Haryana Vet. (September, 2023) **62(SI-2),** 42-45 ISSN 0033-4359 (P)

Research Article

DETECTION OF BIOFILM FORMING *STAPHYLOCOCCUS AUREUS* FROM MASTITIC MILK

SHIKHAR K. VERMA, PARUL, BARKHA SHARMA[1], UDIT JAIN, AMBIKA SHARMA[2], MEENA GOSWAMI[3], PRACHI SINGH, AASHIMA, ABHISHEK RATHEE and ISHTA AGNIHOTRI
Department of Veterinary Public Health, [1]Department of Epidemiology and Preventive Medicine, [2]Department of Veterinary Biochemistry, [3]Department of Livestock Product Technology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura-281001, U.P.

Received: 03.10.2022; Accepted: 04.05.2023

ABSTRACT

Microbial biofilms are microbial aggregates and embedded in matrices of exopolymers. Biofilm production is a natural mechanism by which microorganisms adhering to a wet surface, secrete extracellular matrix and get embedded inside it. Many food-borne outbreaks have been associated with dairy products as main vehicle for transmission. Among those outbreaks, biofilm forming *Staphylococcus aureus* (*S. aureus*) were well documented as major causative agent and constitute serious threat for consumers' health. In this study, a total of 56 samples of raw milk were taken from cows exhibiting clinical symptoms of mastitis from dairy farms and gaushalas, screened by California Mastitis test. On analysis, all the samples (100%) were found positive for mastitis. Occurrence of *Staphylococcus* spp. and *S. aureus* was 58.92% and 51.78%, respectively. The *S. aureus* isolates were screened for their biofilm formation property by three different methods, i.e., Congo Red Agar (CRA) assay, Tube Method (TM) and Tissue Culture Plate (TCP) method. On analysis, 27.59% *S. aureus* were positive for CRA, 86.20% were positive for tube method and 96.55% were positive for TCP. Strong biofilm forming *S. aureus* were subjected to antibiotic sensitivity test (ABST). The isolates exhibited high resistance against oxacillin (73.68%), methicillin (52.63%), ofloxacin (52.63%) and ciprofloxacin (47.36%), whereas high sensitivity was observed against cefoxitin (94.73%), ceftazidime (84.21%), vancomycin (84.21%), imipenem (78.94%), streptomycin (78.99%) and gentamicin (73.68%). A total of 42.10% strong biofilm former *S. aurues* were MDR. The indiscriminate use of antibiotics for prophylactic as well as therapeutic purpose could be the reason for increased antibiotic resistance of *S. aureus*.

Keywords: Biofilm, *S. aureus*, TCP, TM, CRA, *nuc* gene, Mastitic milk

How to cite: Verma, S.K., Parul, Sharma, B., Jain, U., Sharma, A., Goswami, M., Singh, P., Aashima, Rathee, A. and Agnihotri, I. (2023). Detection of biofilm forming *Staphylococcus aureus* from mastitic milk. *Haryana Vet.* **62(SI-2)**: 42-45.

Staphylococcus aureus is a common bacterium that has been recognized as a challenge in both human and veterinary medicine (Bissong and Ateba, 2020). The pathogen is also associated with food poisoning in human, staphylococcal scaled skin syndrome (SSSS), operative wound infections, inflammation of lungs (pneumonia), nosocomial bacteremia in human, clinical and subclinical mastitis in bovine (Torres *et al.*, 2019). *S. aureus* produces a wide range of virulence factors like toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin (SE), Panton-Valentine Leukocidin (PVL) (Hoque *et al.*, 2018). The other important virulence factor of *S. aureus* is its ability to form biofilm *in vivo* which greatly influence its pathogenicity (Andrade *et al.*, 2021). The biofilm producing bacteria exhibit high resistance to disinfectants, antibiotics and host immune system clearance (Hait *et al.*, 2021). Biofilms reduce the effectiveness of treatments and increase morbidity, mortality and health care cost (Akindolire *et al.*, 2015). The biofilm forming ability of the isolates can be characterized by phenotypic methods, i.e., CRA, TM and TCP methods and genotypic characterization can be performed by screening of biofilm forming genes (Panda *et al.*, 2016). Maintenance of quality milk production by developing countries like India is a major problem and is due to lack of efficient hygienic techniques for milking, storing of milk and maintaining herd conditions (Singh *et al.*, 2022). Thus, colonization of dairy herd and subsequent contamination of raw milk by *S. aureus*, especially biofilm producing and MDR remains an important issue for both the dairy producer and public health. Keeping this in view, the present investigation was undertaken for detection of biofilm forming *S. aureus* from mastitic milk.

