar town, Gujar- Khan, and Taxila) in the of district Rawalpindi, in the Punjab. The basis for sampling was the extensive production system instituted by the local farmers in the selected areas. The raw milk samples were randomly collected in sterile tubes from the whole udder from various private flocks comprising 25 to 60 animals. The collection of samples was carried out with visual observation by the veterinary practitioner and the farmer's complaint of reducing milk production. The affected flocks showed typical signs of mastitis including fever, depression and udders that were found to be hot, swollen, redness, painful, hard with enlarging teats and abnormalities found in milk were clots or pus, flecks and wateriness. A total of 10-15 ml of raw milk was collected from infected animals, transferred to the laboratory on ice and submitted for bacteriological examination.

Bacteriological Examination of Milk Samples: In brief, a 0.1 ml milk sample was cultured on blood agar (Oxoid, UK) using the spread-method, and the inoculated plates were incubated at 37^{o}C aerobically overnight. After that, possible *S. aureus* colonies were cultured on to mannitol salt agar (MSA, Oxoid UK) at 37^{o}C for 24 hours. Bacterial colonies were then isolated on MSA and identified using standard microbiological procedures such as colony morphology, gram staining, hemolytic patterns on blood agar, catalase and coagulase tests.

Molecular Characterization of Staphylococcus aureus: DNA from 2 ml milk samples stored in 1.5 ml DNase and RNase free Eppendorf tubes at -40^{o}C was isolated from a total of 100 samples using DNA extraction Macherey Nagel kit (Macherey-Nagel, Milan, Italy) according to the manufacturer's protocol. The obtained DNA was quantified with NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). Bacterial identification and the sequencing of selected isolates were performed using the previously described method (Watts *et al.,* 2000) 16S rRNA primers listed in Table 1.

The *16SrRNA* gene was amplified in a total volume of 25 µl reactions composed of 30 ng gDNA, ten pmol of each forward and reverse primer, DNTPs (0.2 mM each), Mgcl (2.5 mM), 1X PCR buffer and 5 unit/µl of

Taq DNA polymerase enzyme (Thermo Scientific, USA). Thermal profile set in thermos cycler (Bio-Rad, USA) contained an initial denaturation at 95 ºC for 5 mins, followed by 35 cycles at 95 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s with a final extension at 72 °C for 10 min and stored at 4 °C. The amplicons were run on 1.2% agarose gel and purified using the Gene JET gel extraction kit (Thermo Scientific, USA). The PCR products were sequenced using an automated sequencer ABI 3130 XL (ABI, Inc., Foster, CA). The *16SrRNA* gene was sequenced with fifteen *S. aureus* isolates representing various geographical regions. The obtained PrP gene sequences were edited, assembled and analyzed for single nucleotide polymorphisms (SNPs) through BLAST and by BioEdit v7 (Hall, 1999). Phylogenetic analysis was inferred using the maximum likelihood method based on the General time-reversible model using MEGA software v7 (Kumar *et al*., 2016).

Table 1 Primers used for *16SrRNA* of *S. aureus*

	PRIMER			TEMPERATURE
Forward primer	5′	AGA GTT TGA TCC TGG CTC AG	3′	55.2
Reverse primer	5′	AAG GAG GTG ATC CAG CCG CA	3′	63.1

Results

This study was performed on raw milk samples isolated from mastitis infected two goat breeds, i.e. Beetal and Teddy. 100 raw milk samples were collected for examination for the occurrence of *S. aureus*. Based on conventional methods i.e. colony morphology, Gram staining and Biochemical tests (catalase and coagulase), 58 raw milk samples were positive for *S. aureus* (Table 2). The positive samples were further confirmed using the molecular method of the amplification of the *16SrRNA* gene. 45 raw milk samples out of 71 and 13 out of 29 of beetal and teddy goats, respectively were positive for the infectious agent *S. aureus*. In the study, ten other raw milk samples were found positive for *S. epidermidis, S. hominis, S. capitis and S. lentus*. The other 32 raw milk samples were found to be negative for any bacterial infection (Figure 1). The percentage of 58 positive *S. aureus* isolates detected in the different regions of Rawalpindi district is as follows; Kahuta (11%), Kotli Sattian (8%), Kallar Syedan (7%), Rawal town (7%), Potohar town (5%), Gujar Khan (15%) and Taxila (5) (Table 3).

