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Molecular characterization of *Staphylococcus aureus* isolated from milk in Udaipur city (Rajasthan)

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Abstract
This study was aimed at the molecular characterization of *Staphylococcus aureus* isolated from milk samples. A total of 100 milk samples comprising of pooled milk (n=25), vendors milk (n=25), pasteurized market milk (n=25) and individual cow milk (n=25) were collected during from Udaipur city, Rajasthan. The *S. aureus* were found in g pooled milk, vendors milk, pasteurized market milk and individual cow milk as 76%, 36%, 0% and 56%, respectively. Out of total 42 *S. aureus* isolates, 10 were multidrug resistant which were further subjected for molecular characterization by PCR. All the 10 isolates were found positive for *16S rRNA* and *nuc* gene. The prevalence of antibiotic resistant genes *mecA*, *ermC* and *aacA-aphD* were 40% (4), 70% (7) and 30% (3), respectively.

Keywords: Molecular characterization, *Staphylococcus aureus*

Introduction
Staphylococcus aureus bacteria are Gram positive cocci of 0.5- 1.5 micrometer diameter, forming grape like clusters and facultative anaerobes. The pathogenicity of *S. aureus* is mainly related to a combination of toxin mediated virulence, invasive capacity and antibiotic resistance (Argudin *et al.*, 2010) [1]. Drinking contaminated milk with preformed toxins of *S. aureus* causes rapid onset (IP = 2-8 hours) of vomiting, nausea, abdominal cramps and diarrhoea. *Staphylococcus aureus* is also known for its multidrug resistance and MRSA is one of the most potent drug resistant bacteria that has been causing nosocomial infections and community associated infections and animal diseases (Aklilu *et al.*, 2020) [2]. From a public health point of view, there is a concern about the risk of zoonotic transmission of livestock associated methicillin resistant *S. aureus* (LA-MRSA) strains in animals and man. It has been reported that animal MRSA isolates were significantly more resistant to ciprofloxacin, gentamicin, and clindamycin as compared to human MRSA isolates (Jayaweera *et al.*, 2020) [3].

The PCR is a rapid and reliable tool for the molecular based diagnosis of *S. aureus* infections. Genus specific 16S ribosomal RNA and species specific thermonuclease gene *nuc* are two important genes to detect *S. aureus*. The frequent and inappropriate use of antibiotics in livestock for therapeutic and growth promoting purpose, results in the emergence of the antibiotic resistance in *S. aureus*. The antibiotic resistance can be easily transferred among healthier commensals and to other animals and humans by close interactions (Sharma *et al.* 2017) [4]. However, multidrug resistance in *S. aureus* is an emerging and important public health threat as there are fewer, effective antimicrobial agents available for infections caused by these MDR (multidrug resistant) strains.

Materials and methods Samples
A total of 100 milk samples comprising of pooled milk (n=25), vendors milk (n=25), pasteurized market milk (n=25) and individual cow milk (n=25) were collected from Udaipur city, Rajasthan. The samples of milk were collected twice in a week from dairy shops, vendors, market and dairy farms from Udaipur city in Rajasthan. The samples were collected in sterile container and transported to the laboratory within 2 hours in chilled condition by using ice packs.

Molecular characterization
Isolation of DNA from pure culture was undertaken using by Nucleo-pore gDNAfungal/bacterial mini kit by following the manufacturer's instructions supplied along

with the kit. Genomic DNA isolated from *S. aureus* isolates were used in the PCR. Published primers were used for the detection of *16S rRNA, nuc, ermC, aacA-aphD* and *mecA* genes in *S. aureus* isolates are described in Table No. 1.

Table 1: The primers used for detection of *16S rRNA, nuc, ermC, aacA-aphD* and *mecA* genes

S. No.	Oligo name	Name sequence (5' - 3')	Size of amplified product (bp)	Reference
1	16S *rRNA* forward	GTAGGTGGCAAGCGTTATCC	228	Loveseth *et al.* 2004 [5]
	16S *rRNA* reverse	CGCACATCAGCGTCAG		
2	*nuc* forward	GCGATTGATGGTGATACGGTT	279	Barski *et al*. 1996 [6]
	nuc reverse	AGCCAAGCCTTGACGAACTAAAGC		
3	*aacA-aphD* forward	TAATCCAAGAGCAATAAGGGC	227	Strommenger *et al.* 2003 [7]
	aacA-aphD reverse	GCCACACTATCATAACCACTA		
4	*ermC* forward	AATCGTCAATTCCTGCATGT	299	Strommenger *et al.* 2003 [7]
	ermC reverse	TAATCGTGGAATACGGGTTTG		
5	*mecA* forward	AAAATCGATGGTAAAGGTTGGC	533	Strommenger *et al.* 2003 [7]
	mecA reverse	AGTTCTGC AGTACCGGATTTGC		

