Epidemiology and Antimicrobial Susceptibility of Methicillin-Resistant *Staphylococcus aureus* in Cattle of Pothohar Region, Pakistan

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**ABSTRACT**

Sub-clinical mastitis has remained the leading cause for decline in production of dairy animals as a silent epidemic all the times in Pakistan. A questionnaire-based cross-sectional study was conducted to assess the prevalence and potential risk factors for sub-clinical mastitis in the Pothohar region of Pakistan in 2018. Total of 104 cattle milk samples were collected from commercial and subsistent dairy farms. CMT positive samples were cultured and biochemical tests were conducted before confirmed on PCR for mecA gene *Methicillin Resistant Staphylococcus aureus* (MRSA) bacteria. *In-vitro* antibiotic susceptibility was also assessed. An overall prevalence of 71.1% was found; where cross-bred was found more susceptible (80.7%) as compared to other breeds. MecA gene-MRSA prevalence based on PCR was 54%. On regression analysis the potential risk factors identified here included; daily milk yield, parity, udder shape, teat morphology, shed type, quarantine of new animals and deworming of animals (OR>1; P-value<0.05). In MRSA confirmed isolates, Penicillin group was found highly resistant (92.5%) amongst all the groups. While amongst individual antibiotics Amoxicillin was found highly (100%) resistant followed by Cefixime and Spectinomycin. Whereas based on sensitivity Quinolone (Moxifloxacin 95%) group was the most sensitive (91.7%) followed by Sulphonamides (Sulphaphenazole 87.5%) and Amino-glycoside (Gentamycin 90%). An emerging pattern of mecA gene MRSA was recorded here with alarming sub-clinical mastitis prevalence in the study area. Immediate preventive measures need to be taken to address the problem. The findings of the current study will assess in control and prevention of subclinical mastitis in Pakistan.

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INTRODUCTION

Mastitis is an imperative disease of bovines worldwide causing large economic losses through intra-mammary infections in lactating animals (Romero et al., 2018). Approximately 140 pathogen species, sub-species, and serovars have been identified in milk samples from lactating animals and classified into contagious, environmental and opportunistic mastitogens (Patterson, 2017). *Staphylococcus aureus* is contagious in nature and causes subclinical, chronic and acute intra-mammary inflammations in lactating animals. Therefore early detection of pathogens is vital for early therapeutic control of subclinical mastitis (Begum et al., 2015).

Pakistan is an agricultural country; with a large population of cattle and buffalo, almost 46.1 million and 38.8 million, respectively. Livestock contributes 56% as subsector in agriculture and adding 11% into the national gross domestic product (Rehman et al., 2017). The dairy industry in Pakistan faces several health hazards all the times; amongst which mastitis is the common problem and risk factor in the development of the dairy sector (Khan et al., 2015). It causes loss of future calf, reduction in milk production, condemnation of milk, replacement of animals, culling and decrease quarter-wise production (Goncalves et al., 2018). *S. aureus* is highly tolerant due to endorsement of a variety of genetic capabilities, amongst one prominent of them is methicillin-resistant *S.*
MATERIALS AND METHODS

Study design and general settings: A cross-sectional survey was conducted in the north-eastern Pothohar region of Rawalpindi, Punjab, Pakistan.

Field screening and data collection: A total of 104 apparently healthy lactating cattle were selected randomly from the target area. The milk samples were collected after the primary screening with California Mastitis Test (CMT) (Patterson, 2017). CMT positive 2ml milk samples were collected in a sterile test tube and transported to the university research laboratory in ice pack for further diagnosis. The data regarding risk factors including (Location, Age, Breeds, Body condition Score, Milk yield, Lactation stage, number of lactations, Udder shape, teat shape, California Mastitis test, infected quarter, other disease, milk leakage, Quarantine of new animals, Quarantine of infected animals, Deworming program, Flies control programs, Type of farm, Type of shed, Animal movement in shed, Source of drinking water, feed type, Feed supplementation, Udder preparation, dipping Used, Time of dipping, Stimulation of Milker, Gender of milker, Milking Techniques, Number of milking by milker, Number of specie Reared, Ratio of Buffalo and Cattle, Animal moved of Premises recently, Number of people attend animals, Animal bedding change, Animal manure change, Sharing of Feed, Source of Animals for owner, Hoof Trimming, Occurrence of Mastitis, doctor availability) were collected on a pre-defined questionnaire. Data regarding geographical location was collected through Android phones by copying the coordinates.