In this study, the *16SrRNA* gene was sequenced with fifteen *S. aureus* isolates representing various geographical regions and used for phylogenetic analysis. The phylogenetic analysis was performed using the Neighbor-Joining method of Pakistani *S. aureus* isolates with other *S. aureus* isolates from India, China, Japan, Turkey and Saudi Arabia retrieved from GenBank. The Pakistani isolates showed similarity and in one clade (Figure 3).

Table 2 Percentage of positive samples of *S. aureus*

Sample source	Positive samples (*S. aureus*)	Other organisms	Negative samples	Total no of samples	Percentage of *S. aureus*
Infected goat's milk	58	10	32	100	58%

Table 3 Biochemical identification of *S. aureus* from milk samples of infected dairy goats

Region	Beetal (Goat)	Cat +	Coa +	*S. aureus* isolates	Teddy (Goat)	Cat +	Coa +	*S. aureus* isolates	Total samples	Total *S. aureusaureus* isolates	% of Total *S. aureus* isolates
Kahuta	15	+	+	09	04	+	+	02	19	11	11
Kotli-Sattian	12	+	+	07	03	+	+	01	5	08	08
Kallar-Syedan	08	+	+	05	06	+	+	02	14	07	07
Rawal-town	06	+	+	04	05	+	+	03	11	07	07
Potohar town	04	+	+	03	03	+	+	02	07	05	05
Gujar-Khan	20	+	+	13	06	+	+	02	26	15	15
Taxila	06	+	+	04	02	+	+	01	08	05	05
Total	**71**	**+**	**+**	**45**	**29**	**+**	**+**	**13**	**100**	**58**	**58**

