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Detection of virulence genes of *Staphylococcus aureus* in raw cow milk from Aizawl district of Mizoram, India

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Abstract
The present study aimed the molecular detection and antibiotic sensitivity of *Staphylococcus aureus* in raw cow milk samples collected from Aizawl district of Mizoram. A total of 90 numbers of raw milk samples were collected randomly from different sources. *Staphylococcus aureus* has been presumptively identified using conventional bacteriological technique and confirmed by molecular detection of species specific *nuc* gene. The antibiotic sensitivity was studied against 20 numbers of antibiotics commonly used in animals and humans. Based on conventional and molecular detection, 39(43.33%) milk samples were found to be contaminated with *S. aureus* out of which 21(53.84%) numbers were found to be positive for staphylococcal enterotoxin gene A (*sea*) and 2(5.12%) were positive for methicillin resistance gene (*mecA*). The *S. aureus* strains were 100% sensitive to amoxyclav and highest resistant to ampicillin and muprirocin (66.67%).

Keywords: Cow milk, *Staphylococcus aureus*, enterotoxin gene, methicillin resistance gene

Introduction
Frequent outbreaks of food borne diseases both in developed and developing countries have attracted extensive attention for food safety and hygiene globally. India is the leading and one of the most economic milk and meat producers in the world. According to Department of Animal Husbandry, Dairy and Fishery (DAHD&F), Government of India [1], India's milk production has increased to 176.30 million tonnes in 2017-18 from 55.60 million tonnes in 1991-92 to meet the increased demand of milk and milk products for 1,366,417,754 billion human population. The ever increasing demand of milk has resulted in intentional or unintentional break in the chain of hygienic production, storage, transportation and sale of raw milk and hence the food safety is being compromised in unorganized sector of milk production. As milk and dairy products are highly nutritive in nature, it serves as a good medium for growth of micro-organisms and so it is susceptible to microbial contamination especially under such production practices prevailing in many parts of India.

Staphylococcus aureus is the third most economically important cause of food borne infection in the world [20]. Staphylococcal food poisoning is caused due to the absorption of the preformed enterotoxins in the food. Besides food borne intoxication, *S aureus* is responsible for different disease conditions like furuncles, cellulitis, impetigo and postoperative wound infections in animals and human as the organism is a commensal and opportunistic pathogen occurring on the skin and mucous membranes of most warm blooded animals including humans. Further the organism has been reported to be associated with fatal bacteraemia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, cerebritis and meningitis. *Staphylococcus aureus* suitably grows at 37 °C with a wide range of temperature ranging between 6 – 48 °C with high range of pH tolerance between 4 –10, a salt concentration of 0 - 20%, water activity range of 0.85 - 0.99 and redox potential more or equal to 200 mV [10]. The enterotoxins (SEs) produced by the organism are the causative agents for staphylococcal food poisoning and these enterotoxins are heat stable and cause food borne intoxication resulting from ingestion of food contaminated with preformed toxins [4]. The five major classical types of staphylococcal enterotoxins are *sea, seb, sec, sed and see* [18].

Methicillin resistant *Staphylococcus aureus* (MRSA) has been found in livestock and it has

been linked to a jump from humans to animals [21]. The prevalence of livestock-associated MRSA (LA-MRSA) in farm animals is increasing gradually and the resulting food products may become contaminated, leading to its zoonotic occurrence in human.

As *S. aureus* is commensal and opportunistic organism found in skin and mucous membranes of all warm blood animal. There is every possibility of contamination during milking, milk handling and production of toxins in improperly heated milk. The studies on *S. aureus* as a foodborne pathogen are scanty in North-East regional states of India including Mizoram. Therefore keeping the above points in view, the present study aimed the molecular detection and antibiotic sensitivity of *S. aureus* in raw cow milk samples collected from Aizawl district of Mizoram.