Microbiological and biochemical characterization: In order to carry out the microbiological examination, CMT positive milk samples were centrifuged at 2000 rpm for ten minutes. While discarding supernatant the sedimentation of milk for direct demonstration was used. Initially, blood agar (containing 5% sheep blood) and Mannitol salt agar was used to identify Staphylococcus aureus. Further biochemical tests including coagulase, catalase and staining were applied for confirmation of the organisms.

Detection of mec-A gene by PCR technique: The MRSA strain was identifying using standard PCR-assay. DNA extraction Kit (Nucleo-Spin tissue, Macherey Nagel) was used to obtain cellular DNA of Staphylococci coloni from the overnight grown samples on Blood agar as per manufacturer’s. Previous reported primers; Forward, 5’-AAAATCGATGGTAAAGGTTGGC-3’ and Reverse, 5’- AGTTCTGGAGTACCGGATTGC 3’ Pairs were used for implication of 533 bp fragments. A volume of 2µl of extracted DNA, 19µl of sterilized water, 3µl of Master Mix (Solis Bio-dyne FIRPoI), 0.5µl of forward primer, and 0.5 µl reverse primer was added making total volume of 25µl. The temperature and time were optimized as for denaturation 95°C for 5 min, followed by 40 cycling as 94°C for 30 sec, 59.7°C for 30 sec, 72°C for 30 sec, and at last phase for extension 72°C for 10 minutes. Electrophoresis (Fig. 1) was used to visualize amplified product by adding 0.5 mg/ml ethidium bromide stain. The positive sample was thenprocess for an in-vitro therapeutic sensitivity test.

Fig. 1 PCR amplification of mecA gene (533 bp) of Staphylococcus aureus strains isolated from bovines and confirmed by PCR.

In-vitro antibiotic susceptibility test: PCR confirmed (n=40) MRSA samples were carried out for antibiotic sensitivity following Kirby-Baur disc diffusion method, aseptically applied on Muller Hinton Agar by following reported procedure (Aqib et al., 2017). The antibiotic sensitivity against MRSA was processed for different classes of drugs used in the field, included penicillin, Cephalosporin, Sulphonamide, Amino-glycoside, and Quinolones group. The zone of inhibition measured with Vernier calipers in millimeters and compared with latest standard zones reported around the world and given the finding as resistant (R), intermediate (I) and susceptible (S) (CLSI, 2017).

Statistical analysis: Data was entered in Epi-Data for cross-checking and validation. Data was cross-checked with the hard copies of questionnaires. Data analysis was performed through SPSS version 22.00. Data normality was assessed through Shapiro-Wilk test in SPSS. On this test, non-significant departure from normality was found. Passing the normality test descriptive and regression analysis of the data was performed.

Chi-square test was performed at 95% confidence interval to assess the prevalence of sub-clinical mastitis and association of factors with the prevalence. Univariable regression analysis was performed to identify the risk association of predicting variables with the outcome variable at a study power of 80%. Variables having P<0.20 at univariable analysis were passed onto final regression model. Multi-collinearity was assessed.
and variables failing the test were excluded from the final model. Final regression model was conducted at 95% confidence interval.

RESULTS

Out of 104 dairy cows of different breed were tested for sub-clinical mastitis performing CMT screening test add the information of positive samples. Overall prevalence of Sub-clinical mastitis was 71.1% (74/104) in Pothohar region of Punjab, Pakistan. Cross-bred was with the highest prevalence of SCM of 80.7% followed by 19.24% in Sahiwal, Jersey, and Holstein Friesian.

Prevalence of Methicillin-Resistant Staphylococcus aureus (MRSA): Among 74 CMT positive milk samples were examined for the presence of MRSA strain by targeting mecA gene on conventional PCR. The overall prevalence of MRSA recorded in this study was 54% (Table 2). Cattle produced more than five liters of milk per day found were significant (P<0.05) at higher risk of SCM (90%) as compared to low yield cattle. Fifty-four (72.9%) samples were produced typical yellow colonies within 24-48hr among 74 CMT positive milk samples that was further confirmed by biochemical test (Fig.2).