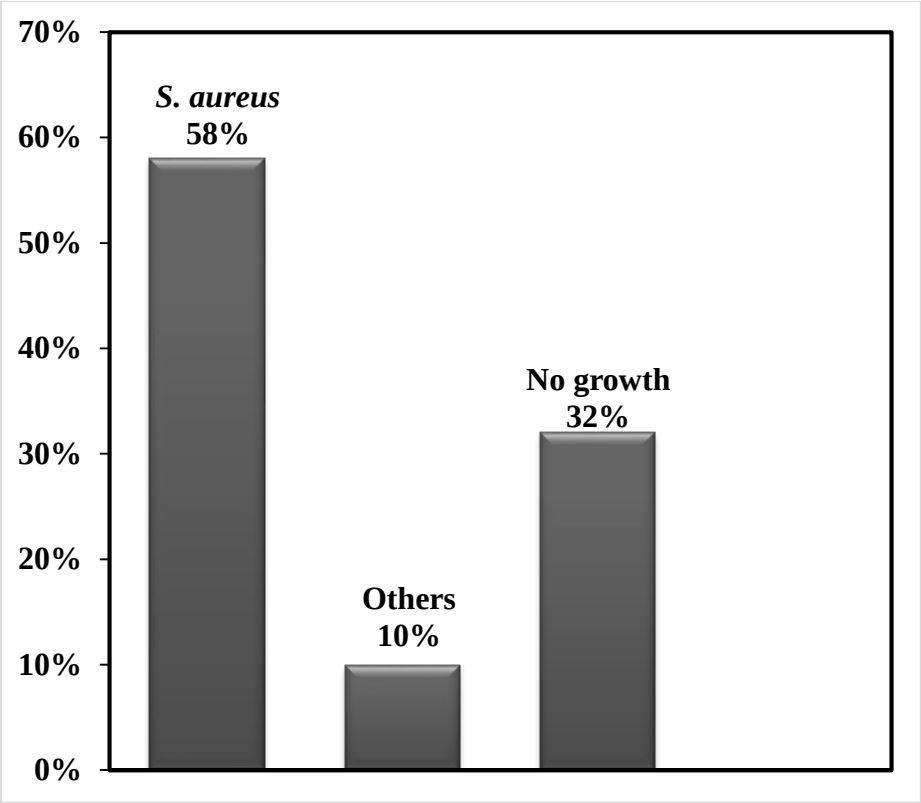

Figure 1 Frequency distribution of *S. aureus* isolates and other microorganisms

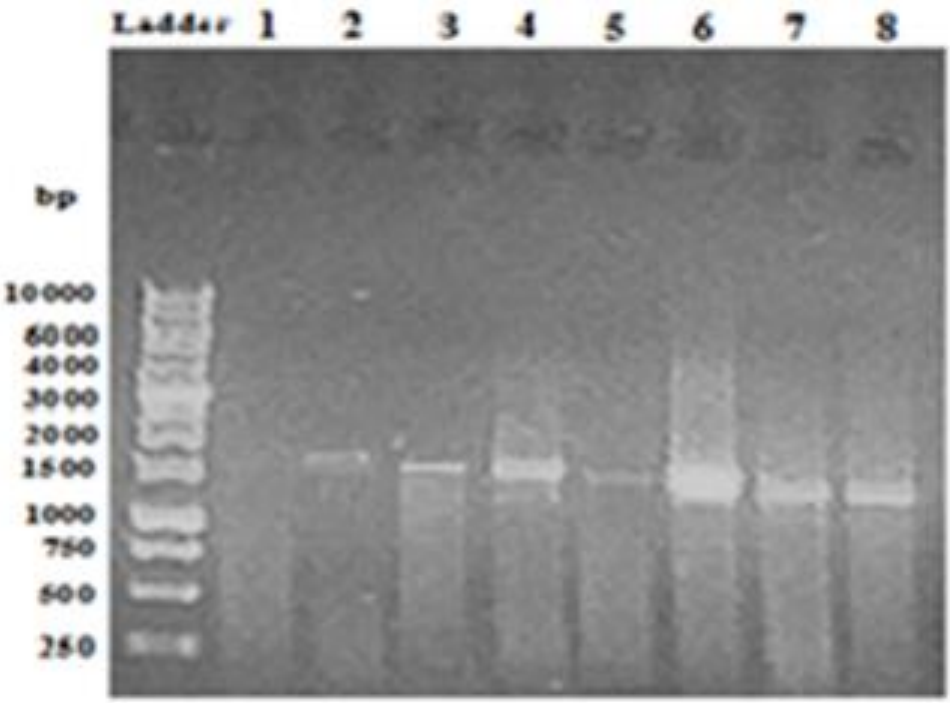

Figure 2 Amplified products of 16S rRNA gene (1500 bp) of *S. aureus* run on 1.2% agarose gel

