Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i7.7000

# METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AND DETECTION OF RESISTANT GENES IN COW MILK FROM SOUTHERN KHYBER PAKHTUNKHWA PAKISTAN

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**Abstract**

The present study was conducted on bovine mastitis in three tehsils of district Karak, Khyber Pakhtunkhwa Pakistan. A total of 124 cow milk samples were collected randomly and were screened for presence of subclinical mastitis through surf field mastitis test (SFMT) and polymerase chain reaction (PCR)and microbiological procedures to isolate *Staphylococcus aureus (S. aureus)*. Furthermore, the disc diffusion technique was applied, and the isolation of positive *S. aureus* was phenotypically assessed for methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA). The overall results showed that out of 124 milk samples, 16.93% (21/124) were found positive for subclinical mastitis on the Surf Field Mastitis Test (SFMT). Further, amongst these 21 positive samples, 8 (38.09%) samples were found positive for *S. aureus* when swabbed on Mannitol Salt Agar (MSA). Through PCR, the resistant genes were amplified and identified. In each of these 8 positive samples, genes were examined for *mecA, tetK, etA, etB, fbe, embP*, and *IS256*. Additionally, good results were found for *mecA, fbe, embP*, and *IS256*. The *S. aureus* isolates displayed 100% resistance to *Erythromycin*,followed by *Cefoxitin, Cefradin* and *Augmentin* (75%); *Ciprofloxacin*(62.5 %);*Gentamicin* (50%);*Clindamycin* (50%) followed by *Amikacin* and *Tigecycline*

who showed 25% resistance. *Vancomycin* and *Linezolid* were the only antibiotics that shown zero resistance and were discovered to be susceptible to study isolates.

**Key words:** Methicillin, *Staphylococcus aureus, mecA*, Subclinical mastitis, Genes, Pakistan

## 1. Introduction

Bovine mastitis is one of the many familiar and expensive disease in the dairy cow business. This disease's etiology generally involves three factors: microbe exposure, host defense systems, and environmental variables (Chishty, M. et al., 2007; Fayazi-Kia, M. T. et al., 2023). Little progress has been made in controlling environmental pathogens, which are regarded as an important opportunistic pathogen and have been connected to outbreaks of mastitis in dairy cows, even though contagious mastitis pathogens have been significantly controlled through improved milking hygiene (Satwik M., et al., 2023)

Agriculture is the largest and most significant sector of the Pakistani economy accounting for 23.3 percent of total GDP ( Bilal, M. et al., 2006; Khan et al., 2021) and livestock which contribute 51.1% is a subsector of agriculture in Pakistan. Pakistan is the world's fifth far-reaching milk producer due to its reliance on agriculture and bovine (cattle and buffalo) population. Approximately 53 million Pakistanis live in rural regions and make their living mostly from livestock through various techniques. They have limited resources for feeding their cattle and utilize whatever is available, resulting in poor health animal productivity and economic losses (Lightner, J. et al., 1988; Miller, G. et al., 1993; Lubna et al., 2023).

Mastitis is a universal issue and affects both animals and human health by the consumption of contaminated milk having pathogens and thus decreasing production resulting with heavy economic losses (Botaro, B.G. et al., 2015; (Maryam S., et al., 2023).

Subclinical mastitis affects 17-93% of cows and 4-48% of buffaloes in Pakistan. The pasteurizing dairy business is suffering because of the high commonness and occurrence of mastitis in dairy cows (Kossaibati, M. et al., 1998;). Mastitis susceptibility is more common in cows with sagging udders rather than in those without sagging (Fayazi-Kia, M. T. et al., 2023). Cows with teat lesions have a higher infection incidence than cows with normal teats.

Antibiotic therapy is the primary treatment approach for illnesses caused by diverse bacteria strains. However, evolving resistant strains have reduced treatment efficacy. Meanwhile, the multidrug resistance of these strains hinders treatment of their illnesses (Ribeiro, M. et al., 2007). Several lines of research have found an alarming surge in resistance (Ahmed et al., 2012).

In this research study, frequency of the bovine clinical mastitis, detection of the resistant bacteria and antibiotics resistance genes in cow milk samples were investigated in district Karak, Khyber Pakhtunkhwa, Pakistan

## 2. Materials and Methods

### *Ethical Approval*

This research project was duly approved by the ethical review committee department of zoology, Abdul Wali Khan University, Mardan Khyber Pakhtunkhwa Pakistan.