Assessing Potential risk factors for spread and transmission of MRSA several factors were found significantly associated. Several health, management and biosecurity factors were identified as risk factors including; daily milk yield, parity, udder, teat morphology, shed type, quarantine of new animal and De-worming. Cattle having cylindrical teat were at higher risk (OR=3.444; C.I=1.323-8.968; P-value=0.00) of getting SCM as compared to cattle having round teat shape. Closed shed type found to be at higher risk (OR=3.508; C.I=1.432-8.594; P-value=0.00) of SCM as compared to open shed. Farms not performing quarantine of newly arrived animals were at higher risk (OR=3.444; C.I= 1.323-8.968; P-value=0.01) Similarly, at farms where teat dipping is not performed were at greater risk (OR=13.091; P-value<0.001) potentially for sub-clinical mastitis. Detail results are depicted in Table 1 & 2.

\[ \text{Represents S. aureus phenotypic identifications: (A) Growth on mannitol salt agar; (B) Gram’s staining; (C) Catalase Positive and (D) Coagulate Positive.} \]

\[ \text{Table 1: Sub-clinical mastitis prevalence in Pothohar Region in Cattle commercial farms} \]

| Variable name     | Description | Positive/ Total | Prevalence | P-Value |
|-------------------|-------------|-----------------|------------|---------|
| Milk yield per day | <5          | 52/80           | 65.0       | 0.00    |
|                   | >5          | 22/24           | 91.7       |         |
| Parity            | First time  | 56/84           | 66.7       | 0.05    |
|                   | 2 or > 2    | 18/20           | 90.0       |         |
| Udder shape       | Cylindrical | 16/22           | 72.7       | 0.20    |
|                   | Non-pendulous | 16/24        | 66.7       |         |
|                   | Pendulous   | 16/18           | 88.9       |         |
|                   | Round       | 26/40           | 65.0       |         |
| Teat shape        | Bowl        | 2/4             | 50.0       | 0.00    |
|                   | Cylindrical | 56/62           | 90.3       |         |
|                   | Round       | 14/38           | 36.8       |         |
|                   | Open        | 38/46           | 82.6       | 0.02    |
| Dipping status    | Yes         | 2/10            | 20.0       | 0.00    |
|                   | No          | 72/94           | 76.6       |         |
| Quarantine of new animal | Yes | 12/24         | 50.0       | 0.01    |
|                   | No          | 62/90           | 77.5       |         |
| Source of animals | By Birth    | 13/21           | 61.9       | 0.10    |
|                   | Purchase    | 18/20           | 90.0       |         |
|                   | Mix         | 43/63           | 68.3       |         |
| De-worming        | No De-worming | 20/22      | 90.0       | 0.01    |
|                   | Yes         | 8/20            | 40.0       |         |
|                   | Once a year | 19/23           | 82.6       |         |
|                   | Twice a year | 15/23        | 65.2       |         |
|                   | Thrice a year | 20/36       | 55.6       |         |

I. Only those variables are reported here which are having significant association with the occurrence of sub-clinical mastitis. 2. Variables having p-value=0.20 were retained in the final regression model.

\[ \text{Table 2: Multivariable logistic regression model for the risk factors associated with the occurrence of MRSA sub-clinical mastitis in commercial Cattle farms} \]

| Variables        | Levels     | Standard error (C.I at 95%) | Odds ratio | P-value |
|------------------|------------|-----------------------------|------------|---------|
| Milk Yield per day | <5         |                             | 0.775      | 5.923(1.297-27.046) | 0.02 |
|                  | >5         |                             | 0.780      | 4.500(0.975-20.775) | 0.04 |
| Parity           | First time |                             | 0.820      | 4.308(0.864-15.489) | 0.02 |
|                  | 2 or > 2   |                             | 0.597      | 13.067(4.058-32.073) | 0.00 |
| Udder Shape      | Round      |                             | 0.474      | 3.508(1.432-8.594)  | 0.00 |
|                  | Pendulous  |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| Text Shape       | Round      |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | Cylindrical |                          | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| Shed Type        | Open       |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | Closed     |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| Dipping Status   | Yes        |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | No         |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| Quarantine of New Animal | Yes |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | No         |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| Source of Animals | By Birth   |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | Purchase   |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
| De-worming       | Yes        |                             | 0.488      | 3.444(1.323-8.968)  | 0.01 |
|                  | No De-worming |                        | 0.488      | 3.444(1.323-8.968)  | 0.01 |

\[ \text{DISCUSSION} \]

