



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

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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 (Viktoría *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

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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* (Muehlherr *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. 1.5×10^8 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,

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

Gene	Oligonucleotide sequence (5'-3')	Amplicon size	Reference
<i>16S rDNA</i>	CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG	420	Strommenger <i>et al.</i> (2003)
<i>mecA</i>	AAA ATC GAT GGT AAA GGT TGG C AGT TCT GCA GTA CCG GAT TTG C	532	
<i>Nuc</i>	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAATAAGC	279	Brakstad <i>et al.</i> (1992)
<i>SCC mec I</i>	GCTTTAAAGAGTGTCTGTTACAGG GTTCTCTCATAGTATGACGTCC	613	Zhang <i>et al.</i> (2005)
<i>SCC mec II</i>	CGTTGAAGATGATGAAGCG CGAAATCAATGGTTAATGGACC	398	
<i>SCC mec III</i>	CCATATTGTGTACGATGCGCC TTAGTTGTCTGTAACAGATCG	280	
<i>SCC mec IVa</i>	GCCTTATTCTGAAGAAACCGC TACTCTTCTGAAAAGCGTCG	776	
<i>SCC mec IVb</i>	TCTGGAATTACTTCAGCTGCAA ACAATATTGCTCTCCCTC	493	
<i>SCC mec IVc</i>	ACAATATTTGTATTATCGGAGA GCTTGGTATGAGGTATTGCTGG	200	
<i>SCC mec IVd</i>	CTCAAAATACGGACCCCAATA CATGCTCCAGTAATTGCTAAAG	881	
<i>SCC mec V</i>	GAACATTGTTACTTAAATGAGC GTGAAAGTTGTACCCTTGACACC	325	
<i>Pvl</i>	ATCATTAGGTAAAATGTCTGGACATGATC CAGCATCAAGTGTATTGGATAGCAAAGC	433	Wang <i>et al.</i> (2012)

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 *Sma*I. 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 sample 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

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

Species	No. of samples	Samples positive for <i>S. aureus</i>	No. of <i>S. aureus</i> isolates	Samples positive for MRSA	No of MRSA positive isolates
Cow	204	32 (15.7%)	64	2 (0.98%)	3
Buffalo	87	10 (11.5%)	16	1 (1.1%)	1
Total	291	42 (14.4%)	80	3 (1.03%)	4

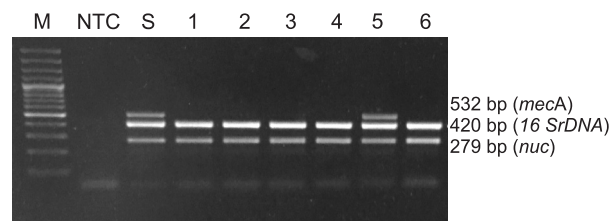


Fig. 1. Gel electrophoresis picture of 16SrDNA (420 bp), nuc (279 bp) and *mecA* (532 bp) gene.

commonly found on teat skin, other body parts as commensal and in different surroundings of animal thus predisposing lactating animals to mastitis (Gleenson 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 handlers 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/carrier 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

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 resistance 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.

Table 3. SCC *mec* typing of MRSA

Species	Sample	SCC <i>mec</i> type	<i>Pvl</i> gene
Cow	Milk	V	+
Cow	Milk	V	-
Cow	Milk	V	-
Buffalo	Milk	V	-

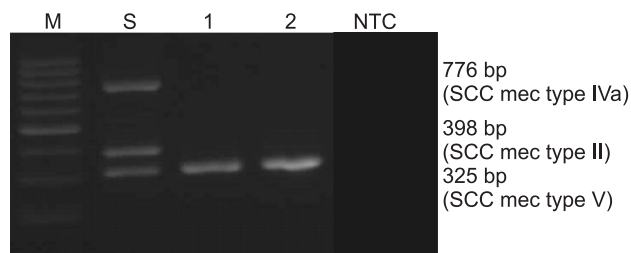


Fig. 2. Gel electrophoresis picture of SCC *mec* typing, SCC *mec* type II (398 bp), SCC *mec* type IVa (776 bp) and SCC *mec* type V (325 bp) gene.

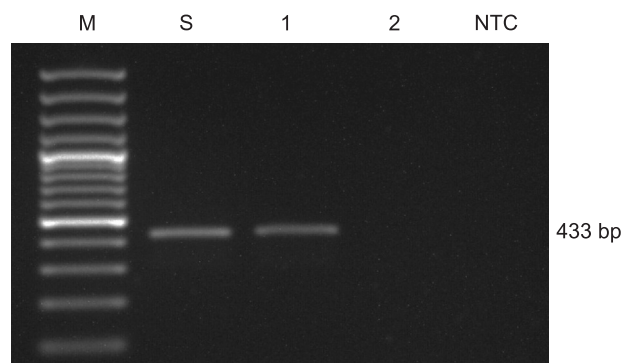


Fig. 3. Gel electrophoresis picture of *pvl* (433 bp).

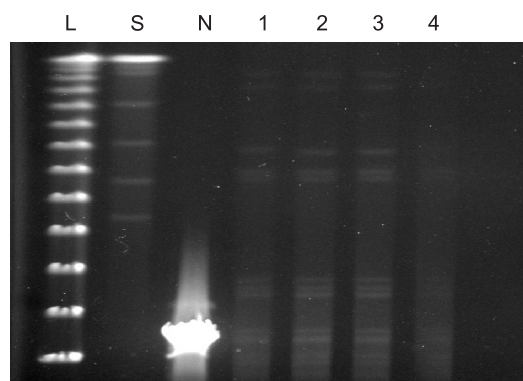


Fig. 4. Pulse field gel electrophoresis of MRSA isolates from raw milk.

PFGE of MRSA: Based on the banding pattern of all the four MRSA isolates, they could be divided into two clusters with two pulsotypes. All the three MRSA isolates from cow were indistinguishable from each other with similar bands pattern were therefore grouped into one pulsotype, whereas one buffalo isolate belonged to other pulsotype (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 PGFE 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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