### *Experimental design and Sampling*

A total of 124 milk samples were randomly collected from thirty local dairy farms located in three tehsils of district Karak, Southern Khyber Pakhtunkhwa, Pakistan during the months of January to June 2022 (**Figure 01**).


**Figure 01:** Map of the study area of district Karak (Red), Khyber Pakhtunkhwa, Pakistan

The collected samples were transported to the Microbiology laboratory, College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University Mardan in a cold container. The procedure for surf field mastitis test (SFMT) was then applied. The samples were refrigerated at -80$^{o}$C until the strains in the samples were separated and purified (Ali, M et al., 2011; Mustafa, Y.S et al., 2011). The suspicious samples were cultured on Mannitol Salt Agar (MSA) media and incubated for 24 hours at 37°C according to the techniques indicated by Bergey's Manual of Systematic Bacteriology to identify Staphylococcus aureus and isolate bovine mastitis (Holt et al., 1994). PCR was done to confirm all susceptible isolates (Mathur, T. et al., 2006).

**Identification and Isolation of *S. aureus***

The suspicious representative samples were cultured on Mannitol Salt Agar (MSA) media and incubated for 24 hours at 37°C by the procedures advised by Bergey's Manual of Systematic Bacteriology to detect *Staphylococcus aureus* and isolate bovine mastitis (Holt et al., 1994). To further establish the presence of *S. aureus*, microscopy, gram staining and biochemical assays such as catalase, coagulase, and mannitol fermentation tests were conducted as previously described by (Walsh, P.S. et al., 2013; Lubna et al., 2023).

**Antimicrobial Sensitivity Test**

According to the recommendations of Clinical and Laboratory Standards Institute 2020 (CLSI 2020), the disc diffusion techniques were used to examine the susceptibility of *S. aureus* isolates (Javed, M.U. et al., 2021). *Augmentin* (30µg), *Cefoxitin*(30 µg), *Ciprofloxacin*(5µg), *Erythromycin*(15µg), *Gentamicin* (10 µg),*Amikacin* (30µg), *Linizolid* (30µg), *Vancomicin* (30µg), *Cefradin* (30 µg) and *Tigecicline*(15µg) were used and was carried out by slanting the antibiotic discs on Muller Hinton Agar; steaked and incubated at 37$^{o}$C for 24 hours.

**Amplification of *mecA, tetK, fbe, embP, etA, etB,* and *IS256* genes by PCR**

Resistant genes were amplified and identified through conventional PCR. DNA was extracted from the samples using the protocols established by Walsh, P.S. et al., 2013. A 25µl solution was prepared for the PCR reaction, which contains 12.5µl master mix, 1µl each of forward and reverse primers; 9.5

µl of deionized water, and 1µl of extracted DNA. The list of the primers used to identify the target genes is shown in **Table 01**.

**Table 01. Primers-sequences, annealing temperature and amplicon size**

| Primers | Sequence (5 to 3) | Annealing Temperature | Ampliconsize in base pairs (bp) | References |
|---|---|---|---|---|
| ***mecA* FW**<br>***mecA* RV** | GGT CCC ATT AAC TCT CAAG<br>AGT TCT GCA GTA CCG GAT TTG C | 55$^{O}$C | 533 | Petinaki, E et al., 2001 |
| ***TetK*FW**<br>***tetK* RV** | TCG ATA GGA ACA GCA GTA<br>CAG CAG ATC CTA CTC CIT | 54$^{O}$C | 361 | Strommenger, B et al., 2003 |
| ***Fbe* FW**<br>***Fbe* RV** | CTACAAGTTCAGGTCAAGGACAAGG<br>GCGTCGGCGTATATCCTTCAG | 55$^{o}$C | 273 | Rohde, H et al 2007 |
| ***embP* FW**<br>***embP* RV** | AGCGGTACAAATGTCAAT<br>AGAAGTGCTCTAGCATCATCC | 57$^{o}$C | 455 | Rohde, H et al 2007 |
| ***etA* FW**<br>***etA* RV** | CTA GTG CAT TTG TTA TTC AA<br>TGC ATT GAC ACC ATA GTA CT | 48$^{o}$C | 119 | Johnson, W.M et al., 1991 |
| ***etB* FW**<br>***etB* RV** | ACG GCT ATA TAC ATT CAA TT<br>TCC ATC GAT AAT ATA CCT AA | 48$^{o}$C | 200 | Johnson, W.M et al., 1991 |
| ***IS 256*FW**<br>***IS 256* RV** | AGTCCTTTTACGGTACAATG<br>TGTGCGCATCAGAAATAACG | 50$^{o}$C | 762 | Chessa, D et al., 2016 |