The PCR procedure to screen the *16S rRNA, nuc, ermC, aacA-aphD* and *mecA* gene in *S. aureus* isolates was standardized as described by Loveseth *et al.* (2004) [5], Barski *et al.* (1996) [6] and Strommenger *et al.* (2003) [7] with certain modifications. Followed by preliminary trials, the reaction mixture was optimized to contain 12.5 µl 2X PCR master mix, 10 nmol of each forward and reverse primer, 10.5 µl nuclease free water and 1 µl of DNA template. The reaction was performed in the thermal cycler with pre-heated lid (lid temp. = 105°C). The cycling conditions of *16S rRNA, nuc, ermC, aacA-aphD* and *mecA* gene were comprised of 30 cycles of denaturation, annealing and extension which are described in Table No 2.

Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

Primers	Cycling conditions				
(Forward and reverse)	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
16S *rRNA* (F)	940C for 5 minutes	940C for 1 minute	540C for 1 minute	720C for 1 minute	720C for 5minutes
16S *rRNA* (R)					
Repeated for 30 cycles					
ermC (F)	940C for 5 minutes	940C for 1 minute	550C for 1 minute	720C for 1 minute	720C for 5minutes
*erm*C (R)					
Repeated for 30 cycles					
aacA-aphD (F)	940C for 5 minutes	940C for 1 minute	550C for 1 minute	720C for 1 minute	720C for 5minutes
aacA-aphD (R)					
Repeated for 30 cycles					
mecA (F)	940C for 5 minutes	940C for 1 minute	580C for 1.5 minute	720C for 1.5 minute	720C for 5minutes
mecA (R)					
Repeated for 30 cycles					
nuc (F)	940C for 5 minutes	940C for 1 minute	580C for 1 minute	720C for 1 minute	720C for 5minutes
nuc (R)					
Repeated for 30 cycles					

Results and discussion

Out of the 42 isolates, 10 MDR *S. aureus* isolates were selected for molecular characterization by targeting the virulence and resistance genes. First of all, detection of *16S rRNA* gene was done by standardizing the PCR protocol as per the method described by Loveseth *et al.* (2004) [5]. Electrophoresis analysis revealed a specific amplification of 228 bp product of the *16S rRNA* gene. *16S rRNA* gene is species specific gene used in identification of *S.aureus* (Monday and Bohach 1999) [8]. In our study, the detection of *16S rRNA* gene revealed its presence in all the 10 MDR isolates recovered from milk and milk products. Similar findings were also reported by Elsayed *et al.* (2015) [9], Can *et al.* (2017) [10], Darwish *et al.* (2018) [11] and Gencay *et al.* (2010) [12] who confirmed all presumptive *S. aureus* isolates by detection of *16S rRNA* gene. For molecular identification of *S. aureus* isolates, molecular targeting of species specific *nuc* gene of *S. aureus* coding for the extracellular thermostable nuclease protein of *S. aureus* was done, which revealed that all the MDR *S. aureus* isolates were positive for *nuc* gene. The detection of the *nuc* gene was carried out as per the method described by Barski *et al.* (1996) [6] and electrophoresis analysis revealed a specific amplification of 279 bp product of the *nuc* gene. Kabir *et al.* (2017) [13] and Javid *et al.* (2018) [14] also reported 100% prevalence of *nuc* gene among *S. aureus* isolates. While, slightly lower prevalence of *nuc* gene among *S. aureus* isolates was observed by Kalorey *et al.* (2007) [15] and Saraiva *et al.* (2018) [16] as 97.29% and 77.94%, respectively. Thus, the simultaneous detection of both *16S rRNA* and *nuc* gene in *S. aureus* should be used for the molecular identification of *S. aureus*.

