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Agroclimatic zone-wise prevalence of biofilm forming methicillin resistant *Staphylococcus aureus* in West Bengal

RAJARSHI BARDHAN[1], ADITYA PAUL[1], INDRANIL SAMANTA[1], KUNAL BATABYAL[1], SAMIR DEY[1], UTTAM SARKAR[1] and SIDDHARTHA NARAYAN JOARDAR[1]✉

West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata, West Bengal 700 037 India

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Staphylococcus aureus is considered as a most common gram-positive commensal bacteria of skin of human and animals, although it is detected in other parts of the body, viz. nostrils, upper respiratory tract and lower reproductive tract of females (Lowry 1998). In addition to mastitis, udder impetigo, wound infections, tick pyaemia, bumblefoot, and botryomycosis in food animals and birds, it can occasionally cause minor skin infections, septicemia, toxic shock, endocarditis, and pneumonia in humans.

Staphylococcus aureus is such a pathogen that is always found in various forms of clinical and sub-clinical mastitis in livestock (Vasudevan *et al*. 2003). In contrary to clinical bovine mastitis, exhibited with cardinal signs such as fever with red and swollen udder, sub-clinical mastitis revealed no visible abnormality in the udder or milk (Khan and Khan 2006), other than the decrease in milk production with an increased somatic cell counts (Abebe *et al*. 2016). Noteworthy is the possibility that antibiotic resistance contributes to *S. aureus*-related mortality in humans and animals, and the rise of methicillin-resistant *S. aureus* (MRSA) strains poses a global threat to clinical care.

In India, 20–40% of the bovine mastitis is related to *S. aureus* infection; although the livestock associated methicillin-resistant *S.aureus* (LA-MRSA) present in food animals and animal-origin food products is poorly characterized except a few reports indicating the occurrence (Joshi and Gokhale 2006, Kumar *et al*. 2010, Bandyopadhyay *et al*. 2015). In Eastern India, especially in West Bengal state, cattle are mostly reared in backyard system with direct contact with human than the intensive farming systems with a high possibility of cross-transmission. Further, the supply of unpasteurized milk directly from the farmers into the food chain increases the occurrence of milk-borne staphylococcal infections or intoxications in human.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections were first detected in nosocomial infection in human hospitals. However, in recent years infections have emerged in community, and from livestock (Stefani *et al*. 2012). Resistance to methicillin is primarily due to intake of the *mecA* gene, not native in this species that codes a modified penicillin-binding protein (PBP2a) with low affinity for β-lactams (Otter and French 2011).

Microorganisms attach to surfaces and develop biofilms. Biofilms are having one or more types of microorganisms that can grow on different inanimate surfaces. Microbes including bacteria, fungi and protists can form biofilms. Within a biofilm, bacteria are more resistant to antibiotics besides major disinfectants for which the biofilm formation is a cause of concern. In addition to the cell surface hydrophobicity, presence of fimbriae and production of extracellular polymeric substance (EPS) can influence the rate and extent of attachment of microbial cells (Donlan 2002).

Occurrence of methicillin-resistant and enterotoxin producing *S. aureus* in raw milk samples collected from the cattle with or without mastitis in different agro-climatic zones of West Bengal was reported earlier (Mahanti *et al*. 2020). In present study occurrence of *nuc* gene is deciphered in *Staphylococcus aureus* from milk samples of different agro-climatic regions of West Bengal, India (Bardhan *et al*. 2023). The present study envisaged the prevalence of biofilm formation by methicillin resistant *Staphylococcus aureus* in cattle milk obtained from different agro-climatic zones of West Bengal, one of the high milk producing state of India is envisaged in current research.

A total of 168 milk samples were collected from cattle, which included animals from both organized farms and backyard cattle, of three different agro-climatic zones (ACZ) of West Bengal, viz. gangetic alluvial (n=87), coastal saline (n=20) and red laterite (n=61). The ACZs selected for the study was based on convenience to collect milk samples from cattle and high cattle population of the places (DAHD 2019). The milk samples were collected from the cattle reared under both types of husbandry practices to avoid biasness associated with hygienic environment.