The DNA was first denatured for 3 minutes at 94 $^{o}$C*mecA,* for *tetK* for 5 minutes at 95 $^{o}$C,*fbe* and *embP* for 5 minutes at 95 $^{o}$C,*etA* and *etB* for 5 minutes at 94 $^{o}$C, and *IS256* for 3 minutes at 94 $^{o}$C the DNA was denaturized at 94 C$^{o}$ for 1 minute.At different temperatures of 55 $^{o}$C, 54 $^{o}$C, 55 $^{o}$C, and 57 $^{o}$C for 30 seconds, the primers *mecA, tetK, fbe,* and *embP* were annealed. The PCR conditions for *IS256* primer was one minute at 54$^{o}$C and for the primer's *etA* and *etB*, it was 2 minutes at 57$^{o}$C. The denaturation temperature was 94 $^{o}$C for one minute for the 35 cycles of *IS256.* The primers *mecA, tetK, fbe,* and *embP* were annealed at temperatures of 55 $^{o}$C, 54 $^{o}$C, 55 $^{o}$C, and 57 $^{o}$C for 30 seconds respectively. For *IS256,* the temperatures were set at 54°C for one minute and 57°C for two minutes for the *etA* and *etB* primers. The extension process was carried out on the *mecA, tetK, fbe,* and *embP* for 30 seconds at 72 $^{o}$C. The *etA, etB,* and *IS256* all underwent an elongation response at 50 $^{o}$C for 1minute and 2 minutes at 72 $^{o}$C respectively. During the last step of amplification, the DNA final extension was polymerized at 72 $^{o}$C for 3 minutes (*mecA*), 4 minutes (*tetK, fbe, embP*), and 5 minutes (*etA, etB,* and *IS256*) (Petinaki, E. et al. 2001; Strommenger, B. et al. 2003; Rohde, H. et al. 2007; Johnson, W.M. et al. 1991; Chessa, D. et al. 2016). Finally, the PCR products were electrophoresed on a 1 gm agarose gel. The gel was colored by using ethidium bromide. In order to analyze the DNA, UV trans illumination was used for visualization.

## 3. Results and Discussion

Antibiotic therapy is a crucial part of contemporary clinical practice, but because of overuse, the prevalence of *S. aureus* strains was invulnerable to antibiotics has dramatically grown, making the healing process extremely challenging (Altaf et al., 2020; Khan et al., 2017). Since these bacteria can infect humans through inappropriate touch or squandering of contaminated milk or meat products, the burgeoning of antibiotic aversion to pathogens has become a severe public concern (Rathi, M. et al., 2015; Caruso et al 2016). The current investigation found a prevalence of sub-clinical mastitis in district Karak was 16.93% while tehsil wise prevalence was 16.36% in tehsil Karak while in tehsil Banda Daud Shah and Takht-e-Nasarati the prevalence was recorded as 17.94% and 16.66% respectively (**Table 02, Figure 03**).

**Table 02. Prevalence of Mastitis in district Karak Khyber Pakhtunkhwa**

| Name of Tehsil | Total samples | Negative samples | Positive samples | Percent Prevalence (%) |
|---|---|---|---|---|
| Karak | 55 | 46 | 9 | 16.36 |

| | | | | |
|---|---|---|---|---|
| Banda Daud Shah | 39 | 32 | 7 | 17.94 |
| Takht-e-Nasarati | 30 | 25 | 5 | 16.66 |

