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Genotypic insights to Panton–Valentine leukocidin positive methicillin-resistant *Staphylococcus aureus* isolated from cattle mastitis

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Research Article

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Abstract

In this research communication we investigate the prevalence and antimicrobial susceptibility of *S. aureus* harboring virulent genes responsible for mastitis in cattle of Punjab, Pakistan. A total of 690 milk samples were collected from commercial dairy farms for analysis of the prevalence of subclinical and clinical mastitis and isolation of *S. aureus*. Virulence ability and methicillin resistance in *S. aureus* (MRSA) was determined by targeting the *pvl* (the gene for Panton–Valentine leukocidin) and *mec*A genes, respectively. A total of 175 *S. aureus* isolates exhibiting prevalence of *pvl* gene (6.28%) and *mec*A gene (22.28%) were determined. Antimicrobial susceptibility testing of *pvl* positive and negative MRSA against different classes of antibiotics revealed 100% resistance against β-lactams while 100% sensitivity towards tylosin and linezolid.

Mastitis, particularly caused by *Staphylococcus aureus* is considered to be one of the most prevalent infectious maladies affecting the dairy industry which leads to severe financial losses in terms of decreased milk yield and quality (Muzammil *et al.*, 2022). The emergence of antibiotic resistance in *S. aureus* has led to limited treatment options and number of resistant strains like methicillin-resistant *S. aureus* (MRSA) has evolved at the animal-human interface (Muzammil *et al.*, 2022). *S. aureus* can produce more than one leukotoxin including Panton–Valentine leukocidin (PVL) that inhibits the immune response, allowing the microorganisms to survive and the inflammatory process to continue in soft tissues (and elsewhere). PVL toxin has been found in the isolates from methicillin-resistant and methicillin-sensitive (MSSA) *S. aureus* of various habitats which include humans (clinical or community-associated isolates), farm animals and animal-derived products (Abboud *et al.*, 2021). This study was planned to estimate the prevalence and antimicrobial susceptibility of *S. aureus* harboring virulent genes and methicillin resistance genes responsible for mastitis in cattle of Punjab province, Pakistan.

Materials and methods

A total of 690 bovine milk samples from smallholder ($n = 149$), commercial ($n = 284$) and corporate ($n = 257$) dairy farms (49 farms in total) in the province of Punjab, Pakistan were collected aseptically to detect subclinical and clinical mastitis. Standardized microbiological procedures were carried out for the isolation and confirmation of *S. aureus* isolates. The phenotypic and genotypic confirmation of methicillin resistance was confirmed by guidelines of (Muzammil *et al.*, 2022). The presence of the *pvl* gene in all confirmed *S. aureus* isolates was done using the following primer for PVL gene (luk-PV-1, ATCATTAGGTAAAATG TCTGGACATGATCCA and, for luk-PV-2, GCATCAATGTATTGGATAGCAAAAGC: Shrivastava *et al.,* 2018). The processing of PCR products was done by multiple sequence alignment by BioEdit software through *Clustal W* multiple alignment to compare the sequence of our isolates with the other highly similar published sequences from different countries. A phylogenetic tree was then constructed *via* maximum likelihood method at 1000 replications bootstrapping technique on MEGA-X software. The sequence of isolates connected by the same internal nodes indicates more closeness as compared to sequences connected by different internal nodes. The *in vitro* susceptibility profiling and prevalence of *pvl* positive and negative MRSA against various antibiotics was also performed, as described by Muzammil *et al.* (2022).

Table 1. Gene pattern of *mecA* and *pvl* in *S. aureus* isolated from clinical and subclinical mastitis

Gene pattern	Cattle		
	Clinical mastitis total and (%), $n = 172$	Sub-clinical mastitis total and (%), $n = 518$	Total and (%) ($n = 690$)
S. aureus	67 (38.95)	108 (20.85)	175 (25.36)
mecA	11 (16.42)	28 (25.93)	39 (22.29)
Pvl	5 (7.46)	6 (5.56)	11 (6.29)
mecA + *Pvl*	3 (4.48)	5 (4.63)	8 (4.57)
None	54 (80.60)	79 (73.15)	133 (76.00)

Results

The data presented in Table 1 reveal an overall total of 175 (25.36%) *S. aureus* isolates from 690 milk samples (subclinical $n = 512$; clinical mastitis = 172). The prevalence of *mecA* and *pvl* gene in *S. aureus* isolates was recorded to be 22.29% and 6.29% respectively, while 133 isolates didn't contain either of these genes. We found the presence of both *mecA* and *pvl* gene in 8 isolates (4.57%), which we labeled as *pvl* positive MRSA. The phylogenetic tree analysis *via* maximum likelihood method at 1000 replication bootstrapping technique revealed that study sequences are more similar with each other as compared to other country's sequences. The bootstrap results showed that sequences from Egypt (FJ821791) and India (MK975993) are comparatively more related to our sequences than other published sequences. In particular, isolates from USA (EF571841), Turkey (FJ895585), Myanmar (MK902786) and China (AB678715) lie in different clades from our isolates whilst isolates from France (EU518770), UK (HM584704) and Japan (AB256039) form out-group showing least similarity with our data (Fig. 1).

All of the 8 *pvl* positive MRSA isolates (Group A) showed 100% resistance towards cefoxitin, oxacillin, and vancomycin while having complete susceptibility against gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, oxytetracycline, tylosin, and linezolid groups. In comparison, the *pvl* negative MRSA isolates (i.e. positive for mecA: Group B) also exhibited 100% resistance against cefoxitin and oxacillin but 100% susceptibility against gentamicin, tylosin, and linezolid (Table 2).