Aminoglycosides resistance in *S. aureus* may occur as a response to the impermeability catalysed by a bifunctional protein encoded by *aacA-aphD* gene. PCR assay for the detection of *aacA-aphD* gene in *S. aureus* was standardized with primers reported by Strommenger *et al.* (2003) [7] with slight modifications and electrophoresis analysis revealed a specific amplification of 227bp product. In our study, 30% prevalence (3/10) of aminoglycosides resistance gene was observed in MDR *S. aureus* which was in line with the reports of Gulzar *et al.* (2018) [17] and Zehra *et al.* (2017) [18] who found prevalence of the *aacA-aphD* gene as 32.5% and 33.3%, respectively. Higher Prevalence was reported by

Adwan *et al.* (2014) [19] and Ruban *et al.* (2017) [20] as 74.5% and 88%, respectively. While, lower prevalence rates were revealed by Hizlisoy *et al.* (2018) [21] and Zehra *et al.* (2019) [22] as 9.4% and 7.69%, respectively.

Macrolides, lincosamides and streptogamin are antimicrobial groups collectively known as MLS agents. These MLS are frequently used for the treatment of staphylococcal food poisoning. The MLS have inhibitory effects on bacterial protein synthesis. The *erm(A)* and *erm(C)* genes are more commonly responsible for the resistance against MLS. PCR assay for the detection of *ermC* gene in *S. aureus* was standardized with primers reported by

Strommenger *et al*. (2003) [7] with slight modifications and electrophoresis analysis revealed

a specific amplification of 299 bp product. In total, out of 10 isolates of MDR *S. aureus*, 7 were found to contain *ermC* gene. Wang *et al.* (2015) [23] reported 92.6% prevalence of *ermC* gene among *S. aureus* isolates. While, Adwan *et al.* (2014) [19] and Asadollahi *et al.* (2014) [24] and Fasihi *et al.* (2016) [25] reported prevalence of *ermC* gene in 54.5%, 57% and 20.5% isolates of *S. aureus*.

Presence of *mecA* gene is considered as a reliable method to detect methicillin resistance. Virulent *S. aureus* strains include methicillin resistant *Staphylococcus aureus* (MRSA) strains, which have become resistant to most antimicrobial agents including beta lactams, aminoglycosides, macrolides and fluoroquinolones. Therefore, the spread of MRSA has now considered as an emerging threat to human health. In our investigation, it was observed that out of 10 MDR *S. aureus*, 4 isolates were found to be positive for *mecA* gene giving a prevalence rate of 40%. PCR assay for the detection of *mecA* gene was standardized with

primers reported by Strommenger *et al.* (2003) [7] with slight modifications and

electrophoresis analysis revealed a specific amplification of 533bp product of the *mecA* gene. Similar results were reported by Mistry *et al.* (2016) [26] and Elkenany (2018) [27] who found 48.71% and 54.5% isolates as positive for *mecA* gene, respectively. While, lower prevalence of MRSA were reported by Hoque *et al.* (2018) [28], Enany *et al.* (2013) [29], Gindonis *et al.* (2013) [30] and Normanno *et al.* (2007) [31] as 20%, 18.18%, 1.8% and 3.75%, respectively. On the other hand, Keyvan *et al.* (2020) [32] reported higher prevalence of MRSA in which they observed 75.4% of the isolates to be positive for *mecA* gene. Thus, MRSA related management should be applied in dairy farms by detecting the changes in the pattern of the methicillin resistance in bovine staphylococci.

