Epidemiology and *in vitro* Drug Susceptibility of *mecA* Positive MDR *S. aureus* from Camel Subclinical Mastitis

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A B S T R A C T

Staphylococcus aureus (S. aureus) is emerging as multiple drug resistant (MDR) pathogens in milk of camels with characteristically zoonotic potential. Current study was planned to investigate epidemiology of MDR *S. aureus* in camel milk, and *in-vitro* therapeutic response to drugs. Two different ecological zones, Cholistan (n=185) and Suleiman Range (n=177) of Pakistan having major population of camel were screened for subclinical mastitis using California mastitis test using convenient sampling technique. Biochemical examination was performed to isolate *S. aureus* that further were tested Penicillin, Glycopeptide, and Cephalosporin classes of antibiotics for confirmation of MDR isolates using disk diffusion method. MDR were tested *in vitro* for response against various antibiotics. A questionnaire was filled for estimation of association of assumed risk factors with prevalence. Nonparametric tests at 5% probability were applied to check significance of results. The study noted overall 33.98% (123/362) of MDR *S. aureus* from subclinical mastitis milk samples while 123 of 192 *S. aureus* isolates (64.06%) were confirmed MDR. Non-significantly higher prevalence of MDR was noted in Cholistan area (38.38%) than to that of Suleiman Range (29.98%) on subclinical mastitis samples basis Similarly 66.36% of *S. aureus* isolates from Cholistan area and 61.18% from Suleiman Range were presented themselves as MDR. Oxacillin, Cefoxitin and Ampicillin faced 100% resistance while Vancomycin, Cefixime and Cefotaxime had >78 % of resistance from MDR isolates from both study areas. However, Trimethoprim and Ciprofloxacin were > 90%, Chloramphenicol >69%, and Gentamycin 61-67.3%, Streptomicin 61-73.07% and Oxytetracycline 69-71.15%, Enoxacin 45.07 and 53.84% and Amikacin 43.6 and 51.92 effective against MDR. Older age of animals, higher California mastitis score, weak body condition, higher milk yield, early lactation, teat injury, higher parity number, tick infestation, self-treatment and higher use of penicillin in general ailments had positive association with MDR prevalence. The study concluded higher prevalence of MDR *S. aureus* in camel milk with significant association of assumed risk factors and some of antibiotics presented higher scope of treatment.

INTRODUCTION

Camel has emerged as 3rd most milk yielding specie in Pakistan despite of 1% of total animal population (Anonymous, 2015). The milk of this species has

individuality of its acceptance for consumption as raw, pasteurized and processed form thus obtaining access to the markets of Gulf countries (El-Agamy and Khatab, 1992) with highest milk yield reported to be 35-40 liters. Camel milk consumption is expected to go double with already ten time higher consumption in comparison to that of meat of this specie. The milk is characteristically blessed with lysozyme and lactoferrin that have antibacterial activity (Beg *et al.*, 1986). Despite of medicinal and nutritive

characteristics of camel milk bacterial contamination resulting into mastitis is rising (Aqib *et al*., 2017a) with *S. aureus* as major pathogen. Adding to this is resistance development against mastitogen. The studies are fewer than needed to address the bacterial isolation and its resistance against antibiotics (Aqib *et al*., 2017b).

Antibiotics used against bacterial isolates are facing resistance which in turn complicates infectious disease management. The complications in disease management are increasing in multitude due to drug resistance (Iqbal *et al*., 2002). This pattern has given rise to concept of multiple drug resistance in bacterial species. The bacteria resisting more than two classes of antibiotics are regarded as multiple drug resistant (MDR). The MDRs are currently amongst salient emerging challenges of mastitis etiologies (Hameed *et al*., 2006).

S. aureus is zoonotic pathogen that gets transmitted to humans through contaminated milk, milk products and coming in contact with contaminated animal skin (Seifu *et al*., 2004). The pathogen presented itself emerging pathogen of mammary gland of dairy animals (Arbeit *et al*., 1998). Intra mammary infection executed by *S. aureus* put animal to chronicity as bacteria hide it in mammary epithelium cells (Yousaf, 2009). Recent studies have demonstrated increased subclinical mastitis in camels with especial perspective of *S. aureus* mastitogen (Ahmad *et al*., 2012; Aqib *et al*., 2017a, b). The lavish and unjustified use of antibiotics has put this bacteria strongly develop resistance thus emerged as MDR *S. aureus.* There has not been study so far addressing MDR *S. aureus* from camel milk. Keeping in view the importance of this pathogen following study was designed to check the prevalence of MDR and its assumed risk factors from camel subclinical mastitis.

