Panton-Valentine leukocidin (PVL) positive methicillin resistant *Staphylococcus aureus* (MRSA) in raw milk in Punjab

AMANDEEP¹, RANDHIR SINGH², SIMRANPREET KAUR³ and J P S GILL⁴

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

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ABSTRACT

The emergence of methicillin resistant *Staphylococcus aureus* (MRSA) as a foodborne pathogen has posed a serious public health issue. Milk is extensively consumed food worldwide. The aim of this study was to estimate the presence of MRSA in raw milk in Punjab, by culture and polymerase chain reaction (PCR) techniques. Raw milk samples (291) were collected from all over Punjab. These 291 samples comprised 204 and 87 cow and buffalo milk samples respectively. The *S. aureus* was isolated on Baird Parker agar and typical colonies were subjected to biochemical tests and PCR for genus (16S rDNA) and species (nuc) specific detection. All the *S. aureus* isolates were further subjected to susceptibility testing to oxacillin and detection of mecA gene for methicillin resistance.

Out of 291 samples, 42 (14.43%) showed contamination of *S. aureus*. Prevalence of *S. aureus* contamination was higher in cow milk (15.68%) than buffalo milk (11.49%). Only, three (1.03%) samples (one from buffalo and two from cow) were positive for MRSA. On SCC mec typing, all the MRSA positive isolates belonged to SCC mec type V, which is characteristic of Community Associated-MRSA (CA-MRSA). One isolate was also positive for *pvl* gene. PFGE showed that all the four isolates were closely related to each other. The results show that MRSA are present in food of animal origin and has potential to spread through it.

Key words: Milk, MRSA, PCR, pvl, SCC mec, *Staphylococcus aureus*

*Staphylococcus aureus* is widely distributed in nature and normal inhabitants of skin and mucous membrane of humans and animals. *S. aureus* is a major foodborne pathogen. Consumption of *S. aureus* contaminated food results in gastroenteritis due to preformed enterotoxins in the food. Unhygienic handling of food is considered as a major risk of contamination, and staphylococcal food poisoning is often associated with manually handled food (Viktoria *et al.* 2001). *S. aureus* is also a major causative pathogen of clinical and subclinical mastitis of dairy domestic ruminants (Loir *et al.* 2003). It can gain access to milk either by direct excretion from udders with clinical or subclinical mastitis or by cross contamination and raw milk processing (Scherrer *et al.* 2004, Jorgensen *et al.* 2005). Its presence in raw milk is a major concern for the safety issue with a potential to cause serious public health problems.

Extensive therapeutic use of antimicrobials in food animal production has resulted in emergence of resistance among animal associated microorganisms (Normanno *et al.* 2007). Resistance to methicillin is of particular relevance because it is conferred by the presence of mecA and such staphylococci are resistant to broad range of important antimicrobials (Kwon *et al.* 2006).

Methicillin resistant *S. aureus* (MRSA) is of public health and veterinary concern globally. Various studies in Punjab reported high prevalence of MRSA infection in hospitals (Jindal *et al.* 2016). Hospital Associated-MRSA (HA-MRSA) typically harbours the SCC mec type I, II or III gene. However, Community Associated-MRSA (CA-MRSA) carry SCC mec type IV or V gene responsible for survival fitness in community settings (Loo *et al.* 2007). CA-MRSA can spread easily in community settings causing serious infections (Lina *et al.* 1999). MRSA had been reported from most food-producing animals and food of animal origin worldwide. Isolation of MRSA from animals was first reported in 1972 following its detection in milk from mastitic cows (Devriese *et al.* 1972).

The transmission of MRSA from animals to humans or back is possible and may contribute to outbreaks in animal and human population (Lee 2003). So far, limited information is available on the prevalence of MRSA in raw milk from dairy herds in Punjab. Therefore the objective of this study was to isolate, identify and characterise MRSA from raw milk in Punjab.

MATERIALS AND METHODS

Sample collection: Raw milk samples (291) were collected from all over Punjab from December 2014 to July 2015.
2015. These 291 samples comprised 204 and 87 cow and buffalo milk samples respectively. All the samples were collected in sterile 50 ml plastic centrifuge tubes, transported at 4°C to the laboratory and processed for isolation on the same day.