*Corresponding author: parulkaler@rediffmail.com

MATERIALS AND METHODS

Collection of milk sample

A total of 56 raw milk samples were collected from cows exhibiting the symptoms of mastitis residing in dairy farms and gaushalas of Mathura region of Uttar Pradesh. Milk samples were taken after cleaning the teat with 70% ethyl alcohol and brought to laboratory under refrigeration and processed immediately.

Screening of samples for detection of clinical mastitis

California Mastitis Test (CMT) was used for screening of clinical mastitis samples as per method described by Schalm *et al.* (1971) with slight modifications. The CMT was conducted to diagnose the presence of clinical mastitis as per Shitandi and Kihumbu (2004). The result was scored based on the gel formation and categorized as negative if there was no gel formation and positive, if there was gel formation ranging from +1 to +3. The mastitic milk samples that showed reactivity in

CMT were considered as positive for clinical mastitis.

Isolation and identification of *S. aureus*

Milk samples were processed for isolation of *S. aureus* as per standard bacteriological procedure given by Kou *et al.* (2021) with slight modifications. Baired parker and Mannitol Salt Agar were used for isolation of *S. aureus* and biochemically confirmed as per the method described by Barrow and Feltham (1993).

Molecular detection of *S. aureus* isolates

Biochemically confirmed staphylococci were further confirmed for *S. aureus* by using species specific primer targeting *nuc* gene of *S. aureus* by using PCR. The sequence of forward and reverse primer for *nuc* gene was (5'GCGATTGATGGTGGATACGGTT3' and 3'AGCCAAGCCTTGACGAACTAAAGC5'), respectively. The DNA extraction of bacterial isolates was done by Gene JET Genomic DNA purification kit. The *nuc* gene was targated as per method given by Brakstad *et al.* (1992). The DNA amplification was carried out using the thermal cycling profile consisted of an initial denaturation at 94 ºC for 5 min, 37 cycles of 94º C for 60s, 55 ºC for 30s and 72 ºC for 90s, with a final step at 72 ºC for 5 min. The resulting PCR products were analyzed in agarose gel electrophoresis as described by Sambrook and Russel (2001). The size of resulted amplicon was 267 bp.

Biofilm production

The *S. aureus* isolates were subjected to observe the biofilm forming capacity *in vitro* by Congo Red Agar (CRA) Assay, Tube Method (TM) and Tissue Culture Plate (TCP) methods as described by Mathur *et al.* (2006). Briefly, in TCP method individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures and plates were incubated for 24 hours at 37° C. Further, plates were washed 4 times with 0.2 ml of PBS (sessile organisms in plate were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. The OD values less than 0.120 were considered as non adherence and weak biofilm former while mean OD values 0.120-0.24 showed moderate adherence and moderate biofilm former while OD > 0.240 showed strong adherence and categorized as strong biofilm former.

Antibiotic Sensitivity Test (ABST)

Antimicrobial susceptibility of the isolates was determined by disc diffusion Bauer *et al.* (1959) method as recommended by clinical and laboratory standards institute (CLSI, 2016) and interpreted as susceptible, intermediate and resistant. A total of 18 antibiotics comprising of different antimicrobial classes, commonly used in the dairy farms were selected in this study (Table 1). *S. aureus* ATCC 25923 were used as control strain. The 100 µl of culture broth of each isolate was inoculated on Muller Hinton Agar plate and spread evenly by using sterilized cotton swabs, the plates were kept upright for an hour so that the culture gets absorbed onto the plates evenly. Antibiotic discs were placed on Mueller Hinton Agar and the plates were incubated at 37º C for 24 hours and the zone of inhibition surrounding the antibiotic discs was measured with the scale.

RESULTS AND DISCUSSION

Screening of samples for mastitis

A total of 56 samples collected from cows with symptoms of mastitis were screened for mastitis by CMT. All the samples showed gel formation within a few seconds, so 100% samples were found positive for mastitis. In studies of Hoque *et al.* (2018), 73.3% were positive by CMT that are slightly lesser in relation to our findings, while Pati and Mukherjee (2016) reported 42.75% positivity that was much lower than the present findings.