MATERIALS AND METHODS

Screening of subclinical mastitis samples

The subclinical mastitis samples based on California mastitis test for current study were practiced from various areas of Cholistan desert of province Punjab and Suleiman Range of Baluchistan. Camel milk (10 ml) was collected aseptically into capped tubes after abandoning of initial few streaks with prior washing and rinsing of teat apices with 70 % alcohol swabs. Using convenient sampling technique subclinical mastitis samples from Cholistan (n=185) and Suleiman Range (n=177) were collected strictly observing regulations of National Mastitis Council. The milk samples were transferred in a cold chain (4ºC) to department of Clinical Medicine and stored at -20ºC until further use.

Biochemical identification of S. aureus

Subclinical mastitis samples were processed to microbiological examination for isolation of *S. aureus* by centrifuging 2 mL milk 2000 g for 10 min resultantly swabbing sedimentation for direct microbiological demonstration on blood agar medium having 5% defibrinated sheep blood and incubated for 24-48 h at 37ºC. The mannitol salt agar was used as selective medium for *S. aureus*. The gram staining was performed and slide was visualized under microscope at 100X with oil emulsion. Further biochemical tests were performed as per flow chart of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The pool observations were used for identification of bacteria.

Risk factors analysis

A dichotomous questionnaire having information of age of animal, California Mastitis Test (CMT) score, body condition, teat health, parity, milking process hygiene, milk yield, lactation stage, tick infestation, treatment approach, and therapeutic drugs use were gathered to assume these as determinants of disease.

Multiple drug resistant S. aureus

Biochemically characterized *S. aureus* were put to antibiotic susceptibility against Oxacillin and Ampicillin (Penicillin), Cefoxitin (Cephalosporin), and Vancomycin (Glycopeptide). The isolates showing resistance to more than two classes of antibiotics were considered as MDR *S. aureus.* These MDR *S. aureus* were then assessed for their susceptibility against various antibiotics including Trimethoprim (25ug), Amikacin (30μg), Oxytetracycline (30μg), Gentamicin (10μg), Ciprofloxacin (5μg), Cefotaxime (30μg), Cefixime (5μg), Chloramphenicol (30μg), Enoxacin (10μg), and Streptomycin (10μg) antibiotics on following guidelines (Bauer *et al*., 1966). The activated 0.5 McFarland culture of *S. aureus* were swabbed on Muller Hinton agar that was kept for five minutes to get dry. The antibiotic discs were aseptically placed on Muller Hinton agar by automatic disc dispenser and kept in incubator at 37°C for 24 h. The zones of inhibition formed around discs were measured by Vernier calipers and were compared with standard provided by Clinical laboratory standards institute (CLSI, 2016). The sensitivity pattern against aforementioned was checked to declare *S. aureus* as multiple drug resistant.

PCR confirmation of MDR S. aureus

The MDR *S. aureus* was confirmed by *mecA* gene identification in *S. aureus*. The specification of *mecA* gene was P1: 59-TGGCATTCGTGTCACAATCG-39 and P2: 59- CTGGAACTTGTTGAGCAGAG-3 (Galdiero *et al*.,

2003). The PCR was performed in a final volume of 20μl consisting of 10μl of TOPreal™ qPCR 2x PreMIX, 2μl of DNA sample and 1μmol of each primer. Reaction was cycled 35 times after initial denaturation at 95°C for 5 minutes with denaturation at 95°C, annealing at 58°C and extension step at 72°C, each step was given 30 seconds, a final elongation at 72°C for 10 min was performed. A positive control and a negative control were included in each PCR run. The PCR products were observed for positive bands against a molecular ladder 100bp on 2% Agarose gel. The bands observed at 310bp level were considered positive.

Statistical analysis

Prevalence of MDR *S. aureus* was calculated by Thrushfield formula (Thrushfield, 2007) while non-parametric statistical analysis was performed to investigate association of risk factors at 5 % probability using SPSS version 22 of statistical computer programme.