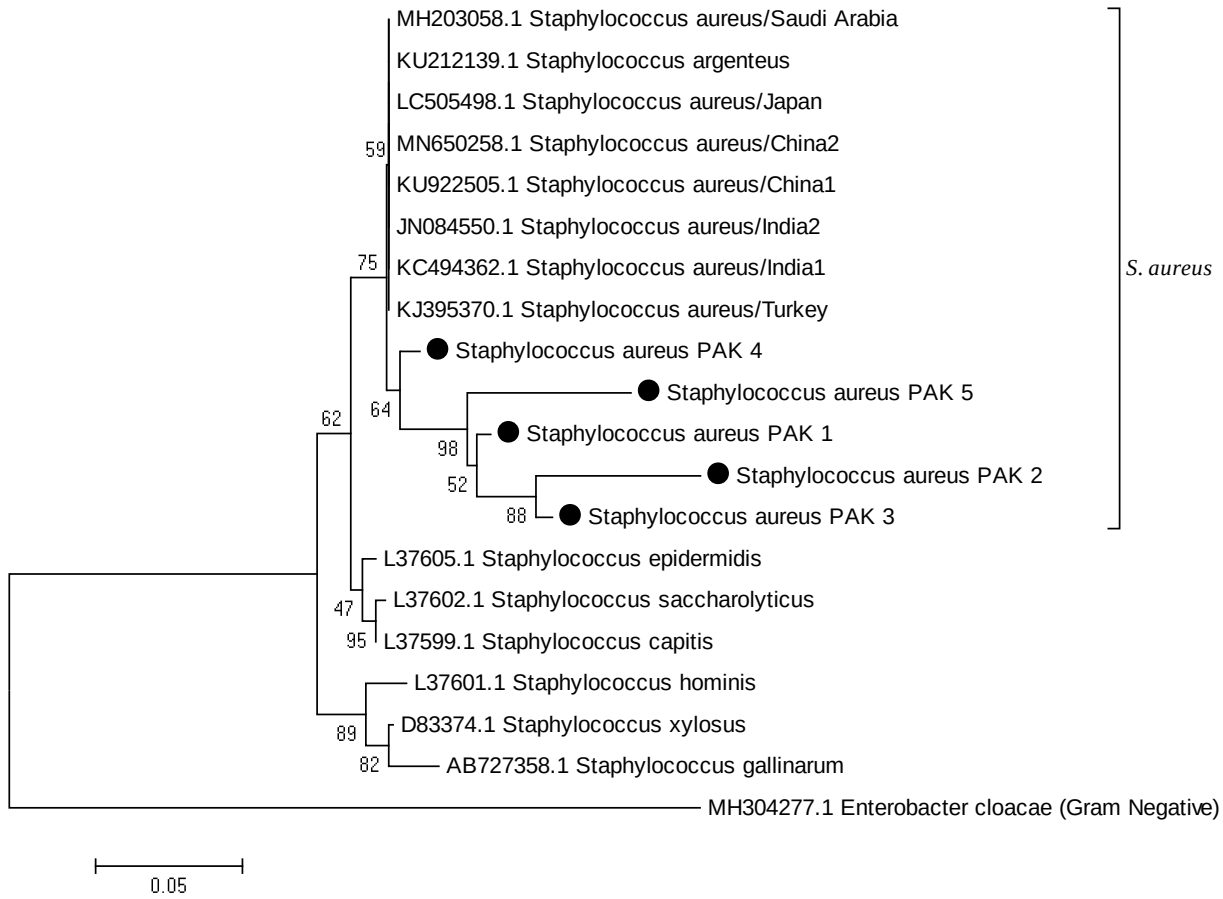

Figure 3 The phylogenetic analysis was performed using the Neighbor-Joining method in MEGA v7. The five included *S. aureus* isolates sequenced in this study are represented with a black circle.

Discussion

Mastitis is the inflammation of mammary glands in dairy cattle that alters the physicochemical properties of milk intended for human consumption. Goats are vulnerable to a variety of bacterial infections, such as *S. aureus*. It is one of the leading pathogens among *Staphylococci* resulting in mastitis infection (Chu *et al.*, 2012). The highest occurrence rate reported in this study is in Gujar Khan region (15%) followed by Kahuta (11%), Kotli Sattian (8%), Rawal Town (7%), Kallar Syedan (7%), Potohar town (5%) and Taxila (5%). Several other studies confirmed varying levels of *S. aureus* in animals afflicted with mastitis. Moroni *et al*. (2005) detected 20.8% of cases of *S. aureus* from chronically infected dairy goats. Our findings differ due to the occurrence of other microorganisms at a higher rate than *S. aureus*. Similarly, 2.9% (4/137) *S.* Chu *et al.* (2012) reported *S. aureus* cases from the raw milk samples of mastitis goats. Najeeb *et al*. (2013) reported a 61.64% higher occurrence of *S. aureus* that is in agreement with our study. However, a difference in the geographical area exists among these studies with a more extensive study area in the present study. Furthermore, this study reported multiple drug resistance in pathogens as a source of mastitis in dairy goats. Abo-Shama (2014) in Sohag Governorate, Egypt, reported 15 (37.5%) of the *S. aureus* isolates from 40 raw goat milk samples. These results differ from the present study plan due to the small sample size. Clinical and subclinical mastitis occurrences reported by Abed and Hamim (2015) were 46 (30.7%) and 60 (40.0%), respectively, in Thi-Qar Province, Iraq.

Mastitis is known to be affected directly by seasonal variations. The molecular analysis of the *16SrRNA* gene of *S. aureus* was detected with an amplicon size of 756bp. On the other hand, the *16SrRNA* gene was also identified, while the amplicon size was 1500bp. The multiplex PCR assay results of 92 *S. aureus* strains of 23S rRNA were obtained from 97 strains Cremonesi *et al.* (2005) in Italy. Only one strain was coagulase-negative *Staphylococcus* and mPCR potentially detected hazardous *S. aureus* from these milk samples. The prevalent cases of *S. aureus* were detected in 205 (96.2%) out of 213 caprine milk samples by Jorgensen *et al.* (2005) in Norway. This occurrence rate is higher in contrast to our results due to more massive herds with poor hygienic conditions leading to increased contamination of milk. In Minnesota farms, USA the occurrence rate of methicillin-sensitive *S. aureus* determined by Haran *et al*. (2011) was 93 (62%) out of 150 milk samples. These results corroborate our findings and the target animal was the goat as well. Chandrasekaran *et al.* (2014) reported *S. aureus* as 116 (49.3%) out of 235 clinical mastitis milk samples. The study of Mitra *et al*. (2013) recorded 173 (58.8%) *S. aureus* strains out of 294 composite milk samples from seven dairy herds located in the southern part of India. Positive strains were also analyzed by partial 16S rRNA gene. Similarly, *S. aureus* reported by Issa *et al*. (2016) was 55.7% out of (88/158) milk samples.