The overall prevalence of sub-clinical mastitis found was 71.1% in cattle similar to earlier reported by Mekonnen et al. (2017) in Ethiopia. The prevalence estimated here is much higher reported in other studies previously from Pakistan (Akhtar and Tanweer, 2016; (Aqib et al., 2019).
The finding shows that overall prevalence of MRSA was 54% in the Pothohar region of Pakistan. Similar high-level MRSA prevalence of 60%, was reported by Locatelli et al. (2017). In contrast to our study lower MRSA prevalence (34%) in Pakistan (2017) was reported: while from other regions of the world a prevalence of 16.7%, and 9.2%, is reported from India (2011), Saudi Arabia (2018), respectively (Kumar et al., 2011; Aqib et al., 2017; El-Deeb et al., 2018). This higher MRSA prevalence in our study area could be due to the difference in management practices and geographical variations. The farm structures and management practices adopted here are below standards which supports the transmission of infectious pathogens. It could also be attributed to several other factors such as climatic factors affecting the susceptibility of animals to pathogens (Khan et al., 2015). MRSA’s contagious nature makes it more lethal when number of animals kept together increases, grazning system (by grazing different farm animals together), lack of awareness (unsatisfactory awareness level in the study area), unhygienic and bio-security measures (especially quarantine of newly arrived animals and diseased animals) (Aqib et al., 2019). The low prevalence reported in previous studies could be due to diagnostic tools creating inconsistency for determination of MRSA prevalence, due to poor appearance of mecA gene or excessive-production of β-lactamase (Haran et al., 2012). mecA documented a most accurate method for MRSA detection. However, some other factor developed a change in phenotypic expression like PH and osmolality of culture media that may lead to inconsistent result (Kumar et al., 2011).

High producing cow have a much larger volume of udder and antibiotic unable to remove the infection from infected tissue (Abera et al., 2010). Cattle with high parity were also at higher risk (OR=4.500; CI=0.975-20.775) of getting SCM (Saifeeuden et al., 2018). Young cows have an energetic immune system with teat functioning properly (Seyoum et al., 2018). Udder and teat morphology showed positive association with SCM (Bharti et al., 2015). Our study also found that distance between floor and teat also play an important role in the development of the infection (Kaur et al., 2018).

Amongst managemental factors including shed type, dipping status and de-worming were found significantly associated with sub-clinical mastitis (Santharan et al., 2016). In closed sheds the frequency of infection spread is into healthy animals because it provides better environment to microorganisms for growth. Milkers hands also act as a source of spread for pathogens, so unhygienic milking practices leads to infection (Seyoum et al., 2018). In developed countries closed herd system exists and several preventive measures are adopted for new entries and replacement (Ramirez et al., 2014). But unfortunately dairy farming in Pakistan does not follow SOPs of management and biosecurity, therefore purchasing new animals and lack of proper deworming strategies increases the risk of SCM at farm level (Zadoks et al., 2011).

The MRSA isolates from the field were highly sensitive to Moxifloxacin (95%), Gentamicyn (90%) and Trimethoprim (87.7%) in line with the reported findings in the previous studies (Aqib et al., 2017). Penicillin groups including: Cloxacillin (100%), Ticarcillin (100%), Amoxicillin (100%), Oxacillin (100%), Ampicillin(100%) and Cefixime (100%) in Cephalosporin group was resistant to these isolates (Kulshrestha et al. 2019). Such higher resistance might have developed due to the prolonged and haphazard use of antimicrobials (Kistler et al., 2018). Which is a result of plasmid transfer and transposons between Staphylococcal species highly prevalent in our environment (Aqib et al., 2017) and excessive production of beta-lactamase enzyme disturbing the function of antibiotic by breaking the beta-lactam ring (Adediran et al., 2018). The non-professional veterinary practice in the field is a serious threat to antibiotic resistance dramatic increase currently and in future if not controlled.

**Conclusions:** We report the presence of MRSA strain in dairy farms of cattle in and around Islamabad, Pakistan. The prevalent MRSA strains were detected in this study and these are resistant to Penicillin group of antibiotics in the field.
Authors contribution: AK: Conducted this study as a principle investigator. AZD: Supervised the study and contributed in study design and write up. AY: Contributed in the study design and laboratory diagnostics. JAK: Contributed in the sample collection and field data collection. MC: Contributed in the study design, sampling and data collection. ZF: Contributed in the write up of the manuscript. AK: Contributed in the data analysis and write up.

REFERENCES

Abera M, Demie B, Aragaw K, et al., 2010. Isolation and identification of Staphylococcus aureus from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. J Vet Med Anim Health 2:29-34.

Aderan S, Sarkar KS, Pratt R, 2018. kinetic evidence for a second ligand binding site on Streptococcus pneumoniae penicillin-binding protein 2x. Biochemistry 57:1758-66.

Akhtar A and Tanweer U, 2016. Prevalence of mastitis and identification of causative pathogens in local and crossbred cows in Dera Ismail Khan. Pak J Sci 64: 265-268.