Materials and Methods

Collection of milk samples

A total of 90 number raw cow milk samples were collected randomly for isolation of *S. aureus* from different unorganized farms/ milk vendors/ shops by following aseptic measures at periodic intervals for a period of one year from July, 2018 to June, 2019 in Aizawl district of Mizoram, India (Fig-1)

Fig 1: Source of milk sample collection

Isolation and identification of *Staphylococcus aureus*

Isolation and identification of *S. aureus* from raw milk was done as per the standard methodology [9] with slight modification. One ml milk sample was mixed with 9ml sterile 0.1% peptone water for enrichment and was centrifuged at 8000 rpm for 2-3 minutes for extrication of the sample in the broth and incubated for 24 hours at 37 °C. A drop of approximately 0.1 ml of broth culture showing cloudy discolouration was streaked aseptically on Baired Parker agar (BPA) plates and incubated at 37 °C for 24 - 48 hours. The suspected colonies on BPA plate were streaked on Mannitol Salt agar (MSA) plate and incubated at 37 °C for 24 hours. The *S. aureus* were phenotypically characterized based on morphological characteristics (colonies of jet black colour with halo zone on BPA) and (yellow colonies with yellow colouration of media on MSA). It is gram positive in reaction, catalase positive and coagulase positive [22].

Molecular detection of *S. aureus* strains by polymerase chain reaction

All the phenotypically positive *S. aureus* isolates were subjected for detection of species specific *nuc* gene by using published primers (F- GCG ATT GAT GGT GAT ACG GTT; R- AGC CAA GCC TTG ACG AAC TAA AGC). The *S. aureus* isolates that were confirmed by *nuc* gene were further screened for *sea* (Staphylococcal enterotoxin A) (F- TAA GGA GGT GGT GCC TAT GG and R - CAT CGA AAC CAG CCA AAG TT) and *mecA* (methicillin resistant *S. aureus* (F- GTA GAA ATG ACT GAA CGT CCG ATA A) and R- CCA ATT CCA CAT TGT TTC GGT CTA A) genes by PCR using published primers. The thermal cycling conditions for amplification of different genes are presented in Table 1.

Table 1: Thermal cycling conditions for different virulence genes of *S. aureus*

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Base Pair	References
nuc	95 – 5 mint	95 – 45 sec	58 –45 sec	72- 45 sec	72– 5mint	279	Brakstad *et al*.,[6]
mecA	95 – 5 mint	95 – 45 sec	53.1–45 sec	72- 45 sec	72– 5mint	310	Cremonesi *et al*.,[11]
sea	94 – 5 mint	94 – 1 mint	58 –1 mint	72- 1 mint	72– 5mint	180	McClure *et al*.,[15]

Detection of antibiotic sensitivity and resistance pattern of *S. aureus* strains

All the *nuc* gene positive *S. aureus* strains were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method [2] on Muller Hinton Agar plates against a panel of 20 antibiotics namely Penicillin G (10 unit), Methicillin (30mcg), Novobiocin (5mcg), Vancomycin (30mcg), Oxytetracyclin (30mcg), Muprirocin (200mcg), Meropenem (10mcg), Tigecycline (15mcg), Minocycline (30mcg), Amox-clav (30mcg), Ampicillin (10mcg), Oxacillin (1mcg), Levofloxacin (5mcg), Linezolid (30mcg), Pip-Taz (100/10 mcg), Cotrimoxazole (25mcg), Cefotoxin (5mcg), Ceftriaxone (30mcg), Ciprofloxacin (5mcg), Cefazolin (30mcg) as per Clinical and Laboratory Standard Institute [8] guidelines. The diameter of zone of inhibition was compared with the standard known value against each specific antimicrobial agent from interpretation guide line (Hi-Media).

Results and Discussion

Detection of *S. aureus* from raw milk

Out of 90samples of raw cow milk from Aizawl district of Mizoram, a total 70 (77.77%) samples were found to be positive for *S. aureus* by the cultural method in which the isolates showed characteristic colour of colonies on different agars such as jet black with halo zone on BPA agar and yellow colonies with yellow colouration of media on MSA, positive Gram staining reaction and positive catalase and coagulase tests [7]. However, 39(43.33%) numbers of bacteriologically positive isolates were confirmed as *S. aureus* on molecular detection of *nuc* gene. From different parts of India, the *S. aureus* was detected in a variable range on detection of *nuc* gene by Sukumar *et al*.,[28] (73.53%), Hamid *et al.* [13] (21. 21%), Begum *et al*.,[3] (92. 31%) and Bhati *et al.*, [5] (63. 80%) from Tirupati, Jammu & Kashmir, Chennai and Rajasthan, respectively in raw milk. The *nuc* gene is a species specific marker of *S. aureus* Ruban *et al.*, [24]. The *nuc* positive 39 isolates were screened for *sea* and *mecA* of which 21(53.84%) and 2(5.12%) were found to be positive, respectively. Sharma *et al.*, [26] detected *sea* gene in 10% of bovine raw milk from North West India. However, the *mecA* gene was recorded comparatively higher in raw milk from different parts of India than the present findings such as Hamid *et al.*, [13] (16.60%), Ridwana *et al.*, [23] (60.00%) and Begum *et al.* [3] (75.00%) from Jammu & Kashmir, Srinagar and Chennai, respectively. Although different virulence encoding genes are detected in *S. aureus*, the present study