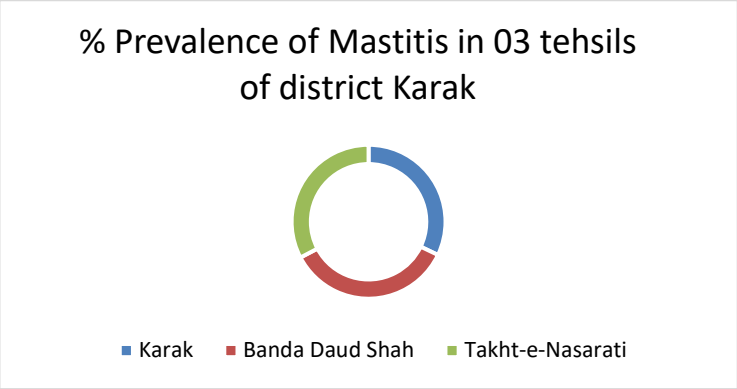


**Figure 03:** The prevalence of Mastitis in 03 tehsils of district Karak

It was found that out of 124 samples, only 21(16.93%) samples were found positive through the surf field mastitis test (SFMT**)** (**Table 03).** It was also found that 8 (38.09%) samples were found positive amongst 21 samples through Mannitol Salt Agar (MSA) during this research work ((**Figure 02, Table 04**). Further, 16.93% (21/124) of the samples were found positive through surf field mastitis test (SFMT) for subclinical mastitis in district Karak. These findings were supported by Memon, J. et al. (2013) who conducted a research experiment at 34 *Staphylococcus aureus* isolates from subclinical mastitis in Eastern China to assess their genotypes, pathogenicity attributes, and antibiotic resistance traits.

**Figure 02:** *Staphylococcus aureus* on MSA media

**Table 03: Identification of positive samples via Surf field mastitis Test (SFMT)**

| Total samples | Negative samples | Positive samples | Prevalence (%) |
|---|---|---|---|
| 124 | 103 | 21 | 16.93 |

**Table 04: Confirmation of positive Samples via Mannitol Salt Agar (MSA)**

| Total samples | Negative samples | Positive samples | Prevalence (%) |
|---|---|---|---|
| 21 | 13 | 8 | 38.09 |

Based on least inhibitory concentration (MIC) values, *Erythromycin* resistance was found in every isolate. Methicillin-resistant *S. aureus* (MRSA; 29%) isolates were alternative conventional to methicillin-sensitive *S. aureus* (MSSA) isolates. Additionally, these isolates had noticeably higher levels of *penicillin*, *oxacillin*, *oxytetracycline*, and *chloramphenicol* resistance. In subclinical mastitis, this analysis shows the appearance of a multidrug-resistant(MDR), highly contagious strain of *S. aureus* (Aqib, A.I., et al., 2017). According to Memon, J. et al. (2013), the research investigation found a substantial impact of parity on the incidence of mastitis. All eight isolates exhibited 100% resistance to *Erythromycin*, followed by *Cefoxitin, Cefradin, Augmentin, Ciprofloxacin, Gentamycin, Clindamycin, Amikac*in, and *Tigecycline*, whereas the isolates were shown to be 100% sensitive to

*Vancomycin* and *Linezolid*. The incorrect usage of this sort of antibiotic has resulted in *S. aureus*and developed resistance to it while 0% sensitivity to *Erythromycin*.

**Identification of the genes *mecA, embP, IS256, fbe, tetK, etA, and etB* in the isolates and antimicrobial sensitivity of the isolates to the antibiotics**

Through PCR, the effectiveness of seven resistance genes, as well as*mecA* and *blaZ*, and thirteen pathogenic components was assessed. The *cna, spaIg, nuc, clfA, fnbpB, hlA*and *hlB* genes were discovered in 35%, 79%, 85%, 59%, 35%, 85%, 71%, and 38% of the isolates, correspondingly. The *spaX* gene was present in each and every isolate. Nine isolates had a total of eight distinct virulence genes. The genes *ermB* and *ermC* for macrolide resistance were also present in all isolates. Methicillin aversion was common even though no isolates examined indubitable for the *mecA*gene. On the other hand, tetK and blaZ were found in 82% and 56% of isolates, correspondingly. The genes *fnbpA, seB, seC, seD, dfrK*, or *tetM* were not present in any isolates.

The investigation of the relationship between physical composition resistance and virulence genes revealed that the genes *clfA, fnbpB, hlB*, and *seA* may be federated with resistance to penicillin G, *ciprofloxacin, methicillin, chloramphenicol, trimethoprim*, and *oxytetracycline* ($P<0.05$). Seven common genotypes (A-G) were found in this area using REP-PCR-based genotyping. Similarly, in the present study, the genes *mecA, embP, IS256, fbe, tetK, etA,* and *etB* have been examined in all 8 samples. Except for the *mecA* gene, which is a *methicillin* resistance gene, rest of these were virulent genes. Additionally, it was pointed out that the PCR results obtained showed the amplification of *fbe* primer with amplicon size 273bp (**Figure 04**)*, embP* primer with amplicon size 455bp (**Figure 05**), *mecA* primer with amplicon size 533bp (**Figure 06**) and *IS256* primers was amplified with amplicon size 762bp (**Figure 07**).