Aqib Al, Ijaz M, Anjum AA, et al., 2017. Antibiotic susceptibilities and prevalence of Methicillin resistant Staphylococcus aureus (MRSA) isolated from bovine milk in Pakistan. Acta Trop 176:168-72.

Aqib Al, Nighat S, Ahmed R, et al., 2019. Drug susceptibility profile of Staphylococcus aureus isolated from mastitic milk of goats and risk factors associated with goat mastitis in Pakistan. Pak J Zool 51:307-15.

Begum M, Hossain M, Ershaduzzaman M, et al., 2015. Study on prevalence and risk factors of subclinical mastitis in lactating dairy cows in Rajshahi and Rangpur division of Bangladesh. Wayamba J Anim Sci 7:1129-37.

Bharti P, Bhakat C, Pankaj PK, et al., 2015. Relationship of udder and teat conformation with intra-mammary infection in crossbred cows under hot-humid climate. Vet World 8:898.

Clinical and Laboratory Standards Institute (CLSI), 2017: Performance standards for antimicrobial susceptibility testing 27th edition: CLSI Document, M02-S26. Informational Supplement, CLSI Document, M02-A12. Clinical and Laboratory Standards Institute, Wayne PA.

El-Deeb W, Fayez M, Elmoslemey A, et al., 2018. Methicillin resistant Staphylococcus aureus among goat farms in Eastern province. Saudi Arabia: Prevalence and risk factors. Prev Vet Med 136:84-90.

Goncalves J, Kamphuis C, Martins C, et al., 2018. Bovine subclinical mastitis reduces milk yield and economic return. Livest Sci 210:25-32.

Harun K, Godden S, Boxrud D, et al., 2012. Prevalence and characterization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, isolated from bulk tank milk from Minnesota dairy farms. J Clin Microbiol Infect 50:688-95.

Kaur G, Bansal BK, Singh RS, et al., 2018. Associations of teat morphometric parameters and subclinical mastitis in riverine buffaloes. J Dairy Res 85:303-8.

Kerar B, Kuyucuoğlu Y and Şeker E, 2012. Antibiotic susceptibility of coagulase-negative staphylococci isolated from bovine subclinical mastitis in Turkey. Pak Vet J 32:390-3.

Khan A, Mustaq MH, Ahmad D, et al., 2015. Prevalence of clinical mastitis in bovines in different climatic conditions In KPK, Pakistan. Sci Int 27: 2289-2293

Kister JM, Thoder JI and Ilyas AM, 2019. MRSA incidence and antibiotic trends in urban hand infections: A 10-Year longitudinal study. Hand (N Y) 14:449-54.

Kulshrestha A, Anamika V, Mritunjay K, et al., 2017. A prospective study on the prevalence and antibiotic sensitivity pattern of methicillin resistant Staphylococcus aureus isolated from various clinical specimen at a tertiary care post graduate teaching institute. Int J Curr Microbiol App Sci 6:1859-69.

Kumar R, Yadav B and Singh R, 2011. Antibiotic resistance and pathogenicity factors in Staphylococcus aureus isolated from mastitic Sahiwal cattle. J Biosci 36:175-88.

Locatelli C, Cremonesi P, Caprioli A, et al., 2017. Occurrence of methicillin-resistant Staphylococcus aureus in dairy cattle herds, related swine farms, and humans in contact with herds. J Dairy Sci 100:608-19.

Magro G, Rebolini M, Beretta D, et al., 2018. Methicillin-resistant Staphylococcus aureus CC22-MRSA-IV as an agent of dairy cow intramammary infections. Vet Microbiol 227:29-33.

Mekonnen SA, Koop G, Melkie ST, et al., 2017. Prevalence of subclinical mastitis and associated risk factors at cow and herd level in dairy farms in North-West Ethiopia. Prev Vet Med 145:23-31.

Patterson C, Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats, Volumes 1 and 2. In: 2017. Ramírez N, Keefe G, Dohoo I, et al., 2015. Study on relationship of udder and antimicrobial susceptibility test of Staphylococcus aureus in bovine subclinical mastitis and its association with bacteria and risk factors in milking cows of Batticaloa District in Sri Lanka. J Agri 4:168-76.

Qureshy MS, Khan MA, Malik SA, et al., 2011. Molecular and morphometric parameters and subclinical mastitis in riverine buffaloes. J Dairy Res 85:303-8.

Kaur G, Bansal BK, Singh RS, et al., 2018. Associations of teat morphometric parameters and subclinical mastitis in riverine buffaloes. J Dairy Res 85:303-8.