**Isolation of S. aureus and its biochemical confirmation:**
About 10 ml of milk sample was mixed with 90 ml buffered peptone water (BPW) and was incubated overnight at 37°C for enrichment. A loopful of enriched milk samples were streaked on the surface of Baird-Parker agar (BPA) supplemented with egg-yolk tellurite emulsion (HiMedia, Mumbai) and incubated at 37°C for 24–48 h. Characteristic appearance of jet black colonies surrounded by a white halo zone considered to be presumptive *S. aureus* (Muelherr et al. 2003). These colonies were further streaked on to a fresh BPA plate and kept for overnight incubation at 37°C. This was followed by another step of purification on TSA plate with same incubation condition. Characteristic colonies were identified by conventional methods, including Gram stain and catalase test as per standard protocol and were subjected to biochemical tests by using HiStaph™ identification kits. Confirmed *S. aureus* isolates were stored at –80°C in TSB containing 20% v/v glycerol.

**DNA isolation:** Genomic DNA was extracted by using snap chill method. *S. aureus* isolates from glycerol stock were streaked on TSA plates for overnight incubation. After incubation, 2–3 colonies were inoculated in 1.5 ml nuclease free water (NFW) and kept for boiling at 100°C for 10 min. Immediately after boiling, cold shock was given by keeping it in ice for 10–15 min. After cold shock, centrifugation was done at 10,000 rpm for 10 min. The supernatant which contained the DNA was aliquoted in a sterile tube and stored at –20°C until further use.

**Detection of 16S rDNA (genus specific), nuc (species specific) and mecA (methicillin resistant) genes by PCR:**
The reaction was carried as per protocol described by Zehra et al. (2017). The sequence of primers used in the procedure are mentioned in Table 1.

**Disc diffusion test:** All the isolates were also screened for susceptibility to antibiotic oxacillin (1 µ disc, Himedia) through disc diffusion method (Bauer-Kirby) for determining phenotypic resistance to methicillin. *S. aureus* strain ATCC 25923 was used as a control strain for AST. The test was performed by applying bacterial inoculums (ca. 1.5×10⁸ CFU/ml or culture turbidity of 0.5 McFarland standard) on the surface of a Muller Hinton agar plates. The antimicrobial disc was placed on the inoculated agar surface within 15 min of inoculation. The plates were incubated for 24 h at 35°C. The zone of inhibition around the oxacillin disc was measured to the nearest millimeter and interpreted as per the manufacturer cut off criteria.

**SCC mec typing and subtyping of MRSA isolates:**
The multiplex PCR for typing and subtyping of *SCC mec* of MRSA isolates was done as per method described by Zehra (2014). *S. aureus* strain with accession no. KR610412 (*SCC mec* type IVa), KT005393 (*SCC mec* type V) and ATCC 335919 (*SCC mec* type II) were used as positive control. The details of primer sequences used are shown in Table 1. The amplification conditions were initial denaturation step at 94°C for 5 min followed by 10 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 45 sec, followed by final extension at 72°C for 5 min.

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**Table 1. Primers used for detection of various genes in S. aureus**

| Gene | Oligonucleotide sequence (5’-3’) | Amplicon size | Reference |
|------|---------------------------------|--------------|-----------|
| 16S rDNA | CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG | 420 | Strommenger et al. (2003) |
| mecA | AAA ATC GAT GGT AAA GGT TGG C AGT TCT GCA GTA CCG GAT TTG C | 532 | |
| Nuc | GCGATTTGATGTGATACGGTT AGGCAAGGCTTTGACGAACACTAAAGCG | 279 | |
| SCC mec I | GCCTTAAGAGGTGTCGTTACAGG GTTCTCTCATAGTAGCAGCTGTC | 613 | Brakstad et al. (1992) |
| SCC mec II | CGTGTGAAGATGATGAAAGCG CGAATACTGGTATTGCAGCC | 398 | Zhang et al. (2005) |
| SCC mec III | CCATATTGTGTACGATGCC CGAATACTGGTATTGCAGCC | 280 | |
| SCC mec IVa | GCCATTATCGAAGAAACCCGC TACCTCTTCTAAAGGCGTGCT | 776 | |
| SCC mec IVb | TCTGGGAATAGTACGACGACTGCA ACAATATTGTATTCCCTTACCA | 493 | |
| SCC mec IVc | ACAATTATTGATTATTGCGGAGA GCTTTGATAGGATTTGCTGG | 200 | |
| SCC mec IVd | CTCAAAATACCGGACCCAAATA TCATACTTATACGAGATCG | 881 | Wang et al. (2012) |
| SCC mec V | GAACATTGTTACTTACGCTGCA GCCATCTTACGAGATCG | 325 | |
| Pvl | 433 | |
65°C for 45 sec, and 72°C for 1.5 min and another 25 cycles of 94°C for 45 se, 55°C for 45 sec, and 72°C for 1.5 min, ending with a final extension step at 72°C for 10 min and followed by a hold at 4°C.

