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Prevalence and Antibiotic Resistance of *Staphylococcus aureus* and Risk Factors for Bovine Subclinical Mastitis in District Kasur, Punjab, Pakistan

Abdul Maalik[1], Shahzad Ali[1,*], Anam Iftikhar[1], Muhammad Rizwan[1], Haroon Ahmad[2] and Iahtasham Khan[1]

[1]*University of Veterinary and Animal Sciences, Lahore*
[2]*Department of Biosciences, COMSATS Institute of Information Technology, Park Road, Chak Shahzad, Islamabad*

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AM, SA, MR and IK conceived and designed the study. AM, HA and IK performed the study and analyzed the data. AM, SA and AI wrote the article. AM, SA, AI, MR, HA and IK proofread the manuscript and approved final version.

Key words
Subclinical mastitis, Risk factors for mastitis, Antibiotic resistance, Bovine, *Staphylococcus aureus*, multi-drug resistant strain.

ABSTRACT

The present study was designed to determine the prevalence and possible risk factors of subclinical mastitis along with antibiotic resistance of one of the causative agent of bovine mastitis, *Staphylococcus aureus*. Ninety milk samples were collected from cows and buffaloes kept at different localities in district Kasur, Punjab, Pakistan. The possible risk factors inquired from farmers were animal type, breeds, urbanicity, age (years), teat washing, bedding area, lactating stage and previous exposure of mastitis. Initially surf field mastitis test (SFMT) was applied for screening subclinical mastitis followed by bacteriological techniques on positive milk samples for confirmatory isolation of *Staph. aureus* as a bovine mastitis causing agent. These *Staph. aureus* isolates were further tested for antibiotic susceptibility. Epidemiological data was analyzed by chi-square methods at 95% confidence interval. The overall prevalence of subclinical mastitis as a result of surf field mastitis test was 42.2%. The prevalence of disease was higher in cows (54.5%) than in buffalo (22.9%). Based on chi-square and P-value the prevalence of mastitis was significantly ($P<0.05$) associated with animal type, urbanicity, age of animal and previous exposure of mastitis. There was no association of the prevalence of bovine subclinical mastitis with breed, teat washing, bedding area and lactating stage. Out of 38 positive milk samples, 13 (34.2%) were positive for *Staph. aureus*. All isolates of *Staph. aureus* were resistant for ten different antibiotics. It was concluded that multi-drug resistant strains of *Staph. aureus* were major causative agent of bovine subclinical mastitis and there were multiple risk factors associated with incidence of mastitis.

INTRODUCTION

Urban and peri-urban dairy production plays an important role in fulfilling the increasing demand of milk and milk products in developing countries. Milk is the essential diet of human being because it has all essential components (Javaid *et al*., 2009; Ajmal *et al*., 2015). Recently, due to increase in world population, liquid milk demand has been increased tremendously (FAO, 2018). Apart from other problems, dairy animals are facing heavy economic losses due to mastitis. Mastitis is known as inflammation of mammary tissue and dairy cattle industry is being affected globally by this ailment (Ali *et al*., 2018; Ashraf and Imran, 2018). The main consequences of mastitis in dairy cattle are altered milk composition, increased number of somatic cells in milk and ultimately low milk production which has now become a serious cause of heavy economic losses (Gianneechini *et al*., 2002; Hogeveen *et al*., 2011). Mainly by changing milk color and inflammation of udder, subclinical mastitis has struck in different countries including Pakistan, Ethiopia and Poland (Mungube *et al*., 2005; Islam *et al*., 2010; Sztachanska *et al*., 2016). A number of risk factors like teat or udder lesions play a significant role in occurrence of mastitis among livestock animals (Hussain *et al*., 2012; Tiwari *et al*., 2013; Iraguha *et al*., 2015). Similarly, a number of microorganisms are responsible for mastitis but among all these agents *Staphylococcus aureus* is a major pathogen which can play vital and significant role in mastitis (Lundberg *et al*., 2014). Moreover, development of antimicrobial resistance in the case of bovine mastitis caused by *Staph. aureus* is increasing day by day (Barkema *et al*., 2006; Gao *et al*., 2012). Keeping in view the economic importance of bovine mastitis in Pakistan, the present study was designed to determine prevalence, associated risk factors and antibiotic resistance of *Staph. aureus* from bovine

* Corresponding author: shahzad.ali@uvas.edu.pk
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subclinical mastitis in district Kasur, Pakistan.