focused on only a small subset of virulence genes. Assays for genes encoding thermonuclease (*nuc*) (Fig:2), methicillin resistant penicillin binding protein (*mecA*) (Fig:2) and staphylococcal enterotoxin A (*sea*) (Fig:3) were included in the present study. Enterotoxigenic *sea* gene is most commonly reported in *S. aureus* isolates obtained from different types of foods Normanno *et al.*, [17]. The human ailments associated with livestock origin MRSA has been significantly increasing from 0% in 2002 to 35% in 2009, Duquette and Nuttall [12] and hence the *mecA gene* was examined in the present study to evaluate the presence of MRSA in bovine raw milk and milk products Kuhl *et al.*, [14].

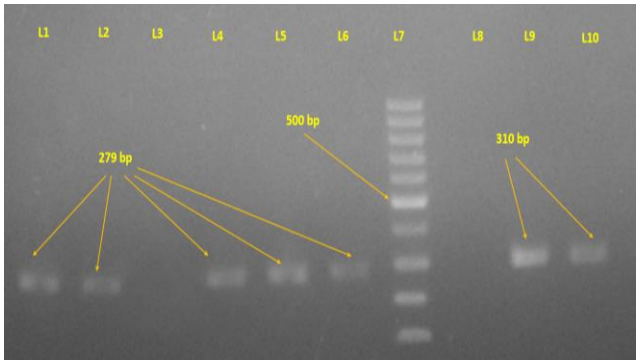

Fig-2: Left lanes of ladder (Lane 7) are *nuc* (279 bp) positive isolates, Lane 3 (negative control), Lane 4 (positive control); and right lanes of ladder are *mecA* (310 bp) positive, Lane 8 (negative control), Lane 9 (positive control)

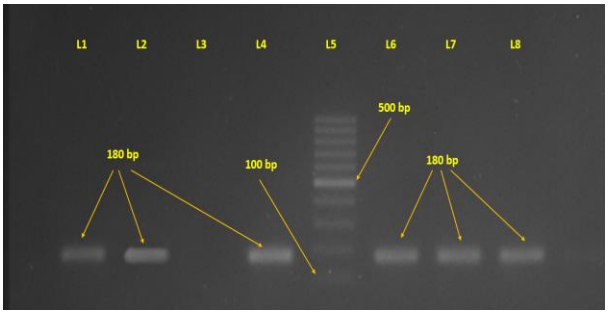

Fig 3: Lane 3 (negative control), Lane 6 (positive control) for *sea* (180bp), Ladder (Lane 5)

Antibiotic susceptibility of *S. aureus* from raw milk

The antibiotic sensitivity and resistance pattern of the 39 strains of *S. aureus* are presented in Table 2. The strains were found to be highest sensitive to amoxyclav (100%) followed bylinezolid, meropenem and tigecycline in (87.17%); cefazolin (82.05%), ceftriaxone (79.48%), pip-taz and oxytetracycline (76.92%), levofloxacin and minocycline (71.70%), ciprofloxacin (69.23%), cotrimoxazole (66.66%), vancomycin (58.97%), penicillin and oxacillin (38. 46%) and ampicillin (33.33%). Mupirocin (33.33%), methicillin and novobiocin (43.58%) were found to be intermediately sensitive (Table-2, Fig-4). In different studies across the country, ampicillin was found to be resistant to *S. aureus* from raw milk as reported by Thaker *et al.*, [29] (40.00%), Sharma and Brinty [25] (50.00%), Sudhanthiramani *et al.*,[27] (74. 42%), Hamid *et al.*,[13] (83. 30%) and Patel *et al.*,[19] (66.66%) from Assam, Gujarat, Tirupati, Jammu and Kashmir and Gujarat, respectively. In some other studies, pencillin was found to be highly resistant to *S. aureus* from raw milk as reported by Thaker *et al.*,[29] (100%), Sharma and Brinty [25] (76.78%), Mohanta *et al.*,[16] (87.50%), Sudhanthiramani *et al.*,[27] (86.04%) and Hamid *et al.*,[13] (94.40%) from different parts of India.