Present address: [1]West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata, West Bengal. ✉Corresponding author email: joardar69@gmail.com

The freshly collected milk samples were transported to the laboratory maintaining cold chain (4°C–8°C), and later incubated at 37°C for 24 h. Further, the overnight growth was inoculated into mannitol salt agar (HiMedia, India) and was incubated at 37°C for 48 h. The characteristic yellow coloured colonies on the plate were selected for biochemical tests such as catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate and urease following the standard procedure (Quinn 1994). The genomic DNA of the bacterial isolates were extracted by heat lysis method (Mohanti *et al*. 2020) and subjected to PCR targeting 16S rRNA gene of *Staphylococcus aureus* species, for the molecular confirmation of the bacteria (Ali *et al*. 2014). The details of the PCR conditions and base-pair size of the target products were depicted in Table 1.

Molecular analysis of methicillin resistance was carried out by PCR of *mecA* gene using a thermal cycler (Eppendorf Germany), following the protocols of Steggar *et al*. (2012) with some modifications. Details about the reaction conditions and base-pair size is described in Table 1.

This test for detection of biofilm producing bacteria was carried out by microtiter plate assay according to Pérez-Conesa *et al.* (2011) with slight modifications. The confirmed *Staphylococcus aureus* isolates were suspended in 5 mL of tryptic soy broth (TSB) and incubated overnight without shaking. After incubation, the stationary-phase culture was vortexed and diluted to 1:100 in TSB with 0.25% glucose, and 200 µL of this solution was incubated in 96-well plates overnight at 37±1°C. Media with suspended bacteria was then removed; it is followed by washing of the plates (four times) and air-drying. Then 200 µL of 0.9% crystal violet solution was added for 15 min. After removing the dye solution and washing with water, the attached dye was made soluble with 95% ethanol. Lastly the O.D. of the solution was determined at 578 nm in ELISA reader (Merilyzer, Germany). The biofilm formation by the bacterial isolates was determined based on an optical density (OD) cut-off, i.e. OD_C (OD of negative control) as described by Stepanovic *et al*. (2007). The interpretation was done as follows.

$$ODi \leq OD_C = \text{Non-biofilm-producing};\ OD_C \leq ODi < 2OD_C = \text{Weak-producing};\ 2OD_C \leq ODi \leq 4OD_C = \text{Moderate-producing};\ 4OD_C \leq ODi = \text{Strong-producing}$$

Where, ODi, Optical density of isolate; ODc, Optical density of control. The OD_C was measured as three standard deviations from the OD mean of the negative control. Identification of biofilm specific genes (*icaA* and *icaD*) in the *Staphylococcus aureus* isolates was carried out by PCR. Details of the PCR conditions, product size and references are provided in Table 1.

Statistical analysis involved frequency distribution of different samples that was performed in MS Excel along with graphical presentation. Chi-square and Likelihood Ratio test was performed to analyze the effect of different zones using IBM SPSS Statistics for Windows, Version 25.0, IBM Corp., Chicago, IL.

In this study, prevalence of biofilm forming, methicillin resistant *Staphylococcus aureus*, isolated from cattle milk as obtained from various agro-climatic zones of West Bengal, was determined. Of the 168 milk samples, the recovered *Staphylococcus aureus* isolates (n=146) showed characteristic yellow coloured colonies in mannitol salt agar, after 48 h of incubation at 37°C and the positive results for biochemical tests and molecular analysis (PCR) confirmed its characteristics (Supplementary Fig. 1A.) When represented zone-wise, the gangetic alluvial zone hold the maximum number of staphylococcal isolates (n=77), followed by red laterite zone (n=53) and Coastal saline zone (n=24) without having statistically significant ($P<0.01$) differences (Table 2).