MATERIALS AND METHODS

Study area

The present study was conducted in District Kasur, Punjab Province, Pakistan. Kasur is a district of province Punjab, Pakistan. It is the 20th most populated city of Pakistan, bordered to the north by Lahore and to the south and east by India.

Sample collection

Total 90 milk samples were collected from cows (n = 56) and buffaloes (n = 34) from various peri-urban and rural areas of district Kasur from September 2016 to February 2017. Before collection of milk samples each teat was thoroughly washed with water, wiped dry with tissue paper and finally disinfected with 70% alcohol. Milk samples were then collected in sterile sampling falcon tubes (15mL) after discarding first two streams of milk from each teat. These samples were transported in ice box (at 4°C) to the Epidemiology and Microbiology Laboratory of University of Veterinary and Animal Sciences, Lahore, Pakistan.

Confirmation of bovine mastitis

The confirmation of bovine subclinical mastitis was done using 3% Surf-solution (Muhammad *et al*., 1995). Briefly, one ml of milk was mixed with equal amount of the surf solution in petri-plate and rotated the mixture for 1 min. The mixture was examined for thickening or any other possible change. The positive milk samples for mastitis showed agglutination or color change and vice-versa.

Isolation of Staph. aureus

The positive milk samples (100µL) were inoculated on Mannitol Salt agar (Lab M Limited, UK) for isolation of *Staph. aureus* and these samples were incubated at 37°C for 24 h. A colony of smooth shaped, golden yellow color was marked as *Staph. aureus,* which was further confirmed by using gram staining technique: a colony of purple, round and clump shaped finally confirmed the presence of *Staph. aureus*. Further confirmation was done by using biochemical tests (Pumipuntu *et al*., 2017).

Antibiotic sensitivity

Disc diffusion method was used to check the susceptibility of *Staph. aureus* isolates according to the instructions of Clinical and Laboratory Standards Institute (2009). Eighteen antibiotics (Bioanalyse, Ankara, Turkey) including Augmentin (30mcg), Ampicillin (10mcg), Cefoxitin (30mcg), Clindamycin (2mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Erythromycin (15mcg), Fosfomycin (200mcg), Gentamycin (10mcg), Kanamycin (30mcg), Oxacillin (1mcg), Ofloxacin (5mcg), Penicillin (10mcg), Rifampicin (5mcg), Tetracycline (30mcg), Teicoplanin (30mcg), Trimethoprim (5mcg), and Vancomycin (30mcg) were used.

Statistical analysis

The data were analyzed by using SPSS software version 22.0. The respective prevalence of bovine mastitis was summarized by taking percentage of positive samples and correlation of possible risk factors with bovine mastitis was calculated by chi-square test on the basis of P-value of each possible risk factor. For the test, $P < 0.05$ was considered statistically significant (Hosseinzadeh and Sei, 2014).

Table I.- Risk factors associated with bovine mastitis in peri-urban and rural areas district Kasur.

Para-meters	Sample			Chi-square value (χ^2)	P-Value
	Collected	Positive	%age		
Animal Type					
Buffalo	35	8	22.9	4.444	0.035
Cow	55	30	54.5		
Breeds					
Local	47	10	21.3	0.178	0.673
Exotic	43	28	65.1		
Urbanicity					
Rural	65	17	26.2	17.778	0
Urban	25	21	84.0		
Age (years)					
≤ 4	24	19	79.1	15.667	0.004
4.1 to 6	28	4	14.3		
6.1 to 8	19	7	36.8		
8.1 to 10	10	6	60.0		
10.1 to 12	9	2	22.2		
Teat washing					
Yes	38	10	26.3	2.178	0.14
No	52	28	53.8		
Bedding area					
Barn	44	26	59.1	0.044	0.833
Brick	46	12	26.1		
Lactating stage					
Early	32	14	43.8	0.267	0.875
Middle	30	16	53.3		
Late	28	8	28.6		
Previous exposure of mastitis					
Yes	37	24	64.9	2.844	0.092
No	53	14	26.9		

RESULTS

The prevalence of bovine sub-subclinical mastitis is given in Table I. Thirty eight (42.2%) out of 90milk samples were found positive for sub-clinical mastitis using surf field mastitis test. Prevalence of sub-clinical mastitis was significantly (P = 0.035) higher in cows (54.5%) than in buffalo (22.9%). The prevalence of sub-clinical mastitis in animals breeds was not statistically significant (P<0.673). This study revealed that mastitis is much more prevalent at urban site (84%) as compared to rural site (26.2%). Interestingly, the maximum prevalence *i.e.* 79.1%was reported in animals with age ≤4 years and lowest in the animals with age 4.1 to 6 years.