RESULTS

Prevalence of multiple drug resistant S. aureus

The study found 33.98% (123/362) of MDR *S. aureus* isolates from subclinical camel mastitis (n=362) samples. There was 29.38% (52/177) of MDR *S. aureus* from subclinical mastitis samples (n=177) in camel from Suleiman Range (Table I). The different areas of Sulaiman range presented significant difference (*p*<0.05) of MDR prevalence with 48.16% prevalence of MDR *S. aureus* in Tuman Buzdar followed by Barthi, Fazla, Baikar, Kharr Buzdar, and Rakni giving 41.467%, 25%, 23.81%, and 6.67%, respectively. The prevalence of MDR *S. aureus* from subclinical camel mastitis was found 38.38% (71/185) from Cholistan desert area with non-significant difference (*p*>0.05) among various areas (Table I). The highest MDR prevalence was noted in Chanan Peer while lowest was found from Thalan Wala area of Cholistan giving rise to 46.43 and 29.17%, respectively among subclinical mastitis samples. Similarly non-significant difference of MDR *S. aureus* was noted from *S. aureus* samples obtained from various areas of Cholistan.

On the other hand from 192 *S. aureus* isolates 64.06% (123/192) were confirmed as MDR (Table I). The study reported 61.18% of MDR from *S. aureus* isolates obtained from camel milk in Baluchistan province (Table I). Higher prevalence in all areas of Suleiman range of province Baluchistan was noted with non-significant difference with in the area (*p*>0.05). However, Rakni area of Sulaiman presented lowest MDR from *S. aureus* while Baikar presented highest MDR from *S. aureus* with 20% and 83.33%, respectively. The overall MDR from *S. aureus* in Cholistan desert area was found 66.36%. Highest MDR from *S. aureus* was noted in Jam Sar while lowest prevalence was found in Chanan Peer areas of Cholistan desert exhibiting 80 and 62.50%, respectively.

Table I.- Prevalence of multiple drug resistant *S. aureus* in camel milk from areas of two distinct ecological zones.

Area	From subclinical mastitis (CMT) basis*				From *Staphylococcus aureus* samples**			
	No examined	Prevalence (%)	CI (95%)	p-value	No examined	Prevalence (%)	CI (95%)	p-value
Cholistan Desert Area								
Malkani	26	38.46	20.2-59.4	0.805	15	66.67	------	0.966
Nagra	28	46.43	27.5-66.1		20	65.00	38.4-88.2	
Din Garh	31	38.71	21.8-57.8		19	63.16	38.4-83.7	
Chanan Peer	33	45.54	28.1-63.6		24	62.50	40.6-81.2	
Noor Sar	17	35.29	14.2-61.7		08	75.00	34.9-96.8	
Thalan Wala	24	29.17	12.6-51.1		11	63.63	30.8-89.1	
Jam Sar	26	30.77	14.3-51.8		10	80.00	44.4-97.5	
Total (a)	185	38.38	31.3-45.8		107	66.36	56.6-75.2	
Suleiman Mountain Range								
Barthi	41	41.46	26.3-57.9	0.014	25	68.00	46.5-85.1	0.356
Tuman Buzdar	27	48.15	28.7-68.1		23	56.52	34.5-76.8	
Fazla	52	25.00	14.0-38.9		21	61.90	38.4-81.9	
Rakni	15	6.670	0.17-31.9		5	20.00	19.5-51.6	
Kharr Buzdar	21	14.28	03.0-36.3		5	60.00	14.7-94.7	
Baikar	21	23.81	8.20-47.2		6	83.33	35.9-99.6	
Total (b)	177	29.38	22.8-36.7		85	61.18	50.0-71.6	
Overall (a+b)	362	33.98	10.9-18.4		192	64.06	56.8-70.8	

p-value<0.05 indicate significant difference. *MDR *Staph aureus* on the basis of subclinical mastitis between Cholistan and Suleiman Range *p*=0.071. ** MDR *Staph aureus* from *Staph aureus* isolates between Cholistan and Suleiman Range *p*=0.458.

Comparison of MDR isolates of *S. aureus* from two distinct zones (Cholistan desert of Punjab and Suleiman Range of Baluchistan) showed non-significant difference (*p*>0.05) with however higher prevalence from Cholistan followed by Suleiman Range with 66.36% and 61.18%, respectively. Similarly prevalence of MDR *S. aureus* from subclinical mastitis samples presented non-significant difference (*p*>0.05) between two distinct zones. The prevalence of MDR *S. aureus* was however higher in camels from Cholistan compared to that of Suleiman Range.