Pulse field gel electrophoresis of MRSA: The genetic relatedness of *S. aureus* isolates was determined by pulse field gel electrophoresis (PFGE) using CDC PulseNet protocol for oxacillin resistant *S. aureus*. Genomic DNA was digested by restriction enzyme Smal. The digested DNA in plug was separated using a CHEF MAPPER (Bio-Rad Laboratories, Hercules, CA, USA) for 21 h by electrophoresis at 200 volts (6 v/cm) with the following conditions: temperature 14°C, initial switch time of 5 sec, final switch time of 40 sec and angle 120. A lambda ladder molecular weight marker (Bio-Rad Laboratories, USA) was included in each gel. After the run, gel was stained with ethidium bromide (0.5 mg/l) for 15 min and then destained for 45 min. The gel was visualized using Gel Documentation system (Syngene) and the results were analysed manually and interpretation was done as per criteria given by Tenover et al. (1995).

RESULTS AND DISCUSSION

MRSA: Out of 291 raw milk samples, 42 (14.4%) were positive for *S. aureus*, as confirmed biochemically and through PCR (Fig. 1). These positive samples comprised 32 (15.7%) and 10 (11.5%) cow and buffalo samples respectively (Table 2). In total, 80 *S. aureus* isolates were obtained from these 42 positive samples, out of which, 76 isolates were phenotypically resistant to oxacillin and only four carried mecA gene. These four mecA positive isolates were designated as MRSA (three from cow and one from buffalo). The overall MRSA positivity in raw milk samples was 1.03% (3/291) and for cow and buffalo milk it was 0.98% (2/204) and 1.1% (1/87), respectively.

*S. aureus* is an important organism that can result in food-borne intoxications. It has been reported as microbial contaminant in raw milk. Normanno *et al.* (2007) found 109 (17%) out of 641 raw milk samples contaminated with *S. aureus*. In another study, a higher level of contamination (48.8%) was reported from the milk samples (Daka *et al.* 2012). Milk samples with lower levels of *S. aureus* had also been reported. Fagundes *et al.* (2010) found 14 (6.7%) milk samples out of 208 from different regions of Brazil positive for *S. aureus*. The variable levels of *S. aureus* presence in milk are indicative of animal hygiene, milking conditions and even milker’s hygiene. This organism is commonly found on teat skin, other body parts as commensal and in different surroundings of animal thus predisposing lactating animals to mastitis (Gleeson *et al.* 2018 and Roberson *et al.* 1994). If the infection is in initial stages, *S. aureus* in apparently normal milk (sub-clinical mastitis) finds its way into the milk intended for human consumption. So, improper washing of the teats/udder, contamination from environment and sub-clinical mastitis are the major factors responsible for *S. aureus* contamination of milk. In addition to that, anterior nares and hands of milkers, animal handlers or food handler are also potent source of *S. aureus* contamination of milk (Adesiyun *et al.* 1997). So, even if animal teats and skin are clean, but infected/crrier milkers or food handlers are around they can still add to the contamination in the production chain.

Antimicrobial resistance in food-borne *S. aureus* is known. They are capable of acquiring resistance to multiple classes of antibiotics and can result in serious life threatening infections. *S. aureus*, resistance to oxacillin/methicillin (MRSA) is a serious public health concern as such resistant isolates on transfer to human can cause rigid infections which are difficult to treat with commonly used antibiotics (Petinaki and Spiliopoulou 2012). There are several reports which have documented presence of MRSA in raw milk (Vanderhaeghen *et al.* 2010, Mistry *et al.* 2016). In one study, low prevalence (0.93%; 11/118) of MRSA close to the level reported in present study was recorded from raw milk samples (Vanderhaeghen *et al.* 2010). Similarly, in another such study, MRSA prevalence of 3% was reported from dairy production chain in Greece (Papadopoulos *et al.* 2018). Contrary to these observations, there are studies which have found even higher levels of MRSA in raw milk samples. Aqib *et al.* (2017) examined both cow and buffalo raw milk samples in Pakistan and found 30 and 38% prevalence of MRSA, respectively. Even higher prevalence of MRSA to the level of 56.1% in *S. aureus* isolates was reported from raw milk and milk products in Uganda. In present study, although 76 isolates showed phenotypic resistance to oxacillin but only four isolates carried mecA and were designated as MRSA. Martineau *et al.* (2000) also reported presence of isolates that were phenotypically oxacillin resistant but negative for mecA gene. Likewise, Pereira *et al.* (2009) reported 38% of *S. aureus* strains resistant to oxacillin but only 0.68% of the isolates showed presence of mecA gene. Such type of antibiotic resistance pattern, where isolates are phenotypically resistant to oxacillin but lacked mecA gene.