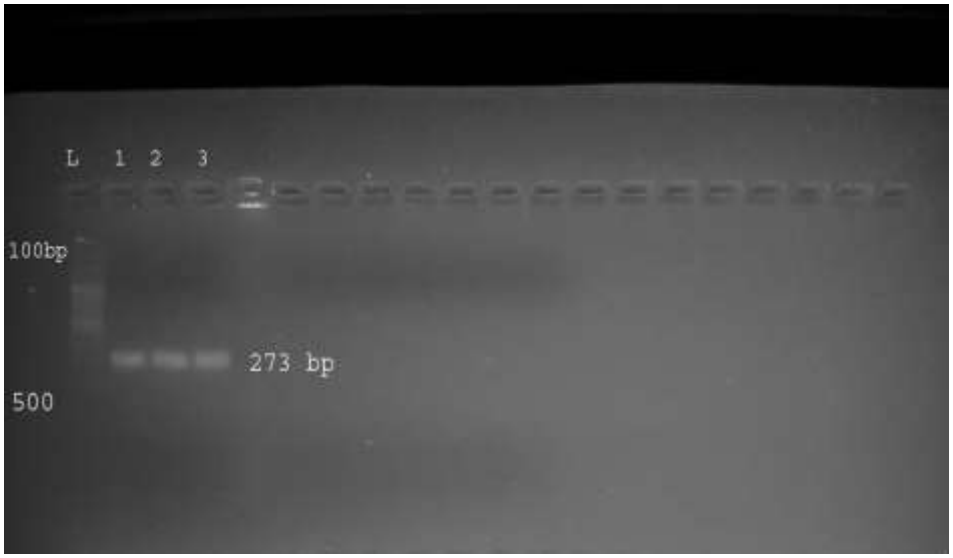


**Figure 04: Amplification of *fbe* (amplicon size 273bp).**

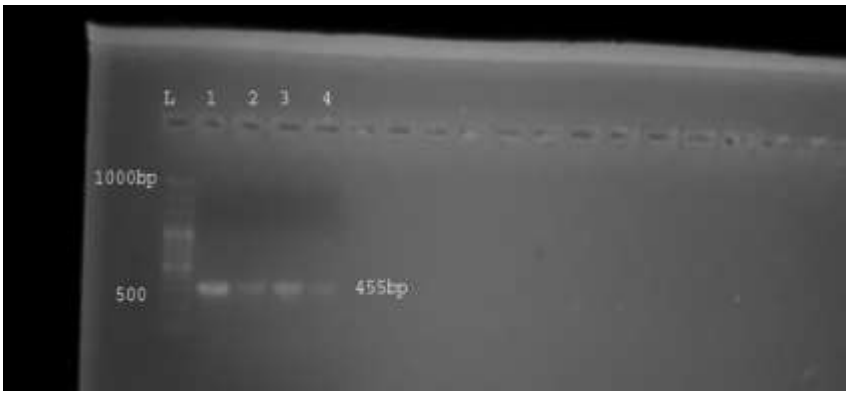


**Figure 05: Amplification of *embP* (amplicon size 455 bp)**

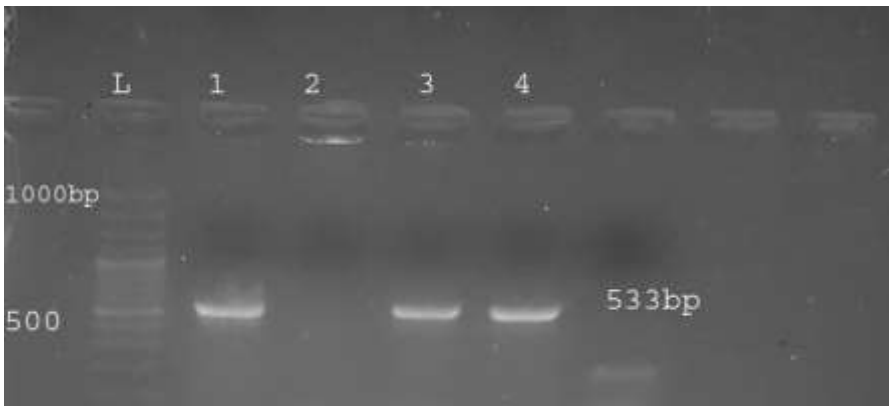


**Figure 06: Amplification of *mecA* (amplicon size 533bp)**

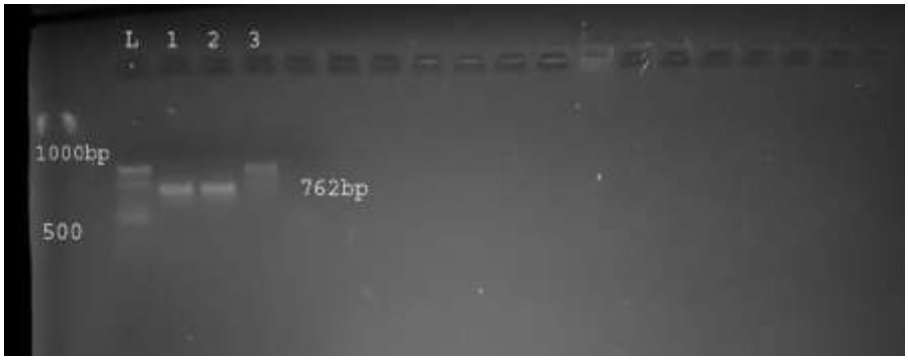


**Figure 07: Amplification of *IS256* (Ampicon size 762 bp)**

All variant of *S. aureus* was found to be extremely resistant to *Erythromycin* (100 %) followed by *Cefoxitin* (75 %) and *Cefradin* (75 %). The overall percent resistance and sensitivity is shown in **table 05**.

**Table 05: Percentage of *S. aureus* isolates of subclinical mastitis showing resistance and sensitivity to antibiotics.**

| S.No | Antibiotic | Resistance (%) | Sensitivity (%) |
|---|---|---|---|
| 1 | *Erythromycin* | 100 | 0 |
| 2 | *Cefoxitin*, *Augmentin* and *Cefradin* | 75 | 25 |
| 3 | *Ciprofloxacin* | 62.5 | 37.5 |
| 4 | *Gentamicin*and *Clindamicin* | 50 | 50 |
| 5 | *Amikacin*and *Tigecycline* | 25 | 75 |
| 6 | *Vancomicin*and *Linezolid* | 0 | 100 |

## 4. Conclusion

Our research revealed that *Staphylococcus aureus* is the primary cause behind persistent mastitis. According to our research, raw milk has a significant risk of dispersing bacteria like *S. aureus* that are antibiotic-resistant. The screened isolates in the current investigation exhibited high levels of resistance to the most beta-lactam drugs, including *Erythromycin*, *Cefoxitin*, and *Cefradin*. Furthermore, the high incidence of beta-lactamase in *S. aureus* revealed high probability of opposing food-borne bacteria infecting individuals through raw milk. Raw milk might contain a disproportionately high number of germs resistant to antibiotics because dairy farmers are frequent and uncontrolled use of these antibiotics in low income countries like Pakistan.