Phenotypic and genotypic identification of *S. aureus*

A total of 33 milk samples produced jet black coloured colonies on Baird-Parker Agar and yellow coloured colonies on nutrient agar and 29 isolates produced yellow coloured colonies on Mannitol Salt Agar surrounded by yellow zone were identified as *S. aureus*. All the isolates were biochemically confirmed by IMViC and also found positive for catalase and coagulase test. Occurrence of *Staphylococcus* spp. and *S. aureus* from various dairy farms and gaushalas are depicted in Table 2. In raw milk taken from mastitic cows, occurrence of *S. aurues* was 51.78% and this value was similar to 47.20% reported in China (Wang *et al.*, 2018). From the clinical and subclinical mastitic milk, isolation rate of *S. aureus* was 61.36% and 75.40% from India and Newzealand, respectively, which were quite higher to current study (Bhattachcharya *et al.*, 2016; Notcovich *et al.*, 2018). Overall, our data indicates that *S. aureus* is common and frequently detected in raw milk of dairy cows with mastitis in Brij region of Mathura. *S. aureus* contaminates many sources of raw milk which are usually related to mastitis or human carrier. Difference in prevalence is largely due to differences in the type of livestock breeding system, animal species, milking method and the surrounding environment conditions.

In the current study, all the phenotypically characterized *S. aurues* were *nuc* gene bearer, thus this house keeping gene was 100.00% prevalent. In the various studies, researchers revealed 100.0%, 85.55% and 85.24% *nuc* genes in *S. aurues* isolated from mastitic milk in India and abroad Bhattacharya *et al.* (2016); Wang *et al.* (2018), Pati and Mukherjee (2016), respectively.

Biofilm production

The twenty nine isolated *S. aureus* were analysed for biofilm formation by three described methods CRA, TM and TCP. On the basis of these assays, the isolates were categorized as strong, moderate and weak biofilm formers and results are depicted in Table 3. In CRA method, 27.59% *S. aureus* produced black colour colonies on CRA and found positive, while rest of the isolates produced red colour colonies and were negative for CRA. In tube method, biofilm forming isolates were produced slime either on bottom or wall or at both the sites of test tube and 86.20% were found positive by tube method. In TCP assay, 65.51% were strong biofilm former with more than 0.240 OD values, 31.03% were moderate biofilm producer, while 13.79% isolates showed OD values less than 0.120 and categorized as weak biofilm producer with overall prevalence of 96.55% biofilm former by TCP. The OD was measured at wavelength of 570 nm by using ELISA microplate reader.

Table 1. Detail of Antibiotic discs used in antibiotyping

S.N.	Antibiotic groups	Name of Antibiotics	Abbrev-iation	Concent-ration (µg)
1	- Lactams	i. Oxacillin	OX	1
		ii. Methicillin	MET	5
		iii. Ampicillin+ Sulbactam	A/S	10
2	Cephalosporins	iv. Cefoxitin	CX	30
		v. Ceftriaxone	CTR	30
		vi. Ceftazidime	CAZ	30
		vii. Cefotaxime	CTX	30
3	Macrolides	viii. Erythromycin	E	15
4	Carbapenems	ix. Imipenem	IPM	10
5	Glycopeptides	x. Vancomycin	VA	30
6	Aminoglycosides	xi. Gentamicin	GE	10
		xii. Streptomycin	S	10
7	Amphenicols	xiii. Chloramphenicol	C	30
8	Quinolones	xiv. Oflxacin	OF	5
		xv. Ciprofloxacin	CIP	50
		xvi. Levofloxacin	LE	5
9	Oxazolidinones	xvii Linizolid	LZ	30
10	Tetracyclines	xviii. Tetracycline	TE	10

Table 2. Occurrence *of S. aureus* in mastitic milk and presence of nuc gene

S.N.	Place of Collection	No of Samples	Prevalence of *Staphylococcus* spp. (%)	Prevalence of *S. aureus* (%)	Prevalence of *nuc* gene in *S. aureus* (%)
1	Dairy Farm 1	8	62.50 (5/8)	50.0 (4/8)	100.0 (4/4)
2	Dairy Farm 2	8	62.50 (5/8)	50.0 (4/8)	100.0 (4/4)
3	Gaushala 1	8	50.0 (4/8)	50.0 (4/8)	100.0 (4/4)
4	Gaushala 2	8	75.0 (6/8)	62.50 (5/8)	100.0 (5/5)
5	Gaushala 3	8	50.0 (4/8)	50.0 (4/8)	100.0 (4/4)
6	Gaushala 4	8	62.50 (5/8)	50.0 (4/8)	100.0 (4/4)
7	Gaushala 5	8	50.0 (4/8)	50.0 (4/8)	100.0 (4/4)
	Total	56	58.92% (33/56)	51.78% (29/56)	100.00% (29/29)

In current study, mastitic milk revealed with high prevalence of biofilm forming *S. aureus* 96.55%, 86.20%, 27.59% by TCP, TM and CRA assay. Comparison of biofilm assay is mentioned in table 3. In accordance with this study 93.4% and 27.17% biofilm forming *S. aurues* were revealed from TCP and CRA assay in mastitis milk from New Zealand as reported by Notcovich *et al.* (2018) and from Brazil 98.9% and 85.0% by the two assays in mastitis milk (Melo *et al.*, 2013). Results of TCP assay are quite similar to our study while there is wide variation in findings of CRA. The discrepancies in the categorization of biofilm phenotypes could result from differences in the interpretation of results. Therefore, standardizing the method and interpretation of biofilm forming is crucial (Atshan and Shamsudin, 2011).

