



Antibiotic resistance gene typing in *Staphylococcus aureus* isolated from bovine mastitis

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ABSTRACT

The study was conducted to determine the antimicrobial resistance pattern of *Staphylococcus aureus* isolates from bovine mastitis. Milk samples (160) collected aseptically from mastitis affected cows at organized dairy farms in and around Jammu were subjected to microbial culture for the isolation and identification of *S. aureus* using Baird Parker Agar. Presumptive *S. aureus* isolates (52) were subjected to molecular confirmation through identification of species specific (*nuc*) gene. *In vitro* antimicrobial resistance pattern of the isolates against a panel of 13 selected antibiotics, using disc diffusion technique, revealed that the isolates were mostly sensitive to enrofloxacin followed by vancomycin, ciprofloxacin, tetracycline, chloramphenicol, streptomycin, ceftriaxone, erythromycin and gentamicin whereas maximum resistance was shown towards penicillin G followed by ampicillin, amoxycylav, methicillin, gentamicin, streptomycin, erythromycin, tetracycline and ceftriaxone. Methicillin resistance (MRSA) was recorded in 32.69% *S. aureus* isolates out of which 41.17% isolates carried *mecA* gene. Among the gentamicin and tetracycline resistant *S. aureus* isolates, 61.53% isolates carried *aacA-aphD* gene and 80% isolates carried *tetK* gene, respectively. Multidrug resistance (MDR) was observed in 71.15% *S. aureus* and 82.35% MRSA isolates. In conclusion, *S. aureus* showed maximum sensitivity to enrofloxacin thereby suggesting the use of this drug for effective treatment of mastitis but the development of resistance against this drug cannot be ruled out in the near future, hence, there is a need for accurate diagnosis of mastitis along with the correct selection of antibiotics to prevent bovine mastitis.

Key words: Antimicrobial resistance, Dairy cows, Genotypic, Mastitis, *Staphylococcus aureus*

Mastitis, although technically defined as any udder injury which results in inflammation of the mammary gland, is mainly caused by the microorganisms that gain entry into the teat canal and mammary tissue. About 200 different organisms are reported to cause bovine mastitis (Blowey and Edmondson 2010). *Staphylococcus aureus* is a contagious mastitis pathogen with a major effect on milk production and bulk tank somatic cell count (SCC) (Keefe 2012). A high prevalence (80.85%) of *Staphylococcus aureus* has been observed in dairy cows affected with sub-clinical mastitis (Yadav 2018). The prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) mastitis in dairy cattle has been recorded as 16.47% (Shrivastava *et al.* 2017).

A selective pressure for the emergence of antimicrobial resistant bacteria is created by the use of antimicrobials in

veterinary medicine and the bacteria selected by this pressure can spread to humans either by direct contact with animals or food products or indirectly via environmental pathways (Da Costa *et al.* 2013). There is a need of determining an efficient tool to use specific antibiotic which can efficiently stop and control bovine mastitis in dairy animals (Hossain *et al.* 2017). Keeping in view these facts and findings, the present study was conceptualized to determine the phenotypic and genotypic antimicrobial resistance of the *Staphylococcus aureus* isolates from mastitis affected dairy cows.

MATERIALS AND METHODS

Collection of mastitic milk samples: Lactating crossbred (HF) dairy cows were screened for clinical and sub-clinical mastitis and 160 pooled milk samples were collected from mastitic cows. After proper disinfection of the teat surface with 70% ethyl alcohol, few streams of milk from each quarter were discarded. Aseptically 10 ml of milk was collected in sterile polyethylene screw capped bottles from all the four quarters. The samples were kept in an ice box and immediately carried to the laboratory for analysis.

Isolation of *Staphylococcus aureus*: Enrichment of milk

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samples was done using sterile peptone water (PW) enrichment broth followed by overnight incubation at 37°C. A loopful of enriched milk sample was inoculated on to a Baird-Parker agar (BPA) plate supplemented with egg-yolk tellurite emulsion and the inoculated plates were incubated at 37°C for 24–48 h.

Identification of *Staphylococcus aureus* isolates: All the presumptive *S. aureus* isolates were subjected to Gram's staining followed by biochemical characterization using catalase, oxidase, DNase and coagulase tests. Molecular confirmation of the *S. aureus* isolates included the detection of the species specific thermonuclease gene (*nuc* gene) using PCR.

In vitro drug sensitivity of *S. aureus* isolates: All the *S. aureus* isolates were examined for their antibiogram pattern against a panel of 13 antibiotics using disc diffusion method as described by Bauer *et al.* (1966).