If various products prepared from the raw milk of small ruminants are consumed then *S. aureus* may affect animal and consumer health. For instance, Merz *et al*. (2016) found *S. aureus* in 60% (n = 34) out of 57 goat bulk milk samples. Further, the findings of Muhlherr et al. (2003), Pisoni *et al.* (2010), and Najeeb *et al.* (2013) agree substantially with our findings due to the percentages of *S. aureus*. However, *S. aureus* isolates of goat milk reported by Elnasri *et al*. (2016) were 11.1% out of 90 isolates, while some Coagulase-negative *Staphylococci* were also found including *S. hyicus* (3.3%), *S. epidermidis* (3.3%) and *S. chromogenes* (3.3%). A decreased percentage of *S. aureus* was found in contrast to the present study because of the higher percentage of other organisms. The occurrence rate of mastitis in milk samples from dairy goats studied by Yuan *et al*. (2017) was 50% (50/100) in various provinces of China. A bacteriological examination of these positive samples indicated 80% culture-negative and 20% culture positive. In the findings by Muhlherr *et al*. (2003), *S. aureus* was found in 109 (31.7%) out of 344 goat's milk samples. The most predominant *S. aureus* reported by Ceniti *et al*. (2017) was 44.44% from the goat's milk sample. Both of the studies determined a low percentage of contamination in milk based on quality standards in comparison to the present study.

Spanu *et al*. (2013) demonstrated higher contamination (60 (76.9%) out of 78 samples obtained from 26 farms) of mastitis in goat's milk than our findings. Subclinical mastitis was positive in 313 (45.82 %) out of 683 dairy goats milk samples in the study of Zhao *et al*. (2015). Among these positive milk samples, only 209 were identified using a multiplex PCR assay. The mPCR results showed 15.24 % *S. aureus,* 59.52% *coagulase-negative Staphylococci,* 11.43%, *Escherichia coli,* and 10.95% *Streptococcus* species. *S. aureus* was found at a lower rate in contrast to our study; however, the higher percentage of other organisms highly infected the goat's milk in this study.

In conclusion, 58% occurrence rate of *S. aureus* was determined in seven regions of Rawalpindi district, Pakistan in the order of Kahuta (11%), Kotli Sattian (8%), Kallar Syedan (7%), Rawal town (7%), Potohar town (5%), Gujar Khan (15%) and Taxila (5%). Some other microorganisms such as *S. epidermidis, S. hominis, S. capitis and S. lentus* were also found in 10 raw milk samples while 32 milk samples showed negative growth. The phylogenetic relationship of these *Staphylococcus aureus* species was compared with *S. aureus* species present at NCBI. This bacterium is more prevalent in goats' raw milk and can lead to the spread of mastitis in various dairy farms that in turn, may cause infections in humans as well. This study is useful in identifying the occurrence of mastitis in dairy goats, and relevant measures must be taken to mitigate its impending impact.

Acknowledgements

The authors are thankful to Virtual University Pakistan for providing the logistic and experimental facilities to conduct this research.

References

Abed KM and Hamim SS 2015. Molecular detection of staphylococci isolated from mastitis in sheep and cows in Thi-Qar province. IJRANSS. 3: 33-44.

Abo-Shama UH 2014. Prevalence and antimicrobial susceptibility of *Staphylococcus aureus* isolated from cattle, buffalo, sheep and goats raws milk in Sohag governorate, Egypt. Assiut Vet. Med. J. 60: 141.

Chu C, Yu C, Lee Y and Su Y 2012. Genetically divergent methicillin-resistant *Staphylococcus aureus* and sec-dependent mastitis of dairy goats in Taiwan. BMC Vet. Res. 8: 39.

Chandrasekaran D, Venkatesan P, Tirumurugaan KG, Nambi AP, Thirunavukkarasu PS, Kumanan K and Vairamuthu S 2014. A study on Methicillin resistant *Staphylococcus aureus* mastitis in dairy cows. Journal of Applied and Natural Science. 6(2): 356-361.

Cremonesi P, Luzzana M, Brasca M, Morandi S, Lodi R, Vimercati C, Agnellini D, Caramenti G, Moroni P and Castiglioni B 2005. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Mol Cell Probes. 19(5): 299-305.