Discussion

The detected prevalence of subclinical and clinical mastitis was reported to be 75.07% and 24.93% in milk samples of cattle which differs from the earlier report of Javed *et al.* (2021). The increased occurrence of subclinical mastitis in dairy livestock is due to multiple predisposing factors like contamination during milking, improper housing and improper disease management of animal (Javed *et al.*, 2021) and in a similar vein the transmission of *S. aureus* between animals occurs with the use of contaminated milk utensils, improper milking hygiene and contaminated milker's hands (Schnitt and Tenhagen, 2020). Studies from Pakistan show that the most common agent causing mastitis is *Staphylococcus* spp. followed by *Streptococcus* spp. (Javed *et al.*, 2021) . The prevalence of *S. aureus* that we recovered from subclinical and clinical mastitis cases was 20.85 and 38.95% respectively, higher than the 15.2% prevalence reported by Shrestha *et al.* (2021).

Our antimicrobial susceptibility testing of *S. aureus* strains showed 100% resistance to cefoxitin and oxacillin, while 22.28% of *S. aureus* isolates were found *mecA* positive (only). This is much higher than the 6.9% reported by Shrestha *et al.* (2021). Methicillin resistance is linked with the existence of the *mecA* gene on the *S. aureus* chromosome, which is responsible for the synthesis of penicillin-binding protein (PBP2a: Muzammil *et al.*, 2022). We observed the presence of the *pvl* gene (alone) in 6.28% of *S. aureus* isolates.

The *pvl* gene has been identified in 10.5% of *S. aureus* isolates in India (Shrivastava *et al.*, 2018) and 41.5% of *S. aureus* isolates in China (Wang *et al.*, 2015). Conversely, some studies could not identify the *pvl* gene in any *S. aureus* isolates from cattle milk (Patel *et al.*, 2021). The *pvl* gene is known to be the most powerful *staphylococcal* leukotoxin that can resist bovine neutrophils (Algammal *et al.*, 2020). Hence, *pvl* may produce resistance by attacking the bovine polymorphonuclear cells and increase pathogenicity against the host (Hata *et al.*, 2010). Our study is the first to determine the occurrence of *mecA* and *pvl* gene in *S. aureus* isolates from cattle milk in Pakistan.

In conclusion, we have demonstrated the presence of *mecA* and *pvl* gene in 22.29% and 6.29% of *S. aureus* isolates, and the combination in 4.57% of milk samples from bovine mastitis in Punjab. The presence of Panton–Valentine leukocidin toxin and methicillin resistance in mastitis-associated *S. aureus* poses a zoonotic threat, requiring awareness and control measures. Antimicrobial susceptibility testing is crucial for effective treatment and to prevent antimicrobial resistance problems locally in Punjab and globally.

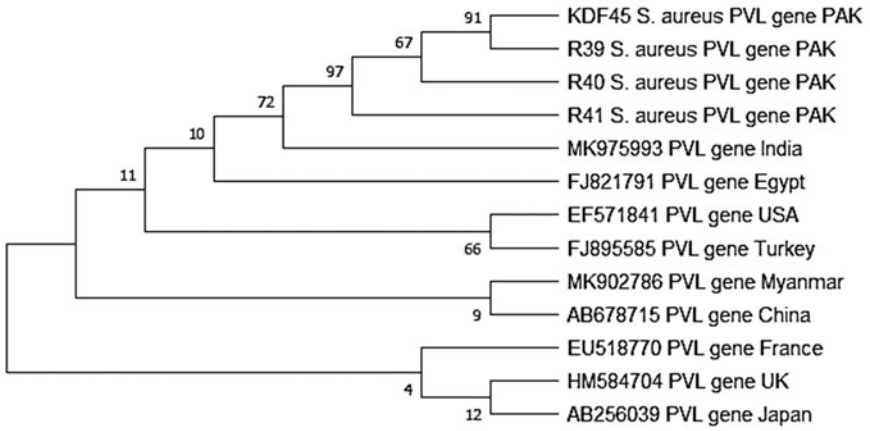

Figure. 1. Phylogenetic tree construction by 1000 bootstrapping replication indicating the relationship of local PVL isolates (PAK) with already reported sequences.

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Table 2. *In vitro* antibiogram profile of different antibiotic groups against *pvl* positive and negative MRSA

Antibiotic group	Antibiotics discs	Group A *pvl* positive MRSA (%)			Group B *pvl* negative MRSA (%)		
		R	I	S	R	I	S
β-Lactam	Cefoxitin (30 μg)	100	–	–	100	–	–
	Oxacillin (1 μg)	100	–	–	100	–	–
Aminoglycosides	Gentamicin (10 μg)	–	–	100	–	–	100
	Amikacin (30 μg)	75	–	25	–	25	75
Quinolones	Ciprofloxacin (5 μg)	–	–	100	–	25	75
	Levofloxacin (5 μg)	–	–	100	25	–	75
	Moxifloxacin (5 μg)	–	–	100	25	–	75
Glycopeptide	Vancomycin	100	–	–	75	25	–
Tetracyclines	Oxytetracycline (30 μg)	–	–	100	25	25	50
Macrolides	Tylosin (30 μg)	–	–	100	–	–	100
Sulfonamides	Trimethoprim + sulfamethoxazole (1.25 μg + 23.75 μg)	75	25	–	25	–	75
Fusidanes	Fusidic acid (10 μg)	75	–	25	75	–	25
Oxazolidinones	Linezolid (30 μg)	–	–	100	–	–	100

R, resistant; I, intermediate; S, sensitive.

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