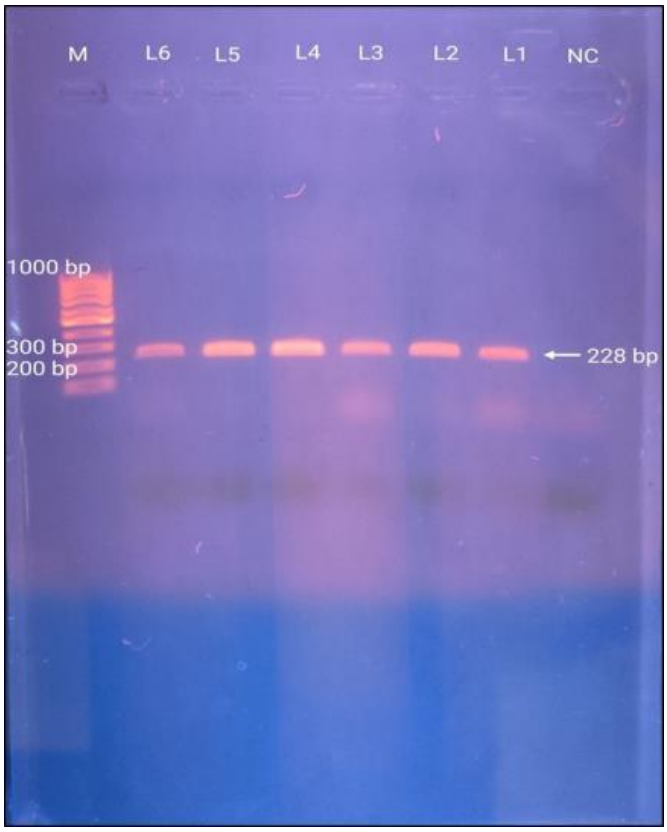

Fig 1: Agarose gel showing PCR amplified product (228 bp) for *16S rRNA* gene in the MDR test isolates. M=1000kb DNA ladder, positive samples (L1= S1, L2=S2, L3= S19, L4=S30, L5=S34, L6=S88, NC= negative control)

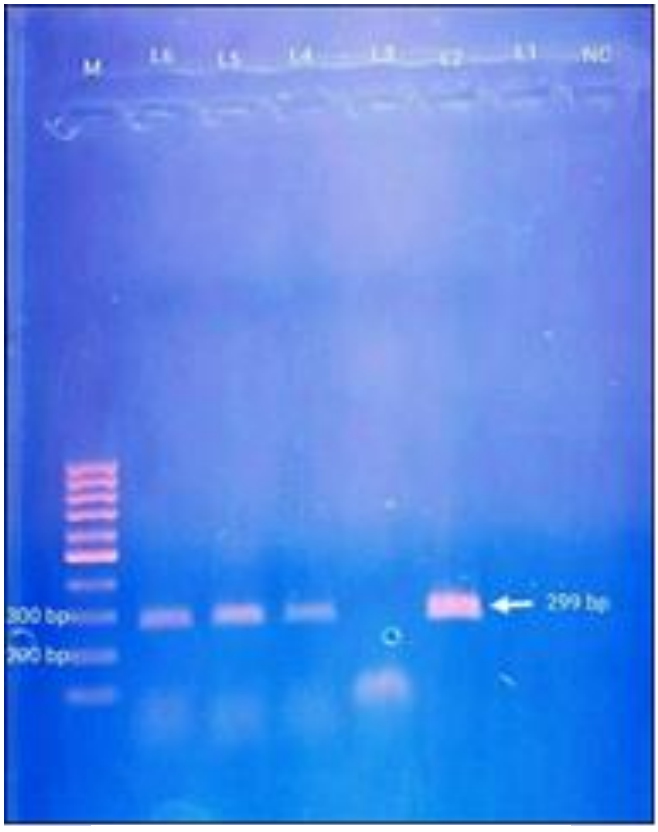

Fig 2: Agarose gel showing PCR amplified product (299 bp) for *ermC* gene in the test isolates. M=1000kb DNA ladder, positive samples (L2=S1, L4=S14, L5= S19, L6=S30, NC= negative control)