Ceniti C, Britti D, Santoro AML, Musarella R, Ciambrone L, Casalinuovo F and Costanzo N 2017. Phenotypic antimicrobial resistance profile of isolates causing clinical mastitis in dairy animals. Ital. J. Food Saf. 6(2): 6612.

Daaloul-Jedidi M, Soudani A and Messadi L 2016. Nasal and rectal carriage of coagulase positive *Staphylococcus* in healthy goats. J. New Sci. 33: 4.

Elnasri HA, El Ayis AA and Almofti YA 2016. Aerobic Bacteria Associated With Subclinical Mastitis in Ewes and Goats in River Nile State, Sudan. Int. J. Sci. Footpr. 4(1):39–47.

Gopal S and Divya KC 2017. Can methicillin-resistant *Staphylococcus aureus* prevalence from dairy cows in India act as potential risk for community-associated infections? A review. Vet. World. 10(3): 311.

Hall TA 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl. Acids. Symp. Ser., 41: 95-98.

Haran KP, Godden SM, Boxrud D, Jawahir S, Bender JB and Sreevatsan S 2011. Prevalence and characterization of *Staphylococcus aureus*, including methicillin resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. J. Clin. Microbiol. 50(3): 688-95.

Issa AI, Duprez JN, Bada-Alambedji R, Djika M, Mainil JG and Bardiau M 2016. A 3-year long study of *Staphylococcus aureus* isolates from subclinical mastitis in three Azawak zebu herds at the Sahelian experimental farm of Toukounous, Niger. Trop Anim Health Pro. 48(2): 321-329.

Jorgensen HJ, Mork T, Hogasen HR and Rorvik LM 2005. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J. Appl. Microbiol. 99(1): 158-166.

Kumar S, Stecher G, Tamura K 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version

7.0 for Bigger Datasets. Mol Biol Evol. 33:1870–1874.

Merz A, Stephan R and Johler S 2016. *Staphylococcus aureus* isolates from goat and sheep milk seem to be closely related and differ from isolates detected from bovine milk. Front. Microbiol. 7: 319.

Mitra SD, Velu D, Bhuvana M, Krithiga N, Banerjee A, Shome R, Rahman H, Ghosh SK and Shome BR 2013. *Staphylococcus aureus* spa type t267, clonal ancestor of bovine subclinical mastitis in India. J. Appl. Microbiol. 114(6): 1604-1615.

Moroni P, Pisoni G, Vimercati C, Rinaldi M, Castiglioni B, Cremonesi P and Boettcher P 2005. Characterization of *Staphylococcus aureus* isolated from chronically infected dairy goats. J Dairy Sci. 88(10): 3500-3509.

Muhlherr JE, Zweifel C, Corti S, Blanco JE and Stephan R 2003. Microbiological quality of raw goat's and ewe's bulk-tank milk in Switzerland. J Dairy Sci. 86(12): 3849-3856.

Najeeb MF, Anjum AA, Ahmad MUD, Khan HM, Ali MA and Sattar MMK 2013. Bacterial etiology of subclinical mastitis in dairy goats and multiple drug resistance of the isolates. J. Anim. Plant. Sci. 23(6):1541-1544.

Pisoni G, Moroni P, Genini S, Stella A, Boettcher PJ, Cremonesi P, Scaccabarozzi L, Giuffra E and Castiglioni B 2010. Differentially expressed genes associated with *Staphylococcus aureus* mastitis in dairy goats. Vet Immunol Immunop. 135(3-4): 208-217.

Spanu V, Scarano C, Virdis S, Melito S, Spanu C and De Santis EPL 2013. Population structure of *Staphylococcus aureus* isolated from bulk tank goat's milk. Foodborne Pathog Dis. 10(4): 310-315.

Watts JL, Lowery DE, Teel JF and Rossbach S 2000. Identification of *Corynebacterium bovis* and other *coryneforms* isolated from bovine mammary glands. J Dairy Sci. 83(10), 2373-2379.

Yuan YG, Peng QL and Gurunathan S 2017. Effects of silver nanoparticles on multiple drug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from mastitis-infected goats: An alternative approach for antimicrobial therapy. Int. J. Mol. 18(3):569.

Zhao Y, Liu H, Zhao X, Gao Y, Zhang M and Chen D 2015. Prevalence and pathogens of subclinical mastitis in dairy goats in China. Trop Anim Health Pro. 47(2): 429-435.