Table 3. Comparison of Biofilm Assays (TCP, TM & CRA)

S. aureus isolates	Biofilm formation	Tissue Culture Plate Assay (TCP) (%)	Tube Method (TM) (%)	Congo Red Agar Assay (CRA) (%)
n=29	Strong	65.51 (19/29)	48.28 (14/29)	27.59 (08/29)
	Moderate	31.03 (09/29)	37.93 (11/29)	-
	Weak/none	3.45 (01/29)	13.79 (04/29)	72.41 (21/29)
	Total Biofilm former (Strong + Moderate)	96.55 (28/29)	86.20 (25/29)	27.59 (08/29)

Antibiotic Sensitivity Test (ABST)

Strong biofilm forming *S. aureus* were subjected for Antibiotic Sensitivity Test (ABST) and high resistance was shown against oxacillin (73.68%), methicillin (52.63%), ofloxacin (52.63%) and ciprofloxacin (47.36%). The high sensitivity was shown against cefoxitin (94.73%), ceftazidime (84.21%), vancomycin (84.21%), imipenem (78.94%), streptomycin (78.99%) and gentamicin (73.68%). The results

of this study are similar with findings of previous antibiotic studies on various types of milk, as oxacillin was 60-100% and 70.0% resistant in study of Akindolire *et al.* (2015) and Naseer *et al.* (2021), respectively, and 44.47% ofloxacin resistant isolates revealed in study of Sharma *et al.* (2015). In accordance to this study, cefoxitin was found 100.0% and 80% sensitive in the study of Marques *et al.* (2017) and Naseer *et al.* (2021), respectively. A total of 42.10% (8/19) strong biofilm former *S. aurues* were multi drug resistant, showing resistant to three or more than 3 class of antibiotics mainly for Penicillins, Quinolones and Macrolides. These biofilm forming MDR strain of *S. aureus* in mastitic milk could be a potential source of antibiotic resistant determinants in environment, animal and human population.

CONCLUSIONS

Biofilm forming attribute of *S. aureus* make it more virulent by increasing adhesiveness on surfaces and that surface continuously act as source of infection in raw milk. Thus, the presence of potential biofilm-producing antibiotic-resistant *S. aureus* in milk intended for human consumption may be cause of severe health risk for human. Consequently, there is a need to enhance control measures, especially in the dairy sector, to curb the spread of pathogenic *S. aureus* and to limit the use of antibiotics. Further studies are required to elucidate other factors that are indulged in biofilm formation.

REFERENCES

Akindolire, M.A., Babalola, O.O. and Ateba, C.N. (2015). Detection of antibiotic resistant *Staphylococcus aureus* from milk: A public health implication. *Int. J. Environ. Res. Public Health.* **12(9)**: 10254-10275.

Andrade, N.C., Laranjo, M., Costa, M.M. and Queiroga, M.C. (2021). Virulence factors in *Staphylococcus* associated with small ruminant mastitis biofilm production and antimicrobial resistance genes. *Antibiotics.* **10(6)**: 633.

Atshan, S.S. and Shamsudin, M.N. (2011). Evaluation of phenotypic and genotypic detection methods for biofilm-forming methicillin sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates. *Ann. Microbiol.* **61**: 825-831.

Barrow, G.I. and Feltham, R.K.A. (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria. (3rd Edn.), Cambridge University Press, Cambridge. pp. 140-43.

Bauer, A.W., Perry, D.M. and Kirby, W.M.M. (1959). Single disc antibiotic sensitivity testing of *Staphylococci*. *A.M.A. Arch. Intern. Med.* **104**: 208-216.

Bhattacharyya, D., Banerjee, J., Bandyopadhyay, S., Mondal, B., Nanda, P.K., Samanta, I. and Bandyopadhyay, S. (2016). First report on vancomycin-resistant *Staphylococcus aureus* in bovine and caprine milk. *Microb. Drug. Resist.* **22(8)**: 675-681.

Bissong, M.E.A. and Ateba, C.N. (2020). Genotypic and phenotypic evaluation of biofilm production and antimicrobial resistance in *Staphylococcus aureus* isolated from milk, North West Province, South Africa. *Antibiotics.* **9(4)**: 156.