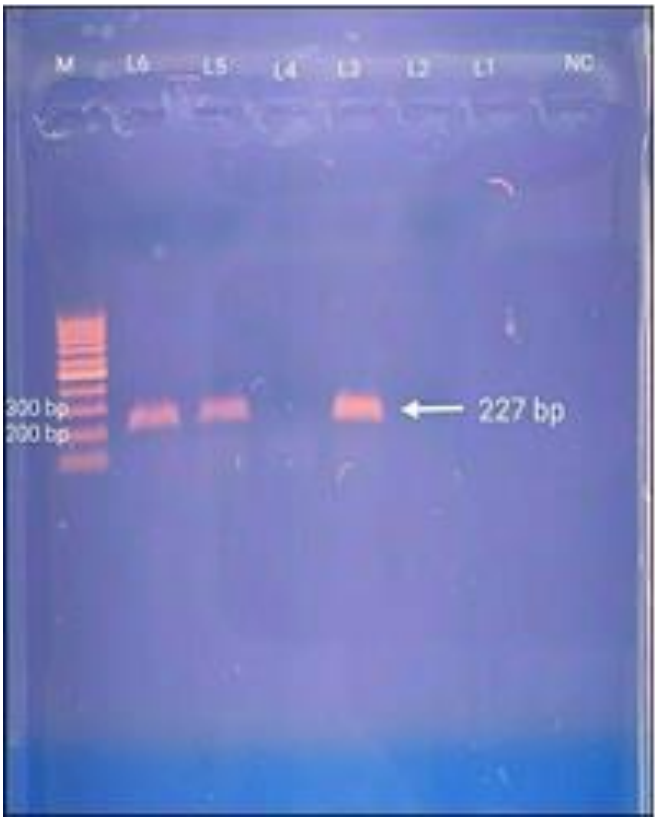

Fig 3: Agarose gel showing PCR amplified product (227 bp) for *aacA-aphD* gene in the MDR test isolates. M=1000kb DNA ladder, positive samples (L3=S88, L5=S90, L6=S95, NC= negative control)

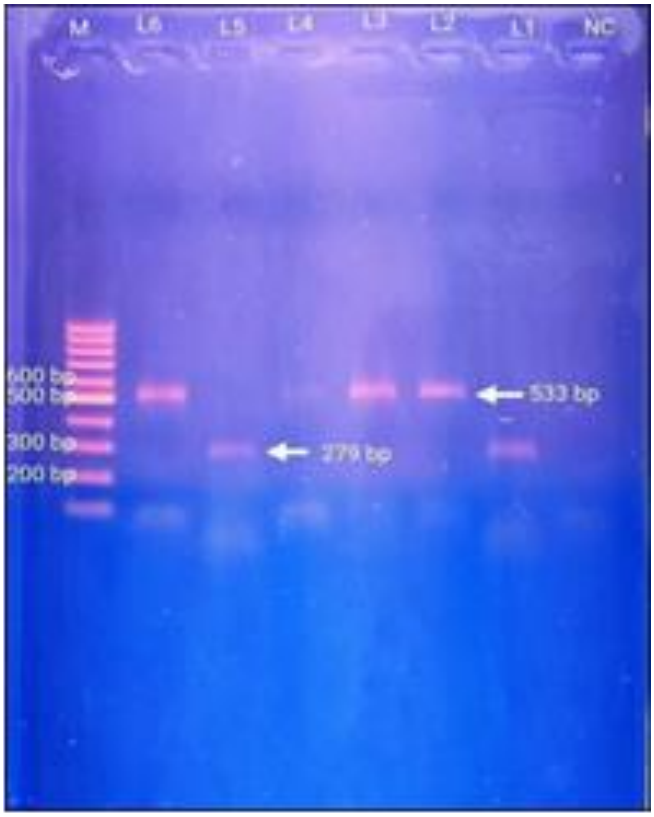

Fig 4: Agarose gel showing PCR amplified product (533 bp) for *mecA* gene and (279 bp) *nuc* gene in the MDR test isolates. M=1000kb DNA ladder, positive samples (L1= S90, L2=S1, L3=S14, L4=S19, L5=S30, L6=S34, NC= negative control)

Conclusion

In the current study, out of the 42 isolates, 10 MDR *S. aureus* isolates were selected for molecular characterization by targeting the virulence and resistance genes. Firstly, detection of *16S rRNA* and *nuc* genes was done by standardizing the PCR protocols. In all the multidrug resistant isolates collected from the different sources of milk samples like pooled milk, vendors milk and individual cow milk, all the MDR isolates (10) were found to be positive for *16S rRNA* and *nuc* genes. Further, the detection of antibiotic resistance genes *aacA-aphD*, *ermC* and *mecA* was carried out. Out of the 10 MDR isolates, only three (30%) isolates were found positive for *aacA-aphD* gene (all in individual cow milk). Similarly, out of the 10 MDR isolates, 7 (70%) were found positive for *ermC* which included four isolates from pooled milk, one from vendors milk and two from individual cow milk. While, among the 10 MDR isolates, four isolates (40%) was found to be positive for *mecA* (three in pooled milk and one in vendors milk). Thus, the high prevalence of multidrug resistant *S. aureus* isolates is a matter of concern for the public health. So, the antibiotics should be used judiciously in animal husbandry practice to prevent the emergence of antibiotic resistant bacterial strains.

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