The prevalence of mastitis in those animals whose teats were being washed before milking was 26.3% while in those whose teats were not being washed before milking was 53.8%. The prevalence measure based on the bedding area was statistically insignificant. The infection was more in those animals which were raised on barn surface (59.1%) than on bricks (26.1%). Lactating stage plays a dynamic and significant role in inflammation of udder. The mastitis was calculated by different lactation stages *i.e.* early, middle and late with prevalence recorded as 43.8%, 53.3% and 28.6%, respectively. Prominent finding from the results was higher prevalence of sub-clinical mastitis (64.9%) in previously exposure animals of mastitis as compared to not exposed (26.9%).

Prevalence of Staph. aureus

Out of 38 positive milk samples, 13 (34.2%) milk samples were found to be positive in bacteriological examination. Only two (25%) out of 8 SFMT positive milk samples of Nili-Ravi buffalo were contaminated with *Staph. aureus.* Out of 30 milk samples of cattle, 11 (36.7%) milk samples were positive for *Staph. aureus*. Out of 10 SFMT positive milk samples of local breeds, 4 (40%) were positive for *Staph. aureus*. Furthermore, from 28 SFMT positive milk samples of exotic breeds, 9 (32.1%) were positive for *Staph. aureus*.

Table II.- Prevalence of *Staph. aureus* in milk of cows and buffaloes.

Parameters	Sample tested	Positive culture	Percentage
Animal species			
Buffalo	8	02	25.0
Cow	30	11	36.7
Animal breed			
Local	10	04	40.0
Exotic	28	09	32.1

Table III.- Breakpoint values of each antimicrobial agent and phenotypic antimicrobial susceptibility profiles of 13 tested *Staph. aureus* isolates used according to CLSI and EUCAST.

Antimicrobial class	Antimicrobial agents	Reference	Conc. (mcg)	Zone diameter (mm)			*S. aureus* (n = 13)		
				S	I	R	S	I	R
Beta-Lactam	Augmentin	1	30	≥20	-	≤ 19	**1**	**0**	**12**
Beta-Lactam	Ampicillin	2	10	≥15	12-14	≤11	**2**	**0**	**11**
Cephalosporins	Cefoxitin	3	30	≥22	16–20	≤ 20	**1**	**0**	**12**
Lincosamide	Clindamycin	2	2	≥21	15–20	≤14	**0**	**1**	**12**
Phenicol	Chloramphenicol	2	30	≥18	13–17	≤ 12	**1**	**1**	**11**
Fluoroquinolone	Ciprofloxacin	2	5	≥21	16-20	≤ 15	**1**	**2**	**10**
Macrolide	Erythromycin	2	15	≥23	14–22	≤ 13	**1**	**1**	**11**
Fosfomycins	Fosfomycin	2	200/50	≥34	-	≤ 33	**0**	**0**	**13**
Aminoglycoside	Gentamycin	2	10	≥15	13–14	≤ 12	**1**	**0**	**12**
Aminoglycoside	Kanamycin	2	30	≥18	14–17	≤13	**0**	**0**	**13**
Beta-Lactam	Oxacillin	2	1	≥22	-	≤ 21	**0**	**0**	**13**
Fluoroquinolone	Ofloxacin	2	5	≥18	15–17	≤14	**2**	**0**	**11**
Beta-Lactam	Penicillin	2	10	≥29	-	≤ 28	**0**	**0**	**13**
Ansamycins	Rifampicin	2	5	≥20	17–19	≤16	**2**	**0**	**11**
Tetracycline	Tetracycline	2	30	≥19	15-18	≤14	**2**	**0**	**11**
Glycopeptides	Teicoplanin	2	30	≥14	11–13	≤ 10	**1**	**2**	**10**
Folate-Pathway Inhibitors	Trimethoprim	2	5	≥16	11–15	≤ 10	**0**	**0**	**13**
Glycopeptides	Vancomycin	4	30	≥15	-	-	**2**	**0**	**11**

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

Antibiotics sensitivity of Staph. aureus

Staph. aureus isolates were completely resistant (100%) to Fosfomycin, Kanamycin, Oxacillin, Penicillin and Trimethoprim (Table III). Twelve (92.3%) isolates were resistant to Augmentin, Cefoxitin, Clindamycin and Gentamycin. Ampicillin, Chloramphenicol, Erythromycin, Ofloxacin, Rifampicin and Vancomycin were 84.6% resistant while Ciprofloxacin and Teicoplanin was 76.9 % resistant (Table III).