Brakstad, O.D.D.G., Aasbakk, K. and Maeland, J.A. (1992). Detection of *Staphylococcus aureus* by Polymerase Chain Reaction Amplification of the nuc Gene. *J. Clin. Microbiol.* **30**: 1654-1660.

Clinical Laboratory Standard Institution. (2016). M100S Performance Standards for Antimicrobial Susceptibility Testing-Approved Standard, (26th Edn.), Clinical and Laboratory Standards Institute: Wayne, PA, USA.

Hoque, M.N., Das, Z.C., Rahmn, A.N.M.A., Haider, M.G. and Islam, M.A. (2018). Molecular characterization of *Staphylococus aureus* strains in bovine mastitis milk in Bangladesh. *Int. J. Vet. Sci. Med.* **6(1)**: 53-60.

Kou, X., Cai, H., Huang, S., Ni, Y., Luo, B., Qian, H., Ji, H. and Wang, X. (2021). Prevalence and characteristics of *Staphylococcus aureus* 'isolated from retail raw milk in Northern Xinjiang, China. *Front. Microbiol.* **12**: 705947.

Marques, V.F., Motta, C.C.D., Soares, B.D.S., Melo, D.A.D., Coelho, S.M.O., Coelho I.D.S. and Souza, M.M.S.D. (2017). Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. *Braz. J. Microbiol.* **48**: 118-124.

Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T. and Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian J. Med. Microbiol.* **24(1)**: 25-29.

Melo, P.D.C., Ferreira, L.M., Nader, F.A., Zafalon, L.F., Vicente, H.I.G. and Souza, V.D. (2013). Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Braz. J. Microbiol.* **44**: 119-124.

Naseer, M.A., Aqib, A.I., Ashar, A., Saleem, M.I., Shoaib, M., Kulyar, M0F.E.A. and Nighat, S. (2021). Detection of altered pattern of antibiogram and biofilm character in *Staphylococcus aureus* isolated from dairy milk. *Pak. J. Zool.* **53(1)**: 191.

Notcovich, S., DeNicolo, G., Flint, S.H., Williamson, N.B., Gedye, K., Grinberg, A. and Lopez-Villalobos, N. (2018). Biofilm-forming potential of *Staphylococcus aureus* isolated from bovine mastitis in New Zealand. *Vet. Sci.* **5(1)**: 8.

Panda, P.S., Chaudhary, U. and Dube, S.K. (2016). Comparison of four different methods for detection of biofilm formation by uropathogens. *Indian J. Pathol. Microbiol.* **59**: 177-179.

Pati, B.K. and Mukherjee, R. (2016). Characterization of *Staphylococcus aureus* isolates of bovine mastitis origin and antibiotic sensitivity pattern from Northern Plains of India. *J. Vet. Res. Ani. Husb.* **1(1)**: 105.

Singh, K., Mishra, K.K., Shrivastava, N., Jha, A.K., Ranjan, R. and Yadav, K. (2022). Sub-clinical mastitis in dairy buffaloes of Rewa district of Madhya Pradesh. *Haryana Vet.* **61**: 26-28.

Sharma, S., Khan, A., Dahiya, D., Jain, J. and Sharma, V. (2015). Prevalence, identification and drug resistance pattern of *Staphylococcus aureus* and *Escherichia coli* isolated from raw milk samples of Jaipur city of Rajasthan. *J. Pure Appl. Microbiol.* **9(1)**: 341-348.

Sambrook, J. and Russell, D.W. (2001). Molecular Cloning-Sambrook & Russel-Vol. 1, 2, 3. Cold Springs Harbor Lab Press: Long Island, NY, USA.

Schalm, O.W.G., Carroll and Jain, N.C. (1971). Bovine Mastitis. Lea and Fibiger, Philadelphia, USA. p. 360.

Shitandi, A. and Kihumbu, G. (2004). Assessment of the California mastitis test usage in smallholder dairy herds and risk of violative antimicrobial residues. *J. Vet. Sci.* **5(1)**: 5-9.

Torres, G., Vargas, K., Sánchez-Jiménez, M., Reyes-Velez, J. and Olivera -Angel, M. (2019). Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms. Heliyon. e02535.

Wang, W., Lin, X., Jiang, T., Peng, Z., Xu, J., Yi, L., Li, F., Fanning, S. and Baloch, Z. (2018). Prevalence and characterization of *Staphylococcus aureus* cultured from raw milk taken from dairy cows with mastitis in Beijing, China. *Front. Microbiol.* **9**: 11-23.