Antibiotic susceptibility against MDR S. aureus

The study found Oxacillin 100%, Cefoxitin 100%, Ampicillin 100%, Cefotaxime84.5% and 71.15%, Vancomycin 78.87 and 78.84%, Cefixime 70.42 and 76.92% facing resistance MDR *S. aureus* obtained from Cholistan and Suleiman Range, respectively (Table II). However, Trimethoprim presented 90.14 and 92.3%, Ciprofloxacin 91.14% and 94.23%, Chloramphenicol 71.83 and 69.23%, Oxytetracycline 69.01 and 71.15%, Streptomicin 61.97 and 73.07%, Gentamicin 61.97 and 67.3%, Amikacin 43.6 and 51.92, and Enoxacin 45.07 and 53.84% effectiveness in inhibiting MDR *S. aureus* isolates of camel mastitis from Cholistan and Suleiman Range, respectively. Some of drugs also showed 5.79-32.39% of intermediate type of response against *S. aureus* isolates.

Risk factors

The assumed risk factors studied for prevalence of MDR *S. aureus* from subclinical mastitis samples from camels dwelling in Suleiman Range and Cholistan desert of Pakistan presented significant association regarding age of animal, CMT score, body condition, teat health, milking hygiene, milk yield, lactation stage, tick infestation, treatment approach, and therapeutic drugs use (Table III).

The increasing age of animal was found more prone toward having MDR *S. aureus* compared to early age of she camels. The camels in Suleiman Range with 7-10 years of age did present higher prevalence of MDR while >10 years of age was found presenting two times more prevalence of MDR *S. aureus*. The increased CMT score was found positively associated with MDR *S. aureus* prevalence. The weak body condition was found more prone to have MDR *S. aureus* in she camels than to normal body condition of camels from both zones. Injured teats were two times more prone toward MDR *S. aureus* prevalence compared to normal teats in both camel rearing areas. The increased parity number was found positively associated with prevalence of pathogen however early lactation presented more prevalence. The camels having tick infestation on their teat had higher prevalence of pathogen. Self-treatment and use of Penicillin was found risk factor for higher prevalence of MDR *S. aureus* in both camel rearing areas.

Table II.- Antibiotic response against MDR *S. aureus*isolates of camel subclinical mastitis.

Drugs used	Potency	Cholistan sampling area (n=71)			Suleiman Mountain Range (n=52)		
		S(%)	I (%)	R (%)	S (%)	I(%)	R(%)
Oxacillin	10μg	0.000	0.000	100.0	0.000	0.000	100.0
Cefoxitin	30 μg	0.000	0.000	100.0	0.000	0.000	100.0
Trimethoprim	25ug	91.54	8.450	0.000	92.30	7.690	0.000
Ciprofloxacin	5 μg	90.14	9.850	0.000	94.23	5.790	0.000
Gentamicin	10 μg	61.97	12.67	25.35	67.30	11.53	21.15
Cefotaxime	30 μg	2.810	12.67	84.5	17.30	11.53	71.15
Vancomycin	30μg	0.000	21.12	78.87	0.000	21.15	78.84
Oxytetracycline	30μg	69.01	9.850	21.12	71.15	9.610	19.33
Cefixime	5μg	16.90	12.67	70.42	15.38	7.690	76.92
Chloramphenicol	30μg	71.83	12.67	15.49	69.23	13.46	17.30
Ampicillin	10μg	0.000	0.000	100.0	0.000	0.000	100.0
Streptomicin	10 μg	61.97	32.39	5.630	73.07	9.610	17.30
Amikacin	30 μg	43.66	18.30	38.02	51.92	21.15	26.92
Enoxacin	10 μg	45.07	36.61	18.30	53.84	19.23	25.00

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

Table III.- Risk factor association with MDR *Staphylococcus aureus* obtained from subclinical camel mastitis from Suleiman Mountain Range and Cholistan Desert Area.