| Species | No. of samples | No. of positive samples | No. of MRSA isolates |
|---------|----------------|-------------------------|---------------------|
| Cow     | 204            | 32 (15.7%)              | 64                  |
| Buffalo | 87             | 10 (11.5%)              | 16                  |
| Total   | 291            | 42 (14.4%)              | 80                  |

Table 2. Prevalence of *S. aureus* in milk samples from cow and buffalo

- Fig. 1. Gel electrophoresis picture of 16SrDNA (420 bp), nuc (279 bp) and mecA (532 bp) gene.
may be due to different reasons, such as hyperproduction of β-lactamases, production of normal PBP (penicillin binding protein) with altered binding capacity or variant of meca gene (Martineau et al. 2000, Wyke et al. 1982, Laurent et al. 2012). Besides presence of S. aureus and MRSA in raw milk samples their presence in milk from clinical mastitis cases had also been observed (Ahangari et al. 2017, Mistry et al. 2016). In one of the studies done in India, that too on mastitic milk, 48.7% (19/39) of the isolates were MRSA (Mistry et al. 2016). Whereas, Ahangari et al. (2017) found only 1.3% of S. aureus isolates were MRSA. These studies again showed variability in the association of MRSA and mastitis. Improper use of antibiotics during farm management practices is the important driver of antibiotic resistant in S. aureus leading to resistance to methicillin/oxacillin and presence of such isolates (MRSA) in raw milk.

In present study although we did not examine our isolates for the enterotoxigenicity or its determinants, but the presence of these virulent factors in S. aureus isolates along with resistance to methicillin is all the more alarming. In one such study, Saka and Gulel (2018) found S. aureus isolates which were not only methicillin resistant but also carried enterotoxin genes. Consumption of food items laden with such combination is a serious public health concern, because not only it can result in food poisoning but also introduces life threatening resistant organisms in the consumers.

**SCC mec typing:** The four MRSA isolates were further typed for SCC mec complex and were found to carrying SCC mec type V (Table 3, Fig. 2) and one isolate even carried pvl gene (Table 3, Fig. 3). Based on the results, presence of meca, SCC mec V and pvl genes in one MRSA isolate, it could potentially be designated as CA-MRSA. Such MRSA isolates from raw milk carrying pvl genes is even more threatening as such combination is rarely reported in animal associated MRSA (Haran et al. 2012). The pvl positive MRSA (CA-MRSA) isolates are frequently reported from community settings (Krishnamurthy et al. 2014). However their presence in milk (food of animal origin) in present study is a possible indicator of contamination of raw milk from human handlers and poses risk of its further transfer to other susceptible human through the food chain. Such isolates on transfer to susceptible human can colonize and turn opportunistic to cause minor skin infection to necrotizing pneumonia (Ebert et al. 2009). Thus, food of animal origin is a potent source of MRSA and can get contaminated from animal source or animal handlers.

| Species | Sample | SCC mec type | Pvl gene |
|---------|--------|--------------|----------|
| Cow     | Milk   | V            | +        |
| Cow     | Milk   | V            | -        |
| Cow     | Milk   | V            | -        |
| Buffalo | Milk   | V            | -        |

**PFGE of MRSA:** Based on the banding pattern of all the four MRSA isolates, they could be divided into two clusters with two pulsortypes. All the three MRSA isolates from cow were indistinguishable from each other with similar bands pattern were therefore grouped into one pulsortype, whereas one buffalo isolate belonged to other pulsortype (Fig. 4). The banding pattern obtained in one buffalo MRSA isolate differed by 3–4 bands from the three cows isolates (Fig. 4). As per the criteria given by Tenover et al. (1995) for interpreting PFGE pattern, these three cow MRSA isolates and one buffalo MRSA isolate were considered closely related to each other. As the results showed that there was not much difference between the cow and buffalo MRSA isolates, therefore, it was difficult to make any broad conclusion on the genetic relatedness of MRSA in raw milk, and also the numbers of MRSA isolates were less in the
present study. However, in some studies based on the PFGE pattern it was possible to figure out the source of MRSA in milk (Papadopoulos et al. 2018). Papadopoulos et al. (2018) in their study found several points in the milk production chain were contaminated with MRSA of same pulsotypes. It was concluded that these contaminated points along the production chain which included human handlers and equipment were responsible for contamination of raw milk and milk products thereby posing health risk to consumers.

In another study done in Italy, high genetic correlation was found between the PFGE pattern of MRSA isolates from cow and human suggesting possible transfer of MRSA strains between human and animals (Alba et al. 2015).

The present study highlights the importance of raw milk as an important source of MRSA to community. Thus hygienic husbandry practices are required to reduce the bacterial contamination of raw milk to prevent further transfer of MRSA to susceptible human.

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