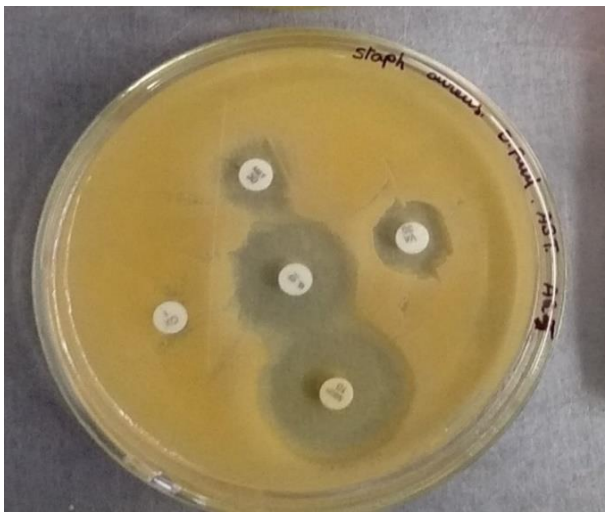

Fig 4: Antibiotic sensitivity pattern *S. aureus* in Muller Hinton Agar

Table 2: Antibiotic Susceptibility of *S. aureus* from raw milk

Sl No.	Antibiotics	No. of isolate	Sensitive (%)	Resistance (%)	Intermediate (%)
1	Amoxiclav (AMC)	39	100.00	0	0
2	Linezolid (LZ)	39	87.17	12.82	0
3	Mereponem (MRP)	39	87.17	12.82	0
4	Tigecilin (TGC)	39	87.17	12.82	0
5	Cefazolin (CZ)	39	82.05	5.12	12.82
6	Ceftriaxone (CTR)	39	79.48	12.82	7.69
7	Pip-Taz (PIT)	39	76.92	12.82	10.25
8	Oxytetracyclin (O)	39	76.92	23.70	0
9	Levofloxacin (LE)	39	71.70	17.94	10.25
10	Minocyclin (MI)	39	71.70	28.30	0
11	Ciprofloxacin (CIP)	39	69.23	17.94	12.82
12	Cotrimoxazole (COT)	39	66.66	33.33	0
13	Vancomycin (VA)	39	58.97	23.90	17.94
14	Penicillin-G (P)	39	38.46	61.53	0
15	Oxacillin (OX)	39	38.46	61.53	0
16	Ampicillin (AMP)	39	33.33	66.66	0
17	Methicillin (MET)	39	0	56.44	43.58
18	Novobiocin (NV)	39	0	56.41	43.58
19	Muprirocin (MUP)	39	0	66.66	33.33
20	Cefotaxin (CTX)	39	7.69	79.48	12.82

The difference in antibiotic sensitivity of *S. aureus* found in various studies indicates that antibiotic resistance pattern of *S. aureus is* changing. Staphylococcus aureus is developing resistance to different antibiotics day by day by adopting different mechanisms. Isolation of antibiotic resistant *S. aureus* from milk samples against these antimicrobial drugs poses a major challenge of human medicine in management of infections as these drugs are commonly used in the treatment of human. The indiscriminate use of antibiotics against S. aureus may lead to further increase in antibiotic resistant *S. aureus*.

Conclusion

The present study indicated the high prevalence of *S. aureus*, a major zoonotic pathogen causing food poisoning in human, in different samples of raw bovine milk from Aizawl district of Mizoram. The presence of the organism in raw milk collected for further processing may be a source of staphylococcal food poisoning when collected, transported and processed under uncooled and unhygienic condition which is an alarming public health threat to the consumers.

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