DISCUSSION

Staph. aureus is an economically important pathogen and can survive in the extreme ranges of temperature. It is responsible for intra-mammary infections in dairy animals. In present study, 42.2% animals were positive for subclinical mastitis which is higher than previous studies conducted in Lahore (22.4%) and Faisalabad (27%), Punjab, Pakistan (Khan and Muhammad, 2005; Mustafa *et al*., 2011).

The prevalence of sub-clinical mastitis was significantly higher (P = 0.035) in cattle (54.5%) than in buffalo (22.9%). In contrast to our findings, Khan and Muhammad (2005) concluded that the overall prevalence of sub-clinical mastitis was 27% in buffaloes while 36% in crossbred cows by using surf field mastitis test. However, a very low prevalence of bovine subclinical mastitis was reported from Egypt (3.9% in cattle and 0% in buffalo) (Osman *et al*., 2009). The prevalence of subclinical mastitis was higher in exotic breeds as compared to local breeds. A higher prevalence was previously reported for exotic (29.2%) breeds as compared to local (23.8%) breed using surf field mastitis test as screening test in Khyber Pakhtunkhwa, Pakistan (Khan *et al*., 2015). Similarly slightly higher prevalence was reported in exotic breed (12.7%) as compared to local (4.2%) in Bangladesh (Hossain *et al*., 2016). Based on urbanicity, the prevalence of bovine subclinical mastitis was higher (84%) in urban areas and lower (26.2%) in rural areas in present study. These findings are in line with Hardenberg (2016), who concluded slightly lower prevalence in rural (36.8%) and higher prevalence in urban (43.9%) area of Bihar India. However, these values are not in line with findings of Bilal *et al*. (2004), who reported the higher prevalence of mastitis in rural areas (25.1%) and lower (19.7%) in urban areas of Faisalabad District, Punjab province, Pakistan. In our study, the prevalence of mastitis with respect to different age groups showed statistically significant (P<0.05) results. There is perfect agreement between our findings and the findings of studies conducted in Ethiopia; the prevalence of bovine subclinical mastitis was statically significant with respect to age (Zeryehun *et al*., 2013). However, Hussain *et al*. (2012) showed no-significant (P>0.05) association of mastitis with age in his study in Pakistan. In case of teat washing, our results were considerably different from the findings of Tesfaye and Abera (2018), who find higher prevalence of mastitis (45.5%) in animals whose udder being washed before milking in Jimma Town Dairy Farms, Western Ethiopia. With respect to bedding area, our study showed the higher prevalence in the animals with barn floor while lower prevalence in the animals with brick floor. Our results considerably differs from the findings of Chishty *et al*. (2007), who observed that the animals which were used to reside on brick floors showed a higher prevalence (36.53%) in case of cattle and (41.53%) buffaloes, and the prevalence on Kaccha floor were the lowest, (10.34%) for cattle and (15.00%) for buffalo in cattle and buffaloes of tehsil Gojra, Pakistan. Our results are consistent with the results of Rahman *et al*. (2009) who reported high prevalence of mastitis on brick floor (30.6%) and low in the animals with barn floor (20%) in dairy cows of various Districts of Sirajganj and Pabna, Bangladesh. From our study the stages of lactation indicated that they are statistically insignificant. The current study does not support the previous research in this area; Tekle and Berihe (2016) reported that lactation stage had significant (P<0.05) effect on the prevalence of mastitis in animals in Ethiopia. This could be due to the delayed inflammation of neutrophils to mammary glands. Even though our findings differ from some previously published literature yet the lactation stage was significantly associated with mastitis (Qayyum *et al*., 2016) in Cholistani cattle of Bahawalpur District, Punjab province, Pakistan. Our findings were in good agreement with Biffa *et al*. (2015), higher prevalence of mastitis (57%) in previously exposed dairy animals as compared to not previously exposed (22%) in Ethiopia.