Parameter	Level	Suleiman Range			Cholistan Desert		
		No examined	Prevalence	p-value	No examined	Prevalence	p-value
Age of animal	4-6 year	74	17.57	0.014	52	19.23	0.000
	7-10 year	41	39.02		97	34.02	
	>10 year	62	37.10		36	77.78	
CMT score	+1	19	10.53	0.000	29	20.69	0.002
	+2	96	27.08		110	34.54	
	+3	62	38.71		46	58.70	
Body condition	Weak	90	43.33	0.000	99	50.50	0.000
	Normal	87	14.94		86	24.42	
Teat Health	Injured	67	55.22	0.000	85	64.71	0.000
	Normal	110	13.64		100	16.00	
Parity	1-2	117	19.66	0.000	79	15.19	0.000
	3-4	15	26.67		50	36.00	
	>4	45	55.56		56	73.21	
Milking process Hygiene	Unhygienic	125	33.6	0.000	135	46.67	0.000
	Acceptable	52	19.23		50	16.00	
Milk Yield	1-2 liters	65	24.61	0.000	80	18.75	0.000
	3-5 liters	87	26.44		40	40.00	
	>5 liters	33	75.76		65	61.54	
Lactation stage	1-4 months	64	32.81	0.49	65	40.00	0.864
	5-7months	84	29.76		88	36.36	
	8-12months	29	20.69		32	40.62	
Tick infestation	Yes	92	39.13	0.003	105	50.48	0.000
	No	85	18.82		80	22.50	
Treatment approach	Self	127	33.86	0.037	130	48.46	0.000
	Professional consultancy	50	18		55	14.54	
Therapeutic drug use	Penicillin in first preference	102	44.12	0.000	110	55.45	0.000
	Case specific	75	9.33		75	13.33	

p-value <0.05 indicate significant difference.

DISCUSSION

Prevalence of MDR S. aureus

Previous studies did not report MDR *S. aureus* prevalence in camel milk. However, higher prevalence of *S. aureus* is positively associated with higher mastitis percentages. Higher prevalence of *S. aureus* of current study was in agreement with findings of Ahmad *et al.* (2012) and Aqib *et al.* (2017b). The current study areas are having higher tick prevalence; thorny bushes and general habit of tying of teats with bushes pave the way to get mammary inflammations (Woubit *et al.*, 2001; Abdurahman, 2006). Structural parts of mammary glands like teat skin, teat orifice, and teat canal are considered as key sites of *S. aureus* adherence (Frost *et al.*, 1977). The animals are frequently exposed to teat injuries leading to inflammation of mammary glands which favors invasion of microbes. The conventional unjustified treatment approaches help spread of *S. aureus* like contagious pathogens with subsequent resistance development against various classes of antibiotics.

Risk factors

The higher prevalence of MDR in older age of animals might be because of increased dilatation of teats (Shittu *et al.*, 2012) partial opening of teat orifice due to repeated lactations (Schroeder, 2010) resulting in repeated exposure of mastitis and reduced physiology of immune system that gives opportunity to bacterial invasions (Abdurahman, 2006). Moreover, older animals' teat orifice left partially open due to repeated lactations which invites environmental and skin bacteria to invade. Early lactation

is reported due to lower resistance of immune system in early age and chances of newer infections in dry periods make intra mammary infection more susceptible. Ticks act as spread of contagious pathogen from one animal to other in addition to injuries inflicted which are source of having bacterial contamination (Seifu and Tafesse, 2010).

The camel herders in Pakistan are habitually unhygienic during milking while *S. aureus* spread during milking process (Dodd, 1983) so unhygienic milking is potential risk factors for spread of this pathogen. Multiparous animals are reported to have higher prevalence of mastitis due to spread of infection from one to next parity (Abdurahman, 2006). The animals in study area are devoid of health care facilities and once infection is established its spread to next parity is inevitable.

Antibiotic susceptibility

The increased resistance of Penicillin group and Vancomycin was in line with findings of Aqib *et al.* (2017b) while contrary to current study 100% resistance of *S. aureus* against Chloramphenicol was noticed in their study. The sensitivity of drugs of current study was also in line with finding of aforementioned previous study. The findings of current study are also in line with Najeeb *et al.* (2013) and Abdulkadhim (2012). Efficacy of Sulphonamides of current study was in agreement with findings of Aqib *et al.* (2017b), Fazlani *et al.* (2011) and Rind and Sheikh (2001). The higher resistance to beta lactam group in current study might be because of unjustified use of penicillin group that was observation of current study. Moreover self-approach of treatment of ailments without proper diagnosis paves way to resistance against antibiotics.

The study found higher prevalence of multiple drug resistant *S. aureus* in both ecological zones. The assumed risk factors were significantly associated with prevalence of pathogen. Trimethoprim, ciprofloxacin, gentamicin, chloramphenicol, streptomycin, oxytetracycline, amikacin and enoxacin were found effective in combating multiple drug resistant *Staph. aureus*. The study demands immediately attention to take effective preventive measures in order to stop spread of this pathogen and to search out effective treatment avenues.

ACKNOWLEDGMENTS

Statement of conflict of interest

The authors declared that they have no conflict of interest.

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