PCR detection of *nuc* and *mecA* genes in *S. aureus* isolates: PCR amplification was performed using a 25 µl reaction containing a final concentration of 0.2 mM dNTPs, 2.5 mM MgCl₂, 1× buffer, 25 µM of each forward and reverse primer, 1 U of *Taq* polymerase, 2 µl template DNA and the sterile nuclease free water was added to make up the reaction volume. PCR amplification was performed in DNA thermal cycler. The cycling conditions included an initial denaturation of DNA at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, followed by a final extension of 7 min at 72°C and hold at 4°C.

PCR detection of tetracycline (*tetK* and *tetM*) and gentamicin resistance genes (*aacA*-aphD) in *S. aureus* isolates: The PCR amplification was carried out in 25 µl reaction volume containing 0.5 µl of dNTPs, 2.5 µl of buffer, 0.5 µl of MgCl₂, 0.5 µl of each primer set containing forward and reverse primers, 0.3 µl of *Taq* DNA polymerase, 2 µl of DNA template and sterilized nuclease free water to make up the reaction volume. PCR amplification was performed using DNA thermal cycler. The cycling conditions included an initial denaturation of DNA at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension of 4 min at 72°C and hold at 4°C.

Amplified PCR products were then electrophoresed on a 1% agarose gel in a 1× tris acetate EDTA (TAE) buffer and the DNA bands were visualized under UV trans-illuminator and photographed with gel documentation system.

RESULTS AND DISCUSSION

Out of 160 pooled milk samples collected from mastitic cows, 52 (32.5%) *S. aureus* isolates were obtained on the basis of colony morphology on Baird Parker agar (BPA) (Jet black, shiny colonies surrounded with a halo zone), Gram's staining (Purple coloured, like bunches of grapes) and biochemical test interpretation (catalase positive, oxidase negative, DNase positive and coagulase positive). Similar results were obtained by Abera *et al.* (2010) who reported the presence of 59 (42.14%) *S. aureus* isolates out

of 140 milk samples obtained from bovine mastitis cases in Ethiopia.

In order to confirm the isolates on molecular basis, all the presumptive *S. aureus* isolates (52) were subjected to the identification of species specific (*nuc*) gene and the results were positive for all the isolates (100%). Similar findings were reported by Kuřma *et al.* (2003) who detected the PCR product, a single DNA band of 270 bp in all the 29 *S. aureus* strains (100%) isolated from milk samples.

The susceptibility pattern of the 52 *S. aureus* isolates determined by the disc diffusion method revealed highest sensitivity towards enrofloxacin (100%) followed by vancomycin (92.30%), ciprofloxacin (88.46%), tetracycline (86.53%), chloramphenicol (84.61%), streptomycin (78.84%), ceftriaxone (73.07%), erythromycin and gentamicin (67.30% each), methicillin (59.61%), amoxycylav (32.69%), ampicillin (13.46%) and penicillin G (11.53%). Isolates showed highest resistance towards penicillin G (88.46%) followed by ampicillin (84.61%), amoxycylav (67.30%), methicillin (32.69%), gentamicin (25%), streptomycin (21.15%), erythromycin (17.3%), tetracycline and ceftriaxone (9.61% each), vancomycin (7.69%), chloramphenicol (5.76%), ciprofloxacin (3.84%) and enrofloxacin (0%). This can be explained by the fact that the newer chemotherapeutic agents like enrofloxacin, vancomycin, ciprofloxacin and chloramphenicol are less commonly used for the treatment of mastitis resulting in higher efficacy of these drugs while the frequent use of some antibiotics like penicillin, streptomycin, amoxycylav and methicillin in animals could be the reason for their ineffectiveness against bacterial isolates. Similar antibiogram pattern was reported by Kumar *et al.* (2011) who observed highest sensitivity to vancomycin (100%). Bhat *et al.* (2017) reported similar antibiotic sensitivity pattern of isolates recovered from clinical mastitis cases in bovines of Jammu region with maximum sensitivity of the isolates towards enrofloxacin, gentamicin, amoxicillin/sulbactam, ceftriaxone/tazobactam, ceftizoxime, ampicillin/sulbactam and least sensitivity towards oxytetracycline and penicillin. Chandrasekaran *et al.* (2014) also reported maximum sensitivity of *S. aureus* isolates towards enrofloxacin (79.8%) and highest resistance towards penicillin (63.5%). In the present study, out of 52 *S. aureus* isolates, methicillin resistance (MRSA) was recorded in only 17 (32.69%) isolates. Susceptibility pattern of the MRSA isolates to other antibiotics is shown in Table 1. The present findings were in accordance with those of Joshi *et al.* (2013) who observed 100% susceptibility of MRSA isolates towards Vancomycin.