A total of 45 *S. aureus* isolates were positive (30.82%) for *mecA* gene, confirmed under PCR analysis (Supplementary

Table 1. Primer sequences with PCR conditions, base pair size and reference

Gene	Gene sequence (5'-3')	PCR conditions	Product size (bp)	Reference
S. aureus 16S rRNA	F: GGAATTCAAAGGAATTGACGGGGC R:CGGGATCCCAGGCCCGGGAACGTATTCAC	Initial denaturation at 95°C for 5 min. Denaturation at 95°C for 30s, Annealing at 55°C for 30s and Extension at 72°C for 60s with final extension at 72°C for 10 min.	479	Ali *et al.* 2014
mecA	F:TCCAGATTACAACTTCACCAGG R : CCACTTCATATCTTGTAACG	Initial denaturation at 94°C for 5 min, Denaturation at 94°C for 1 min, Annealing at 59°C for 1 min, and 1 min at 72°C, Final elongation at 10 min at 72°C.	162	Stegger *et al.* 2012
icaA	F: CCT AAC TAA CGA AAG GTA G R: AAG ATA TAG CGA TAA GTG C	Denaturation at 92°C for 45 s, Annealing at 49°C for 45s, Elongation at 72°C for 1 min, Final extension at 72°C for 7 min	1315	Vasudevan *et al.* 2003
icaD	F: AAA CGT AAG AGA GGT GG R : GGC AAT ATG ATC AAG ATA C	Denaturation at 92°C for 45 s, Annealing at 49°C for 45s Elongation at 72°C for 1 min, Final extension at 72°C for 7 min	381	Vasudevan *et al.* 2003

Table 2. Total number of *Staphylococcus aureus* isolates obtained from the milk samples

Zone	*Staphylococcus aureus* isolates obtained*
Gangetic alluvial	77
Red laterite	53
Coastal saline	24
Total	154

*No significant difference observed in the isolates (P<0.01).

Fig. 1B). For zone-wise distribution, the most prevalent zone was in terms of mecA positive isolated was gangetic alluvial, (22/77, 28.57%), followed by red laterite 16/53, 30.19%) and coastal saline (7/24, 29.16%) without having significant differences (P<0.01) (Table 3). Similar kind of findings has been reported by Shah *et al.* (2020) where a total 20 out of 80 *S. aureus* isolates, recovered from 150 mastitic milk samples of local market, were *mecA* positive (25%) in PCR. Methicillin resistant *S. aureus* was also isolated from the samples of milk and dairy products from South Italy, where 8.26% of the isolates were *mecA* positive (Basanisi *et al*. 2016). A total of 170 *S. aureus* isolates were recovered from composite milk samples of cattle in Brazil. PCR analysis had shown that 10 out of 170 samples were *mecA* positive (5.88%) (Santos *et al*. 2016). Mistry *et al.*(2016) had characterized 48.72% of *mecA* positive *S. aureus* obtained from milk of southern states of India, viz. Andhra Pradesh, Telengana and Tamil Nadu. Prashant *et al*. (2011) reported 29.42% of *mecA* positive *Staphylococcus aureus* from milk of Andhra Pradesh, a southern state of India. In contrast, our earlier study in West Bengal, we reported occurrence of 9.6% MRSA in collected composite milk samples from different agro-climatic zones (Mahanti *et al*. 2020). Occurrence of MRSA in raw milk and its products varied widely depending on husbandry practices of cattle, biosecurity of the farms and detection technique of the bacteria.

Staphylococcus aureus isolates were also observed to be biofilm formers. Phenotypic assay using microtiter plate had shown that a total of 14 isolates were strong biofilm producers, 79 isolates were moderate, 47 were weak and 06 were non biofilm producers. PCR studies of biofilm specific genes, viz. *icaA* (Fig. 1A) and *icaD* (Fig. 1B) revealed that 30 isolates were *icaA* positive (20.55%), and 25 isolates were *icaD* positive (17.12%) (Table 3). Zone wise prevalence rate of *icaA* and *icaD* positive *Staphyloccus aureus* has been shown in Table 3.

Table 3. Zone-wise occurrence of *mecA, icaA* and *icaD* positive *Staphylococcus aureus* isolates

Zone	*mecA*	*icaA*	*icaD**
Gangetic alluvial	22	11	19
Red laterite	16	15	02
Coastal saline	07	04	04
Total	45	30	25

*Significant difference observed in the isolates (P<0.01).

