RESEARCH ARTICLE

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

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A B S T R A C T

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. aureus* (MRSA). MRSA

has been recognized as a contagious zoonosis, by its transmission to humans from animals and vice-versa (Magro *et al*., 2018). MRSA strain boosts the virulence of *S. aureus* intra-mammary infection and escape from host defense system (Kenar *et al*., 2012). Hit and trial use of antibiotics develops resistance (Kistler *et al*., 2018) to ß-lactams by hydrolysis of ß-lactam ring (Adediran *et al*., 2018). Regular monitoring is required for such rising concerns on human as well as animal side. MRSA causing sub-clinical mastitis are originated into animal body exposed to bad management and contaminated environment (Aqib *et al*., 2017).

Prevalence of sub-clinical mastitis, specific sensitivity of antibiotics against MRSA and identification of its risk factors is important to improve the udder health in lactating animals. The research data regarding mec-A gene MRSA strains is limited in Pakistan. Considering this the current study was designed to understand the epidemiology of MRSA along with *in-vitro* therapeutic sensitivity in the Pothohar region of Punjab, Pakistan.

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′-AGTTCTGGAGTACCGGATTTGC 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 FIRPol). 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 95C° 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.
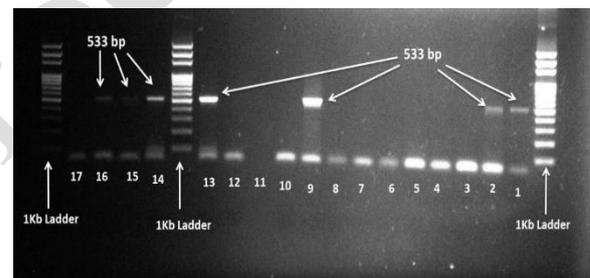
Fig. 1 PCR amplification of mecA gene (533 bp) of *Staphylococcus aureus* strains isolated from bovines and confirmed by PCR.

***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 ware 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=13.067; P-value<0.001) 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.

***In-Vitro* antibiotic sensitivity test:** Forty confirmed PCR MRSA isolates were subjected to antimicrobial sensitivity tests against commonly in use antibiotics in field and veterinary Hospital. Accordingly, class-wise results showed that MRSA is highly resistant to Penicillin group (92.5%) and sensitive to Quinolones group (91.7%) in the study area, while at individual levels, Moxifloxacin (95%) was highly sensitive followed by Levofloxacin (90%), Cefrofloxicine (90%), Gentamycin (90%), Trimethoprim (87.5%), Sulphaphenazole (87.5%) antibiotic (Table 3).

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 (Aqib *et al.*, 2019; Akhtar and Tanweer, 2016).
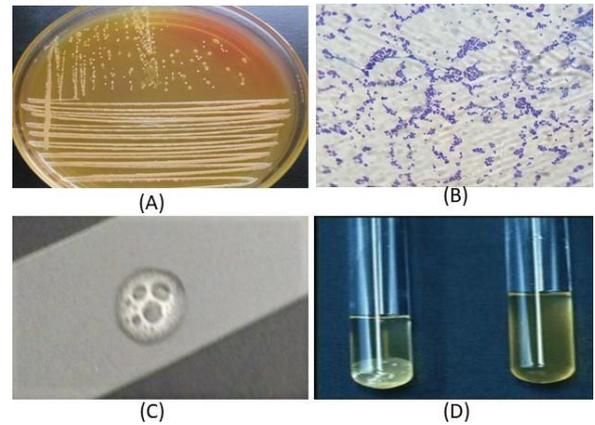
Fig. 2: Representative S. aureus phenotypic identifications (A) Growth on mannitol salt agar; (B) Gram's staining; (C) Catalase Positive and (D) Coagulase Positive.

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	
Shed Type	Closed	38/46	82.6	0.02
	Open	36/58	62.1	
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	62/80	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
	Once a year	19/23	82.6	
	Twice a year	15/23	65.2	
	Thrice a year	20/36	55.6	

1. 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.

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	Odds ratio (C.I at 95%)	P-value
Milk Yield per day	<5		Ref	
	>5	0.775	5.923(1.297-27.046)	0.02
Parity	First time		Ref	
	2 or > 2	0.780	4.500 (0.975-20.775)	0.04
Udder Shape	Round		Ref	
	Pendulous	0.820	4.308 (0.864-15.489)	0.02
Teat Shape	Round		Ref	
	Cylindrical	0.597	13.067 (4.058-32.073)	0.00
Shed Type	Open		Ref	
	Closed	0.474	3.508 (1.432-8.594)	0.00
Dipping Status	Yes		Ref	
	No	0.827	13.091 (2.587-46.241)	0.00
Quarantine of New Animal	Yes		Ref	
	No	0.488	3.444 (1.323-8.968)	0.01
Source of Animals	By Birth		Ref	
	Purchase	0.793	4.186 (0.885-17.806)	0.03
De-worming	Thrice a year		Ref	
	No De-worming	0.861	5.333 (0.986-21.844)	0.02

Table 3: *In-vitro* antibiotic sensitivity against *MRSA* Sub-clinical mastitis in cattle.

Antibiotics Class	Antibiotic	Disk content	CLSI AZD (mm)	MRSA isolates	Individual drug (%)			Antibiotic class wise % response (n / N%)		
					R	I	S	R	I	S
Penicillin	Cloxacilin	5µg	20-26	40	100	-	-			
	Ticarcillin	75µg	18-22	40	100	-	-			
	Amoxicillin	25µg	20-28	40	100	-	-			
	Oxacillin	1µg	18-24	40	100	-	-	92.5	-	7.5
	Ampicillin	10µg	27-35	40	100	-	-			
	Amoxicillin & Calvalunic Acid	10/20µg	28-36	40	80	-	20			
	Piperacillin & Tazabactam	75/10µg	24-32	40	65	-	35			
	Ticaracillin & calvalunic Acid	75/10µg	20-28	40	95	-	5			
Cephalosporin	Cefixime	10µg	26-30	40	100	-	-			
	Cefotaxime	30µg	25-31	40	25	-	75	76.25	-	23.75
	Cefuroxime	30µg	27-35	40	87.5	-	12.5			
	Ceftazidine	30µg	16-20	40	92.5	-	7.7			
Sulponamide	Sulphamethazole	300µg	24-34	40	22.5	-	77.5			
	Sulphaphenazole	300µg	24-34	40	12.5	-	87.5	13.4	2.5	84.1
	Trimethoprime	5µg	19-26	40	5	7.5	87.5			
Amino-glycoside	Gentamycine	10µg	19-27	40	10	-	90			
	Spectinomycine	100µg	19-27	40	25	-	75	13.5	6.7	80
	Amikacine	30µg	20-28	40	5	20	75			
Quinolones	Cefrofloxicine	5µg	20-30	40	10		90			
	levofloxacin	5µg	20-30	40	10		90	8.3		91.7
	Moxifloxine	5µg	28-34	40	5		95			

n=Positive samples; N=Total samples; R=Resistance; I=Intermediate; S=Sensitive; CLSI= Clinical laboratory standard Institute, AZD= Acceptable Zone Diameter (mm).

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, grazing 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 ß-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; C.I=0.975-20.775) of getting SCM (Saifudeen *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 (Sanotharan *et al*., 2016). In closed sheds the frequency of infection spread isinto healthy animals because it provides better environment to microorganisms for growth. Milker 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 (Ramírez *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%), Gentamycin (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*. 2017). 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 were 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.

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