Multidrug resistance (MDR) i.e. resistance to 3 or more than 3 antibiotics in *S. aureus* and MRSA isolates is shown in Table 2. MRSA strains are multidrug resistant (MDR) owing to the carriage of other resistance genes on the cassette chromosome harbouring the *mecA* gene (Holmes and Zadoks 2011). Our findings are in accordance with those of Kozerski *et al.* (2014) who reported that 12 (24.50%) isolates (7 *S. aureus* and 5 CoNS) were resistant to more

Table1. Antibiotic sensitivity pattern of MRSA (17 isolates) recorded phenotypically

Antibiotic	Resistant		Intermediate		Sensitive	
	n	%	n	%	n	%
Tetracycline (TE)	3	17.64	2	11.76	12	70.58
Erythromycin (E)	6	35.29	4	23.52	7	41.17
Ceftriaxone (CTR)	5	29.41	4	23.52	8	47.05
Gentamicin (GEN)	7	41.17	2	11.76	8	47.05
Vancomycin (VA)	0	0	0	0	17	100
Chloramphenicol (C)	0	0	3	17.64	14	82.35
Ciprofloxacin (CIP)	2	11.76	1	5.88	14	82.35
Penicillin G (P)	16	94.11	0	0	1	5.88
Amoxyclav (AMC)	13	76.47	0	0	4	23.52
Ampicillin (AMP)	16	94.11	0	0	1	5.88
Enrofloxacin (EX)	0	0	0	0	17	100
Streptomycin (S)	2	11.76	0	0	15	88.23

Table 2. Multi-drug resistance (MDR) pattern of *S. aureus* isolates and MRSA strains

No. of antimicrobials	<i>S. aureus</i> (52 isolates)		MRSA (17 isolates)	
	n	%	N	%
3	13	25	4	23.52
>3	24	46.15	10	58.82
Total	37	71.15	14	82.35

than three classes of antibiotics. On the contrary, Umaru *et al.* (2016) reported higher (96.4%) prevalence of MDR in *S. aureus* isolates. Methicillin-resistant *Staphylococci* frequently show multiple resistance to several classes of antimicrobial agents, including aminoglycosides, clindamycin, macrolides, quinolones, sulfonamides and tetracycline (CLSI 2012), leaving only vancomycin as the drug of choice against these agents.

Genotypically, methicillin resistance was detected in only 7 (41.17%) isolates out of 17 MRSA isolates detected phenotypically. It was based on the PCR amplification of the *mecA* gene at 532 bp. Our findings corroborate with those of Koupahi *et al.* (2016) who observed the presence of *mecA* gene in 105 (47.72%) out of 220 *S. aureus* isolates. Similar findings were reported by Havaei *et al.* (2015) who identified 10 (18.52%) *mecA* positive out of 54 *S. aureus* isolates. These results suggest the presence of some other genes like *mecC* responsible for methicillin resistance in MRSA isolates as reported by Paterson *et al.* (2014). Other reason includes the over production of β -lactamase enzyme (Sancak 2000, Olayinka *et al.* 2009).

Among the 13 *S. aureus* isolates which were phenotypically resistant to gentamicin, *aacA-aphD* gene was observed in only 8 (61.53%) isolates. The gene *aacA-aphD* codes for a bifunctional enzyme that shows acetyl transferase and phospho transferase activity and confers resistance to gentamicin, kanamycin and tobramycin. It is speculated that intrinsic antibiotic resistance, being the naturally low permeability of the bacterial cell wall, which limits uptake of many antibiotics including amino-

glycosides, is responsible for the other 5 isolates that did not show amplicons of the investigated target gene in this study. These findings corroborate with those of Pekana and Green (2018) who found that among the 15 *S. aureus* isolates which were phenotypically resistant to gentamicin, only 5 (33.3%) isolates carried gentamicin resistance genes. Similarly, Amandeep (2016) found that out of the 83 *S. aureus* isolates, 47 isolates were positive for *aacA-aphD* gene by PCR yielding 227 bp amplicon.

For tetracycline resistance, out of the 5 phenotypically resistant isolates, *tetK* gene was found in only 4 (80%) isolates while as none (0%) of the isolates carried *tetM* gene. These results were in accordance with those of Shamila-Syuhada *et al.* (2016) who found *tetK* gene in only 33.3% of the phenotypically resistance tetracycline *S. aureus* isolates while as *tetM* gene was not detected at all. Similar to this, Amandeep (2016) found 25 *S. aureus* isolates positive for tetracycline resistant genes, among which 24 isolates were *tetK* and 1 isolate was *tetM* positive.

The present study concludes that *S. aureus*, the major causative agent for contagious bovine mastitis, is highly sensitive towards enrofloxacin and hence this drug is a good choice for treatment of bovine mastitis. However, presence of methicillin resistant *S. aureus* (MRSA) and detection of multidrug resistance (MDR) in *S. aureus* and MRSA isolates indicates a major zoonotic threat that needs to be addressed for the prevention of mastitis in dairy cows.

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