About 72.5% of the *S. aureus* isolates, recovered from milk samples were both *icaA* and *icaD* positive, and 25% were only *icaD* positive, as determined by PCR (Basanisi *et al.* 2017). *S. aureus* strains (n=100), isolated from the mastitis milk samples of cattle were selected of which 17 (17%) isolates possessed both *icaA* and *icaD* (Salina *et al*. 2020). In contrast, 100% positive for *icaD*, and 98.91% positive for both *icaA* and *icaD* genes were noted in *Staphylococcus aureus* isolates from bovine mastitis in New Zealand (Notcovich *et al*. 2018).

Significant differences were noted in prevalence rate of *icaD* positive *Staphylococcus aureus* when studied zone-wise (P<0.01) (Table 3). However, no significant difference could be detected in the zone-wise study of *icaA* positive *Staphylococcus aureus* isolates. It can be concluded that differences in agro-climatic conditions influence at least the prevalence rate of specific gene like *icaD* in *Staphylococcus aureus* that warrants further studies for its proper explanations. The milk samples collected from three different agro-climatic zones of southern part of West Bengal were found to harbour the methicillin resistant, biofilm forming *Staphylococcus aureus* isolates which is an alarming situation on public health point of view.

SUMMARY

The present study envisages the prevalence of biofilm producing, methicillin resistant *Staphylococcus aureus*,

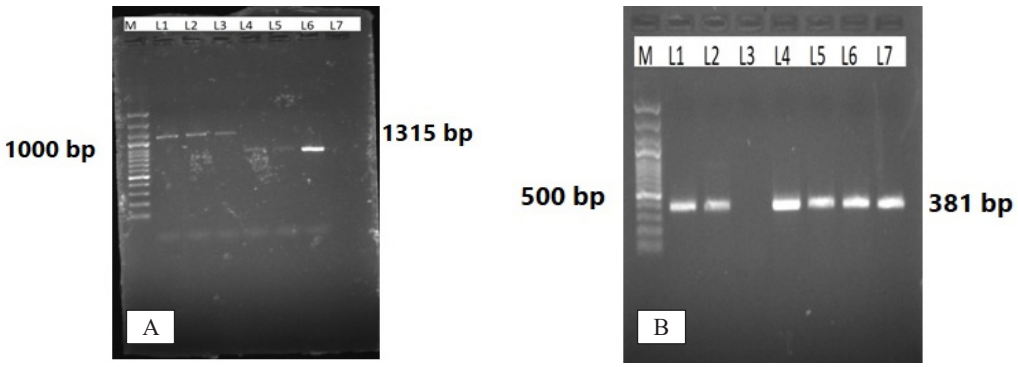

Fig. 1. Agarose gel electrophoresis showing PCR amplicon of; A. *icaA* gene (1315 bp) obtained from *S. aureus*; M: 1 kb ladder, L1-L3: Positive representative samples, L4-L7: Negative sample; B. *icaD* gene (381 bp) obtained from *S. aureus* L1, L2, L4-L7: Positive representative samples, L3: Negative sample.

isolated from cattle milk of different agro-climatic zones of an eastern state of India, West Bengal. The milk samples (n=168) were collected from three predominant agro-climatic zones of South Bengal, viz. new alluvial, coastal saline and red laterite. A total of 146 *Staphylococcus aureus* isolates were recovered, confirmed by biochemical tests and polymerase chain reaction (PCR). Through PCR of *mecA* gene, it was found that 45 strains were methicillin resistant, encompassing 28.57% new alluvial zone, 30.19% red laterite region and 29.16% coastal saline region. Determination of biofilm formation was done, both phenotypically by microtiter plate method and genotypically by PCR of *icaA* and *icaD* genes, specific for biofilm formation in *Staphylococcus aureus*. Phenotypic study revealed that 14 isolates were strong biofilm producers, 79 were moderate and the 47 were weak biofilm producers. PCR studies also showed that a total of 30 isolates were *icaA* positive and 25 isolates were *icaD* positive. Although no significant difference was found in the occurrence of *icaA* positive isolates, the possession of *icaD* by the *Staphylococcus aureus* isolates varied significantly according to different agro-climatic zones.

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