Therefore, it is crucial for human health to improve milk safety and apply excellent manufacturing procedures. It is necessary to pasteurize raw milk, prevent cross-contamination, to clinch the harmlessness of milk and dairy outcome, store raw milk at a low temperature, put in place sufficient authority oversight, and establish regulatory monitoring on the use of antibiotics in dairy cattle farms. *Erythromycin* has a higher level of resistance than other drugs used which show the effectiveness for the farmer to be followed in treating mastitis. Future studies can concentrate on the main point of entry for resistant bacteria into raw milk to find out whether these bacteria are incorporated in milk during or after milking, or whether they enter the milk through the cow's udder. To confirm the genetic variation of opposing bacteria, it is advised that the assay be perform on food pathogenic isolate in subsequent study.

## Acknowledgments

The authors are thankful to the supporting staff of both the Department of Zoology and College of Veterinary Science & Animal Husbandry (CVS&AH), Abdul Wali Khan University Mardan, Pakistan for their assistance during this research study.

## Novelty Statement

The research and experimental work on "Methicillin-resistant *Staphylococcus aureus* (MRSA) and Detection of Resistant Genes in Cow milk from Southern Khyber Pakhtunkhwa Pakistan" is original and new in the field of clinical and veterinary medicine in Khyber Pakhtunkhwa, Pakistan.

## Author's Contribution

Mubasher Ullah: Investigation, Writing-original draft preparation; Asad Ullah: Supervision; Tayyaba Ilyas: Project administration; Sohrab Ahmad: Conceptualization; Tahira Tayyeb: Data Curation; Mansoor Ahmad & Rafiq Ullah: Formal analysis; Aziz Ullah Khan: Methodology; Muhammad Hanif: Resources; Muhammad Owais Khan & Muneeb Islam: Validation; Raheela Taj: Software; Ali Gohar: Visualization; Muhammad Sadeeq: Writing-review and editing.

## *Statement of conflict of interest*

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication with the work submitted.

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