In present study, *Staph. aureus* is a prominent pathogen constituting 34.2% of SFMT positive milk samples; our results are slightly higher than the findings of Zeryhum *et al*. (2013), who reported 28.7% isolates of *Staph. aureus* and their primary role in bovine subclinical mastitis in small holder dairy farms of Addis Ababa, Ethiopia.

Resistance in all isolates of *Staph. aureus* against Penicillin has been reported from cow milk in Afar, Ethiopia (Beyene, 2016). Resistance against Penicillin was also reported in *Staph. aureus* isolates from subclinical mastitis in dairy cow from Germany (74.28%) (EI-Behiry *et al*., 2012). Resistance in *Staph. aureus* isolates against Oxacillin has been reported from buffalo infected with mastitis in Brazil (50%) (Garcia *et al*., 2017). *Staph. aureus* isolated from mastitic milk of cows were also resistant against Trimethoprim in Ethiopia (21%) and Kososvo (11.1%) (Abera *et al*., 2013; Hamidi *et al*., 2016). *Staph. aureus* isolates from cattle were found resistant

to Kanamycin in Romania (28%) and China (17.37%) (Brinda *et al*., 2010; Shi *et al*., 2010). Similarly, previous work from Brazil on buffaloes affected with subclinical mastitis showed high antimicrobial resistance (97.98%) (Cuhna *et al*., 2006). However, comparatively low (57%) resistance was recorded against Gentamycin from bovine mastitis milk samples in India (Pati and Mukherjee, 2016). Interestingly, in case of *Staph. aureus* resistance against Vancomycin, our findings are in line (88.89%) with a study conducted on cattle and buffaloes in India (Sharma *et al*., 2015). However, comparatively very low resistance (6%) was reported in dairy products in Malaysia (Sasidharan *et al*., 2011). Our results differ from some previous studies (Szweda *et al*., 2014; Hamidi *et al*., 2016), in which comparatively low resistance against Ampicillin was reported from cows infected with mastitis (46.1% from Poland and 22.8% from Kosovo). Surprisingly, the findings of Goa *et al*. (2012) are in line with our study; which showed very high resistance (98.1%) of *Staph. aureus* isolates against Tetracycline from bovine mastitis in single herd in China. Tetracycline however revealed moderately low resistance (26%) in cows with sub clinical mastitis in Poland (Malinowski *et al*., 2002). Our results validate the findings of Leigue *et al*. (2017) who declared that *Staph. aureus* have resistance (52%) against Erythromycin in cattle with mastitis in Brazil. The results of our study supports previous research in this area: Tetracycline showed comparatively high resistance (61.9%) against *Staph. aureus* isolated from bovine mastitis in Algeria (Saidi *et al*., 2015). In contradiction with earlier findings; El-Behiry *et al*. (2012) who described resistance of *Staph. aureus* showed a very low resistance (2.85%) against Chloramphenicol in cows with subclinical mastitis in Germany. This study is in good agreement with the results of Abraha *et al*. (2018) in which Chloramphenicol was found highly resistance (81.8%) against *Staph. aureus* isolated from raw cow milk in Ethiopia. In case of Clindamycin, we found much higher resistance than Kumar *et al*. (2010) who found a low resistance (14.1 %) against *Staph. aureus* isolates from milk of mastitic crossbred cattle in India. The findings of Younis *et al*. (2018) showed relatively low resistance (41.25%) as compared to our study against Clindamycin in dairy cows affected with bovine clinical and sub clinical mastitis in of Egypt.

The results of the present study proved that bovine subclinical mastitis is prevalent in cattle and buffalo of Pakistan and *Staph. aureus* is the major pathogen associated with this disease. Multiples risk factors *i.e.* species, urbanicity, age and previous exposure of animal with mastitis were associated with bovine subclinical mastitis. High antibiotic resistance was investigated in mastitis causing *Staph. aureus* isolates. This is an alarming condition regarding presence of multi-drug-resistant *Staph. aureus* strains in food (*i.e.* milk). The results of the present study and knowledge of disease causing pathogen and their antibiotic resistance patterns against various drugs will be very helpful in mastitis control, and will play a vital and significant role in the improvement of health status of dairy herds in Pakistan.

Statement of conflict of interest

The authors declare no conflict of interest.

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